METHOD DEVELOPMENT FOR IDENTIFICATION OF TACHYKININS AND THEIR POST-TRANSLATIONAL MODIFICATIONS AND DISCOVERY WITH IN SILICO ANALYSIS OF BIO-INFORMATIC DATABASES.

GEORGIA DELICONSTANTINOS

THIS THESIS IS BEING SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF KINGSTON UNIVERSITY FOR THE AWARD OF A DOCTOR OF PHILOSOPHY, UNDERTAKEN AT KINGSTON UNIVERSITY AND ROYAL HOLLOWAY UNIVERSITY OF LONDON.

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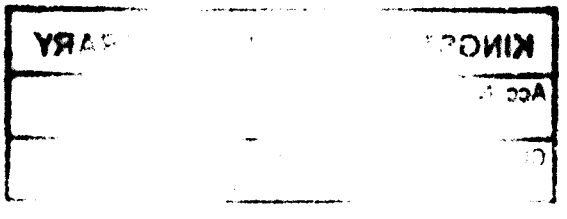

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I declare that the work reputed in this Thesis is entirely my own and has been carried out at Kingston University and the Royal Holloway, University of London, U.K.

This Thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Abstract.

Tachykinins are signalling peptides released by the processing of preprotachykinin precursors, which are subject to post-translational modifications including amidation. Tachykinins act on the three tachykinin receptors NK1, NK2 and NK3. Differential processing of the preprotachykinin precursors can lead to the formation of a diverse range of tachykinins (including extended forms). Our previous findings have shown increased proteolytic cleavage of the human TAC3 and TAC4 preprotachykinin precursors by the placenta in pathological conditions such as pre-eclampsia comparable to that found in the brain. This study has developed an experimental strategy for the capture and detection of tachykinins combining peptide extraction, enrichment by immunoaffinity purification, RP-HPLC separation and MALDI-TOF. The combined application of these methods in rat brain identified mHK-1 as an amidated decapeptide and also captured and detected SP. The detection of a modified form of mHK-1 in the brain matching the mass of an additional acetyl group (+42 a.m.u.) indicates the existence of a neuropeptide–specific post-translational modification. The biological role of acetylation is to provide greater stability for the peptide and affinity of binding for its receptor. The methodology described in this research could be applied for the capture of tachykinins expressed in normal, pre-eclamptic and IUGR placentae, in order to investigate the changes that occur in precursor processing during disease states such as pre-eclampsia and to identify post-translational modifications. A parallel *in silico* analysis of the publicly accessible NCBI and Ensembl databases was conducted to identify tachykinin precursors. Multiple sequence alignment of retrieved preprotachykinin sequences was conducted and the phylogenetic relationship between the identified species investigated. Collectively, the results expand the number of known or predicted tachykinins and tachykinin gene-related peptides. Moreover, they separate the preprotachykinin precursors into three distinct groups. The analysis also sheds light on the evolution of the tachykinin precursor cleavage sites (*e.g.* the N-terminal monobasic cleavage site of human EKA/B). Overall, this study has developed technologies for identifying tachykinin precursor post-translation modifications that may serve as a tool for determining different peptide physiologies between neuronal and peripheral tissues and different disease states.

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Abbreviations.

amu	atomic mass unit
AU	arbitrary units
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
C14TKL-1	Chromosome 14 tachykinin-like peptide 1
Ci-TK-R	*Ciona intestinalis* tachykinin receptor
CID	collision induced dissociation
cDNA	complementary DNA
CRF	corticotrophin releasing factor
CRH	corticotrophin releasing hormone
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide
DTKR	*Drosophila melanogaster* tachykinin receptor
EDTA	ethylenediaminetetraacetic acid
EK-1	endokinin-1
EKA	endokinin A
EKB	endokinin B
EKC	endokinin C
EKD	endokinin D
ELISA	enzyme-linked immunoassay
ESI	electrospray ionisation
ESTs	expressed sequence tags
GPCRs	G protein-coupled receptors
GnIH	gonadotrophin-inhibitory hormones
HCCA	α-Cyano-4-hydroxycinnamic acid
HELLP	haemolysis, elevated liver function enzymes and low platelets
hHK-1	human hemokinin-1
mHK-1	mouse hemokinin-1
ID	internal diameter

IUGR	intra-uterine growth restriction
LC	liquid chromatography
m/z	mass to charge ratio
MS	mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MW	molecular weight
NCBI	National Centre for Biotechnology Information
NK1	neurokinin 1 receptor
NK2	neurokinin 2 receptor
NK3	neurokinin 3 receptor
NKA	neurokinin A
NKB	neurokinin B
NPγ	neuropeptide gamma
NPK	neuropeptide kappa
nr/nt	non-redundant/ nucleotide
NT-PTA	N-terminal pro-tachykinin A
NSS	normal sheep serum
OG	octyl β-D-glucopyranoside
ORF	open reading frame
PAM	peptidylglycine α-amidating monooxygenase
PBS	phosphate buffered saline
PNPP	p-nitrophenyl phosphate
PC	prohormone convertase
POMC	proopiomelanocortin
PPT	preprotachykinin
RFRP	RF-related peptide
RP-HPLC	reversed phase-high pressure liquid chromatography
RPM	rotations per minute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STKR	*Stomoxys calcitrans* tachykinin receptor
SP	substance P

TOF	time-of-flight
TFA	trifluoroacetic acid
TKRPs	tachykinin related-peptides
UV	ultraviolet
UTKR	*Urechis unitinctus* tachykinin receptor

Chapter 1. Introduction.

Tachykinins are a vast family of signalling peptides, involved in pleiotropic functions, found in a large number of animal species (Severini *et al.*, 2002). The field of tachykinin research was introduced with the discovery of substance P (SP) by von Euler and Gaddum in 1931. They described as "preparation P" (P as the initial of the word "powder") an active semi-purified substance extracted from equine brain and intestine capable of acting as a vasodilator and as a contracting agent on rabbit jejunum. The term "preparation P" or "substance P" remained in the literature as no clear biological role for this preparation of substance(s) existed. Forty years later, Chang and colleagues purified and sequenced SP from bovine hypothalamus as an amidated undecapeptide (Chang *et al.*, 1971). Following the initial discovery of SP, two more peptides physalaemin and eloidosin were discovered in amphibia and octopus, respectively, with similar structure to SP and between them, also capable of causing fast (*tachy*-) contractions (*-kinin*) on muscles (Anastasi & Espramer, 1962; Anastasi *et al.*, 1964). Kimura and colleagues (1983) isolated the "next" mammalian tachykinin peptide, neurokinin A (NKA) initially designated as neuromedin L or substance K and a "third" mammalian tachykinin, neurokinin B (NKB) initially designated as neuromedin K from porcine spinal cord. Independently from Kimura and colleagues, a group by Kangawa and colleagues (1983) the same year isolated NKB from porcine spinal cord and characterised it as a substance with a stimulant activity on guinea pig ileum and rat uterus. Tachykinins were initially considered having restricted expression in neuronal tissues and found in the periphery only by release from nerve endings, involved in pain perception and other neurological functions, thus the term "neurokinins" was coined (Patacchini *et al.*, 2004). However, nowadays, this initial perception has been challenged with the finding of the widespread peripheral distribution of tachykinins (as well as tachykinin-like peptides, found in invertebrates) outside of the nervous system that cause a plethora of tachykinin-mediated biological responses (Satake & Kawada, 2006).

1.1) Tachykinin peptides and their receptors in mammals.

The *classical* members of the tachykinin family in mammals are SP, NKA and NKB (Table 1.1) showing complete identity among mammalian species (Severini *et al.*, 2002). The members of the tachykinin family comprise a common highly conserved signature C-terminal motif –FXGLM-NH_2, where X is traditionally thought to be a bulky, hydrophobic residue valine (V), isoleucine (I) or aromatic phenylalanine (F), tyrosine (Y) (Page, 2005). Tachykinins in their mature forms comprise 10 or 11 amino acid residues while there are also the N-terminally extended forms of NKA namely neuropeptide K (NPK) (Tatemoto *et al.*, 1985) and neuropeptide gamma (NPγ) (Kage *et al.*, 1988) that comprise 36 and 21 residues, respectively. Recently, the family of mammalian tachykinin peptides was expanded to include the endokinins (or hemokinins), a group of tachykinins with peripheral tissue distribution and potential endocrine or paracrine roles (Page, 2004). This group of endokinin/hemokinins was introduced with the discovery of the tachykinin mouse hemokinin-1 (mHK-1) in B-lymphocytes (Zhang *et al.*, 2000). A diverse range of endokinin peptides was demonstrated with the discovery of more endokinin peptides namely human hemokinin-1 (hHK-1) (Page *et al.*, 2003) and the extended forms EKA and EKB in humans (Page *et al.*, 2003) or truncated forms namely hHK-1 (4-11) (Kurtz *et al.*, 2002) and endokinin-1 (EK-1) in rabbit. In contrast to the mammalian tachykinins SP, NKA and NKB, which are identical among species, the human HK-1 peptide does not show complete homology with the mouse or rat orthologues (Page *et al.*, 2004). Furthermore, Page and colleagues (2003) have identified the human tachykinin gene-related peptides EKC and EKD that comprise the divergent C-terminal motif FQGLL-NH_2, expressed by the same gene as the human endokinins (EKA/B).

The hydrophobic C-terminal tachykinin signature motif FXGLM-NH_2 activates, through binding interactions, the three mammalian tachykinin receptors NK1 (cloned by Yokota *et al.*, 1989), NK2 (cloned by Masu *et al.*, 1987) and NK3 (cloned by Ingi *et al.*, 1991) that belong to the family of G protein-coupled receptors (GPCRs), while the divergent more hydrophilic N-terminal domain of each tachykinin determines receptor specificity (Pennefather *et al.*, 2004; Page *et al.*, 2005). Amino acid substitution studies have shown Phe^6 is a crucial amino acid for tachykinin-receptor binding as the aromatic ring plays an important role in establishing interactions,

namely the π*-π* orbital interactions, with other aromatic amino acids of the receptor (Warner *et al.*, 2002). Moreover, the C-terminal methionine was found crucial for tachykinin receptor activation (Ikeda *et al.*, 1999) while the C-terminal amidation is a key post-translational modification for the activation of the three receptors (Patacchini *et al.*, 1993; Oldham *et al.*, 1997). However, studies have shown the existence of a C-terminally truncated form of SP (1-7; RPKPQQF) which is also capable of producing a pharmacological response through binding to the NK1 receptor both *in vivo* and *in vitro* (Michael-Titus *et al.*, 2002).

Each receptor has a preferred ligand, NK1 for SP, NK2 for NKA and NK3 for NKB, however, there is cross-reactivity among the three tachykinins for each of the receptor types (Maggi, 1995). The order of potency of these receptors for each tachykinin is as follows: NK1, SP ≥NKA >NKB; NK2, NKA >NKB >SP and NK3, NKB >NKA >SP (Ebner *et al.*, 2009). Two different isoforms of the NK1 receptor have been described; one with a truncation of the C-terminus (Fong *et al.*, 1992; Baker *et al.*, 2003). Different structural foldings of the NK1 receptor are also proposed that result in up to three conformations the "classic", "septide-sensitive" and "new NK1-sensitive" receptor (Beaujouan *et al.*, 2004; Page, 2005). The "classic" NK1 receptor binds only SP, while the "septide-sensitive" subtype is activated by SP (and extended forms NPγ, NPK), septide (a C-terminal analogue of SP, the hexapeptide [pGlu6, Pro9] SP6-11), also truncated forms of SP *i.e.* SP6-11, SP5-11, SP3-11 and NKA, NKB (Beaujouan *et al.*, 2004). The "new NK1-sensitive" receptor subtype is activated by SP (and extended forms NPγ, NPK) and NKA (Beaujouan *et al.*, 2004; Page, 2005). The three well-studied tachykinins SP, NKA and NKB act as agonists on each of the three mammalian receptors NK1, NK2 and NK3, with different potencies, while the endokinins are also full peripheral agonists at the NK1 receptor when SP is not expressed (Page *et al.*, 2003; Pennefather *et al.*, 2004). On the other hand, the tachykinin gene-related peptides EKC and EKD remain orphan acting as weak agonists at the NK3 receptor (Page *et al.*, 2003, Bellucci *et al.*, 2004). The NK1, NK2, and NK3 receptors induce both elevation of intracellular Ca^{2+} and production of cAMP (Almeida *et al.*, 2005; Page, 2006).

Table 1.1. The sequence of *classical* tachykinins, tachykinin gene-related and tachykinin-like peptides.

[1]Nomenclature and source	[2]Primary sequence	Reference
SP Mammalian	RPKPQQF**F**GLMa	von Euler & Gaddum, 1931
NKA Mammalian	HKTDSF**V**GLMa	Kimura *et al.*, 1983
NPK Mammalian	DADSSIEKQVALLKALYGHGQISHKRHKTDSF**V**GLMa	Tatemoto *et al.*, 1985
NPγ Mammalian	DAGHGQISHKRHKTDSF**V**GLMa	Kage *et al.*, 1988
NKB Mammalian	DMHDFF**V**GLMa	Kangawa *et al.*, 1983
NKB (50-91) Mammalian	LYDSRSISLEGLLKVLSKASVGPKETSLPQKRDMHDFF**V**GLMa	Lang *et al.*, 1995
Mouse HK-1	RSRTRQF**Y**GLMa	Zhang *et al.*, 2000
Human HK-1	TGKASQF**F**GLMa	Page *et al.*, 2003
Human EKA	DGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKTGKASQF**F**GLMa	Page *et al.*, 2003
Human EKB	DGGEEQTLSTEAETWEGAGPSIQLQLQEVKTGKASQF**F**GLMa	Page *et al.*, 2003
Human HK-1 (4-11)	ASQF**F**GLMa	Kurtz *et al.*, 2002
Human EKC	KKAYQLEHTF**Q**GL**L**a	Page *et al.*, 2003
Human EKD	VGAYQLEHTF**Q**GL**L**a	Page *et al.*, 2003
Physalaemin (frog)	QADPNKF**Y**GLMa	Anastasi *et al.*, 1964
Eloidosin (octopus)	QPSKDAF**I**GLMa	Anastasi & Erspamer, 1962
Locustatachy-kinin I (Locust)	GPSGF**Y**G**V**Ra	Schoofs *et al.*, 1990a
Locustatachy-kinin II (Locust)	APLSGF**Y**G**V**Ra	Schoofs *et al.*, 1990a
Sialokinin (*Aedes Aegypti*)	NTGDKF**Y**GLMa	Champagne *et al.*, 1994

1. The name of the species-derived tachykinin peptide; 2. The primary sequence of the tachykinin peptide showing the amidation as the common post-translational modification of the signature motif; the variable amino acid comprised in the signature motif is highlighted in bold, showing the diversity of the tachykinin motif.

1.2) Tachykinin-related peptides and their receptors in invertebrates.

In invertebrates, the tachykinin-like peptides, (also termed tachykinin related peptides; TKRPs), comprising the conserved consensus signature motifs FX_1G/AX_2R-NH_2 are predominantly expressed (Satake *et al.*, 2003; Clynen *et al.*, 2009). The field of tachykinin-like peptides was introduced with the discovery of the locustatachykinins (Table 1.1) from the extracts of the locust, *Locusta migratoriu* (Schoofs *et al.*, 1990a; Schoofs *et al.*, 1990b). Since then, multiple TKRPs in species belonging to the anthropods (insects, crustaceans), molluscs, echiuroid worms, nematodes and octopus have been identified (reviewed in Van Loy *et al.*, 2010). The discovery in certain invertebrate species of the "invertebrate tachykinins" such as eloidosin, the tachykinin expressed in octopus *Eledone moschata*, and sialokinin (Table 1.1), the tachykinin in the salivary gland of the mosquito *Aedes Aegypti*, which hold the classic tachykinin motif FXGLM-NH_2 raises two hypotheses on the evolution of TKRPs, invertebrate tachykinins and mammalian tachykinins. The one hypothesis giving the most acceptable explanation on the evolution of tachykinins and TKRPs, suggests that the putative original TKRP gene possessed the "tachykinin-type" structural organisation comprising one or two peptide sequences. This *ancestral* TKRP sequence underwent species-specific multiplication followed by various divergences of the respective precursor sequences during the evolution of protostomes. The chordate tachykinin genes conserved the original organisation; however, the C-terminal Arg was replaced by Met, while Leu as the second residue from the C-terminus was conserved. The other hypothesis suggests the putative common ancestral gene giving rise to the TKRPs, invertebrate tachykinins and mammalian tachykinins possessed a "TKRP-type" structural organisation encoding multiple TKRP sequences. The characteristic of multiple sequences encoded in the TKRP precursors, was essentially conserved in the evolutionary process of protostome TKRP genes with minor species-specific alterations in the sequences and the copy numbers of peptides; whereas truncations of sequence copies occurred during the evolution of chordates (Satake *et al.*, 2013).

Similarly to the field of tachykinins the number of identified tachykinin-like receptors is lagging to the number of identified tachykinin-like peptides. Hitherto, only five invertebrate receptors are known, namely Ci-TK-R, expressed in the protochordate

Ciona intestinalis (Satake *et al.*, 2004), DTKR and NKD, cloned from the fruit-fly *Drosophila melanogaster* (Li *et al.*, 1991; Monnier *et al.*, 1992), STKR cloned from the fly *Stomoxys calcitrans* (Guerrero, 1997) and UTKR, cloned from the echiuroid worm *Urechis unitinctus* (Kawada *et al.*, 2002). UTKR, DTKR and NKD show similar homology as the mammalian tachykinin receptors NK1, NK2 and NK3 suggesting tachykinin receptors and tachykinin-like peptide receptors derive from a single common ancestor via gene duplication affected by-epigenetic processes (modifications of chromatin) in the evolutionary process (reviewed in Satake *et al.*, 2006; Van Loy *et al.*, 2010). The NK1, NK2, NK3, and Ci-TK-R receptors share high sequence homology and are identical in their gene structure of 5 exons and 4 introns; inferring the evolution of these tachykinin receptors from a common ancestral GPCR gene (reviewed in Satake, 2013).

1.3) Tachykinin genes.

The mammalian tachykinins are encoded on three genes, namely in humans *TAC1* (Nawa *et al.*, 1983), *TAC3* (Kotani *et al.*, 1986) (*Tac2* in rodents; Kako *et al.*, 1993) and *TAC4* (Page *et al.*, 2003) or *Tac4* in mouse and rat (Zhang *et al.*, 2000; Kurtz *et al.*, 2002). Alternative splicing of the *TAC1* gene gives rise to four α, β, γ and δ *TAC1* gene transcripts that can produce the tachykinins, SP, NKA and the extended forms of NKA, namely, NPK and NPγ (Page, 2005). The SP and NKA tachykinins were initially thought to be produced from two different tachykinin genes (Nawa *et al.*, 1983); however, the following years, it was reported that a distinct gene segment encodes NKA through the alternative splicing of the *Tac1* gene (Nawa *et al.*, 1984; Krause *et al.*, 1987; Carter & Krause, 1990). The human *TAC3* gene expresses the three α, β, γ, alternative transcripts (with three further variants v1, v2, v3 of each transcript found) (Page *et al.*, 2009). The α and β TAC3 transcripts both express NKB while the γ transcript does not contain any tachykinin (Page *et al.*, 2009). An expressed transcript that does not encode a tachykinin is potentially a mechanism of controlling NKB expression (Helke *et al.*, 1990) or an evolutionary throwback where an ancestral TAC3 gene precursor encoding two tachykinins, gave rise to two tachykinin gene homologues. The murine *Tac4* gene was identified in mouse pre-B cells encoding mHK-1 (Zhang *et al.*, 2000). This was followed by the characterisation of the orthologue human (*TAC4*), rat and rabbit *Tac4* cDNAs (Kurtz *et al.*, 2002;

Page, 2004). In contrast to the human *TAC4* gene, the murine *Tac4* gene gives rise to a single transcript (Kurtz *et al.,* 2002).The *TAC4* gene in humans generates four transcripts (α*TAC4* with two variants v1 and v2, β*TAC4*, γ*TAC4*, δ*TAC4*) that encode the tachykinins EKA and EKB, human HK-1 (a shortened form of EKA and B), and the tachykinin-gene related peptides EKC and EKD that have the alternative motif, FQGLL-NH_2 (Page, 2005).

1.4) Mechanism of tachykinin precursor processing.

Similarly to other prohormones that undergo proteolytic cleavage to release active peptide-hormones (Perone *et al.,* 1997), the expressed mammalian tachykinin gene transcripts are also translated into "precursors". Precursors encoding mammalian tachykinins are known as preprotachykinins (ranging from fewer than 100 to approximately 300 amino acid residues in length). Each precursor comprises a signal peptide sequence, with up to two encoded tachykinins or a tachykinin with a tachykinin gene-related peptide, where each putative peptide is flanked by either a monobasic or dibasic cleavage sites *i.e.* residues of lysine (K), or arginine (R) or dibasic KK or RR (Page *et al.,* 2006). The TKRP preprotachykinins comprise not only a signal peptide sequence, but also possess several copies of the same or different TKRPs separated by spacer arms and flanked by dibasic cleavage sites (Satake *et al.,* 2003). The "fate" of pre-processed precursors after their synthesis is subjected to the following three events; transport to subcellular localisation sites, processing (or degradation) and post-translational modification (Nakai, 2001). The signal peptide guides the newly synthesised precursor to the endoplasmic reticulum, where it is rapidly cleaved off forming the pro-tachykinin (Nelson & Bost, 2004). The pro-tachykinin is packed into secretory granules in the Golgi apparatus followed by transport and exocytotic release (Hokfelt *et al.,* 2000; Pennefather *et al.,* 2004). Inside the granules the tachykinins are released through the proteolytic cleavage of the pro-tachykinin by enzymes known as pro-hormone convertases (otherwise termed as proprotein convertases). These enzymes recognise dibasic or monobasic (or potentially multibasic) K, R, KK, KR, RK, RR cleavage sites (Cain *et al.,* 2002). Prohormone convertases are a family of seven endoproteases, which include furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7 (Seidah & Prat, 2002). Each convertase is initially synthesized as an inactive zymogen that undergoes a two-stage autocatalytic

processing event to yield an active convertase (Seidah *et al.*, 2008). Prohormone convertases recognise the consensus motif (K/R)-(X)*n*-(K/R)↓ (the site of processing is indicated by ↓), where *n* is 0, 2, 4 or 6 and X is any residue and cleavage of the precursor takes place at the C-terminal side of the K/R cleavage site yielding the processed peptide(s) (Rholam & Fahy, 2009). Nevertheless, i*n vivo* cleavage occurs preferentially at KR and RR cleavage sites and weakly at KK or RK cleavage sites (Seidah & Prat, 2002). The TAC1 preprotachykinin precursor comprises the tachykinins SP and NKA, flanked by dibasic processing sites that are fully processed for the release of these peptides or remain unprocessed at the N-terminus to release the extended forms NPK and NPγ (Page, 2005). The TAC3 preprotachykinin precursor comprises the tachykinin NKB, identical in most mammalian species, flanked by dibasic KR cleavage sites, where fully processing releases the NKB decapeptide while partial processing at the N-terminus releases the extended form of NKB (Page, 2005). The TAC3 preprotachykinin precursor also comprises dibasic cleavage sites at its N-terminus where processing releases the N-terminal part of the TAC3 precursor (residues 50-79; Lang & Sperk, 1995). The mouse TAC4 preprotachykinin precursor comprises mHK-1, which is flanked by dibasic cleavage sites releasing the peptide by full processing of the precursor (Zhang *et al.*, 2000). The human TAC4 preprotachykinin precursor was found to comprise the hHK-1 undecapeptide (Table 1.1). The hHK-1 was found not to be identical with the mouse, and rat homologues. Moreover, the tachykinin is flanked by a dibasic cleavage site at its C-terminus capable of releasing the amidated hHK-1 undecapeptide, however at its N-terminus a single base substitution was observed that changes the R residue to T, hence destroying the dibasic cleavage site at the N-terminus (Page *et al.*, 2003). The human TAC4 preprotachykinin precursor also lacks additional dibasic residues on its N-terminus; hence cleavage at the signal peptide cleavage site would predict extended forms of hHK-1, namely EKA/B comprising 47 or 41 residues (Page *et al.*, 2003).

1.5) Tissue-specific prepropeptide precursor processing.

The cleavage sites of a precursor protachykinin are not always fully processed *in vivo*, thus tissue-specific processed "mature" or partially processed "extended" forms of the tachykinins are released; this alternative processing of tachykinins has also been implicated in pathological conditions *e.g.* pre-eclampsia (Page, 2006). The placenta is

the archetype for differential processing of precursor peptides. It has been reported for many precursors, *e.g.* proopiomelanocortin (POMC) that a processing mechanism exists, operating in a tissue-specific manner, releasing alternative peptides (Raffin-Sanson *et al.,* 2003). The processing of POMC in the placenta is unique, leading to the release of significant amounts of both unprocessed and partially processed precursors, as well as the peptides adrenocorticotrophin, β-lipotrophin, α-melanocyte-stimulating hormone and β-endorphin. Furthermore, the POMC derived from the placenta is post-translationally modified with phosphocholine, implicated in immunomodulation (reviewed in Bicknell, 2008). A previous report provides evidence of increased precursor processing of the corticotrophin releasing hormone (CRH) in the human placenta in pathological conditions such as pre-eclampsia. Most CRH in the human normotensive placenta exists in either or three forms *i.e.* as an unprocessed pro-CRH, or partially processed pro-CRH, or traces of the fully processed form of the CRH_{1-41}. However, in the pathological condition of pre-eclampsia the fully processed form of CRH_{1-41} (a form predominantly found in the hypothalamus), is present (Ahmed *et al.,* 2000).

1.6) Post-translational modifications of tachykinins.

The preprotachykinin precursors are cleaved by signal peptidases, which remove the signal peptide and release the protachykinin (Page, 2006). The protachykinin is processed by the hormone convertases to release the tachykinin peptides. The released peptides are further post-translationally modified with enzymes called carboxypeptidases that remove the dibasic residues from the carboxyl terminal of the released peptide. Next, the tachykinin acquires its bio-activity through the effect of the only known mammalian glycine converting enzyme, peptidylglycine α-amidating monooxygenase (PAM) (Suzuki *et al.,* 1990), which converts the exposed glycine into an amide catalysing the breakdown of the Cα-NH_2 bond (Abou-Mohamed *et al.,* 2000; Ozawa *et al.,* 2007;). The C-terminal amidation is a post translational modification consistently occurring in many peptides *e.g.* oxytocin/vasopressin (Yin *et al.,* 2011) including the tachykinin peptides (Figure 1.1.) (Page, 2004). Cleaved peptides may be further post-translationally modified in a profoundly tissue specific manner (Hokfelt *et al.,* 2000). An important post-translational enzymatic modification of the processed peptides found to occur specifically in neuropeptides is acetylation,

e.g. the acetylation of endorphins (Wilkinson, 2006). Lovell and colleagues (2007) have reported that apart from the C-terminal amidation, another possible post translational modification on NKB secreted in the placenta is the addition of phosphocholine on the fourth aspartyl residue of the peptide. This modification suggests a possible role for NKB in modulating the immune system of the mother for the prevention of placental rejection (Lowry, 2008).

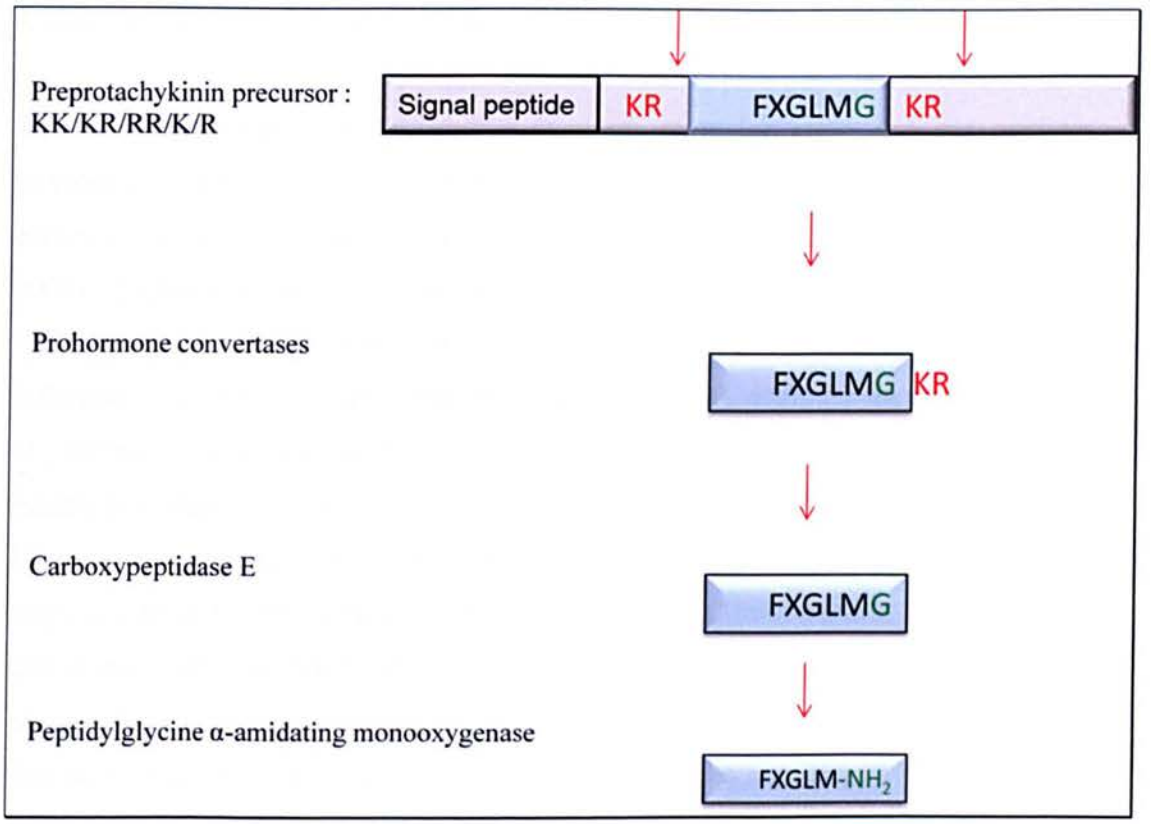


Figure 1.1. The schematic diagram of the synthesis of a tachykinin from a TAC precursor. Processing of the preprotachykinin precursor occurs on the C-terminal of the K/R cleavage sites (indicated with an arrow) by the enzymes prohormone convertases which release the extended or truncated forms of the tachykinin peptide. Carboxypeptidases then act to remove the C-terminal K/R residues bound to the peptide. The peptidylglycine α-amidating monooxygenase acts to convert the remaining C-terminal glycine (G) residue into an amide.

1.7) Biological role of the tachykinins.

Tachykinins have multiple physiological roles, as major brain and gut peptides (reviewed in Severini *et al.,* 2002), as endocrine or paracrine hormones in the chordates (Page, 2006) and also as exocrine factors in amphibians (Liu & Burcher, 2005). Tachykinins are involved in pain (Naono, 2008; Naono-Nakayama *et al.,* 2010) smooth muscle contraction (Mechiche *et al.,* 2011; Kong *et al.,* 2009), inflammation (Metwali *et al.,* 2004; Makino *et al.,* 2012), neurodegeneration (Chen *et al.,* 2008) also in neuroprotection (Chu *et al.,* 2011). The central nervous system is an established major source of SP and NKA, while NKB is expressed mainly in the hypothalamus and the dorsal horn of the spinal cord, a distribution attributing an original role as neurotransmitters for the three peptides (Marksteiner *et al.,* 1992). The tachykinins SP and NKA play a significant role in nociception, as shown in homozygous mutant knock-out mice for *Tac1* (Cao *et al.,* 1998). The response of double knock-out mice to moderate to intense pain was significantly reduced; also neurogenic inflammation, which results from peripheral release of SP and NKA, was almost absent in the mutant mice. Hence, the release of tachykinins from the primary afferent pain-sensing receptors (nociceptors) was required to produce moderate to intense pain (Cao *et al.,* 1998). The role of mHK-1 in pain and nociception has been investigated in mice intrathecally administering mHK-1 and comparing the effects with that of SP. Intrathecal administration of mHK-1 as well as SP caused pain-related behaviour such as scratching (Endo *et al.,* 2006). Similarly, another investigation reported effects of mHK-1 in pain modulation at supraspinal level in mice using the tail-flick test. Intra-cerebroventricular administration of the peptide dose-dependently induced potent analgesic effect; when co-injected with a selective NK1receptor antagonist, the analgesic effects of mHK-1 were fully antagonised (Fu *et al.,* 2005, 2006, 2007a, Watanabe *et al.,* 2010).

Extensive research has been performed on the effects of mHK-1 in immunological regulation and the development of B-cells and T-lymphocytes (Zhang *et al.,* 2000; Zhang & Paige, 2003). Mouse HK-1 was originally reported as an autocrine factor regulating the lymphopoiesis of B cells and potentially confined only in hematopoietic cells (Zhang *et al.,* 2000). Later studies suggested a similar role of mHK-1 in the development of T cells as well (Zhang & Paige, 2003). The peptides

SP and mHK-1 also play a role in neuro/immuno-modulatory activity; the mHK-1 and SP tachykinins were found to stimulate Ca^{2+} flux in the human astrocytoma cell line U-251 MG, expressing the NK1 receptor and showed no difference in their pharmacological responses (Berger & Paige, 2005). Moreover, the research on the biological role of the mHK-1 was extended to the cardiovascular system (Bellucci *et al.*, 2002; Long *et al.*, 2007). An attempt was made to investigate the effect and mechanism of action of mHK-1 on systemic arterial pressure after intravenous injections in anesthetised rats and compare it with the effects of SP. The injection of mHK-1 lowered systemic arterial pressure dose-dependently; this effect was significantly blocked by pre-treatment with SR140333 (a selective tachykinin NK1 receptor antagonist). The results suggest that the mechanism of the depressor response caused by SP was similar to mHK-1 (Fu *et al.*, 2007b). In the above studies, mHK-1 was chemically synthesised by a solid phase peptide synthesiser as it was predicted by Zhang and colleagues (2000) to be an amidated undecapeptide (Table 1.1). However, no hard evidence presently exists on the sequence of the naturally occurring peptide.

1.8) The role of tachykinins in the physiology and pathophysiology of reproduction.

Page and colleagues (2002) have reviewed that in normal pregnancies placental NKB secretion could be responsible for haemodynamic adaptations occurring during gestation such as contraction of the hepatic portal vein and venoconstriction of mesenteric beds as well as increased heart rate and also vasodilation of the placental blood vessels. Tachykinins play a significant role in the physiology and pathophysiology of mammalian reproduction and pregnancy (reviewed by Candenas *et al.*, 2005). All mammalian tachykinin peptides, their precursor genes, and tachykinin-metabolising enzymes (*e.g.* neprilysin) are expressed widely in the mammalian female genital tract, suggesting an important role for tachykinins in intercellular communication in reproduction (reviewed by Candenas *et al.*, 2005). The *TAC3* and *TAC4* (and *Tac4*) are expressed in the human (and mouse) uterus and myometrium and their expressed peptides (NKB, hHK-1 and mHK-1) have a significant stimulatory effect in the myometrium of pregnant and non-pregnant women and female mice (Patak *et al.*, 2003). Pennefather and colleagues (2006) have shown that the NK2 receptor is the main tachykinin receptor involved in contractions of the human uteri in response to NKA and suggest that the tachykinins NKA and

hHK-1 might have a role in pre-term labour. The tachykinin mHK-1 has been suggested to be involved in the regulation of female reproductive function (Pintado *et al.*, 2003). *Tac4* gene expression has been detected in cells from mouse uteri and embryos (Pintado *et al.*, 2003). Here and in blactocysts, the mHK-1 peptide may be involved in the implantation stage as the *Tac4* gene is up-regulated in blastocyst stage embryos, when the embryo comes into direct contact with the uterus (Pintado *et al.*, 2003). Further studies have supported the expression of the *Tac4* and *Tac3* genes in the mouse uterus and have involved the observation of uterotonic effects during the oestrus cycle and pregnancy following mHK-1 and NKB peptide treatment (Patak *et al.*, 2005; 2008). In terms of male reproduction Ravina and colleagues (2007) were the first to show the presence of the three tachykinin receptors NK1, NK2 and NK3 on human sperm and have suggested that the tachykinins SP, NKA, NKB and hHK-1 play a role in sperm motility in a dose-dependent manner.

Arck and colleagues (1995) have investigated the role of SP in stress-induced abortions in mice and suggest that SP increases the rate of abortion either by directly inducing smooth muscle contraction of the myometrium and vasodilation of the mouse uteri, or acting on trophoblast cells. NKB secreted by hypothalamic nuclei and its preferred receptor NK3 have been found to be essential regulators of the reproductive axis through the pulse-release of gonadotropin release hormone (Topaloglu *et al.*, 2009). Therefore, loss-of-function mutations on the *TAC3* and *TACR3* genes were found in individuals affected by familial hypogonadotrophic hypogonadism, a condition characterised by the failure of sexual maturation, impaired gametogenesis, and infertility (Topaloglu *et al.*, 2009). The effects of NKB in the regulation of the reproductive axis are also confirmed by the expression of *TAC3* and *TACR3*, kisspeptin (*KISS1*; a known peptide hormone) and its receptor (*KISS1R*) in the human uterus, ovary, and oviduct. Hence, these hypothalamic peptides have a potential modulatory role in the control of gonadotrophin-releasing hormones at peripheral reproductive tissues (Cejuno-Roman *et al.*, 2012).

1.9) Tachykinins, pre-eclampsia and intra-uterine growth restriction.

Pre-eclampsia is a pathologic condition that affects a high percentage (3-10%) of pregnancies (Page & Lowry, 2000; Gilbert *et al.*, 2008; Poon *et al.* 2009) and a major cause of maternal morbidity and mortality (Shankar *et al.*, 2004). Pre-eclampsia is a two-stage disease (reviewed in Page, 2002; Hladunewich *et al.*, 2007). The first stage is asymptomatic and characterised by the abnormal development of the placenta resulting in placental ischemia and hypoxia during the first trimester of the pregnancy. This leads to excessive amounts of placental material being released into the maternal circulation. The second stage is characterised by symptoms such as hypertension, renal impairment, proteinuria (glomerular endotheliosis) and potentially the HELLP syndrome (haemolysis, elevated liver function enzymes and low platelets) as well as eclampsia (convulsions) and other end-organ damage symptoms occurring after the 20^{th} week of pregnancy (Page & Lowry, 2000; Hladunewich *et al.*, 2007). Symptoms of severe pre-eclampsia have been recorded even in the case of a molar pregnancy, as the case where no viable foetus is formed, but merely disordered placental tissue (hydatidiform mole) has developed in the uterus (Page & Lowry, 2000; Hladunewich *et al.*, 2007). The removal of the placenta terminates the symptoms of pre-eclampsia and the only method for the eradication of the symptoms is early delivery. Page and colleagues (2002) have reported that placental peptides can be used as markers of gestational disease. Since the manifestation of the symptoms of pre-eclampsia and IUGR occurs when the disorder is well advanced, the identification of markers of disease onset is of great value for the early prevention of the condition (Shankar *et al.*, 2004).

Pre-eclampsia is one of the conditions created as a result of insufficient implantation of the trophoblast, where the myometrial portions of the spiral arteries of the uterus fail to invade the tissue (Page *et al.*, 2000b; Page & Lowry, 2000). Inter-uterine growth restriction (IUGR) is another condition resulting from incomplete placentation where restricted development of the foetus occurs due to insufficient flow of oxygen and nutrients to it (Shankar *et al.*, 2004). Many factors contribute to the development of IUGR (including life style habits of the mother) however, the condition can be also associated with pre-eclampsia (Shankar *et al.*, 2004). Page and colleagues (2000a, b) have shown that the tachykinin peptide NKB could be an agent causing the pre-

eclamptic symptoms. HPLC and radioimmunoassay analysis of placental extracts as well as the plasma of pre-eclamptic women revealed that NKB peptide levels were elevated (Page *et al.*, 2000a). Later, it was shown that the expression of *TAC3* was also significantly higher in pre-eclamptic term placenta than in normal placenta (Page *et al.*, 2006b). Geissbuehler and colleagues (2007) confirmed these observations by determining elevated amounts of NKB in the plasma of pre-eclamptic women through an enzyme-linked immunoassay (ELISA) and radio-immunoassay. In addition, elevated plasma concentrations of NKB have been detected in women in cases of IUGR (D'Anna *et al.*, 2004). Torricelli and colleagues (2007) have reported NKB expression to be higher in placenta collected after pre-term labour than in those collected after term labour and significantly higher than the NKB expression found in placenta collected after Caesarean section. This observation suggests an involvement of NKB in the mechanisms of parturition.

1.10) Processing of preprotachykinins in pre-eclampsia.

In vivo, not all putative cleavage sites of a precursor preprotachykinin may be processed, thus the partially processed *i.e.* the "extended" forms of the products of the TAC3 and TAC4 precursors are potentially expressed in the placenta in normotensive pregnancies compared to diseased cases such as pre-eclampsia or the brain where the fully processed forms of NKB and hHK-1 are potentially expressed (Page, 2006). Moreover, in contrast to NKB, which is flanked by dibasic KR cleavage sites, hHK-1 (rather than mHK-1) has evolved to possess a monobasic cleavage (K) site immediately upstream, inferring differential processing between the two precursors (Page *et al.*, 2003). Initially, Page and colleagues (2000) reported the cloning of the *TAC3* gene from human placenta, whose expression was confined predominantly to the outer syncytiotrophoblast cells of the tissue and reported higher expression of the *TAC3* gene in the placenta compared to other tissues. Moreover, they showed that significant concentrations of the NKB peptide was measurable in the plasma of women with pregnancy-induced hypertension and pre-eclampsia, indicating that the elevated levels of NKB in early pregnancy could be a potential indicator of pre-eclampsia. The fully processed variant of NKB *i.e.* the amidated decapeptide was detected in the pre-eclamptic placenta (Page *et al.*, 2001). In contrast in the normotensive placenta NKB-like immunoreactivity of a larger, in terms of MW,

peptide was measured potentially corresponding to an N-terminally extended form of NKB, inferring partial processing in spite of the dibasic KR cleavage site (Page *et al.*, 2009). Later observations reported that the TAC4 precursor is expressed in the placenta as well (Page *et al.*, 2003). Similarly to the NKB processing significant EKB-like immunoreactivity corresponding to the fully N-terminally processed amidated hHK-1 undecapeptide was found in the pre-eclamptic placenta in contrast to the normotensive placenta where EKB-like immunoreactivity was measured as a larger peptide potentially an N-terminally extended, or a partially processed form of EKB (Page, 2006). In the above studies, the processing variants of the TAC3 and TAC4 precursors was determined by means of separation by size-exclusion chromatography and immunoassays, while no reports exist on the definitive identification of the MW or sequencing of the peptides by mass spectrometry. The potential biological significance of the alternative processing is the increased release of active NKB and hHK-1 during the diseased state of pre-eclampsia perhaps in order to increase vasodilatory stimulation of the poorly perfused pre-eclamptic placenta and increase of the blood flow to the fetus (Page, 2006).

1.11) Identification of tachykinin peptides using analytical chromatography in combination with bio-assays.

The three well-studied tachykinins SP, NKA and NKB were originally isolated and sequenced using various biochemical techniques and bio-assays. The initial extraction of tachykinin peptides was performed using alcoholic extracts from equine brain and intestine, mincing the fresh tissue in a volume of alcohol five times its weight. The concentrated semi-purified extracts caused contractions of the longitudinal muscle of the rabbit's isolated intestine and a hypotensive action when intravenously administered in anaesthetised rabbits (von Euler & Gaddum, 1931). These effects were attributed to an agent distinct from the then known gut-stimulating compounds, named as SP (von Euler & Gaddum, 1931). In a following study by Chang & Leeman (1970), bovine hypothalami were homogenised in acidified acetone. More advanced analytical methods were applied for the separation of SP *i.e.* gel filtration in combination with cation-exchange chromatography and high voltage paper electrophoresis providing a sample of the peptide of higher purity (Chang *et al.*, 1971). Moreover, the following year the sequence of the peptide was elucidated using

Edman degradation, identifying SP as an amidated undecapeptide (Chang *et al.*, 1971). Later, Kimura and colleagues (1983) purified the tachykinins NKA and NKB from pig spinal cord, homogenising the tissue in acetified acetone, followed by centrifugation of the homogenised tissue in buffer and separation of the peptidome using gel filtration. Fractions were collected and then assayed for bioactivity on guinea-pig ileum. The bioactive fractions were further concentrated using reversed phase-high pressure liquid chromatography (RP-HPLC) on a C8 column eluted with a gradient of acetonitrile with trifluoroacetic acid (TFA) and fractions were again collected and assayed for bio-activity on guinea pig ileum. The bio-active fractions were re-chromatographed and the purified peptides NKA and NKB, collected in separate fractions, were sequenced with Edman degradation. Kangawa and colleagues (1983) also isolated and sequenced NKB extracting the peptidome of the pig spinal cord and purifying the peptide using size exclusion chromatography. After separation according to molecular weight, the fractions containing a substance of the MW range 800-2000 Da, were found able to cause fast contractions on guinea-pig ileum and rat uterus. These fractions were re-chromatographed using RP-HPLC with a linear gradient of acetonitrile with TFA and fractions were collected. The bio-active fractions were sequenced, using micro-sequencing, revealing NKB as an amidated decapeptide (Table 1.1) comprising the signature tachykinin motif, while a role as a neurotransmitter was attributed to the newly-identified peptide.

The identification of tachykinins using biochemical methods and assays was continued by the studies of Tatemoto and colleagues (1985) who purified NPK (the 36 residue extended form of NKA) from porcine brain using size exclusion chromatography and successive rounds of separation by means of RP-HPLC until a pure (a sharp chromatographic peak) preparation was obtained capable of contracting guinea-pig gall bladder *in vitro*. The purified NPK was sequenced using Edman degradation as an amidated peptide comprising 36 residues (Table 1.1). Kage and colleagues (1988) purified NKA and NPγ (the 21 residue extended form of NKA) from rabbit intestine using RP-HPLC. In contrast to previous studies using tissues in bio-assays for the identification of active compounds, in this particular study, the usage of antibodies raised against the C-terminal sequence of NKA for the identification of the peptide was exploited. Hence, the chromatographic peaks corresponding to the purified extracts were identified by their immunoreactivity for

antiserum raised against the C-terminal sequence of NKA. The two purified peptides were sequenced using Edman degradation (Table 1.1.). Lang and colleagues (1995) purified from rat brain the peptide preprotachykinin B (50-79) *i.e.* a non-tachykinin peptide encoded on the *Tac2* gene located immediately upstream from NKB and flanked by dibasic RR and KR cleavage sites. Brain regions were individually dissected and homogenised in acetic acid or perchloric acid; the mixture of homogenised brain in buffer was centrifuged and the peptide was purified by means of RP-HPLC while immunoreactivity was detected in the purified fractions using an iodinated antibody raised against the preprotachykinin B (50-79) peptide that did not cross-react with NKB, SP or NKA. The highest concentration of the preprotachykinin B (50-79) and also NKB was found in the hypothalamus and the interpenducular nucleus.

1.12) Identification of tachykinin peptides using molecular cloning methodologies and in silico analysis of bioinformatic databases.

The identification of the hemokinins/endokinins was achieved with molecular cloning techniques (Zhang *et al.*, 2000; Page *et al.*, 2003). The tachykinin mHK-1 was identified by cloning of the mouse *Tac4* gene, isolated from haematopoietic cells. The sequence of the mHK-1 peptide was inferred from the sequence of the cDNA, although the translated peptide has not yet been purified from mouse tissues (Zhang *et al.*, 2000). Page and colleagues (2003) aligned the newly-published mouse *Tac4* gene sequence on the human genome database, and revealed the human *TAC4* gene homologue. Then, the human *TAC4* gene encoding the hemokinins namely hHK-1, EKA/B, EKC/D was cloned from human tissues and its existence verified. The bioinformatics era commenced (in 1988) with the development of information systems *e.g.* the nucleotide and protein databases of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov), generated and structured to be searched for the identification of gene homologues (Wheeler *et al.*, 2006). Moreover, multiple tools such as the BLAST algorithm were generated to effectively perform homologous motif and gene identification searches (Altschul *et al.*, 1990). The blastn and tblastn algorithms were generated to query sources *e.g.* the nucleotide collection (nr/nt) or the expressed sequenced tags (ESTs) databases and blastp to query the non-redundant protein sequences (nr) database. Hence, preprotachykinin precursors have

been identified inferring the existence of more bio-active peptides, by aligning known precursor sequences on the above databases (Christie *et al.*, 2008). The construction of commonly used and publicly accessible protein databases such as X! Tandem (Craig *et al.*, 2004), Mascot (Perkins *et al.*, 1999) and SwePep (Falth *et al.*, 2006) allows the rapid identification of protein or peptide sequences or post-translational modifications from mass spectrometry data. The above mentioned tools use the existing knowledge on prepropeptide processing by endogenous peptidases, or protein processing by *e.g.* trypsin, to predict possible peptide sequences. Identification of protein and peptides is performed matching the measured molecular masses from mass spectrometry data, against the theoretical masses in the sequence collection. Nowadays, mass spectrometry and bioinformatic algorithms (peptide mass fingerprinting) have become essential tools for the identification of many endogenous peptides (Menschaert *et al.*, 2010).

1.13) Peptidomic analysis by HPLC and mass spectrometry.

The terms peptidome and peptidomics referred to the heterogeneous entity of small proteins, peptide hormones, neuropeptides or transient fragments of protein degradation expressed in a cell or tissue (Schulz-Knappe *et al.*, 2001; Verhaert *et al.*, 2001). Peptides having different physicochemical properties from large proteins, such as size and motility, render the classical well-established methods used in proteomics, such as SDS-PAGE, unsuitable and inapplicable in the field of peptidomics for separation and detection (Schrader *et al.*, 2001). Moreover, the high complexity of the cellular peptidome and the low abundance of many of the peptides necessitate highly sensitive analytical techniques (Aebersold & Mann, 2003). Separation of complex peptide extracts by RP-HPLC and detection by mass spectrometry have been established as high efficiency methods of identifying novel peptides or tissue-specific post-translational modifications (Svensson *et al.*, 2007; Hummon *et al.*, 2006). These two methods along with applicable detection systems in peptidomics fulfil the analysis criteria; high sensitivity (reaching particularly low detection limits; parts per billion) permitting small sample analyses (*e.g.* milligram quantities of tissue), high mass resolution (the ability to differentiate between similar molecular weights), mass accuracy (as the extent to which a mass analyser reflects the "true" mass-to-charge ratio (m/z) values, measured in atomic mass units; a.m.u.) and the ability of further

analysing and identifying peptides *e.g.* by means of sequencing (Steen & Mann, 2004; Svensson *et al.,* 2007). Dissociation of the identified peptides with collisions with an inert gas (as applied during collision-induced dissociation; CID) offers the sequencing of the peptides for identification or for determination of lower detection limits (Qin & Yuan, 2004; Lange *et al.,* 2008).

The development of chromatography in the past 50 years has established the modern HPLC as an essential tool for the purification and separation of peptides and proteins (Snyder, 2000). A reversed phase HPLC system consists of a stationary phase (*e.g.* a hydrophobic C18 column), where the analyte (*e.g.* peptides) absorbs, according to hydrophobicity (Snyder, 2000). Then a mobile phase *i.e.* an organic modifier elutes the adsorbed peptides from the reversed phase column (Vissers *et al.,* 1997). Each peptide elutes at a unique retention time and is detected (commonly used detectors, U.V., fluorescence, electrochemical detector; Vissers *et al.,* 1997). The peptide bond absorbs strongly in the far ultraviolet (U.V.) region of the spectrum, hence, this detection method is the most commonly used for the detection of peptides and proteins in conjunction with HPLC (Vissers *et al.,* 1997). Reversed phase liquid chromatography (LC) is suitable for coupling to ESI (electrospray ionisation) and mass spectrometry for use in peptidomics/proteomics (Gygi & Aebersold, 2000; Whitehouse *et al.,* 1985). Mass spectrometry (MS) has undergone many instrumental improvements in the past decades, such as in the efficiency of the ionisation source, the mass analyser and mass identification software (reviewed in Hardouin, 2007). A mass spectrometer consists of an ion source that nebulises and ionises the analyte, a mass analyser that measures the m/z of the ionised analytes and a detector that registers the number of ions at each m/z value (Aebersold & Mann, 2003). Electrospray ionisation (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two most typically used techniques applied to volatise and ionise peptides for mass spectrometry analysis (Aebersold & Mann, 2003; Lim & Elenitoba-Johnson, 2004). MALDI as a gentle ionisation method proposed in the late 1980s (Karas & Hillenkamp, 1988) has become increasingly applicable for the study of biological molecules (Aebersold & Goodlett, 2001). Desorption/ionisation as a technique transforms bio-molecules into vapour-phase ions, producing almost exclusively intact protonated species (Karas & Hillenkamp, 1988). An energy-absorbing matrix is uniformly mixed with the sample, mediating both desorption and

ionisation processes by pulsed-laser radiation, forming generally singly-charged ions in the positive ion mode (Li *et al.,* 2000). MALDI ionisation sources are typically coupled with a time-of-flight (TOF) analyser; the advantages of a MALDI-TOF configuration are the large mass range, the high sensitivity and the tolerance to Na^+ and K^+ adducts (Hardouin *et al.,* 2006). MALDI-TOF because of its high-sensitivity offering the ability to detect low concentrations of peptides, the soft ionisation which prevents decomposition of the bio-molecules, and easily interpretable spectra, has also been used in conjunction with immunoaffinity purification for the capture of SP from rat brain peptide extracts with the purpose of estimating the concentration levels of the peptide (Suresh Babu *et al.,* 2004).

1.14) Aims and strategy of the project.

Tachykinins as signalling peptides are initially expressed as a part of a preprotachykinin precursor that undergoes cleavage by signal peptidases to release the protachykinin, which comprises the active tachykinin peptides. Alternative proteolytic cleavage of the protachykinin by specific prohormone convertases releases alternative forms of the tachykinin peptide-hormones, which then acquire bioactivity through the action of enzymes such as carboxypeptidases and peptidylglycine α-amidating monooxygenase (Page, 2005). The tachykinin precursors comprise a signal peptide sequence, with up to two encoded tachykinins or a tachykinin with a tachykinin gene-related peptide, where each putative peptide is flanked by a monobasic or dibasic cleavage site *i.e.* residues of lysine (K), or arginine (R) or dibasic KK or RR cleavage sites (Page *et al.,* 2006). Enzymatic cleavage occurs with the enzymes prohormone convertases, releasing the elongated or normal (or potentially truncated) forms of these peptides. In the presence of a proline adjacent to a dibasic cleavage site (as is found at the N-terminus of the SP sequence) there is resistance to enzymatic cleavage by proprotein convertases at the C-terminal of the RR, which causes cleavage between the two RR residues (Page *et al.,* 2005). The mHK-1 was identified from the cloning of the mouse *Tac4* gene, isolated from B-lymphocyte (Zhang *et al.,* 2000). The sequence of the mHK-1 peptide was inferred from the sequence of the cDNA (as an amidated undecapeptide, Table 1.1), while the translated peptide has not been purified from mouse/rat tissues (Kurtz *et al.,* 2002). We suggest cleavage of the TAC4 precursor occurs at the C-terminal of the KR dibasic cleavage sites flanking mHK-1

releasing an amidated decapeptide (SRTRQFYGLM-NH_2, sequence shown in Table 2.4).

Accordingly, the aims of this study were:

1) To capture the peptides mHK-1, SP, hHK-1 and NKB from tissues with potential expression in order to investigate the presence of fully processed forms, or the partial processed forms of these peptides and potential post-translational modifications.

2) To use specific antibodies raised against tachykinins for the immunoaffinity purification of tachykinins.

3) To perform reversed phase HPLC in order to separate the captured peptides.

4) To detect immunoaffinity captured peptides using MALDI-TOF.

5) To identify by *in silico* analysis of the NCBI and Ensembl databases novel tachykinin peptides and to elucidate the phylogenetic relationship between the identified species. Particularly of interest is to elucidate why in humans and primates or in mammalian species where pre-eclampsia occurs there may be differential processing at the N-terminal dibasic cleavage site of NKB and EKA/B (where in the later the dibasic cleavage site has been lost).

Chapter 2. The development of an affinity chromatography purification system to capture tachykinin peptides.

2.1) A brief introduction to affinity chromatography and immunoassays.

Cuatrecasas and colleagues (1968) introduced the term "affinity chromatography" to first describe an efficient procedure for purifying high yields of enzymes. This procedure was performed using the strong reversible association of the enzymes for their inhibitors that were immobilised on cyanogen bromide-activated Sepharose. To date, affinity purification (or immunoaffinity purification) still employs the immobilisation of a ligand onto a solid support matrix (*e.g.* cyanogen bromide-activated Sepharose) to purify antibodies from complex mixtures *e.g.* antiserum. It can also be used for removing proteases and other unknown contaminating agents from complex mixtures. The procedure offers the advantage of producing an enriched antibody population of known specificity (Ayyar *et al.*, 2012; Lee & Lee, 2003). The principal stages of immunoaffinity purification are the immobilisation of corresponding ligand molecules onto specific matrices that are packed into chromatographic columns. This is followed by the loading of analyte mixtures (*e.g.* antiserum) onto matrix-ligand complexes, the washing off of weakly bound analyte molecules, and the elution of high affinity analyte molecules and finally the regeneration of the column (Roque *et al.*, 2007; Huse *et al.*, 2002; Yarmush *et al.*, 1992). Elution of the captured analyte (*e.g.* antibody) is a reversible procedure usually achieved by disrupting the interactions between antibody-ligand with a buffer of low pH or high salt concentration (Firer, 2001). Inversely, immobilisation of antibodies onto agarose matrices offers an affinity medium capable of enriching the antigen from biological samples (Fang & Zhang, 2008; Muronetz & Korpela, 2003). For example, the enzyme placental leucine aminopeptidase was isolated from human placentae using antibodies raised against the protein and then immobilised on an agarose resin (Nakanishi *et al.*, 2000).

Immunoassays are defined as analytical methods employing antibodies for the detection of sample components based on the specificity of the antibodies for its antigen (Lipman *et al.*, 2005). The specificity of antibodies developed against an

antigen, the functionality of the immunopurified antibodies or proteins and their relative concentration can be shown with the use of immunoassays. Jin and colleagues (2009) raised mouse monoclonal antibodies against the mHK-1 peptide with the functionality of the antibodies proven by enzyme-linked immunosorbent assay (ELISA). Different types of immunoassay have been developed according to their detection label. A common immunoassay technique is ELISA, which makes use of an antibody linked to an enzyme for high-sensitivity detection (Hage, 1999). Peptides and antibodies require covalent immobilisation via reactive amines ($-NH_2$) with a spacer arm for attachment onto chemically-activated microtitre surfaces (*e.g.* Immobilizer™ Amino). This reaction enhances the orientation of peptides to prevent steric hindrance *i.e.* the prevention of an efficient interaction with detection by the antibody because of spatial structure (Esser, 1988a). Antibodies being large molecular weight molecules can be immobilised on microtitre wells through non-covalent interactions *e.g.* passive absorption, where multiple weak molecular interactions form a stable bond between the molecule and the surface (Esser, 1988b). Non-specific or weakly bound reactants are washed off. Typically during this step a detergent is included to eliminate steric hindrance caused by reactant accumulation on the immobilisation surface and for blocking excess surface after coating with analyte or the primary antibody. Detergents being molecules consisting of distinct hydrophobic and hydrophilic parts (*e.g.* Tween, Triton) disperse hydrophobic molecules in buffer. Their blocking effect is based on the ability to compete with other molecules for both hydrophobic and hydrophilic binding sites (Esser, 1988a). The enzyme-labelled antibodies that are used in ELISA assays are detector molecules, which provide physicochemical properties that can be directly measured. For example, an alkaline phosphatase-labelled secondary antibody that recognises the primary antibody in its Fc fragment according to the species it was raised in (*e.g.* anti-sheep antibody) can act as an amplifier increasing the sensitivity of the assay (Hage, 1999).

2.1.1) Antisera production and affinity purification of tachykinins.

Previous studies have developed antibodies against tachykinins, and then purified using immunoaffinity purification for their use in immunoassay (Page *et al.*, 2003). The immobilisation of antibodies raised against tachykinins on a Sepharose matrix can be employed as a means of capturing and identifying the mature peptide sequence

or the post-translationally modified versions of naturally occurring tachykinins in cells and tissues (Lovell *et al.*, 2007). Monoclonal antibodies were raised against SP in mice and purified from mouse antiserum by passing them through a protein G affinity column; protein G is capable of capturing IgG antibodies from their Fc domains. The reactivity of the purified antibodies was checked with a radioimmunoassay, which was shown to react with the N-terminal of SP (1-6). The captured anti-SP (1-6) antibodies were immobilised on cyanogen bromide activated Sepharose and the Sepharose-antibody conjugate was effective in capturing SP expressed in macrophages. The presence of SP in the eluent was proven by HPLC and radioimmunoassay (Pascual & Bost, 1990). Lovell and colleagues (2007) developed antibodies against NKB (1-6) in sheep, then immunopurified by immobilising the peptide on Sepharose beads, before passing the serum through the beads and eluting the captured antibodies with a gradient of higher to lower pH buffer. In the same study antibodies were raised against the corticotrophin releasing factor (CRF), a peptide released from the human placenta which were immunopurified and immobilised on a Sepharose beads and used as an affinity medium to capture the CRF peptide from human placental extracts and investigate the presence of post-translational modifications specifically phosphocholine on the peptide. In another study, the distribution of NKB and SP expressing neurons in the rat brain was illustrated by immunochemistry (Marksteiner *et al.*, 1992). This was performed using antibodies specific for NKB and SP (that showed no cross-reactivity with NKA or with themselves) that were raised in rabbits inoculated with the synthetic peptides and immunoaffinity purified from antiserum by passing it through a protein A-Sepharose column. Protein A is a bacterial protein, which has the ability to capture antibodies through its Fc domains (Marksteiner *et al.*, 1992).

2.2) Rational of application of immunoaffinity purification for this study.

The scope of the experimental procedures described in this Chapter was, firstly, the development a Sepharose-tachykinin peptide affinity medium for the capture of tachykinin antibodies from ovine antisera and secondly, the development of an anti-tachykinin antibody-Sepharose affinity medium capable of capturing tachykinin peptides from a repertoire of peptides found in a tissue extract. Hence, the following range of procedures was performed. Page and colleagues (2003) raised the polyclonal

antibodies used in the present study with the inoculation of four individual sheep with the peptide EKA/B, GKASQFFGLM-NH_2 and their sera were annotated as BO14, BO15, BO16 and BO17. The cross-reactivity and specificity of anti-GKASQFFGLM-NH_2 antibodies for the four peptides mHK-1, SP, hHK-1 and NKB was determined by antibody titre curves. The synthetic peptides mHK-1, SP, hHK-1 and NKB were immobilised on a Sepharose matrix (Section 2.3.1) and the functionality of the Sepharose-peptide column was investigated (Section 2.3.2). The individual sheep antiserum was run through the Sepharose-peptide column and antibodies specific for the peptides mHK-1, SP, hHK-1 and NKB were immunoaffinity purified (Section 2.3.3) and immobilised on a Sepharose matrix (Section 2.2.5). The functionality of the immunopurified antibodies was proven with antibody titre curves.

2.3) Methods.

2.3.1) Testing of the antisera for anti-tachykinin antibodies production.

A bicarbonate buffer (0.1 M Na_2CO_3 with 0.1 M $NaHCO_3$, see Appendix 1 for reagent supplier) was prepared for the dilution of peptides, while antibodies were diluted in an albumin-phosphate buffer (0.04 M Na_2HPO_4 with 0.01 M NaH_2PO_4 and 0.5% (w/v) BSA). Bicarbonate buffer (0.1 M Na_2CO_3 with 0.1 M $NaHCO_3$) was prepared by transferring into a 500 ml Duran bottle 5.3 g of Na_2CO_3 and 4.1 g of $NaHCO_3$. The volume of the solution was adjusted to 500 ml with the addition of distilled H_2O followed by gentle stirring to ensure full dissolving of the salts. Adjustment of the pH of the solution was not required and the bicarbonate buffer (pH 9.6; measured with a pH meter) was stored at room temperature. Albumin-phosphate buffer (0.04M Na_2HPO_4 with 0.01M NaH_2PO_4 and 0.5% (w/v) BSA) was prepared by transferring into a 500 ml Duran bottle, 2.8 g of Na_2HPO_4 with 0.75 g of NaH_2PO_4 followed by dissolving in distilled H_2O and the pH of the solution was measured as pH 7.42. BSA (2.5 g) was added and left to dissolve with gentle agitation and the volume of the solution was brought up with distilled H_2O to 500 ml. Excess peptides or antibodies were removed using a wash buffer containing 0.9% (w/v) NaCl with 0.01% (v/v) Triton X-100. To prepare 500 ml of buffer, 4.5 g of NaCl were transferred into a 500 ml Duran bottle and distilled H_2O added to the 500 ml mark. A stock of 10% (v/v) Triton X-100 was made up with the dilution of 5 ml of ~100% Triton X-100 into 45 ml of distilled H_2O. 500 µl of the 10% (v/v) Triton X-100 stock was pipetted into the 0.9% (w/v) NaCl solution. The buffer was stored at 4°C. For the detection of alkaline phosphatase activity in the immunoassay p-nitrophenyl phosphate (PNPP) was used as a substrate; 20 ml of PNPP (SigmaFast) solution were prepared by dissolving one Trizma base tablet (SigmaFast) and one PNPP tablet (SigmaFast) in 20 ml of distilled H_2O. The hydrolysis of PNPP to p-nitrophenol (PNP) was terminated with the addition of 50 µl of 2 M NaOH solution, prepared by dissolving 4 g of NaOH in 50 ml of distilled H_2O.

A stock solution, 20 mg/ml, of each synthetic peptide mHK-1, SP, hHK-1 and NKB was made up in 99.8% DMSO (Fischer Scientific D/4120/PB08). An amount of SP (1.2 mg; Designer Biosciences) was weighed out and dissolved in 60 µl DMSO;

similarly synthetic NKB (1.2 mg; Designer Biosciences) was dissolved in 60 µl DMSO, mHK-1 (1.1 mg; Designer Biosciences) was dissolved in 55 µl DMSO and hHK-1 (1.1 mg; Designer Biosciences) was dissolved in 55 µl DMSO. Each peptide was further diluted to 2 ng/µl in bicarbonate buffer (0.1 M Na_2CO_3 with 0.1 M $NaHCO_3$) and then dispensed at 100 µl per well into a Nunc Immobilizer™ Amino plate. Plates inoculated with the peptide solutions were wrapped in cling film and placed at 4°C overnight to enable the synthetic peptides to covalently couple to the wells on the plate. The next morning the peptide solution was removed and unbound peptide was washed-off three times with 400 µl of wash buffer (0.9% (w/v) NaCl with 0.01% (v/v) Triton X-100).

2.3.2) Determination of antibody titre curves.

Frozen 50 ml stocks of the four anti-tachykinin antisera (BO14, BO15, BO16 and BO17) and normal sheep serum (stored at -20°C) were placed overnight at 4°C. The thawed antisera were mixed by gentle inversion and an aliquot (1 ml) of each antiserum was transferred into a 1.5 ml Eppendorf tube. Dilutions in albumin-phosphate buffer (0.04 M Na_2HPO_4 with 0.01 M NaH_2PO_4 and 0.5% (w/v) BSA) were prepared as shown in Table 2.1. The antisera dilutions 1:1000 to 1:8000 were dispensed at 100 µl per well in duplicate into the Nunc Immobilizer™ Amino plates, previously pre-coated with either of the synthetic peptides mHK-1, SP, hHK-1 or NKB. The immobilised peptides were incubated with the primary antibody *i.e.* the anti-tachykinin antiserum for 3 hours at room temperature. The wells were washed three times with 400 µl wash buffer (0.9% (w/v) NaCl with 0.01% (v/v) Triton X-100) to remove any excess or weakly bound antibodies. Donkey anti-sheep IgG-alkaline phosphatase conjugate (Sigma-Aldrich) in albumin-phosphate buffer was added (100 µl) at a dilution of 1:5000 into each well. The plates were left for 2 hours at room temperature. The solution was removed and excess and the weakly bound IgG-alkaline phosphatase secondary antibodies were washed off three times with 400 µl of wash buffer (0.9% (w/v) NaCl with 0.01% (v/v) Triton X-100). The substrate p-nitrophenyl phosphate (PNPP; Sigma-Aldrich) was added at 200 µl per well for the detection of immunoreactivity. The plates were left in the dark for 15 min for the development of a yellow colour. The reactions were stopped with 50 µl of 2 M NaOH. Absorbance was measured at 405 nm using a Varian spectrometer (Varian

Cary, 50 MPR Microplate Reader). Absorbance measurements were plotted against dilutions and the standard deviation between duplicates was calculated.

Table 2.1. Serial dilutions prepared from the 1 ml aliquots of the stock solution of the ovine antisera.

Dilution[a]	Ovine antiserum[b]	Buffer added[c]
1:10	100 µl taken from antiserum stock (1 ml)	900 µl
1:1000	20 µl taken from the 1:10 dilution	1980 µl
1:2000	1000 µl taken from the 1:1000 dilution	1000 µl
1:4000	1000 µl taken from the 1:2000 dilution	1000 µl
1:8000	1000 µl taken from the 1:4000 dilution	1000 µl

[a]The final antibody dilution factor produced compared to the stock of antiserum. [b]Volume of stock of antiserum used from each diluted antibody stock. [c]Volume of the albumin-phosphate buffer added to produce the final dilution.

2.3.3) Development of a Sepharose-tachykinin conjugate.

The following method was adapted from the product information for cyanogen bromide-activated matrices by Sigma-Aldrich. Cyanogen bromide-activated Sepharose 4B (1 g; Sigma-Aldrich) was swollen into a 3.5 ml gel with 25 ml of 1 mM HCl. After 30 min incubation at 25°C, the mixture was centrifuged and the supernatant was discarded. Lactose was removed from the Sepharose gel by repeating eight washing cycles of adding 25 ml of 1 mM HCl to the gel, mixing well into a homogenous mixture and centrifuging at 168 x g (1000 RPM) for 10 min. The swollen Sepharose gel was then washed with 200 ml of distilled H_2O repeating the above procedure of adding 25 ml aliquots of distilled H_2O to the resin gel, mixing and centrifuging at 168 x g (1000 RPM) for 10 min and discarding the supernatant.

A volume of coupling buffer (0.1 M $NaHCO_3$ with 0.5 M NaCl and 0.5% (v/v) Tween 20, pH<9) was added to the 3.5 ml of Sepharose gel pellet to produce a 10 ml homogenous mixture. The diluted Sepharose gel in coupling buffer (10 ml) was divided equally among four tubes. 25 µl from each stock of 20 mg/ml of peptide in DMSO was dissolved in 1 ml of the coupling buffer (0.1 M $NaHCO_3$ with 0.5 M NaCl and 0.5% (v/v) Tween 20, pH<9). Each 1 ml solution of peptide in coupling buffer was added into a respective tube of 2.5 ml of Sepharose gel in the coupling buffer (0.1 M $NaHCO_3$ with 0.5 M NaCl and 0.5% (v/v) Tween 20, pH<9). The four tubes were

labelled (according to the tachykinin peptide added) before placed in agitation by gentle shaking (in a Labnet DS211 incubator) overnight at room temperature. This allowed the cross-linkage of the peptide with the cyanogen bromide-activated Sepharose beads (Figure 2.1).

The following morning, the four 50 ml Falcons (containing the mixtures of activated Sepharose with the peptide in coupling buffer) were taken out of gentle shaking and the unbound reactive binding sites were blocked with 10 ml of blocking agent (0.2 M glycine, pH ~8). Then, the tubes were returned to gentle shaking for 2 hours at room temperature. The blocking agent was removed by centrifuging the tubes at 168 x g (1000 RPM) for 10 min and discarding the supernatant. The Sepharose-peptide complexes were washed twice with distilled H_2O (20 ml), once with coupling buffer (20 ml) and twice with 0.5 M NaCl, pH ~4 (20 ml). The Sepharose-peptide complexes were stored in 5 ml of storage buffer (1 M NaCl with 0.02% (w/v) NaN_3 and 1 mM EDTA disodium) at 4 °C.

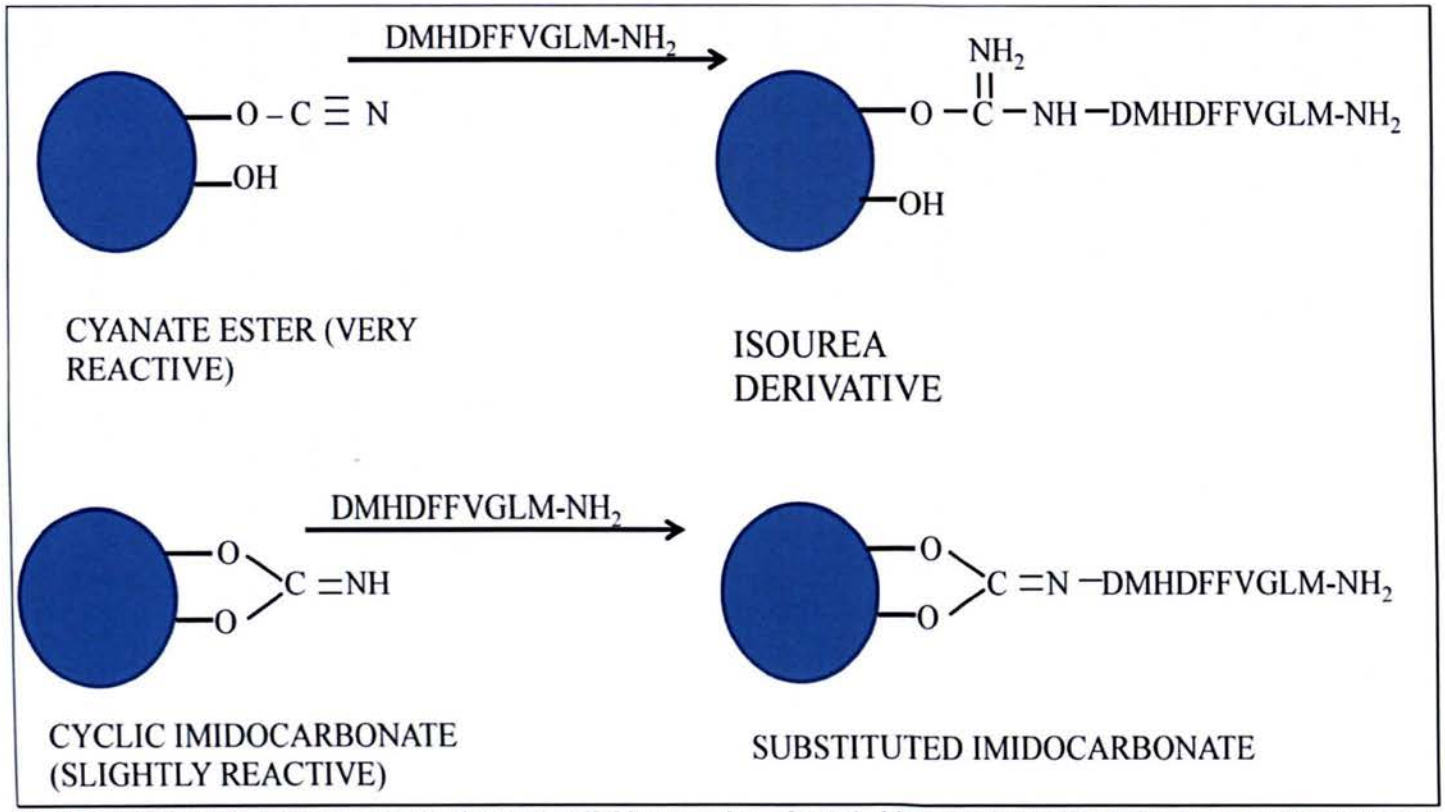


Figure. 2.1. The diagrammatic representation of the cross-linking reaction of a tachykinin peptide, using NKB as an example to cyanogen bromide-activated Sepharose beads. Cyanogen bromide reacts with hydroxyl groups on agarose to form cyanate esters or cyclic imidocarbonates. Both groups react readily with primary amines comprised on the N-terminus or C-terminus of the peptide. The end result is the covalent coupling of peptide to the agarose matrix forming an isourea derivative or a substituted imidocarbonate, which has no net charge. Adapted from product information for C9142 (www.sigmaaldrich.com; and Kohn & Wilcheck, 1984).

2.3.4) Making of the Sepharose-peptide conjugate column and testing of its functionality.

An Econo-column (BioRad) was fitted with a flow adaptor (BioRad) and 30 cm of silicon tubing (BioRad) ending into a waste beaker. The four Sepharose-peptide gels (~0.8 ml each) in storage buffer (1 M NaCl with 0.02% (w/v) NaN_3 and 10 mM EDTA disodium) were mixed together (final volume ~3.2 ml of Sepharose-peptide gel) and poured into the Econo-column. The flow-adaptor was opened and the storage buffer (1 M NaCl with 0.02% (w/v) NaN_3 and 10 mM EDTA disodium) was allowed to run through the column until a final volume of ~3.2 ml Sepharose-peptide gel bed was made. An "empty run" was made with ten times the volume of the column (30 ml) elution buffer (0.1M glycine pH ~2.5), before the Sepharose-peptide conjugate was brought to "binding conditions" by running through the column 30 ml of binding buffer (PBS with 0.01% (v/v) Tween-20). The stock of BO17 showed a high concentration of anti-tachykinin antibodies that cross-reacted with the four peptides of this study's predominant interest. The antiserum was chosen to be used as a trial for the investigation of the functionality of the Sepharose-peptide column. The antiserum (~50 ml) stored at -20 °C, was thawed at 4 °C overnight. The following morning, the antiserum was mixed with gentle inversion and a protease inhibitor cocktail tablet (serine and cysteine protease inhibitors; Roche, #11836170001); 0.18 g EDTA disodium and 2% (w/v) NaN_3 were transferred into the tube before the stock had thawed completely. An aliquot of this prepared antiserum (6 ml) was passed through the Econo-column at a flow rate of 0.5 ml/min to allow a longer binding time between the antibodies and the Sepharose-peptide gel. The column was washed with 30 ml of PBS with 0.01% (v/v) Tween-20 followed by 15 ml of distilled H_2O. The captured antibodies were eluted with 24 ml of 0.1 M glycine pH ~2.5 and twelve 2 ml fractions were collected and immediately neutralised with 140 µl of 0.1 M KH_2PO_4 pH ~9.

The column was "regenerated" with 50 ml of 0.1 M citric acid pH ~2.5 and re-equilibrated with 50 ml of binding buffer. The experiment was repeated with ~42 ml of BO17 antiserum, loaded on the column at a flow rate of ~0.5 ml/min (to allow a longer binding time) between the antibodies and the Sepharose-peptide conjugate. The flow-through was collected into a fresh 50 ml Falcon tube. The 30 cm of silicon tubing was re-

connected to the column before the washing steps to increase the flow rate to ~2.5 ml/min for the removal of contaminant protein and to minimise the loss of bound antibodies. The washing steps were performed by running through the column 30 ml of 1x PBS with 0.01% (v/v) Tween-20 followed by 15 ml of distilled H_2O. The tubing was then removed and captured antibodies were eluted with 0.1 M citric acid pH ~2.5 (flow rate 0.5 ml/min). The void was discarded before the collection of twenty-four 2 ml fractions. The fractions were neutralised with 30 µl of 10 M NaOH. The column was "regenerated" with 50 ml of 0.1 M citric acid pH ~2.5 and re-equilibrated with 50 ml of binding buffer (PBS with 0.01% (v/v) Tween-20). The flow-through (~40 ml of the antiserum passed through the column and re-collected) was loaded again on the column and re-collected; the washing procedure was performed as above. Three 6 ml elutions were collected after the pass-through of the antiserum, then neutralised with 100 µl of 10 M NaOH. Finally, the Sepharose-peptide gel was "regenerated" with ~17 volumes of elution buffer 0.1 M citric acid pH ~2.5 and re-equilibrated with ~17 volumes of binding buffer PBS with 0.01% (v/v) Tween-20. The ~40 ml of flow-through (the antiserum passed through the column and recollected) were loaded another three times, repeating the washing steps and the collection of three 6 ml elutions.

Two experiments were performed, the first by passing a 6 ml aliquot of the BO17 antiserum through the Sepharose column and eluting with 0.1 M glycine pH 2.5 and a second running the 42 ml of the BO17 serum through the column and eluting with 0.1 M citric acid pH 2.5. The titre of antibodies collected in the elution fractions from the two experiments was tested. The elution fractions were diluted in 1x PBS (as shown in Table 2.2). The dilutions (100 µl) were immobilised onto the wells of a Nunc plate (through passive absorption). The plate was then blocked with 300 µl of 1% (w/v) BSA in 1x PBS. Excessive material was washed off three times with 300 µl of 1x PBS and 0.01% (v/v) Tween. The secondary antibody anti-sheep IgG-alkaline phosphatase conjugate was diluted to 1:5000 in 1x PBS with 0.1% (w/v) BSA and 0.01% (v/v) Tween-20. The secondary antibody solution (100 µl) was added to each well and incubated for 1 hour. Excessive and weakly bound antibody was again washed off (with 1x PBS with 0.01%

(v/v) Tween) and alkaline phosphatase was detected with the addition of PNPP substrate at 100 µl per well, as described above.

Table 2.2. Serial dilutions of the fractions of purified antibodies.

Dilution[a]	Purified antibodies[b]	Buffer[c]
1:10	20 µl of fraction of purified antibodies	180 µl of 1x PBS
1:100	20 µl taken from 1:10 dilution of purified antibodies	180 µl of 1x PBS
1:1000	20 µl taken from 1:100 dilution of purified antibodies	180 µl of 1x PBS

These were collected from the two experiments of passing BO17 ovine antiserum through an Econo-column and eluting either in low pH 0.1 M glycine or 0.1 M citric acid buffer as described in the experimental Section 3.2.4. [a]The final dilution produced compared to the stock of purified antibodies. [b]Volume of fraction of purified antibodies used or of diluted sample. [c]Volume of 1x PBS buffer added to produce the final titre volume.

2.3.5) Purification of anti-tachykinin antibodies from BO16 antiserum.

The stock of BO16 antiserum (50 ml) kept at -20 °C, which also showed a high titre of anti-tachykinin antibodies, was thawed at 4°C overnight. A serine and cysteine protease inhibitors cocktail tablet (Roche), 1 mM EDTA disodium and 2% (w/v) NaN_3 were transferred into the tube before the stock thawed completely. The BO16 antiserum (50 ml) was run through the column at a flow rate of ~0.5 ml/min. The flow-through containing the unbound anti-tachykinin antibodies was collected into a 50 ml Falcon tube. The 30 cm silicon tubing was reconnected to the column before running through 50 ml of wash buffer (flow rate 2.5 ml/min). Non-specifically bound proteins present in the serum *e.g.* proteases and weakly bound antibodies were washed off the column. The eluted wash buffer was discarded. The column was further "washed" with 15 ml of distilled H_2O for the removal of detergent and salt and the silicon tubing removed. For the elution of the captured antibodies, 7 ml of elution buffer 0.1 M citric acid pH ~2.5 was added to the column. The void volume (1 ml) was discarded. The flow adaptor was closed and the Sepharose-peptide-antibody complex was incubated in the buffer for 5 min. The flow adaptor was opened and the following 6 ml of purified antibodies in buffer was collected (flow rate 0.5 ml/min). The elution procedure was repeated three times in

total and three 6 ml elutions were collected. The elutions were immediately neutralised with 100 µl of 10 M NaOH. The 30 cm of silicon tubing was reconnected to the column and elution buffer (50 ml) passed through the column to regenerate the Sepharose-peptide. The column was re-equilibrated with 50 ml of binding buffer (PBS with 0.01% (v/v/) Tween-20).

The antiserum (~50 ml) was re-loaded on the column at a flow rate of 0.5 ml/min and flow-through was re-collected. The washing step was repeated with 50 ml of wash buffer (flow rate 2.5 ml/min) before 15 ml of distilled H_2O was added. The captured anti-tachykinin antibodies were eluted with 7 ml of elution buffer 0.1 M citric acid pH ~2.5 (passed at a flow rate of 0.5 ml/min). Three elutions (6 ml) were collected; and the fractions were neutralised with 100 µl of 10 M NaOH. The column was regenerated with 17 column volumes of 0.1 M citric acid pH ~2.5 and brought to "binding conditions" with 17 column volumes of binding buffer. The antiserum was re-loaded (and collected) for a third time. The washing and elution procedure was performed as above. Three elutions of 6 ml were collected and neutralised with 100 µl of 10 M NaOH. Aliquots (0.5 ml) of the nine elutions (6 ml) were kept. The aliquots (0.5 ml) and stocks (~6 ml) of purified anti-tachykinin antibodies were stored at -70°C. The column was regenerated with 50 ml of elution buffer (0.1 M citric acid pH ~2.5) and equilibrated with 50 ml equilibration buffer (1 x PBS with 0.01% (v/v) Tween-20). The column was stored in 1 x PBS with 0.01% (v/v) Tween-20 and 0.02% (w/v) NaN_3 at 4 °C. For storage at -20°C the 3 ml of Sepharose-peptide gel was transferred into a 50 ml Falcon tube and mixed with an equal volume of glycerol.

A Bradford reagent protein assay (Bradford, 1976) was used to measure the concentration of total IgG in the nine elution fractions of BO17 immunopurified antibodies. A stock of 10 mg of IgG antibody (I5131; Sigma-Aldrich) was reconstituted in 1 ml of 1x PBS; 50 µl of the 10 mg/ml stock was brought to 500 µl in 1x PBS. Antibody IgG standards were made up in 0.1 M sodium citrate monobasic pH ~7 (Sigma-Aldrich) (see Table 2.3). An aliquot (40 µl) of each IgG standard concentration and purified antibodies sample was plated in triplicate into the wells of a 96-well Nunc plate. 200 µl of Bradford reagent

(Sigma-Aldrich) were added into the well. The colour was left to develop for 5 min. Absorbance was measured at 620 nm.

Table 2.3. Preparation of IgG standard curve.

Final IgG concentration [a]	IgG [b]	0.1M Sodium citrate [c]
0.50 mg/ml	200 µl of 1 mg/ml stock	200 µl buffer
0.25 mg/ml	200 µl of 0.50 mg/ml sample	200 µl buffer
0.125 mg/ml	200 µl of 0.25 mg/ml sample	200 µl buffer
0.06 mg/ml	200 µl of 0.12 mg/ml sample	200 µl buffer
0.03 mg/ml	200 µl of 0.06 mg/ml sample	200 µl buffer
0.015 mg/ml	200 µl of 0.03 mg/ml sample	200 µl buffer

[a]The final concentration of the IgG standards. [b]The volume of stock or of each standard used to make the next sample. [c]The volume of sodium citrate buffer used to make the dilution.

2.3.6) Immobilisation of purified anti-peptide antibodies on cyanogen bromide-activated Sepharose.

The BO16 immuno-purified anti-tachykinin antibodies were immobilised on cyanogen bromide-activated Sepharose. In Section 2.3.5 it was described that the serum was passed three times through the Sepharose-antibody column (*i.e.* three purification experiments) and that three of 6 ml elutions were collected after each run. The first elution (6 ml) from the first purification experiment contained the greatest amount of immunopurified antibodies (as determined by the Bradford assay) while the following eight elutions were merged into a ~45 ml mixture adding up to contain an amount of immunopurified antibodies in the same range as the first elution fraction. The pH of the first 6 ml elution and the 45 ml elution pool was pH ~8.5. An aliquot (0.5 ml) of the mixture was kept and stored at -20 °C. Three vials of the cyanogen bromide-activated Sepharose 4B (1 g) were swollen in parallel and washed with 1 mM HCl (for the removal of lactose), followed by washes in distilled H_2O and coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 9.0) using an identical procedure as stated in Section 2.3.3. The first elution fraction (6 ml) of immunopurified BO16 antibodies were transferred into 10 ml of activated cyanogen bromide-activated Sepharose (in coupling buffer) in a 50 ml Falcon tube. The

pool of the next nine elutions (~45 ml) was transferred into a second tube with ~ 5 ml cyanogen bromide-activated Sepharose (in coupling buffer) in a 50 ml Falcon tube. The two 50 ml Falcon tubes containing the reactions of Sepharose with immunopurified BO16 antibodies were incubated overnight at room temperature by gentle shaking in an incubator (Labnet DS211). The following morning, the two Falcon tubes were spun at 168 x g (1000 RPM) for 10 min. The Sepharose-antibody complex produced a pellet and the supernatant was decanted and retained for checking for the presence of any uncoupled antibodies. The un-reacted groups of the cyanogen bromide-activated Sepharose were blocked with 0.2 M glycine pH 8.0. The same washing procedure as in Section 2.3.3 was followed.

A third Sepharose-antibody complex was made with the uncoupled antibodies collected in the supernatant after the immobilisation of the antibodies in the first elution fraction. The ~7.5 ml of unbound antibodies was incubated with 3.5 ml of swollen and "washed" cyanogen bromide-activated Sepharose 4B. The mixture was placed overnight on a platform with gentle shaking at room temperature. The un-reacted groups were blocked and the Sepharose-antibody complex was washed as in Section 2.3.3. The three Sepharose-antibody complexes were stored in storage buffer, 1 M NaCl with 10 mM EDTA disodium and 0.02% (w/v) NaN_3 (~5 ml) in 50 ml Falcon tubes at 4°C.

2.4) Results.

2.4.1) Cross-reactivity of antibodies raised against synthetic EKA/B (GKASQFFGLM-NH_2) with mHK-1, SP, hHK-1 and NKB.

The polyclonal antibodies used in the present study were raised with the inoculation of four individual sheep with the peptide EKA/B, GKASQFFGLM-NH_2 and their sera were annotated as BO14, BO15, BO16 and BO17. In the present study, the antisera that were raised against the synthetic tachykinin sequence EKA/B (GKASQFFGLM-NH_2) were tested for their ability to cross-react against the four tachykinin peptides of predominant interest, mHK-1 (SRTRQFYGLM-NH_2), SP (RPKPQQFFGLM-NH_2), hHK-1 (TGKASQFFGLM-NH_2) and NKB (DMHDFFVGLM-NH_2) (Table 2.4). The cross-reactivity of the four antisera and relative concentration of specific antibodies for each peptide was demonstrated with a series of dilution antibody titre experiments (Figures 2.2 to 2.5). In each well of the Nunc amino plates, 200 ng of each respective synthetic peptide was immobilised and a dilution of the antiserum was added (in the range of 1:1000 to 1:8000). The ability of the four antisera (namely BO14, BO15, BO16, BO17 according to the ear tag of the inoculated sheep) to recognise mHK-1 is illustrated in Figure 2.2. Among the four antisera, the BO16 and BO17 appear to show the highest amount of antibodies recognising mHK-1. Figure 2.2 illustrates BO15 antiserum showing a relatively high concentration of antibodies cross-reacting with mHK-1 as well, while in the BO14 antiserum produced the lowest concentration of antibodies specific for this peptide. Moreover, Figures 2.2 to 2.5, show that the "control" serum *i.e.* the serum collected from the sheep not exposed to the tachykinin antigen did not show any immunoreactivity for the four tachykinin peptides.

The cross-reactivity of the four antisera for SP is illustrated in Figure 2.3. The BO17 antiserum shows the highest amount of antibodies specific for SP; almost twice the amount of antibodies contained in the other three antisera. The antisera BO14, BO15 and BO16 were shown to contain a concentration of antibodies specific for SP within a similiar titre range. The cross-reactivity of the four antisera for NKB is illustrated in Figure 2.4. It was shown that the antisera BO15 and BO17 contained the highest amount

of antibodies specific for the peptide NKB while the antisera BO16 possessed a lower amount of antibodies specific to NKB. The antiserum BO14 was not much different from the normal sheep serum control. Figure 2.5 illustrates the reactivity of hHK-1 for the four antisera. The antisera BO17 and BO16 contained the highest amounts of antibodies specific for the peptide hHK-1; the antisera BO15 and BO14 also contain a high amount of antibodies specific for the peptide, however, these were lower around half the amount when compared to BO16 and BO17. Table 2.4 shows the homology between the five tachykinin peptides; the sequence of hHK-1 was published by Page *et al.*, (2003), the sequence of mHK-1 is shown as an amidated decapeptide as suggested by this study.

Table 2.4. Alignment of the five tachykinin peptide sequences, investigated in this study.

EKA/B		G	K	A	S	Q	F	F	G	L	M-NH_2
[1]hHK-1	T	G	K	A	S	Q	F	F	G	L	M-NH_2
SP	R	P	K	P	Q	Q	F	F	G	L	M-NH_2
[2]mHK-1		S	R	T	R	Q	F	Y	G	L	M-NH_2
NKB		D	M	H	D	F	F	V	G	L	M-NH_2

1. Sequence published by *Page et al.*, (2003). 2. Sequence of mHK-1 as proposed by this study.

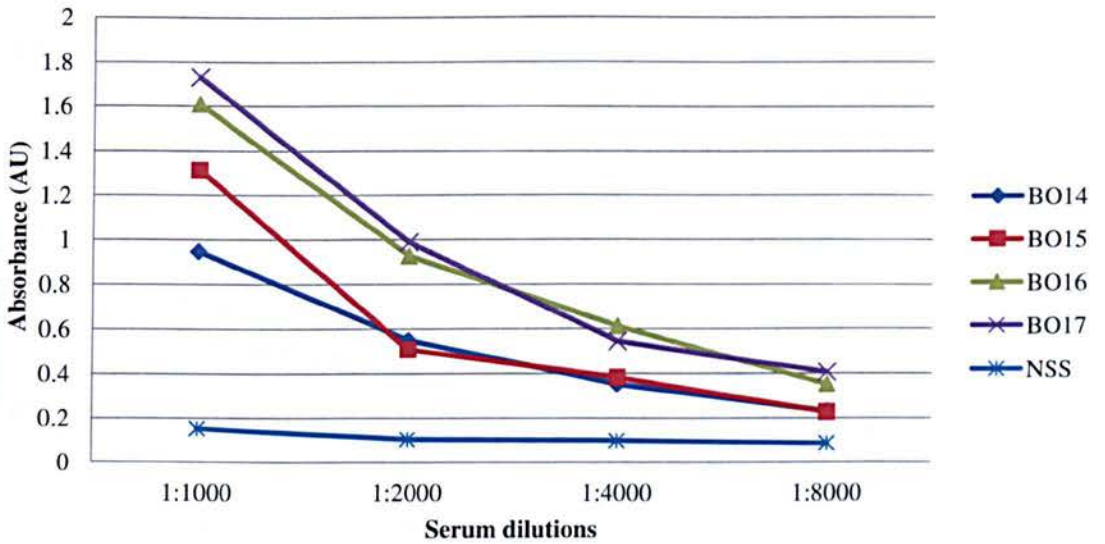


Figure 2.2. Antibody titre curves of the four antisera against mHK-1. The synthetic peptide mHK-1 was plated at 200 ng per well. The serial dilutions of the four antisera were added onto the immobilised peptide. Antibody titre curves are shown in a range from 1:1000 to 1:8000 dilutions of the four antisera (BO14-BO17) and normal sheep serum (NSS). A secondary anti-sheep IgG antibody-alkaline phosphate conjugate was added. The absorbance of the breakdown of PNPP by alkaline phosphatase was determined at 405 nm.

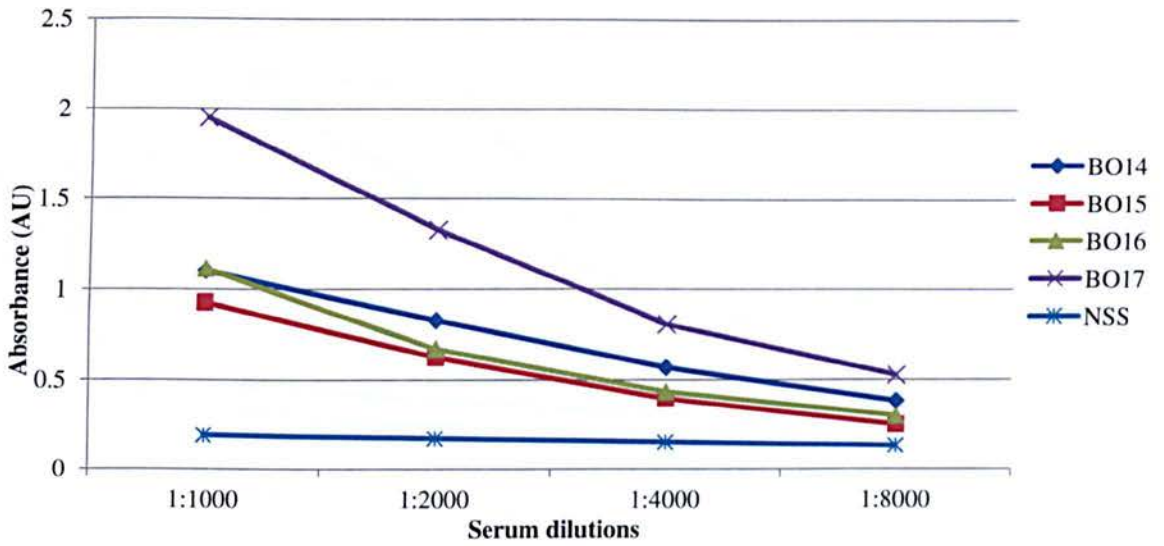


Figure 2.3. Antibody titre curves of the four antisera against SP. The synthetic peptide SP was immobilised (200 ng) per well. The serial dilutions of the four antisera were added onto the immobilised peptide. Antibody titre curves are shown in a range from 1:1000 to 1:8000 dilutions of the four antisera (BO14-BO17) and normal sheep serum (NSS). A secondary anti-sheep IgG antibody-alkaline phosphate conjugate was added. The absorbance of the breakdown of PNPP by alkaline phosphatase was determined at 405 nm.

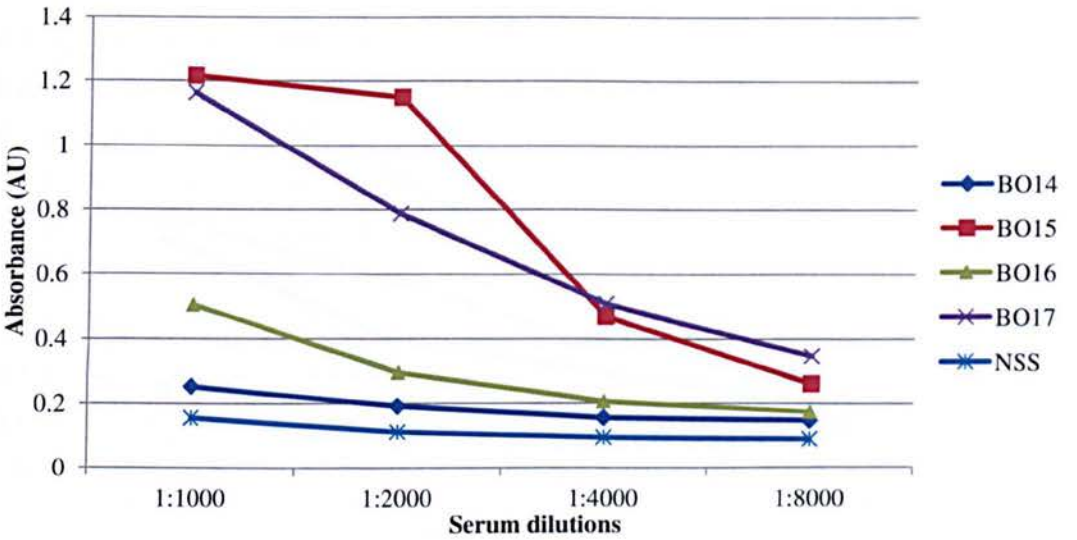


Figure 2.4. Antibody titre curves of the four antisera against NKB. The synthetic peptide NKB was plated at 200 ng per well. The serial dilutions of the four antisera were added onto the immobilised peptide. Antibody titre curves are shown in a range from 1:1000 to 1:8000 dilutions of the four antisera (BO14-BO17) and normal sheep serum (NSS). A secondary anti-sheep IgG antibody-alkaline phosphate conjugate was added. The absorbance of the breakdown of PNPP by alkaline phosphatase was determined at 405 nm.

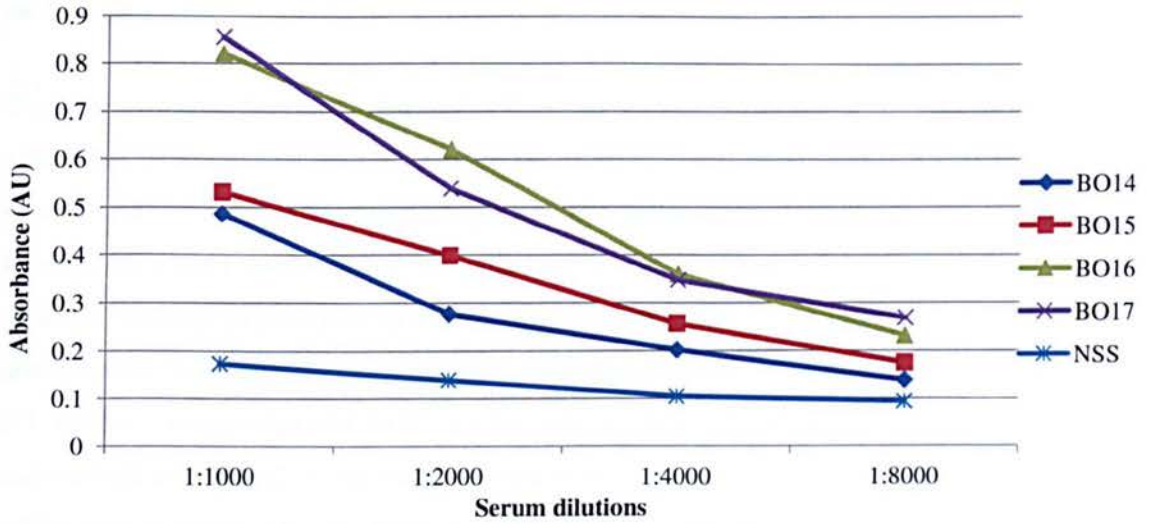


Figure 2.5. Antibody titre curves of the four antisera against hHK-1. The synthetic peptide hHK-1 was plated at 200 ng per well. The serial dilutions of the four antisera were added onto the immobilised peptide. Antibody titre curves are shown in a range from 1:1000 to 1:8000 dilutions of the four antisera (BO14-BO17) and normal sheep serum (NSS). A secondary anti-sheep IgG antibody-alkaline phosphate conjugate was added. The absorbance of the breakdown of PNPP by alkaline phosphatase was determined at 405 nm.

2.4.2) Functionality of the Sepharose-peptide column and the purification of the anti-tachykinin specific antibodies.

The four synthetic peptides were immobilised on Sepharose to purify the anti-tachykinin antibodies from the four sera. Cyanogen bromide-activated Sepharose was chosen as the matrix to immobilise the synthetic peptides. The advantages of Sepharose usage are the hydrophilic matrix consisting of beads that allow minimal non-covalent interactions with proteins, which provide a porous network for the efficient flow of buffer; also, the spacer arm between the beads and the peptide-ligand which minimises steric hindrance (Figure 2.1; Product information of C9142; Sigma-Aldrich, 2011). To obtain a pure population of anti-tachykinin antibodies and remove impurities present in the antiserum, 0.5 mg of each of the four synthetic peptides mHK-1, SP, hHK-1 and NKB (see Appendix 1 for reagent supplier) were immobilised on a cyanogen-bromide activated Sepharose. A 3.5 ml Sepharose-peptide column was built and its' functionality was investigated by passing through it an aliquot of the BO17 antiserum. The stock of BO17 antiserum showed a high concentration of anti-tachykinin antibodies cross-reacting with all four tachykinins of this study's predominant interest, hence, this serum was chosen to be used as a trial for the investigation of the functionality of the Sepharose-peptide column. A low pH buffer, 0.1 M glycine (pH 2.5), was applied to disrupt the antibody-ligand interactions; and twelve 2 ml fractions were collected. Three dilutions in the range of 1:10 to 1:1000 of each fraction were prepared in buffer and immobilised with passive absorption into the wells of a Nunc plate. The presence of anti-tachykinin antibody was detected in each fraction (and in the three dilutions). In Figure 2.6 is shown the immunoreactivity of the twelve fractions plated. The gradual decrease of immunoreactivity in each fraction (in combination with the decrease in the immunoreactivity among the various dilutions), indicates the anti-tachykinin specificity and functionality of the purified antibodies. The Sepharose-peptide column effectively captured the anti-tachykinin antibodies from the sheep antiserum. The column was regenerated with ~17 column volumes of 0.1 M citric acid pH 2.5 and brought to "binding" conditions *i.e.* pH in the range of 7-8 and optimal salt concentration for the establishment of peptide-antibody interactions. We investigated the number of fractions that contain a significant concentration of anti-tachykinin

antibodies to collect. The BO17 antiserum (42 ml) was passed through the column and captured antibodies were eluted with 0.1 M citric acid pH 2.5. The void was discarded and then twenty four 2 ml fractions were collected. The results from the procedure showed that the captured antibodies eluted predominantly in the first 9 fractions (Figure 2.7).

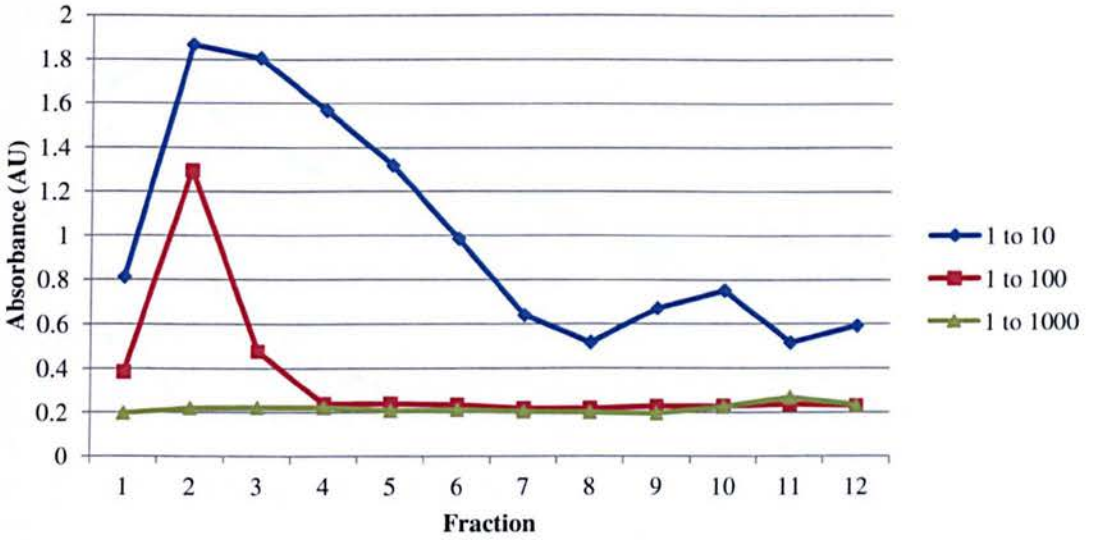


Figure 2.6. Application of low pH glycine buffer for the elution of anti-tachykinin antibodies from the Sepharose-peptide column. An aliquot of the BO17 serum (6 ml) was passed through the column. Weakly bound antibodies and contaminant proteins were washed off with high salt buffer. The captured anti-tachykinin antibodies were eluted with glycine buffer pH 2.5 and collected in twelve fractions, then neutralised. The above dilutions (range 1:10 to 1:1000) were prepared and transferred onto an ELISA plate (immobilised with passive absorption) for the detection of the presence of antibodies in the fractions. Absorbance was measured at 405nm. The functionality of the Sepharose-peptide column is shown.

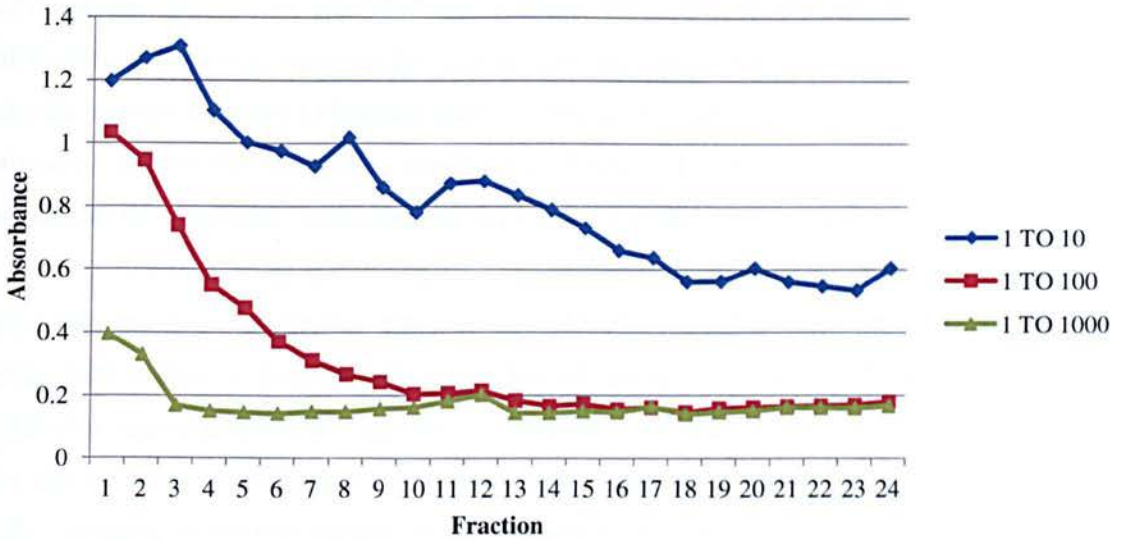


Figure 2.7. Application of low pH citric acid buffer for the elution of anti-tachykinin antibodies from the Sepharose-peptide column. BO17 serum (42 ml) was passed through the column. Weakly bound antibodies and contaminant proteins were washed off with 1x PBS with Triton-X buffer. The captured anti-tachykinin antibodies were eluted with citric acid buffer pH 2.5 and collected in twenty-four (2 ml) fractions, immediately neutralised with 10 M NaOH. The above dilutions (range 1:10 to 1:1000) were prepared and transferred onto an ELISA plate (immobilised with passive absorption) for the detection of the presence of antibodies in the fractions. Absorbance was measured at 405 nm.

The reversible interactions (electrostatic, hydrophobic interactions, van der Waals' forces and hydrogen bonding) between the captured antibodies and the peptide-Sepharose gel were disrupted with a low pH elution buffer (citric acid or glycine). High and low affinity antibodies against the tachykinin motif were eluted and collected. As the aim was to immobilise the captured antibodies on Sepharose for the capture of known and novel tachykinin peptides from tissues of interest, antibodies of different affinities for the tachykinin motif were purified using a low pH elution buffer rather than a gradient elution with buffers of different pH or salt. The advantage of the usage of a low pH citric acid buffer instead of a low pH glycine elution buffer was that glycine blocks the reactive sites during the immobilisation of antibody on cyanogen bromide-activated Sepharose beads, resulting in the interference of the immobilisation of the purified anti-tachykinin antibodies.

The anti-tachykinin antibodies were purified from the antiserum annotated BO16, with the purpose of the particular antibodies to be immobilised on the activated Sepharose. The titre curves shown in Figures 2.2 to 2.5 illustrated the antiserum contained a high titre of antibodies specific for mHK-1, and hHK-1 and a lower but still relatively strong titre of antibodies specific for SP and NKB. The antiserum was passed through the Sepharose-peptide column (named experiment 1), and unbound protein was washed off and captured antibodies were eluted with 0.1 M citric acid (pH 2.5). Three elution fractions of 6 ml were collected and annotated as "experiment 1", "elution 1", "elution 2", and "elution 3" respectively. The column was regenerated and brought to binding conditions before the antiserum was passed through the column for a second time "experiment 2". Three 6 ml elution fractions, 1-3 were again collected. The serum was passed through the Sepharose-peptide column for a third time "experiment 3" with the collection of the anti-tachykinin antibodies in another three 6 ml fractions. The total amount of times the BO16 antiserum was passed through the Sepharose-peptide column and the elution fractions collected are shown in Table 2.5.

Table 2.5. The immunoaffinity purification experiment and the elution fractions collected from antiserum BO16.

	First loading of the BO16 antiserum: "Experiment 1"	Second loading of the BO16 antiserum: "Experiment 2"	Third loading of the BO16 antiserum: "Experiment 3"
First elution (6 ml)	Experiment 1, elution 1	Experiment 2, elution 1	Experiment 3, elution 1
Second elution (6 ml)	Experiment 1, elution 2	Experiment 2, elution 2	Experiment 3, elution 2
Third elution (6 ml)	Experiment 1, elution 3	Experiment 2, elution 3	Experiment 3, elution 3

2.4.3) Functionality of purified antibodies using antibody titre curves.

The BO16 anti-tachykinin antibodies were chosen for immobilisation on a Sepharose matrix, showing a high titre of specific antibodies for the four peptides of this study's predominant interest (albeit lower for NKB). The functionality of the anti-tachykinin antibodies after purification from the BO16 antiserum was investigated preparing a set of antibody titre curves. In Figure 2.8 the antibody titre curves of the purified anti-tachykinin antibodies present in the elution fraction "experiment 1 elution 1" from the BO16 antiserum, are shown in the range of 1:1000 to 1:8000. The four peptides, mHK-1, SP, hHK-1 and NKB were immobilised individually on Nunc amino plates before adding with the purified anti-tachykinin antibodies. The antibody titre curves illustrate the purified antibodies recognised the four peptides after purification which shows antibody functionality. The concentration of the purified anti-tachykinin antibodies found in the nine elution fractions of was measured using a Bradford reagent assay. Concentrations in the range of 0.5 mg/ml to 0.015 mg/ml of sheep total IgG antibody were prepared to create a calibration curve. As the amount of antibodies found in the elution fraction "experiment 1, elution 1" was in the same range as the amount of antibodies found in each of the next 8 elution fractions, they were merged to produce an ~45 ml pool of purified antibodies (BO16 mixture). In Table 2.6 the concentration of antibodies in the elution fraction "experiment 1, elution 1" and in the fraction BO16 mixture is shown.

Table 2.6. The antibody concentrations of elution fractions BO16 "experiment 1, elution 1" and BO16 mixture.

Fraction	Mean concentration (mg/ml) [a]	Standard deviation [b]	Fraction volume [c]
BO16, experiment 1, elution 1	4	1.03	6 ml
BO16 mixture	0.15	0.07	48 ml

[a] The mean concentration of 3 measurements are shown, their [b] standard deviation and [c] the volume of the elution fraction.

Subsequently, the amount of antibodies in the elution fraction BO16 "experiment 1, elution 1" (~24 mg) were immobilised on a Sepharose matrix and in parallel the amount of antibodies in the fraction "BO16 mixture" (7.2 mg) were immobilised on a second Sepharose matrix. The antibodies present in the elution fraction BO16 "experiment 1, elution 1" were of lower affinity having eluted first from the Sepharose-peptide column, while the polyclonal antibodies present in the other eight elution fractions *i.e.* in the combination of antibodies "BO16 mixture" were a population of antibodies of different affinity for the tachykinin motif. A Bradford reagent assay was performed to measure the amount of antibodies present in the supernatant after the immobilisation procedure. It was found that only half of the amount of the antibodies present in the elution fraction BO16 "experiment 1, elution 1" was immobilised on the Sepharose matrix. Therefore, the antibodies present in the supernatant were immobilised on a third Sepharose matrix. Three Sepharose matrix-antibody conjugates were created. The ability of the Sepharose-antibody conjugates to effectively capture tachykinin peptides was demonstrated with the incubation of the three Sepharose-antibody complexes with synthetic peptide and these are described in the next chapter (Section 3.5).

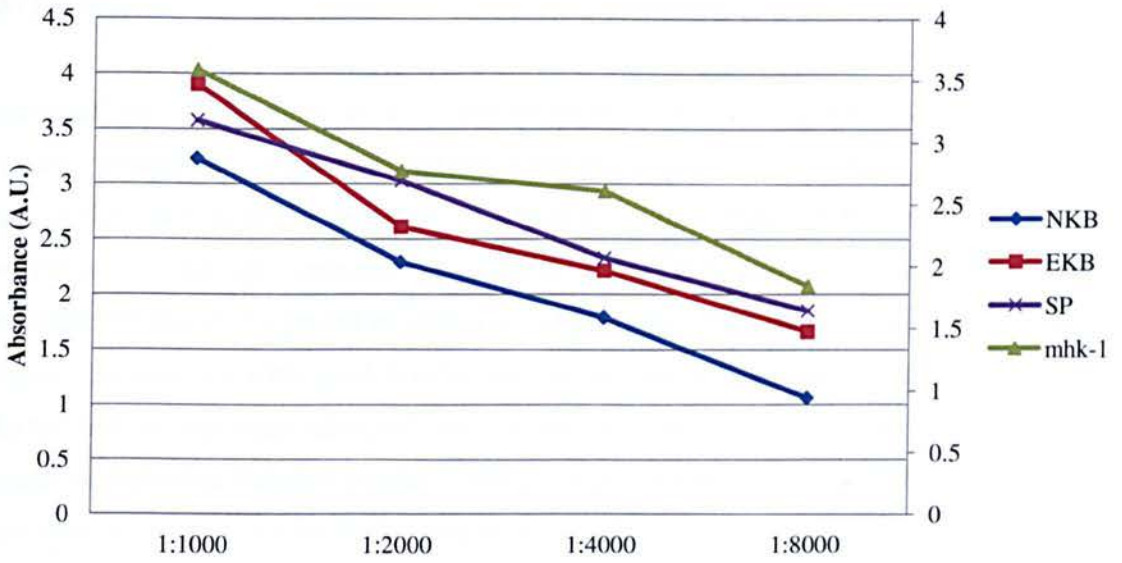


Figure 2.8. Titre of affinity purified BO16 "experiment 1, elution1" antibodies. The four synthetic peptides (NKB, hHK-1, mHK-1 and SP) were plated at 200 ng per well. Serial titre curves of the purified antibodies BO16 from "experiment 1, elution 1" are shown in a range from 1:1000 to 1:8000. A secondary antibody IgG-alkaline phosphatase conjugate was added to the immobilised peptide-primary antibody conjugate. Absorbance was determined at 405 nm by measuring the breakdown of PNPP by alkaline phosphatase.

2.5) Discussion.

Immunoaffinity purification as an efficient method of isolating specific antibodies, peptides, proteins or cellular membrane proteins has many biochemical and clinical applications, such as in the discovery of biomarkers (Ackermann & Berna, 2007). The bottleneck in the procurement of reagents for immunoaffinity purification is predominantly the development of antibodies of good functionality and specificity for an antigen. An antibody with good functionality is the one with the ability to recognise a selected peptide, showing sufficient affinity, and with an antibody off-rate that is short enough to retain the targeted peptide during sample handling. The antibody must also show low cross-reactivity to high abundance peptides in tissues that normally compete with the targeted peptide for binding.

In the present study, four individual sheep were inoculated with the synthetic tachykinin EKA/B. Their antisera were collected and investigated for cross-reactivity to the four tachykinins mHK-1, SP, hHK-1 and NKB. Similar dilution experiments (as for the anti-tachykinin antisera) for normal sheep serum (Figure 2.2 to Figure 2.5) showed the lack of immune-reactivity of the serum from the non-inoculated sheep for any of the tachykinins containing the FXGLM-NH_2 motif. The four sheep were injected with the same antigen; however, they responded differently in the titres of antibodies they produced specific for each of the four tachykinin peptides. The antibodies raised against EKA/B were likely to cross react with the FXGLM-NH_2 motif especially as it contains a charged amide group with the potential to make this region very immunogenic (Page, 2010). This makes such antibodies raised to complete tachykinin sequences extremely useful tools for the isolation of naturally occurring tachykinins. In contrast, raising antibodies to the unique N-terminal regions of the tachykinins has proved to be problematic in the past as these regions are not only very short but appear not to be very immunogenic (Page, 2004). Moreover, such an approach would not produce antibodies with a wide selectivity property, in order to capture as complete as possible repertoire of the tachykinins (and their potential variants) from a range of cell and tissue types.

The tachykinins are short linear peptides, that form an α-helix in their secondary structure (Mantha *et al.*, 2004), and possess an immunogenic backbone on the FXGLM-NH_2 motif. This study successfully showed the reactivity of the polyclonal antibodies raised against the synthetic tachykinin EKA/B (Page *et al.*, 2003), which recognised and responded to the tachykinin peptides hHK-1, SP, mHK-1 and NKB (the alignment of the four sequences is showed in Table 2.4). Previous studies using synthetic derivatives of SP for the determination of SP-specific antibody cross-reactivity showed that the C-terminal pentapeptide *i.e.* the FFGLM-NH_2 is the smallest sequence that was able to cross-react with SP-C-terminally raised antisera (Sandberg *et al.*, 1981). A previous study used chemical derivatives of SP replacing specific amino acids and investigated the effect on the biological activity of the derived peptide. The residues of SP recognised by the specific antibodies, showed that phenylalanine^7 and glutamine^5 and glutamine^6 are central amino acids essential for recognition of the SP peptide by anti-SP antibodies (Creminon *et al.*, 1995). A study investigating the antigenicity of the SP residues in the hexapeptide $SP_{6\text{-}11}$ (a synthetic truncated form of SP which still retains biological activity) suggested that the combination of residues phenylalanine^7 and phenylalanine^8 formed an epitope whose antigenicity was completely abolished in synthetic analogues where the two residues were replaced with α, β –dehydroxyphenylalanine and glycine^9 with histidine; it was reported that the role of glycine was to maintain a distance between the side chains of phenylalanine^7 – phenylalanine^8 and leucine^{10} – methionine^{11} (Janecka *et al.*, 1987).

The C-terminal amidation of the tachykinin peptides plays an important role in the development of antibodies including anti-tachykinin antibodies (Page, 2010; Conzelmann & Jekely, 2012). Page and colleagues (2010) tested commercial antibodies developed against NKB with the intention of investigating whether they could recognise a non-amidated form of NKB using synthetic glycine C-terminally extended NKB (DMHDFFVGLMG). However, this synthetic undecapeptide showed no significant cross-reactivity with the commercial NKB antibodies, suggesting that C-terminal amidation is crucial for the recognition of the natural epitope. Other studies have also compared the effect of C-terminal amidation and N-terminal acetylation on the immunogenicity of a peptide (*e.g.* derived from a neurotoxin specific for a T-cell receptor

subunit). It was reported that the N-terminal acetylation increased the immunogenicity of the peptide. However C-terminal amidation did not increase immunogenicity *per se* but had a significant effect on the specificity of the antibodies. The antibodies raised against the amidated peptide recognised the amidated peptide as antibody titres showed, however the recognition of the antibodies for the non-amidated version of the same peptide was completely abolished and *vice versa* (Maillere & Herre, 1997). C-amidated peptides have been reported in terms of antibody production as having the advantages of increased specificity in the recognition of the peptide and protection from degradation, thus offering a better implication in eliciting an efficient immune response (Blanchet *et al.*, 2001). X-ray crystallography studies on amidated di-peptides have confirmed that the amide group generates (in the presence of chlorine ions as well) strong hydrogen bonds that are potentially necessary for the bioactivity of the C-amidated peptides and play a strong role in specific interactions of the peptides with antibodies and receptors (In *et al.*, 2002). From our antibody titre curve experiments (Figures 2.2 to 2.5) aiming to investigate the cross-reactivity of the anti-tachykinin antibodies, we assume that there is a tachykinin backbone which the antibodies recognise comprising residues of both the common motif and the variable N-terminal sequence and the C-terminal amide of course plays a critical role in recognition.

When developing antibodies to a specific antigen, the "antibody affinity", termed as the strength of a single antigen–antibody bond that is produced by the attractive and repulsive forces developed between the two molecules as well as the "avidity" of an antibody for its antigen, determined by the sum of all of the individual interactions taking place between individual antigen-binding sites of antibodies and determinants on the antigens, must be accounted (Lipman *et al.*, 2005). Affinity can be determined using a surface plasmon resonance experiment (Lipman *et al.*, 2005). Antibodies with a high affinity bind larger amounts of antigen with a greater stability in a shorter time than those with a low affinity and are preferable for use in immunochemical techniques (Lipman *et al.*, 2005). An antibody with high affinity for its antigen can function more effectively in the immune system and usually increases during an immune response, called "affinity maturation", *in vivo*. In our immunoaffinity purification experiment, low pH elution

buffer in combination with a longer incubation time during elution was applied, eluting antibodies of different affinities for tachykinin peptides, as it was aimed to capture apart from the forms of the peptides shown in Table 2.4, the extended or truncated forms as well as those with an unknown affinity for the anti-tachykinin antibodies. The purified antibodies were subsequently immobilised on a Sepharose matrix. Antibodies react with the cyanogen bromide-activated beads through amine groups (Figure 2.1), hence, the immobilisation procedure might result in the inactivation of antibodies, predominantly IgG, depending on their orientation after attachment. Bivalent IgG antibodies can covalently bind the Sepharose beads through their Fc domain in which case they remain fully active as their Fab domains sustain their antigen-binding ability (Jung *et al.*, 2008). In the case, the IgG antibodies immobilise through one of the two Fab domains, they remain partially active being able to recognise and bind peptides (antigens). However, if both of the two Fab domains covalently bind to the Sepharose beads they are rendered inactive (Jung *et al.*, 2008). Taking these factors into consideration, the three Sepharose-antibody conjugates built in the experiments described in this Chapter were challenged with a mixture of the four synthetic peptides to test their binding ability. In Chapter 3 (Section 3.5) the experiment to test the ability of the three Sepharose-antibody conjugates to bind the peptides is described.

Previous publications have employed the immobilisation of antibodies raised against a peptide motif on a Sepharose matrix as a means of capturing the mature sequence of naturally occurring peptides and identifying its' molecular weight using MALDI-TOF (Tobari *et al.*, 2011). Similarly to the tachykinins which comprise the FXGLM-NH_2 motif, the gonadotrophin-inhibitory hormones (GnIH) are a family of peptides also possessing a characteristic C-terminal motif LPXRF-NH_2, where X is L or Q. The GnIH peptides belong to the larger family of RFamide peptides comprising the kisspeptins and the RFamide-related peptides which comprise the motif RX-NH_2, where X is F or Y (Tsutsui *et al.*, 2000). Antibodies raised against the motif LPXRF-NH_2 in quail cross-react for LPXRF-NH_2 peptides of other species as shown with ELISA titres; anti-LPXRF-NH_2 antibodies have been purified from antiserum and immobilised on cyanogen bromide-activated Sepharose (Ubuka *et al.*, 2012). Two novel endogenous peptides with

a neuro-endocrinological role, comprising the RF-related peptide motif namely the RFRP-1 and RFRP-3 were identified in hamster brain using the Sepharose-antibody conjugate as an affinity medium to capture the peptides and confirm their structure as predicted from cDNA sequences, using MALDI-TOF (Ubuka *et al.*, 2012). A similar methodology was employed to identify their orthologs in humans (Ubuka *et al.*, 2009), rat brain (Ukena *et al.*, 2002), fish (Sawada *et al.*, 2002), and other peptides namely the growth hormone-releasing peptide-related peptides-1, -2, -3, comprising the motif LPXRF-NH_2 (Ukena *et al.*, 2003). Immunoaffinity purification was also used to immobilise on cyanogen bromide-activated Sepharose anti-RX-NH_2 antibodies raised against the rat peptide kisspeptin-17 (regulator of the reproductive axis) which comprises a C-terminal motif (RY-NH_2) common for all kisspeptins. The affinity medium was used to capture and identify a novel peptide, gonadotrophin-releasing hormone-I from zebra finch brain (Tobari *et al.*, 2010).

In conclusion the experiments described in this Chapter show a methodology to construct a Sepharose-peptide conjugate efficient to purify anti-tachykinin antibodies from ovine antisera and immobilise the purified antibodies on a Sepharose matrix to construct a conjugate capable of capturing peptides from tissue extracts. In the subsequent Chapters the application of the Sepharose-antibody conjugates for the capture and identification of tachykinin peptides is described.

Chapter 3. A reverse phase HPLC method for the separation of mHK-1, SP, hHK-1 and NKB and their detection using MALDI-TOF.

3.1) Introduction.

A detailed description of the procedure for the development of a Sepharose-anti-tachykinin antibody conjugate, for the purpose of capturing, enriching and separating both synthetic and endogenous tachykinin peptides was given in Chapter 2. This chapter gives a description of the development of an analytical method using reverse phase high pressure liquid chromatography (RP-HPLC) for the separation and detection of the four tachykinin peptides *i.e.* mHK-1, SP, hHK-1 and NKB. This chapter also describes their identification using matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF). Moreover, two peptide extraction methods were compared, the first using an acidified methanol procedure and the second method using Bennett's solution (1 M HCl with 5% (v/v) formic acid, 1% (v/v) TFA and 1% (w/v) NaCl) (Bennett *et al.*, 1981).

3.1.1) Peptide extraction methods and assessment of their efficiency with RP-HPLC and immunoassays.

The efficiency of peptide extraction buffers was assessed with RP-HPLC with immunoassay as a quantitative method (Brodin *et al.*, 1994). In example, previous studies have used acidified methanol for the extraction of the repertoire of peptides from rat brains (Sturm *et al.*, 2010, Dowell *et al.*, 2006) and also whole organisms such as the pacific white shrimp (Ma *et al.*, 2010). The protocols use acetic acid contained in methanol that neutralises the polar groups of peptides rendering them more organic solvent-soluble (Ma *et al.*, 2010). However, a further step for the removal of proteins and lipids using a methanol/chloroform extraction has been used (Kurihara *et al.*, 1977) and was also performed in the present study. Nevertheless, aqueous acidic buffers like Bennett's solution (Bennet *et al.*, 1981) or even acetic acid have also been used for the extraction of peptides from rat brain (Dowell *et al.*, 2006) or human placenta (Lovell *et*

al., 2007). Aqueous acetic buffers have the advantage of extracting fewer lipids from the tissue and allow the usage of a larger amount of tissue when necessary.

Tachykinin peptides have been separated from complex repertoires of peptide extracts, then detected or quantified using RP-HPLC (Troger *et al.*, 2001; Lorenz *et al.*, 2008). Having a hydrophobic C-terminal, for their retention a system employing as a stationary phase a reversed phase C12 or a C18 column was used and a mobile phase of acetonitrile with trifluoroacetic acid (TFA) was applied as a hydrophilic to hydrophobic gradient for their elution, while a U.V. detector was incorporated to measure the absorbance of the peptide bonds in the range of λ 205 to 215 nm. A relevant study for the quantification of the levels of SP and secretoneurin (a 33-amino acid neuropeptide) in the vitreous fluid, fractionated on a C18 column the repertoire of peptides extracted from the fluid, before quantifying immunoreactivity in an immunoassay using an antibody specific for SP or secretoneurin (Troger *et al.*, 2001; Lorenz *et al.*, 2008). The RP-HPLC system used combined a preparative C18 column and a mobile phase of acetonitrile with TFA/water, applied as a gradient, which was previously calibrated with synthetic peptides (Troger *et al.*, 2001; Lorenz *et al.*, 2008). The levels of NKA and NKB in the human retina and iris-ciliary body complex and the rat trigeminal nerve have been quantified, at fmol/mg wet weight concentrations, by immunoassay after separation into fractions of the tissue extracts (on a C18 column eluted with a gradient of acetonitrile with TFA/water). The antibody raised against the two tachykinins and used in the immunoassay showed a 63% cross-reactivity between the two peptides co-expressed in the eye tissues (rendering immunohistochemistry impractical); hence the repertoire of peptides was first fractionated on a preparative C18, and then the two peptides were detected and quantified with the immunoassay (Schmid *et al.*, 2005; Schmid *et al.*, 2006). A reversed phase C18 stationary phase and a gradient of acetonitrile with TFA/ water mobile phase was also applied to elute the retained N-terminal fragment of the pro-tachykinin A precursor peptide (NT-PTA) from the repertoire of peptides found in human blood and lumbar cerebrospinal fluid (CSF) (Ernst *et al.*, 2008). The NT-PTA fragment is a potential surrogate for the quantification of SP with a sandwich immunoassay in disease samples since SP is rapidly degradable *in vitro* (Ernst *et al.*, 2008). This study suggests complete

processing of the TAC1 precursor at the dibasic arginine (RR) cleavage site and contradicted earlier reports that suggest the expression of NKA in the CSF (Toresson *et al.*, 1993).

3.1.2) Combination of liquid chromatography online or offline with mass spectrometry for the detection of tachykinin peptides.

Reversed phase liquid chromatography (LC) on-line with an electrospray ionisation (ESI) source and peptide detection with MS offers the advantage of high sensitivity, distinct peptide identification and quantification (Svensson *et al.*, 2003). An approach was applied by Svensson and colleagues (2003) of combining sample preparation, and MS peptide profiling that enabled the simultaneous detection of more than 550 endogenous neuropeptides in 1 mg of hypothalamic extracts. LC-ESI-MS with a C18 stationary phase and a mobile phase of acetonitrile/water applied as a gradient was part of an analytical method for the detection of the classical tachykinins SP and NKA in the rat hypothalamus and the discovery of novel peptides (Svensson *et al.*, 2003). The tachykinins SP and the TAC1 (AA 72–94) were also detected in the suprachiasmatic nucleus of rats by means of solid phase extraction and LC-MS/MS in an effort to elucidate the peptidomic contents of the brain nucleus (Lee *et al.*, 2010).

LC off-line with MALDI-TOF has been commonly applied for the detection not only of tachykinins but also for the discovery of novel peptides in the rat brain. MALDI-TOF had been used in combination with the C18 ZipTips (Millipore), which are pipette tips with a C18 tip with the ability of further concentrating samples before spotting and analysis. The ability of C18 ZipTips (Millipore) to adsorb compounds for their detection off-line on MALDI-TOF was used by Hatcher and colleagues (2008) to collect the neuropeptides secreted by the suprachiasmatic region (site of the master circadian clock) with an *in situ* collection strategy, *i.e.* the direct collection of the peptide contents of the brain structure using the C18 ZipTips and immediate elution with spotting on the MALDI-TOF plate. NKB was detected in the brain nucleus and novel peptides were also identified in this manner. In the same study, lyophilised rat brain extracts (with solid phase extraction) were separated on RP-HPLC and the fractions were concentrated with C18 ZipTips

(Millipore) before spotting on MALDI-TOF plates; SP and NKB were identified among the neuropeptides, as contributors to the circadian clock (Hatcher *et al.*, 2008). The approach of peptide extraction, fractionation by LC and detection on MALDI-TOF has also been used in the analysis of the tachykinin-related peptides from *e.g.* insects expressing tachykinin-related peptides. The peptidome of the central nervous system of the species *Delia radicum* was analysed, identifying novel tachykinin-related peptides (Audsley *et al.*, 2011). The tissue was homogenised in acetified methanol, followed by fractionation on a C18 column and spotting of fractions on MALDI-TOF (Audsley *et al.*, 2011).

3.2) Scope for the development of a RP-HPLC method in combination with MALDI-TOF in the present study.

The scope of this Chapter was to develop a RP-HPLC method for the separation of the four tachykinin peptides (mHK-1, SP, hHK-1, NKB). RP-HPLC in combination with an ELISA assay, were used as a comparative method of the efficiency of an acidified methanol extraction buffer and Bennett's solution to extract natively occurring tachykinins from rat brain. A reversed phase C12 column was employed to separate tachykinins extracted from two rat brains using two different extraction buffers 1) Bennett's solution and 2) acidified methanol extraction buffer. The peptide extracts were then separated into fractions and the immuno-reactivity of each fraction was checked using immunopurified anti-tachykinin antibodies, as a comparative methodology to estimate extraction efficiency. An analytical method was also developed on a reversed phase C18 column for the separation of the four synthetic peptides (mHK-1, SP, hHK-1, NKB). Fractions of the eluted peptides were collected and the elution fraction of each peptide was confirmed with MALDI-TOF (Section 3.4). Another procedure described in this Chapter (Section 3.5) was the assessment of the recovery of the acidified methanol/chloroform extraction and enrichment of peptide using the Sepharose anti-tachykinin antibody conjugate, also investigating its efficiency. The presence of the four peptides in the immunoaffinity purification eluent was confirmed with MALDI-TOF.

3.3) Methods and Results.

3.3.1) Rat brain homogenisation and peptide extraction.

Bennett's solution consisting of 1 M HCl 5% (v/v) formic acid, 1% (v/v) TFA and 1% (w/v) NaCl was prepared in a 500 ml graduated Duran bottle by adding 5 g of NaCl (Sigma-Aldrich, S-3014) into 41 ml of 12.07 M HCl (Riedel-de Haen #07102), followed by the addition of 25 ml of ~100% formic acid (BDH, 101155F) and 5 ml of ~100% TFA (Sigma-Aldrich). The final volume of the buffer was made up to 500 ml with distilled H_2O. Acidified methanol buffer consisting of 90% (v/v) methanol with 9% (v/v) acetic acid was prepared in a 500 ml Duran bottle, by adding 450 ml ~100% methanol (VWR) and 45 ml of ~100% acetic acid (Sigma- Aldrich, 33209).

The procedure detailed here is summarised in Figure 3.1. Two rat brains weighing ~1 g each (Charles River; kept at -70 °C) were thawed on ice for a few minutes. The two rat brains were cut into smaller pieces using a scalpel (Fisher) and homogenised into sub-cellular components by pushing the pieces through a 10 ml syringe (BD Plastipak; without a needle) and collected into two 2 ml eppendorf tubes. Into the two Eppendorf tubes with the homogenised rat brain tissue, either 1 ml of the Bennett's solution or 1 ml of acidified methanol buffer was added. A scalpel was fitted into the tube and used to blend the homogenised brain with the extraction buffer until a homogenous mixture was formed. The tubes were placed at 4°C overnight. The following morning, the two 2 ml Eppendorf tubes were centrifuged at 7,558 g (13,000 RPM) for 15 min in a table-top centrifuge at room temperature for the homogenised tissue to form a pellet. From the supernatant, 1 ml from each tube was recovered and transferred into two fresh 1.5 ml Eppendorf tubes. Into the remaining homogenised tissue pellet, 1 ml of extraction buffer was added used to re-suspend the tissue pellet and the remaining peptide was extracted by incubating the formed homogenates for 2 hours at 4°C. Following incubation, centrifugation of the two 2 ml tubes was performed and another 1 ml of the supernatant from each 2 ml tube was collected and transferred into two fresh 1.5 ml Eppendorf tubes.

The four 1.5 ml Eppendorf tubes with the supernatants from the two extraction procedures were centrifuged at 7558 g (13,000 RPM) for 15 min for any remaining cell debris present to pellet. In total ~1.8 ml of supernatant from the incubation of the rat brain tissue with Bennett's solution was collected. The supernatant (0.9 ml) from each of the two tubes with the methanol extraction was collected and transferred into two fresh 2 ml tubes. An equivalent volume (0.9 ml) of ~100% chloroform was pipetted into the two 2 ml tubes, which were vortexed for 15 min until a homogenous mixture was formed. The two 2 ml tubes containing the homogenous mixture of peptide in methanol buffer and chloroform were centrifuged at 7558 g (13,000 RPM) for 10 min. Three phases were formed, a lower chloroform phase containing lipids, an intermediate phase with denatured proteins and an upper methanol phase with peptides and sugars. From each 2 ml tube, 0.8 ml of the upper phase was recovered taking care not to disrupt the interphase. Approximately 1.6 ml of upper methanol phase (containing a repertoire of peptides) was combined in a fresh 2 ml Eppendorf tube. The 1.8 ml pool of supernatant collected from the incubation of the tissue with Bennett's solution and the 1.6 ml pool of upper phase collected from the acidified methanol procedure after chloroform extraction were each diluted ten-fold in 0.1% (v/v) aqueous TFA.

Two C18 SepPak cartridges (Waters, WAT051910) were primed with ~ 100% methanol (10 ml) and equilibrated with 0.1% (v/v) TFA (5 ml). A 10 ml syringe was attached onto each cartridge as a funnel and ~5 cm of silicon tubing (BioRad) was attached to establish a ~1 ml/min flow rate. The diluted peptide extracts were passed through the two cartridges using the above set up. Then, each cartridge was washed by pushing through 10 ml of 0.1% (v/v) TFA followed by 10 ml of distilled H_2O. The bound peptide was eluted from the cartridges by pushing through 1 ml of elution buffer (described in Table 4.1) and collecting the eluent into a fresh 1.5 ml Eppendorf tube. Binding buffer (50 µl) consisting of 1 x PBS containing 10 mM EDTA with 0.01% (w/v) octyl β-D-glucopyranoside (O.G.) and 0.02% (w/v) NaN_3 (used as a bacteriostat) plus serine and cysteine proteases inhibitors was added into the tube containing the methanol eluent. Each sample was concentrated to 50 µl by blowing a "slow" stream of nitrogen gas over the sample.

Table 3.1. The C18 Sep Pak cartridge elution buffers.

[a] Cartridge	[b] Sample	[c] Elution buffer
Cartridge 1	~ 18 ml of the diluted supernatant from Bennett's solution extraction	60% (v/v) acetonitrile with 0.1% (v/v) TFA
Cartridge 2	~ 16 ml of the diluted supernatant from methanol extraction	~ 100% methanol

[a] The respective cartridge used for the binding/elution of peptide. [b] The total sample volume passed through each respective cartridge. [c] The elution buffer pushed through each cartridge to elute the bound peptide.

3.3.2) Reversed-phase HPLC for the separation of the extracted peptides

The HPLC instrument (Varian ProStar, Model 210) system employed comprised a stationary reversed phase C12 column, of 250 mm x 2 mm I.D. and particle size 4 micron (Phenomenex). The mobile phases A, ~100% acetonitrile with 0.085% (v/v) TFA and B, distilled H_2O with 0.085% (v/v) TFA were prepared, measuring 1 L of either solvent in a 1 L Duran bottle and pipetting into 850 µl of ~100% TFA (Sigma-Aldrich, 302031). Absorbance was measured at 216 nm using a U.V. detector (Varian ProStar).

The repertoire of peptides extracted from the two rat brains using the two extraction procedures were separated into fractions on the above RP-HPLC system. Into the 50 µl of concentrated eluent from the two extractions, 50 µl of 20% (v/v) acetonitrile with 0.085% (v/v) TFA (initial phase) was added to dissolve any undiluted peptide. 20 µl of the diluted sample was injected into the reversed phase C12 column. The peptides were eluted according to the gradient shown in Table 3.2. Fractions were collected in thirty 1.5 ml centrifuge tubes labelled 1 to 30. A fraction collector (Agilent, Model 1200 fraction collector) was set up to collect 200 µl fractions every minute in the labelled tubes from 10 min to 40 min of the method running time. Next, 20 µl of the synthetic sample of 1 ng/µl of mHK-1 with 20 ng/µl of SP and 1 ng/ µl NKB (simulating the approximate concentrations of the three peptides in the brain) was injected into the system.

Table 3.2. The gradient for the elution of the C12 column.

Time (min)	Flow rate (ml/min)	Mobile phase A: acetonitrile with 0.085% (v/v) TFA	Mobile phase B: distilled H_2O with 0.085% (v/v) TFA
Pre-run	0.2	20%	80%
5	0.2	20%	80%
10	0.2	30%	70%
35	0.2	50%	50%
36	0.2	20%	80%
50	0.2	20%	80%

3.3.3) Testing the 30 fractions for the presence of tachykinin peptide.

Into each 1.5 ml Eppendorf tube containing the 200 µl fractions, 150 µl of bicarbonate buffer was added (the buffers used in this section were prepared as described in Section 2.3.1). The 350 µl of each neutralised sample was transferred into an individual well of a Nunc Peptide Immobilizer Amino strip plate. The strips were wrapped in cling film and left at 4 °C overnight. The following day, the fractions were removed. The un-reactive sites on the wells were blocked with 400 µl of blocking buffer (0.1 M Na_2CO_3 containing 0.1 M $NaHCO_3$, pH ~9.6 with 10 mM ethanolamine) prepared by pouring into a 50 ml Falcon tube, 50 ml of the bicarbonate buffer and adding 30 µl of 16.6 M ethanolamine (BDH, 28306). The strips were incubated for 1 hour on the bench. Each well of the plate was washed three times with 400 µl of wash buffer (0.9% (w/v) NaCl with 0.01% (v/v) Triton X-100). 350 µl of a 1 to 2000 dilution of the immuno-purified "*BO16*" anti-tachykinin antibody (Section 2.3.5) in albumin-phosphate buffer, 0.04 M Na_2HPO_4 with 0.01 M $NaH_2PO_4.2H_2O$ with 0.5% (w/v) BSA buffer was added into each well of the peptide-coated Nunc strips. The strips with the immobilised peptide and the anti-tachykinin antibody were left at room temperature for 3 hours. Each well was washed three times with 400 µl of wash buffer. A 1 to 5000 dilution of the anti-sheep IgG-AP conjugate in albumin-phosphate buffer, 0.04 M Na_2HPO_4 with 0.01 M $NaH_2PO_4.2H_2O$ with 0.5% (w/v) BSA buffer (350 µl) was added into each well. The wells were incubated with the anti sheep secondary antibody at room temperature for approximately 30 min.

The secondary antibody was removed and each well of the Nunc plate was washed three times with 400 µl of wash buffer to remove excess IgG-AP conjugate. pNPP substrate solution prepared in distilled H_2O (350 µl) was added to each well. The wells were left in the dark for 10 min. The reaction was stopped by pipetteing into each well 50 µl 2 M NaOH solution straight into the 350 µl of reaction mixture. The absorbance was read at 405 nm.

3.3.4) Preparation of samples for their detection on MALDI-TOF.

A Bruker Autoflex III MALDI-TOF mass spectrometer was used for further identification of peptide molecular mass. The instrument used the FlexControl software. Polypropylene tubes (0.6 ml) and yellow tips along with the MALDI-TOF stainless steel plate were rinsed with methanol and left to air dry. A fresh solution of α-Cyano-4-hydroxycinnamic acid matrix (HCCA, Agilent Technologies #G2037A) used for the crystallisation of the analyte was prepared by transferring into a clean micro-centrifuge tube an appropriate volume of the α-HCCA matrix (6.2 mg/ml). A volume of 100% ethanol with 1% (v/v) TFA was added to dilute the matrix (matrix: ethanol/ TFA, 2:1 volumes ratio). Then, into 0.6 ml polypropylene tubes, 4 µl of matrix in ethanol with TFA were aliquoted and 1 µl of sample was added. 1 µl of the sample in matrix was taken up from the 5 µl mixture and three spots were made on the MALDI-TOF plate (MTP 384 ground steel Bruker S/N 21761). Also 1 µl of a 5 µg/ml trypsinised BSA stock (kept at -20 °C and left to thaw on ice) was mixed with 4 µl of the matrix in 67% ethanol with 0.7% TFA. 1 µl of the 1 µg/ml BSA in matrix mixture was taken up to make three spots on the MALDI-TOF plate.

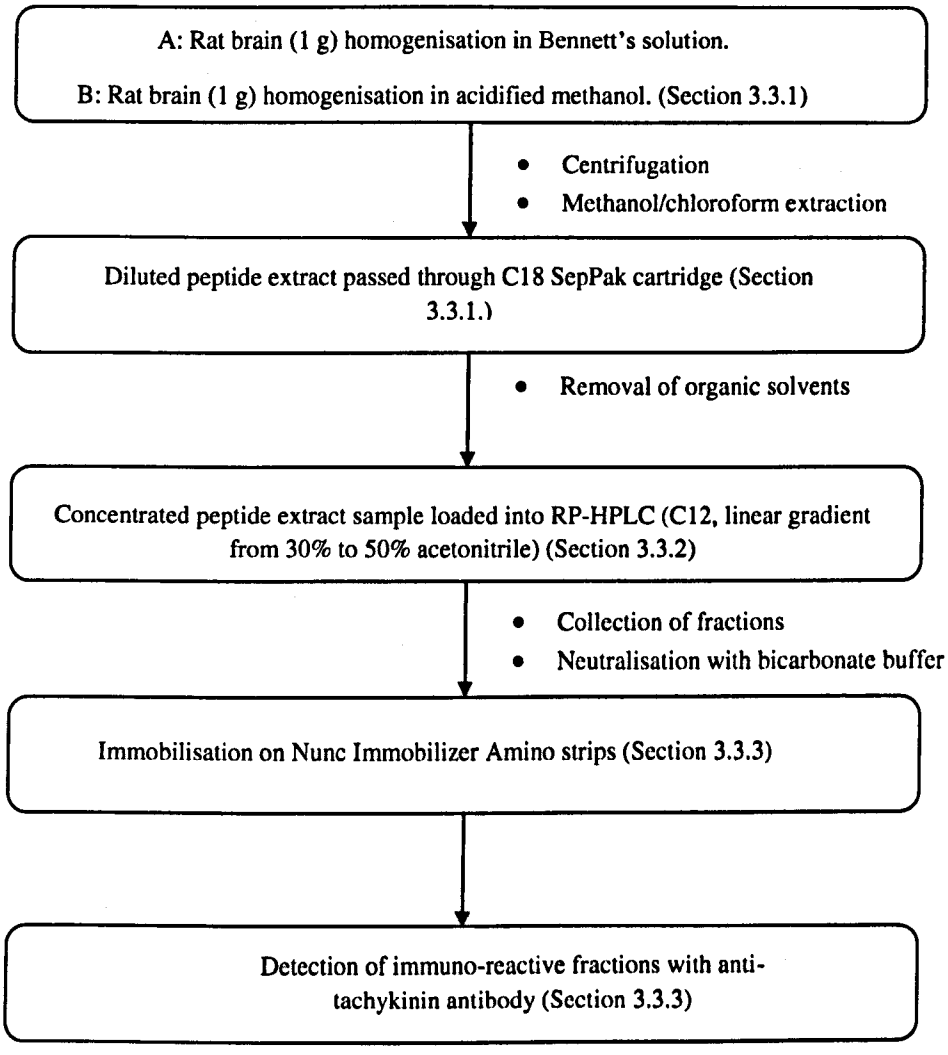


Figure 3.1. The schematic overview of the experimental procedure followed to compare the efficiency of the two peptide extraction procedures.

3.3.5) Investigation of extraction buffer efficiency.

The concentration of SP in the rat brain has been reported as approximately 350 ng per g of brain tissue (Wei *et al.*, 2006) and the concentration of NKB has been reported almost 20 times lower *i.e.* ~18.5 ng per g of brain tissue (Merchenthaler *et al.*, 1992) while currently no reports exist on the concentration of mHK-1 in the brain. A synthetic peptide sample consisting of 1 ng/µl of mHK-1 with 20 ng/µl of SP and 1 ng/µl of NKB (*i.e.* containing 20 times higher concentration of SP than NKB) was prepared and separated by HPLC on a reversed phase C12 column followed by the collection of fractions and immunoassay. The sample (20 µl) was injected into the C12 column and eluted with a gradient ranging from 30% to 50% (v/v) acetonitrile/water with 0.085% (v/v) TFA over 25 min at a flow rate of 0.2 ml per min; thirty fractions (200 µl) were collected, neutralised and immobilised on wells. The chromatogram from the separation of the three peptides is illustrated in Figure 3.2A. This shows the separation of the three synthetic peptides with retention time for mHK-1, 18.20 min, for SP, 22.85 min, and for NKB, 28.75 min. Immuno-reactivity in the collected fractions immobilised onto the wells of the Amino® plate was detected using the immunopurified anti-tachykinin "*BO16*" antibody (also immobilised on Sepharose beads). The separation on reversed-phase HPLC of the mixture of the three synthetic peptides (mHK-1, SP, NKB) followed by immunoassay revealed two peaks. In Figure 3.2B are shown the elution position of mHK-1 (Fractions 10-12, peaking in Fraction 11) and SP (Fraction 13-17, peaking in Fraction 15= 16). The expected elution position of NKB (Fractions 18-20) is represented as a shoulder at the end of the SP profile. The two peaks corresponded to mHK-1 and SP while NKB immunoreactivity was covered by the intensity of the immunoreactivity of SP; also as shown experimentally using MALDI-TOF (Section 3.3.5).

The repertoire of neuropeptides expressed in the rat brain was extracted using two extraction buffers, *i.e.* Bennett's solution and acidified methanol. Separation of the repertoire of the endogenous peptides extracted was performed by RP-HPLC using the same analytical method as described for the separation of the mixture of synthetic peptides. The sample (20 µl) from the extraction using Bennett's solution was divided

into 30 fractions (200 µl), then neutralised and immobilised into the wells of an Amino® plate for the detection of immunoreactivity using the purified anti-tachykinin antibody "*BO16*". The result from the immunoassay is shown in Figure 3.3B. In the "immuno-gram" three peaks were revealed. The peak of higher intensity (relative absorbance of ~0.9 arbitrary units) which appeared in the same position as of synthetic SP (Fractions 14-17) and two peaks corresponding to the other two peptides mHK-1 and NKB, present as shoulder peaks, (mHK-1, Fractions 11-13) and NKB (shoulder peak, Fractions 18-20) were detected. Two additional smaller peaks which could correspond to the oxidised forms of the three peptides or potentially different processed forms of the peptides (Fractions 25-27, and 28 and 30) were also observed (Figure 3.3B).

Next, the sample (20 µl) from the extraction using acidified methanol was run on RP-HPLC using the same analytical method as for the separation of the synthetic peptide mixture and sample from extraction using Bennett's solution. In Figure 3.4A the chromatogram is shown; many peaks are detected with lower relative absorbance (compared to the Bennett's solution extract). The immunoreactivity detected in the 30 fractions collected is illustrated in Figure 3.4, B. Three peaks were detected in the same position, as the three synthetic peptides, mHK-1 (Fractions 11-13), SP (Fractions 15-18) and NKB (Fractions 19-22). Peptides were successfully extracted using both of the peptide extraction buffers *i.e.* the Bennett's solution or the acidified methanol with chloroform extraction. However, it was observed that the peaks corresponding to mHK-1, SP and NKB on the chromatogram from the run of the three synthetic peptide sample (Figure 3.2,A) compared to the peaks of the three peptides on the "immuno-gram" showing the immuno-reactivity of the fractions (Figure 3.2,B) are out of synchronisation by 1.5 min. This was attributed to the tubing connecting the HPLC instrument to the fraction collector which diluted the peptide and elongated its elution from the instrument. Therefore, another analytical method was developed using a C18 column on a HPLC system with more efficient tubing for the collection of fractions (described in Section 3.3).

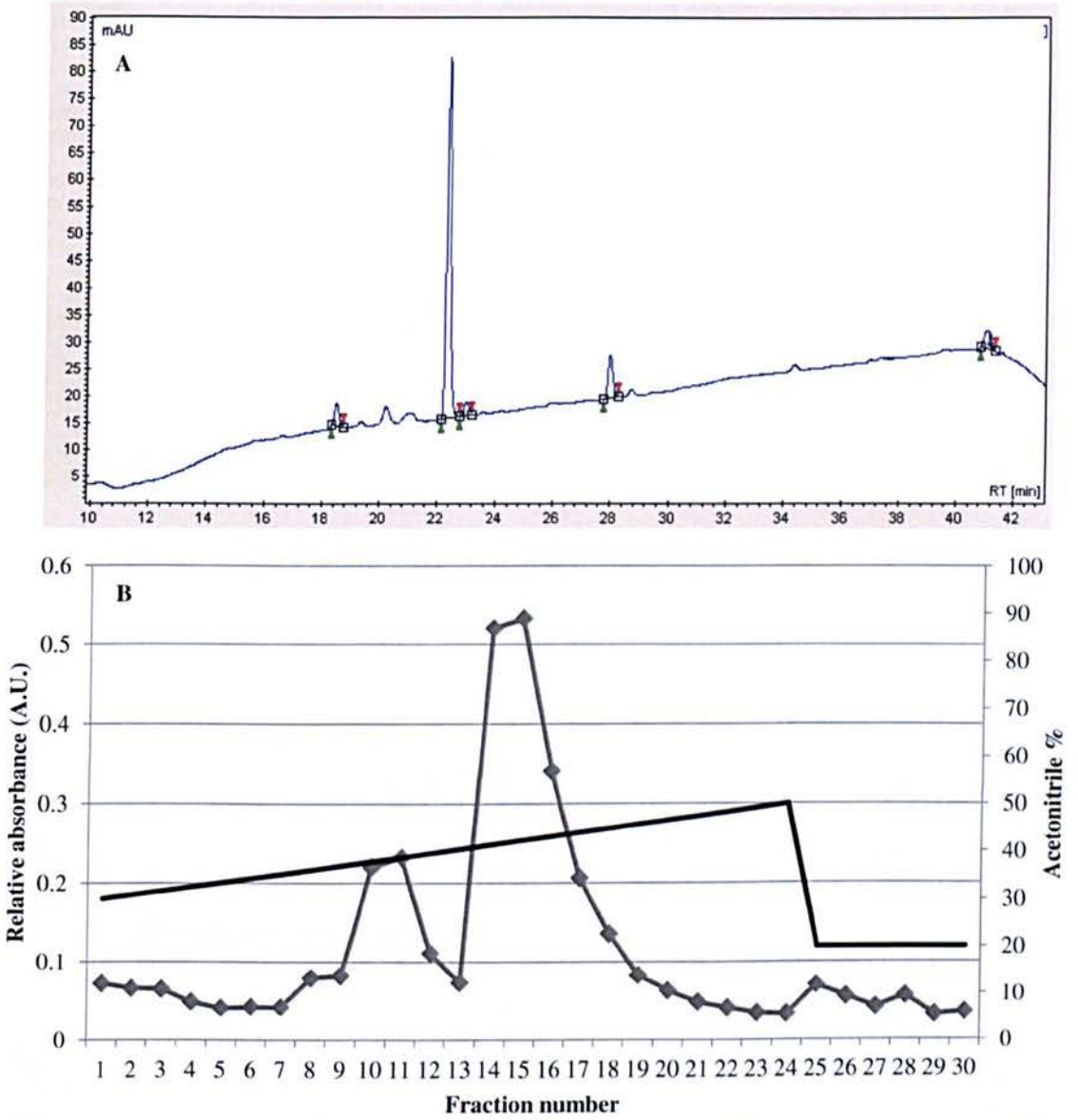


Figure 3.2. Chromatogram of the synthetic peptides mHK-1, SP and NKB and immuno-reactivity of the collected fractions. (**A**) The chromatogram of the separation of the sample (20 µl) of synthetic 1 ng/µl of mHK-1 with 20 ng/µl of SP and 1 ng/µl NKB. The sample was injected into a C12 column and eluted with a gradient of acetonitrile with 0.085% (v/v) TFA/water. The applied gradient started from 30% (v/v) to 50% (v/v) acetonitrile with 0.085% (v/v) TFA over 25 min, returning to 20% (v/v) acetonitrile with 0.085% (v/v) TFA and remaining constant for 14 min. The flow rate was set to 200 µl per min. The absorbance was measured at 216 nm. The retention time of the mHK-1 was 18.21 min, of SP was 22.85 min and for NKB was 28.75 min. (**B**) Immuno-reactivity in the fractions collected from the separation of the synthetic peptides. The black line indicates the gradient profile (percent acetonitrile, right ordinate). Thirty fractions (200 µl) were collected, neutralised in bicarbonate buffer and immobilised into wells, immuno-reactivity was determined using anti-tachykinin antibody BO16. The relative absorbance of the immunoreactivity (left ordinate) and the fraction number is illustrated. The elution position of mHK-1 (fractions 10-12, peaking in fraction 11) and SP (fractions 13-17, peaking in fraction 15= 16) are indicated above. The expected elution position of NKB (fractions 18-20) is represented as a shoulder at the end of the SP profile.

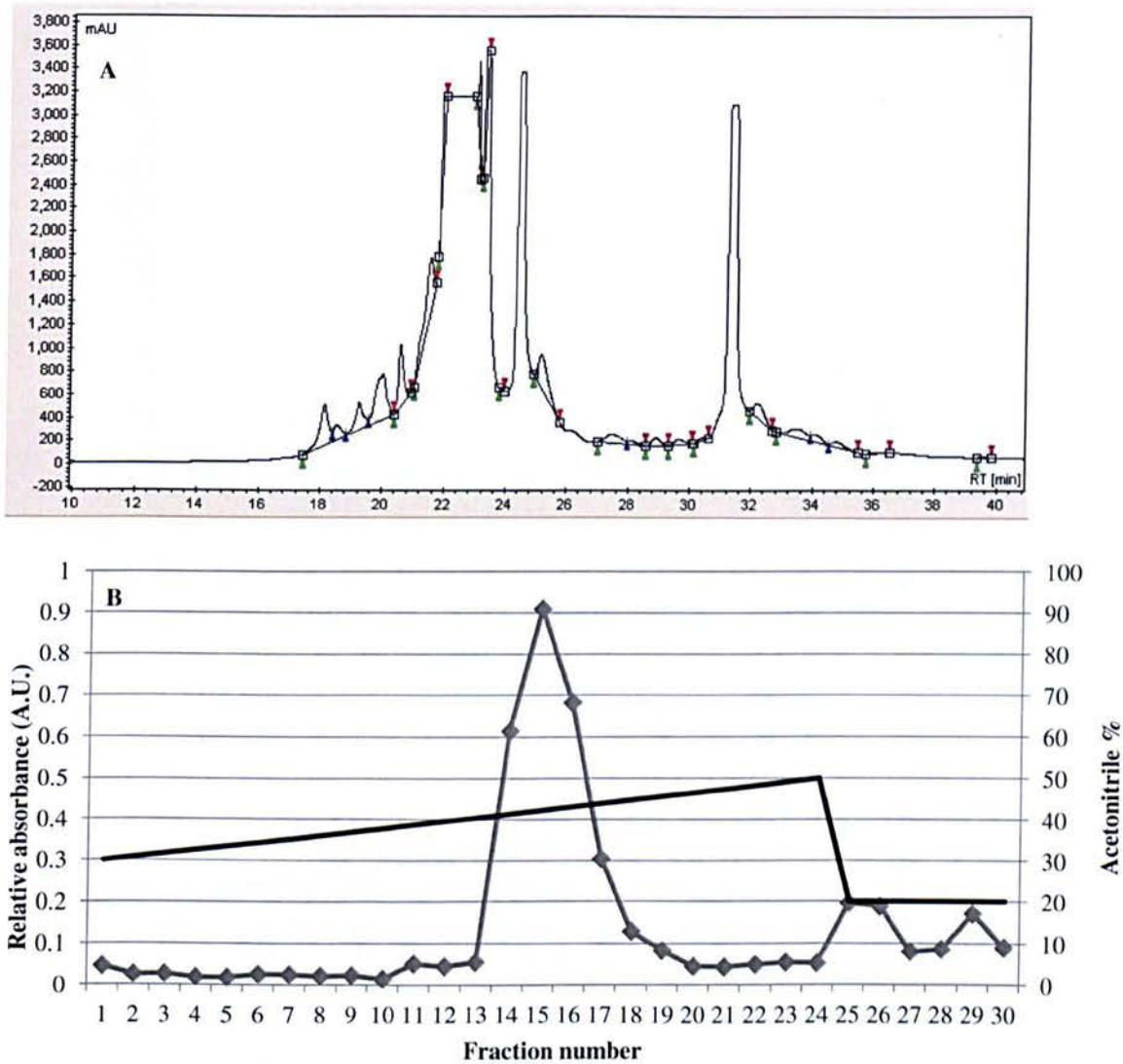


Figure 3.3. Bennett's solution extraction. (**A**) Chromatogram of the separation of the rat brain acid extract. 20 µl from the acid extract of rat brain was loaded into a reversed phase C12 column and eluted with a gradient of acetonitrile with 0.085% (v/v) TFA/water. The sample was eluted with a gradient of 30% to 50% (v/v) acetonitrile with 0.085% (v/v) TFA over 25 min, returning to 20% (v/v) acetonitrile with 0.085% (v/v) TFA and remaining constant for 14 min. The flow rate was set to 0.2 ml/min. The absorbance was measured at 216 nm. The retention time was measured in min. The chromatogram illustrates the repertoire of endogenous peptides, and potential products of protein degradation along with contaminant proteins soluble in the acid buffer following centrifugation. (**B**) The black line indicates the gradient profile (percent acetonitrile, right ordinate). Tachykinin immunoreactivity in the fractions collected from the separation of the rat brain acid extract. Thirty fractions (0.2 ml) were collected, neutralised in bicarbonate buffer and immunoreactivity was determined using the anti-tachykinin antibody BO16. The relative absorbance of the immunoreactivity (measured in arbitrary units, left ordinate) and the fraction number is illustrated. The elution position of SP is indicated above (fractions 14 -17). The expected elution position of NKB (fractions 18 = 20) and mHK-1 (fractions 11- 13) are in the same fraction number as the synthetic peptides. The immunoreactivity detected in fractions 25-26 and 29 is potentially the oxidised forms of the peptides or alternatively processed forms of these tachykinins.

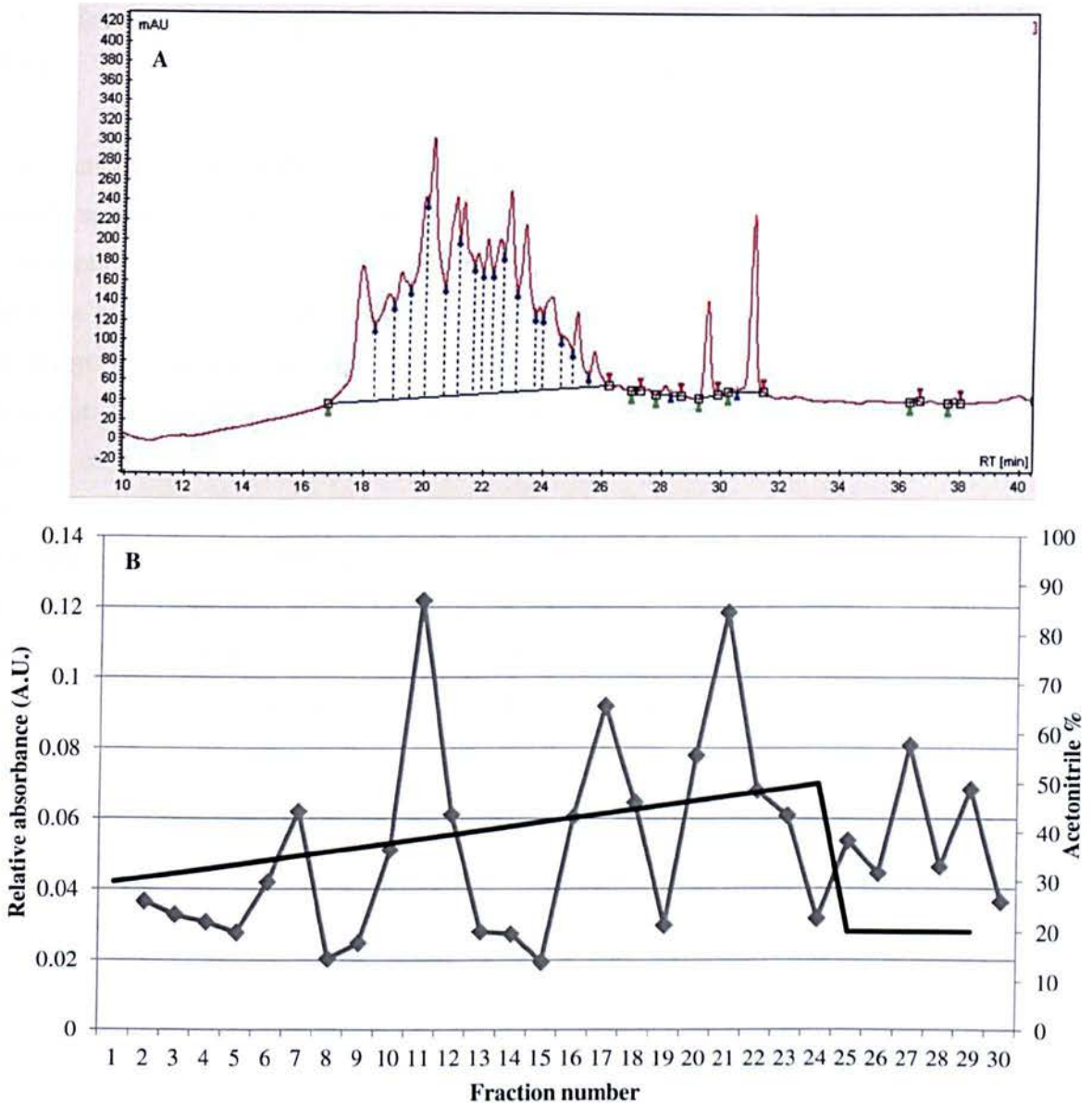


Figure 3.4. Acidified methanol extraction. (**A**) Chromatogram of the separation of the rat brain following acidified methanol extraction and chloroform purification. 20 µl of the rat brain methanol extract was loaded into a reversed phase C12 column and eluted with a gradient of acetonitrile with 0.085% (v/v) TFA/water. The sample was eluted with a gradient of 30% to 50% (v/v) acetonitrile with 0.085% (v/v) TFA over 25 min, returning to 20% (v/v) acetonitrile with 0.085% (v/v) TFA and remaining constant for 14 min. The flow rate was set to 0.2 ml per min. The absorbance was measured at 216 nm. The retention time was measured in min. The relative absorbance of endogenous peptides, potential peptide fragments from protein degradation was much lower in the methanol extract. This is possibly because more contaminating polypeptides were removed during the chloroform extraction stage. (**B**) The black line indicates the gradient profile (percent acetonitrile, right ordinate). Immunoreactivity in the fractions collected from the separation of the rat brain acidified methanol/chloroform extract. Thirty fractions (0.2 ml) were collected, neutralised in bicarbonate buffer, immobilised on wells and immunoreactivity was determined using anti-tachykinin antibodies BO16. The relative absorbance (A.U.) of the immunoreactivity (left ordinate) and the fraction number (below) are illustrated. The elution position of SP (fractions 15-17), NKB (fractions 19-22) and mHK-1 (fractions 10-12) are indicated in the above peaks.

3.3.6) Spotting and detection by MALDI-TOF of synthetic peptides fractionated by HPLC.

The fractions 15 and 19 (Section 3.3.5, Figure 3.2) from the separation of the synthetic peptide sample that contained 1 ng/µl of mHK-1 with 20 ng/µl of SP and 1 ng/µl NKB were spotted (Section 3.3.4). The MALDI-TOF instrument was calibrated by measuring the mass of the peptides yielded from the trypsinisation of BSA and aligning them with the theoretical masses. The laser intensity was set to 50% and 200 shots were made on each spot. The fractions 15 and 19 collected from the separation of the synthetic peptide mixture on RP-HPLC were spotted on the MALDI-TOF plate. In Figure 3.5A, SP (m/z 1347.758 amu) was detected in high abundance in Fraction 15, also the oxidised form SP (O) (m/z 1363.747 amu) and a peak with m/z 1311.657 amu potentially SP with loss of two water molecules. In Figure 3.5,B the peaks detected in Fraction 19 are shown. Peaks corresponding to NKB (m/z 1210.588 amu), NKB (O) (m/z 1226.579 amu) also the NKB Na^+ adduct (m/z 1232.572 amu) and the NKB K^+ adduct (m/z 1248.548 amu) were detected.

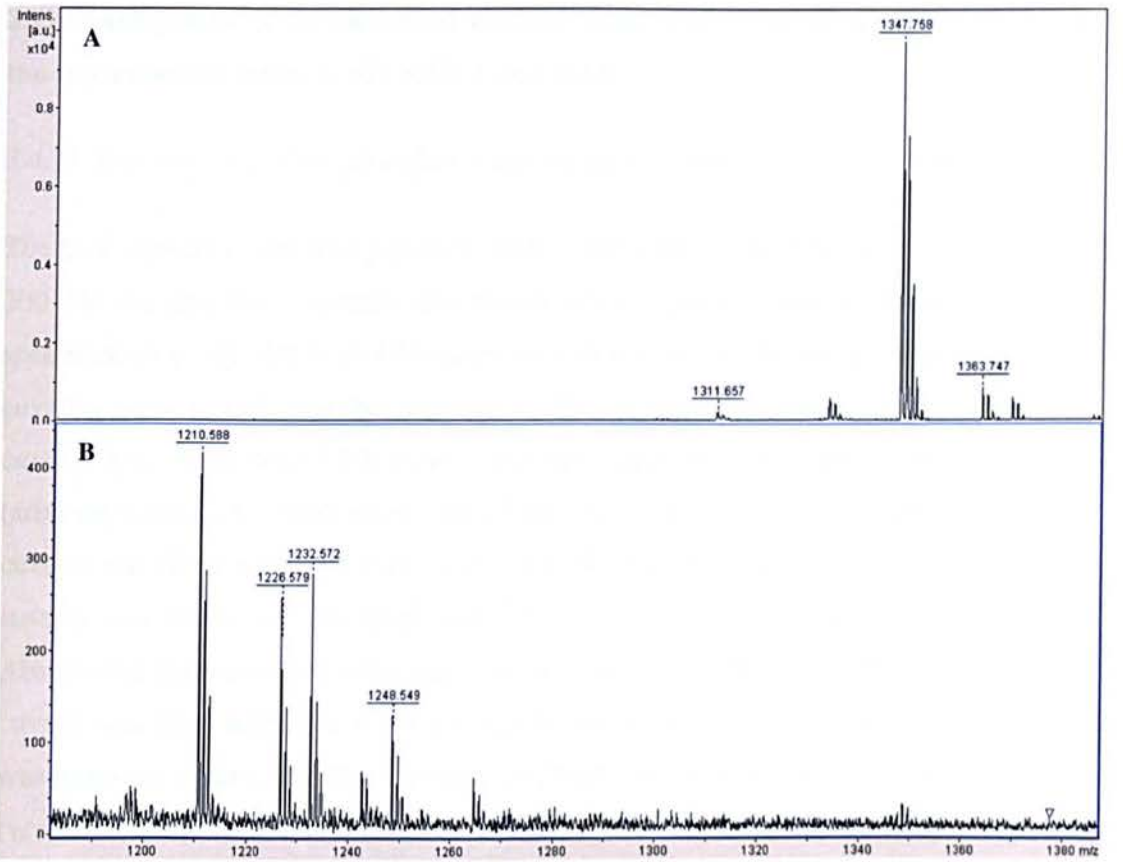


Figure 3.5. MALDI-TOF spectra of the synthetic peptides collected in Fractions 15 and 19. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity of the molecular ions. Laser intensity was set to 50%, and 200 shots per spot. (A) Fraction 15 the peak at m/z 1347.758 amu corresponds to SP, the oxidised form of SP (O) (m/z 1363.747 amu) and SP (m/z 1311.657 amu) with potential loss of two water molecules detected. (B) Fraction 19 a potential fraction corresponding to NKB. NKB (m/z 1210.588 amu), NKB (O) (m/z 1226.579 amu), NKB $+Na^+$ adduct (m/z 1232.572 amu) and the NKB $+K^+$ adduct (m/z 1248.549 amu) were detected.

3.4) Development of an analytical method using a reversed phase C18 column for the separation of mHK-1, SP, hHK-1 and NKB.

3.4.1) Measurement of the absorbance wavelength of mHK-1, SP, hHK-1 and NKB.

The U.V. spectra of the four peptides mHK-1, SP, hHK-1 and NKB were recorded from 200-240 nm and the maximum absorbance of each peptide was determined. The UV-spectrometer (Cary 100 Scan UV-spectrometer) was set to 200 nm and two empty glass cuvettes were placed into the instrument. The background was set to zero. The first cuvette was filled with 25% (v/v) acetonitrile and measurements of the absorbance (arbitrary units, A.U.) were taken every 2 nm from 200 nm to 240 nm. Then, the second cuvette was filled with 25% (v/v) acetonitrile the background was set to zero. The first cuvette was taken out and filled with 25% (v/v) acetonitrile with 0.04% (v/v) TFA. Absorbance measurements were taken every 2 nm, from 200 nm to 240 nm. The second cuvette was filled with 25% (v/v) acetonitrile with 0.04% (v/v) TFA and the background was again set to zero. The first cuvette was filled with sample 1 μg/ml of mHK-1 in 25% (v/v) acetonitrile with 0.04% (v/v) TFA. Absorbance measurements were taken as above. The procedure was repeated for all four peptides *i.e.* 1 μg/ml of SP in 25% (v/v) acetonitrile with 0.04% (v/v) TFA, 1 μg/ml of hHK-1 in 25% (v/v) acetonitrile with 0.04% (v/v) TFA, 1 μg/ml of NKB in 25% (v/v) acetonitrile with 0.04% (v/v) TFA (Figure 3.6). In Table 3.3, the wavelength (λmax) that each peptide maximally absorbs is shown. This was used to determine the optimal wavelenghth for the detection of the four peptides with the HPLC U.V. detector.

Table 3.3. The λmax absorbance for each of the five samples.

Sample	Absorbance λmax
25% (v/v) acetonitrile with 0.04% (v/v) TFA	200 nm
mHK-1 in 25% (v/v) acetonitrile with 0.04% (v/v) TFA	208 nm
SP in 25% (v/v) acetonitrile with 0.04% (v/v) TFA	208 nm
hHK-1 in 25% (v/v) acetonitrile with 0.04% (v/v) TFA	208 nm
NKB in 25% (v/v) acetonitrile with 0.04% (v/v) TFA	208 nm

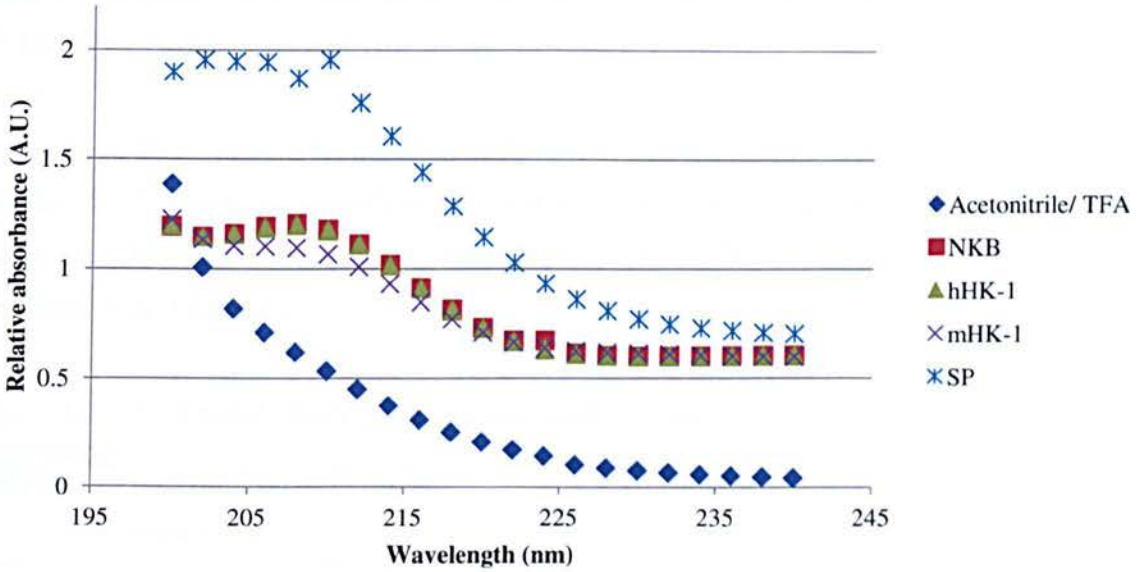


Figure 3.6. The relative absorbance of the four peptides SP, NKB, hHK-1, mHK-1 and of the mobile phase 25% (v/v) acetonitrile with 0.04% (v/v) TFA. The relative absorbance (measured in arbitrary units) is shown against the set wavelength (measured in nm) for each peptide. Absorbance measurements were taken every 2 nm from 200 nm to 240 nm using a Cary 100 Scan UV-spectrometer. The maximal absorbance for each of the five samples was measured in the wavelength 208 nm.

3.4.2) Preparation of stocks and working samples of the mHK-1, SP, hHK-1 and NKB for RP-HPLC method development.

A sample of 20 μg/ml and 80 μg/ml of each peptide and a 20 μg/ml mixture of the four peptides in 25% (v/v) acetonitrile with 0.04% (v/v) TFA was prepared as described in Table 3.4. The samples of peptides were kept stored at -20 °C and thawed at room temperature until required.

Table 3.4. A detailed description of the sample preparation for HPLC method development.

[a] Tube no.	[b] Concentration of sample stock	[c] Peptide	[d] Buffer
1	80 μg/ml of mHK-1	1 μl of the 20 mg/ml of stock in DMSO	250 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
2	80 μg/ml of SP	1 μl of the 20 mg/ml of stock in DMSO	250 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
3	80 μg/ml of hHK-1	1 μl of the 20 mg/ml of stock in DMSO	250 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
4	80 μg/ml of NKB	1 μl of the 20 mg/ml of stock in DMSO	250 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
5	20 μg/ml of mHK-1	50 μl of the 80 μg/ml sample of mHK-1	150 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
6	20 μg/ml of SP	50 μl of the 80 μg/ml sample of SP	150 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
7	20 μg/ml of hHK-1	50 μl of the 80 μg/ml sample of hHK-1	150 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA
8	20 μg/ml of NKB	50 μl of the 80 μg/ml sample of NKB	150 μl of 25% (v/v) acetonitrile with 0.04% (v/v) TFA

[a] Numbering of tube set up for the preparation of the sample. [b] Final concentration of the peptide. [c] Volume taken from each stock (1-4) or sample (5-8) and dissolved in solvent. [d] Volume of solvent used to prepare the final concentration of the peptide.

3.4.3) Development of an isocratic method on RP-HPLC for the elution of the four peptides.

The reversed phase HPLC analysis was performed on a C18 column (Vydac). This was connected to a Waters 600E system controller (Millipore) with a Waters 600E pump and Rheodyne 7725i manual injector and Waters 486 Tunable UV absorbance detector. In the first instance, reversed phase HPLC analysis of the four peptides (mHK-1, SP, hHK-1, NKB) with an isocratic elution system was performed, as a preliminary investigation of the elution conditions of each peptide. The separation system consisted of a reversed-phase C18 column (Vydac) and an isocratic elution system of acetonitrile with 0.04% (v/v) TFA in distilled water. The flow rate was set to 0.45 ml per min and absorbance was measured at 205 nm. An injection of 20 µl of the samples 5 to 8 listed in Table 3.4 (concentration 20 µg/ml of synthetic peptide in 25% (v/v) acetonitrile with 0.04% (v/v) TFA and 0.1% (v/v) DMSO) was made. In Table 3.5 the percentage of acetonitrile in the mobile phase run isocratic through the reversed phase C18 column and the retention time of each peptide is shown. Then, a gradient method for the elution of the four peptides was developed.

Table 3.5. The composition of the mobile phase run isocratic through the C18 column and the retention time of the eluted peptides.

	[b] Retention time (seconds)				
[a] Mobile phase	DMSO	mHK-1	SP	hHK-1	NKB
21%	-	650	-	-	-
23%	-	570	1030	-	-
25%	530	460	730	920	-
27%	-	450	610	760	Not eluted
29%	-	420	540	600	1670
31%	-	-	540	Not retained	920
33%	-	-	-	Not retained	684
35%	-	-	-	-	600

[a] % (v/v) acetonitrile with 0.04% (v/v) TFA. [b] The retention times of the peaks observed are reported in seconds, (-) signifies that the specific peptide was not injected using the concentration of mobile phase.

3.4.4) Development of a gradient method on RP-HPLC for the detection and separation of mHK-1, SP, hHK-1 and NKB.

The mobile phase A, 50% (v/v) acetonitrile with 0.04% (v/v) TFA was prepared by measuring into a 1 L Duran bottle; 500 ml ~100% acetonitrile (Sigma-Aldrich #34851) and bringing the volume to 1 L with distilled H_2O. Next, 4 ml of 10% (v/v) TFA was pipetted into the solution. The mobile phase B, distilled H_2O with 0.04% (v/v) TFA was prepared by measuring into a 1 L Duran bottle; 1 L of distilled H_2O and pipetting in 4 ml of 10% (v/v) TFA. A gradient elution method on the HPLC system was applied using a reverse-phase C18 column (Vydac) with a mobile phase A of 50% (v/v) acetonitrile with 0.04% (v/v) TFA and a mobile phase B of distilled H_2O with 0.04% (v/v) TFA. This was developed for the separation of the four peptides. The gradient is shown in Table 3.6. The absorbance was measured at 205 nm. The samples of the four peptides were injected in the order of their polarity (most to least polar, listed in Table 3.7). The polarity of each peptide was previously experimentally determined by RP-HPLC (on a RP C12 column as shown in Section 3.3.2).

Table 3.6. The HPLC gradient method applied for the elution of the four peptides.

Time (min)	Flow rate (ml/min)	A% [a]	B% [b]
INITIAL	0.45	20	80
10	0.45	20	80
40	0.45	80	20
50	0.45	80	20
55	0.45	100	0
56	0.45	20	80

[a] 50% (v/v) acetonitrile with 0.04% (v/v) TFA; [b] distilled H_2O with 0.04% (v/v) TFA

The chromatogram of the blank sample, which was composed of 0.1% (v/v) DMSO in 25% (v/v) acetonitrile with 0.04% (v/v) TFA is shown in Figure 3.7. It was shown that 0.1% (v/v) DMSO was not retained and eluted as a single peak at 530 s. The gradient applied is also illustrated with a black line. The chromatograms from the elution of the four peptides are shown in Figures 3.8 to 3.11; The retention time of mHK-1 was 1221

sec (~20 min), for SP, 1730 s (~29 min), for hHK-1, 1760 s (~29.5 min) and for NKB, 2196 s (retention time ~36.6 min). A chromatogram from the separation of a mixture of the four peptides is shown in Figure 3.12. The four peptides were separated and demonstrated individual peaks having unique elution times.

Table 3.7. Peptide samples injected into the RP-HPLC.

[a] Injection Order	[b] Sample	[c] Concentration
1.	Sample 1	0.1% (v/v) DMSO in 25% (v/v) acetonitrile with 0.04% (v/v) TFA
2.	Sample 2	20 µg/ml of mHK-1 [d]
3.	Sample 3	20 µg/ml of SP [d]
4.	Sample 4	20 µg/ml of hHK-1 [d]
5.	Sample 5	20 µg/ml of NKB [d]
6.	Sample 6	20 µg/ml of mHK-1, SP, hHK-1, NKB [e]

[a] Order of injections. [b] List of samples. [c] Concentration of sample. [d] In solvent, 0.1% (v/v) DMSO in 25% (v/v) acetonitrile with 0.04% (v/v) TFA . [e] In solvent, 25% (v/v) acetonitrile with 0.04% (v/v) TFA and 0.4% (v/v) DMSO

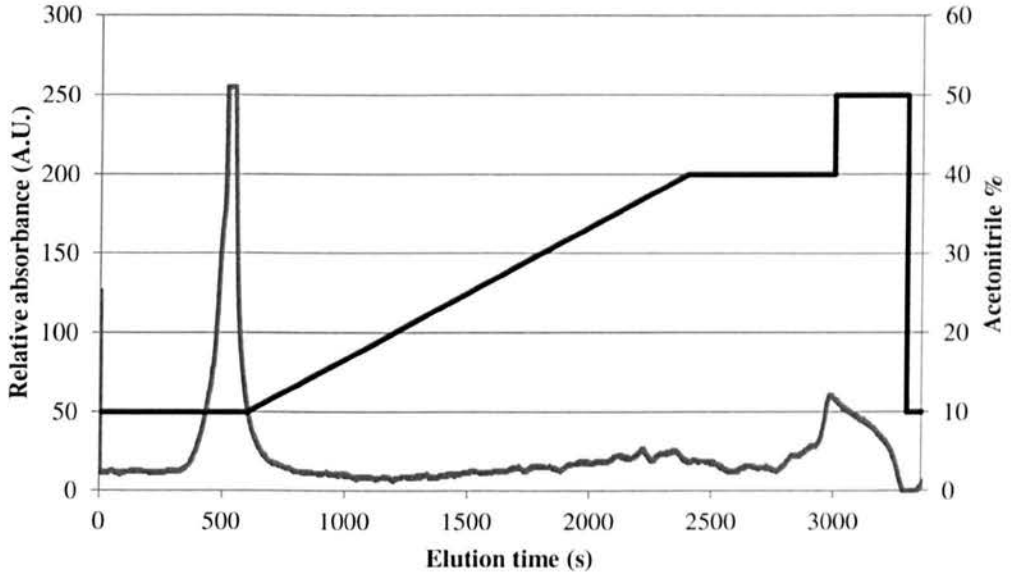


Figure 3.7. The chromatogram of the baseline. 20 µl of the 0.1% (v/v) DMSO sample was injected into the C18 column. The black solid line illustrates the linear gradient, set as 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min, 40% (v/v) acetonitrile isocratic for 10 min and then at 50% (v/v) acetonitrile isocratic for 5 min before returning to 10% (v/v) acetonitrile (right ordinate). The flow rate was set to 0.45 ml/min. Absorbance was monitored at 205 nm (left ordinate). The peak detected at ~530 s is the "front" *i.e.* 0.1% (v/v) DMSO unretained by the column.

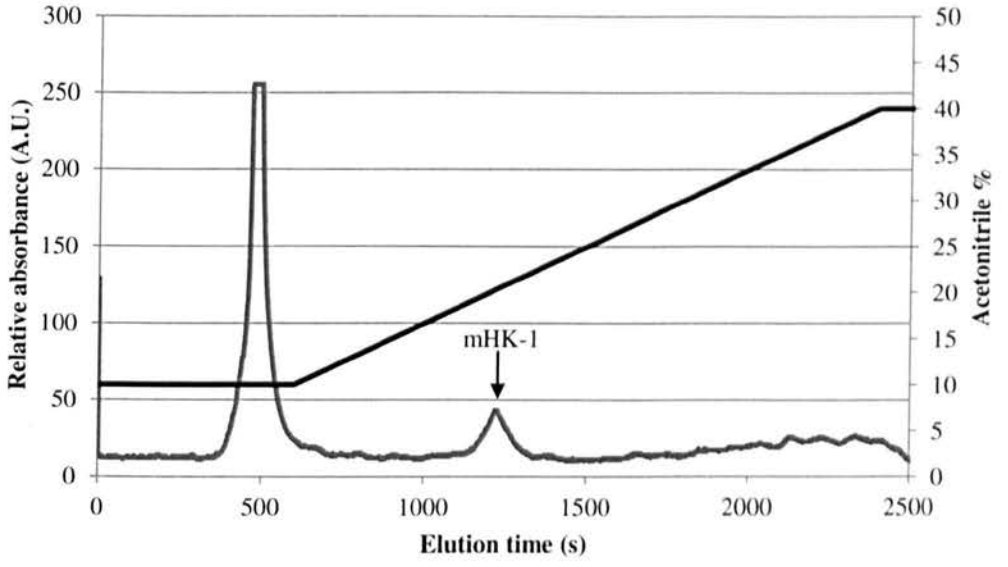


Figure 3.8. Chromatogram of mHK-1. 20 µl of the 20 µg/ml synthetic mHK-1 sample was injected into the reverse phase C18 column. The peptide was eluted with a linear gradient of 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min (right ordinate). The flow rate was set to 0.45 ml/min. Absorbance was monitored at 205 nm (left ordinate). The retention time of synthetic mHK-1 is indicated with an arrow. mHK-1 eluted at 1221 s. The first peak at 530 s corresponds to unretained DMSO.

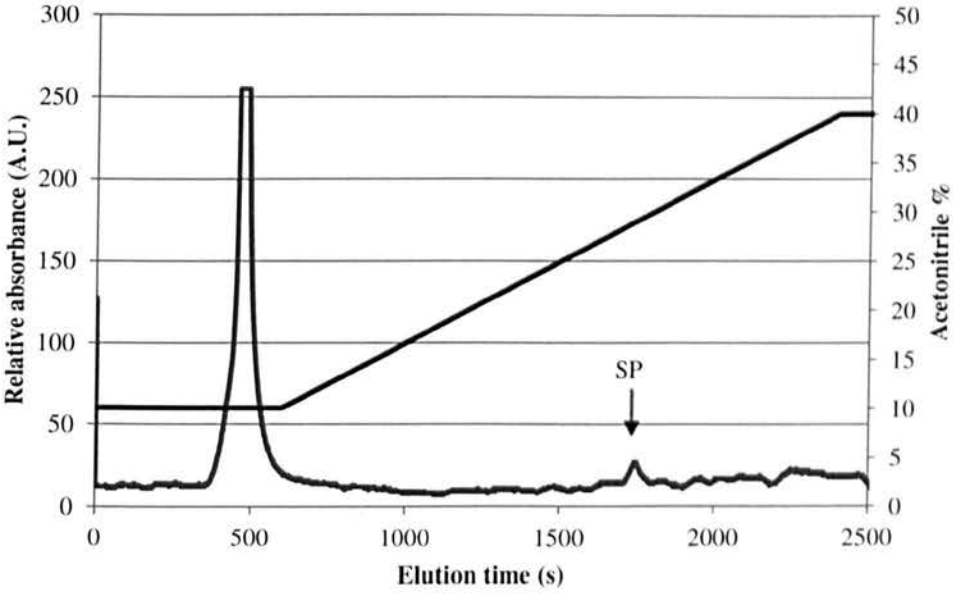


Figure 3.9. Chromatogram of SP. 20 µl of the 20 µg/ml synthetic SP sample was injected into a reverse phase C18 column. The peptide was eluted with a linear gradient of 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min (right ordinate). The flow rate was set to 0.45 ml/min. Absorbance was monitored at 205 nm (left ordinate). The retention time of synthetic SP is indicated with an arrow, eluting at 1730 s.

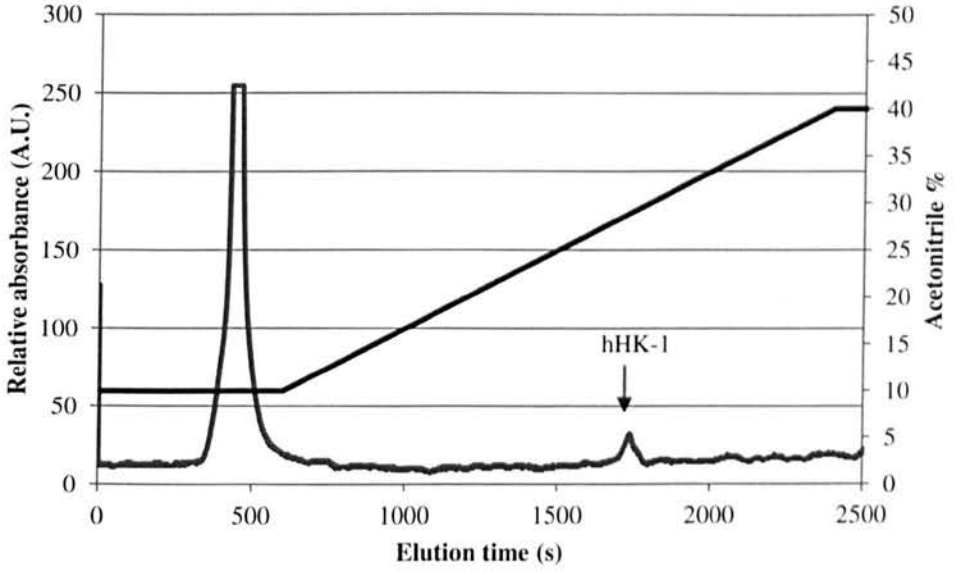


Figure 3.10. Chromatogram of hHK-1. 20 µl of the 20 µg/ml hHK-1 sample were injected into the reverse phase C18 column. The peptide was eluted with a linear gradient of 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min. The flow rate was set at 0.45 ml/min. Absorbance was monitored at 205 nm. The retention time of synthetic hHK-1 peptide is indicated with an arrow and was 1760 s.

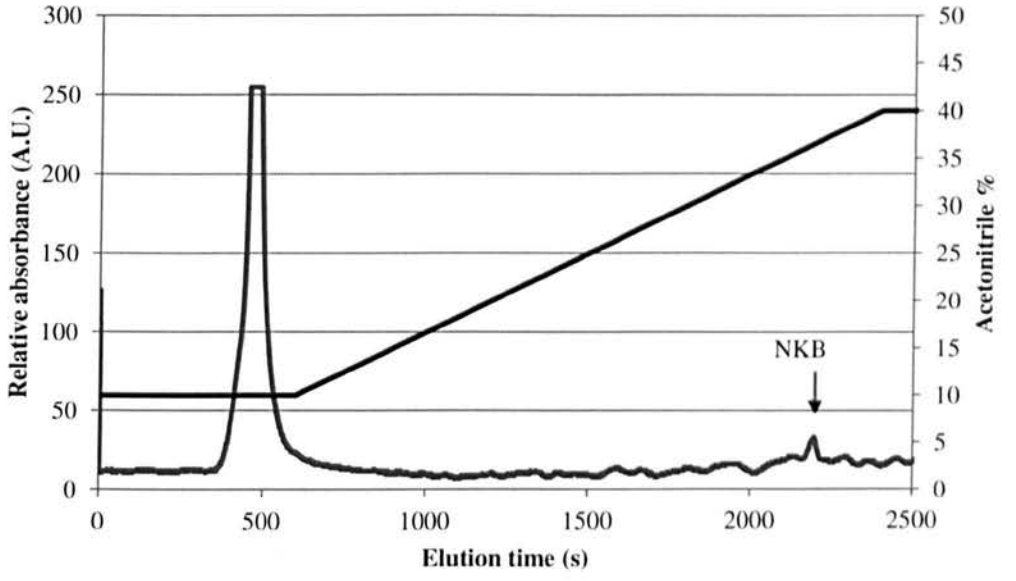


Figure 3.11. Chromatogram of NKB. 20 µl of the 20 µg/ml NKB sample were injected into the reversed phase C18 column. The peptide was eluted with a linear gradient of 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min. The flow rate was set at 0.45 ml/min. Absorbance was monitored at 205 nm. The retention time of synthetic NKB peptide is indicated with an arrow. NKB eluted at 2196 s.

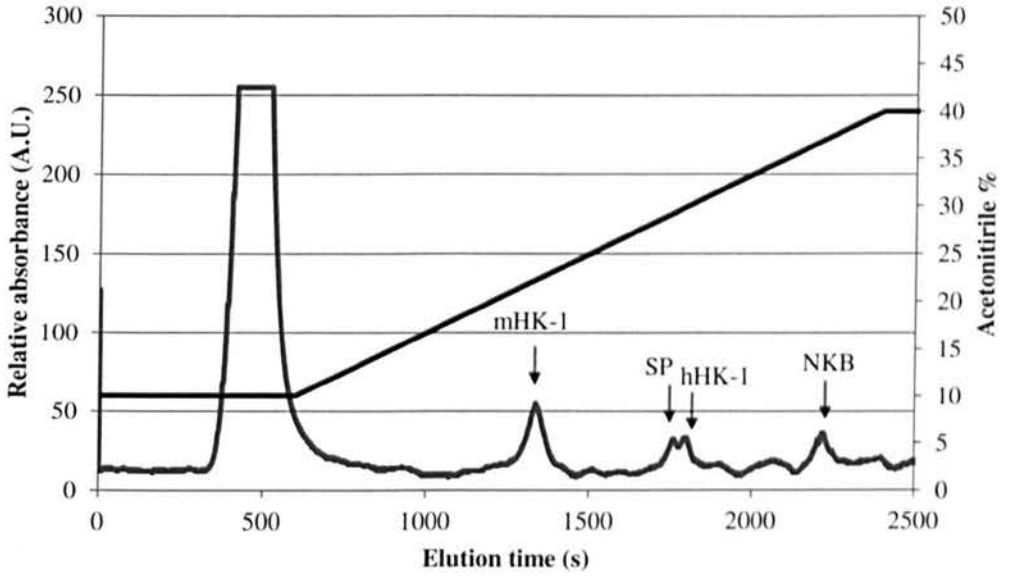


Figure 3.12. Chromatogram of the mHK-1, SP, hHK-1 and NKB mixture. 20 µl of the 20 µg/ml mixture of the four peptides was injected into the reverse phase C18 column. The peptides were eluted with a linear gradient of 10% (v/v) acetonitrile to 40% (v/v) acetonitrile with 0.04% (v/v) TFA over 30 min (right ordinate). The flow rate was set at 0.45 ml/min. Absorbance was measured at 205 nm (left ordinate). The retention times of the four peptides are indicated with arrows, mHK-1 (retention time 1221 s), SP (retention time 1730 s.), hHK-1 (retention time 1760 s), and NKB (retention time 2196 s).

3.4.5) Separation of the four synthetic peptides on RP-HPLC, collection of fractions and identification by MALDI-TOF.

The RP-HPLC method (shown in Table 3.6) was modified to reduce the elution time of the four peptides (as shown in Table 3.8). In particular, the initial conditions were increased to 15% (v/v) acetonitrile and then taken to 35% (v/v) acetonitrile over 20 min. The gradient was taken to 50% (v/v) acetonitrile and kept isocratic for 5 min before returning to 15% (v/v) acetonitrile. 20 µl of the sample of 20 µg/ml of mHK-1, SP, hHK-1 and NKB in 25% (v/v) acetonitrile with 0.04% (v/v) TFA and 0.4% DMSO was injected into the RP-HPLC system and eighty fractions (~225 µl) were collected from 5 min to 45 min (one fraction every 30 s). The chromatogram from the separation of the four peptides using the gradient method in Table 3.8 is illustrated in Figure 3.13. The four peptides eluted according to their polarities the retention time of mHK-1 was ~834 s, SP ~1635 s, hHK-1 ~1656 s and NKB ~2046 s. Each of the eighty fractions collected from the separation of the synthetic peptide mixture were spotted on a MALDI-TOF plate.

Table 3.8. The HPLC gradient method applied for the elution of the four peptides.

Time (min)	Flow rate (ml/min)	Mobile phase A%: 50% (v/v) acetonitrile with 0.04% (v/v) TFA in water	Mobile phase B%: distilled H_2O with 0.04% (v/v) TFA
INITIAL	0.45	30	70
10	0.45	30	70
30	0.45	70	30
35	0.45	100	0
40	0.45	100	0
41	0.45	30	70

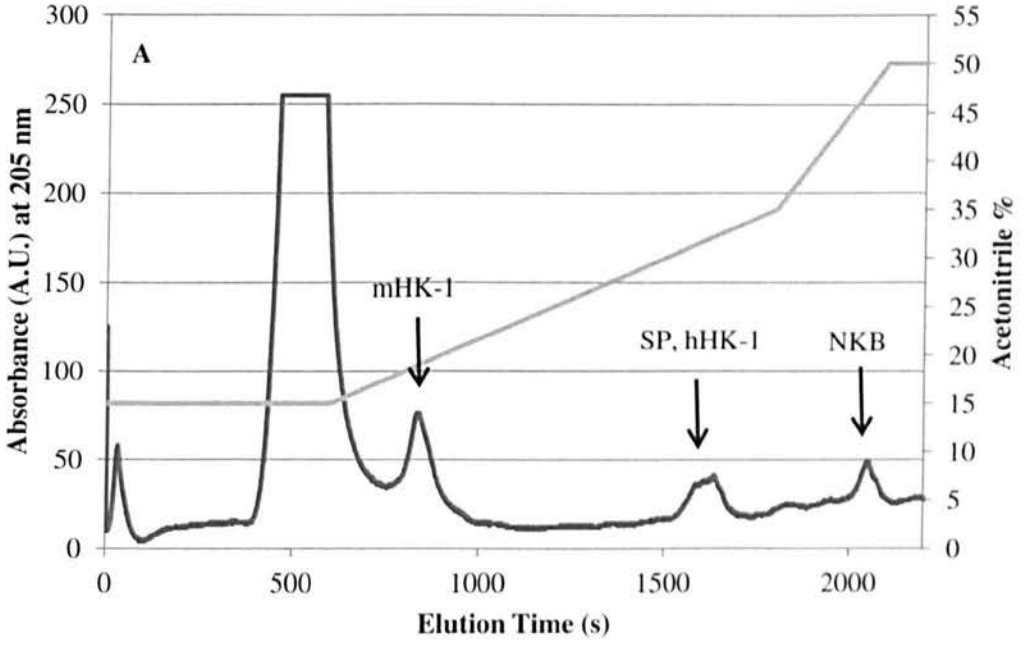


Figure 3.13. Chromatogram of mHK-1, SP, hHK-1, and NKB separation. 20 µl of the 20 µg/ml mixture of the four synthetic peptides was injected into the reverse phase C18 column. The peptides were eluted with a linear gradient of 15% (v/v) acetonitrile to 35% (v/v) acetonitrile with 0.04% (v/v) TFA over 20 min (right ordinate). The gradient was taken to 50% (v/v) acetonitrile and kept isocratic for 5 min before returning to 15% (v/v) acetonitrile (right ordinate). The flow rate was set at 0.45 ml/min. Absorbance was measured at 205 nm (left ordinate). The retention times of the four peptides are indicated with arrows, mHK-1 (retention time 834 s), SP (retention time 1635 s), hHK-1 (retention time 1656 s), and NKB (retention time 2046 s).

The α-HCCA matrix was diluted in 2:1 in ethanol with 1% (v/v) TFA and 1 µl of each of the 80 fractions was mixed with 4 µl of the matrix in ethanol with TFA (as described in Section 3.3.4). The MALDI-TOF instrument was calibrated with the peptides yielded from the trypsinisation of BSA. The laser intensity was set to 50% and 200 shots were made on each spot. The calculated and observed masses of the four peptides $[M+H]^+$ and their oxidised forms $[M+O]^+$ (ΔM +15.999 amu), or sodium or potassium adducts $[M+Na]^+$ (ΔM +21.989 amu) or $[M+K]^+$ (ΔM +37.963 amu) are listed in Table 3.9. The MALDI-TOF spectra of the ion peaks detected from fractions 9, 25, 27, 46 are illustrated in Figure 3.14. mHK-1 was found to elute in Fraction 9 (Figure 3.14, A), the annotated peak with m/z 1257.66 corresponds to mHK-1 $[M+H]^+$. The peak at m/z 1273.644 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.572 (ΔM -36.02 amu) is mHK-1 with potential water loss from serine and threonine residues (Harrison, 2012). The peptide SP elutes in Fraction 25 (Figure 3.14, B); the peak detected at m/z 1347.691 corresponds to SP $[M+H]^+$. The peak detected at m/z 1363.675 (ΔM +15.999 amu) corresponds to SP where methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. The peptides SP and hHK-1 have similar polarities and hence using this analytical method they eluted in the same fractions (*i.e.* 25, 26 and 27). The peak in fraction 27 detected at m/z 1185.567 corresponds to hHK-1 $[M+H]^+$, the peak detected at m/z 1201.535 (ΔM +15.999 amu) corresponds to hHK-1 where methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure and the peak at m/z 1223.53 (ΔM +37.963 amu) is the K^+ adduct of hHK-1 (Figure 3.14, C). The peptide NKB eluted in fraction 46 (Figure 3.14, D); the peak detected at m/z 1210.511 corresponds to NKB $[M+H]^+$. The peaks detected with m/z 1232.51 (ΔM +21.989 amu) and 1248.494 (ΔM +37.963 amu) are the Na^+ and K^+ adducts respectively of the NKB peptide. In Figures 3.15, 3.16, 3.17, 3.18 the chemical structures of the four peptides are shown as designed using the program ChemSketch®. In Figure 3.18 the chemical structure of hHK-1 is shown where the a/b/c/x/y/z ions are also shown. Table 3.9 lists the masses of the four peptides detected (with Na^+ and K^+ adducts) and the theoretical masses as well. The discrepancy between the values could be attributed to the calibration of the instrument; and also to inaccuracy in the annotation of peaks with the FlexAnalysis software.

Table 3.9. Masses of the four peptides and their Na^+ and K^+ adducts.

Peptide	Mass detected / amu	Mass calculated / amu	Adduct (ΔM)
hHK-1	1185.567	1185.609	-
hHK-1	-	1207.59	Na^+ (+21.989 amu)
hHK-1	1223.53	1223.564	K^+ (+37.963 amu)
NKB	1210.511	1210.538	-
NKB	1232.51	1232.52	Na^+ (+21.989 amu)
NKB	1248.494	1248.49	K^+ (+37.963 amu)
mHK-1	1257.66	1257.652	-
mHK-1	-	1279.634	Na^+ (+21.989 amu)
mHK-1	-	1295.608	K^+ (+37.963 amu)
SP	1347.691	1347.735	-
SP	-	1369.717	Na^+ (+21.989 amu)
SP	-	1385.691	K^+ (+37.963 amu)

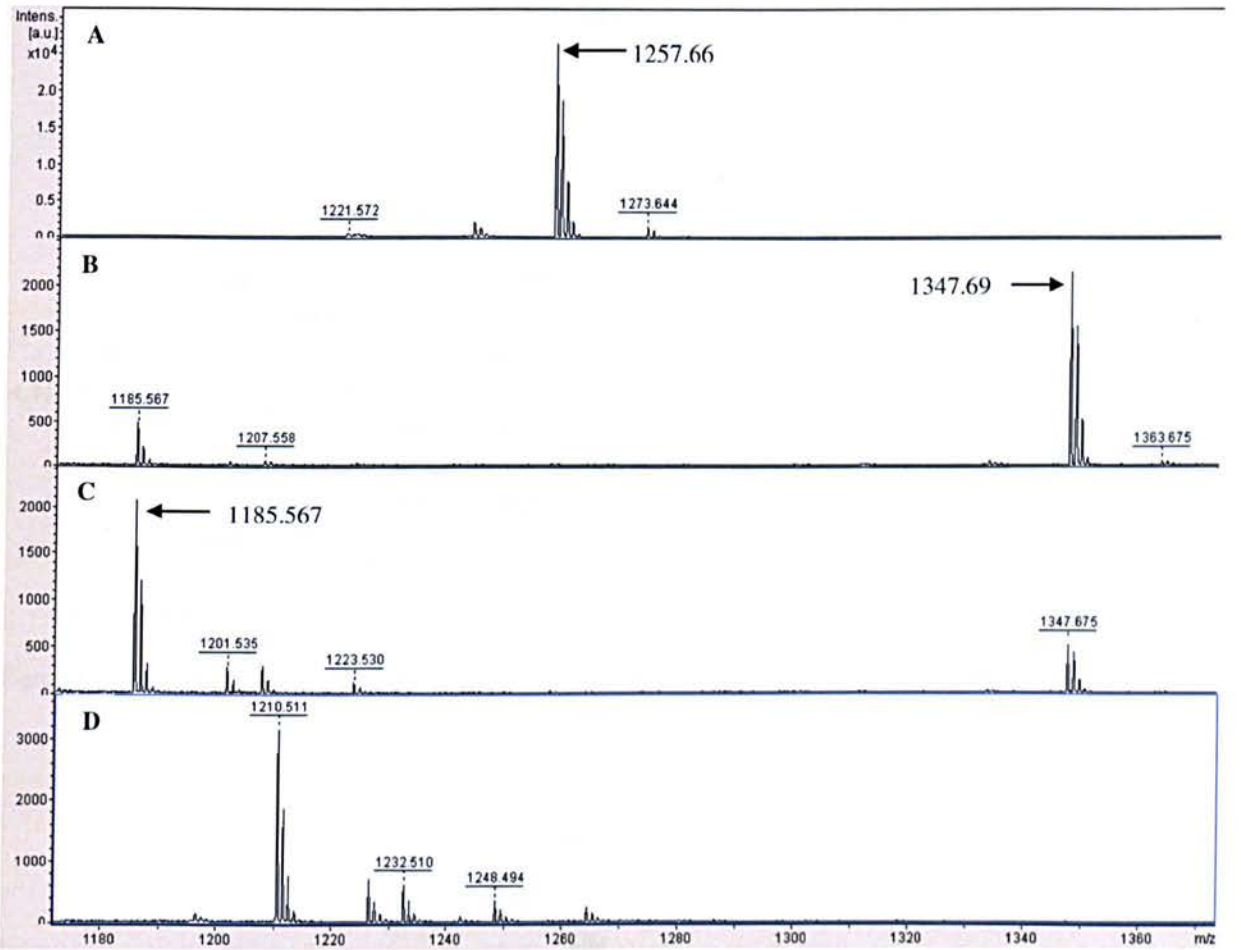


Figure 3.14. MALDI-TOF spectra of the synthetic peptides collected in Fractions 9, 25, 27 and 46 after separation of their mixture on RP-HPLC. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1170 to 1370 is shown. (**A**) Fraction 9: the annotated peak with m/z 1257.66 corresponds to mHK-1. The peak at m/z 1273.644 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.572 (ΔM -36 amu) is mHK-1 with water loss of serine and threonine residues. (**B**) Fraction 25: the peak detected at m/z 1347.691 corresponds to SP. The peak detected at m/z 1363.675 (ΔM +15.999 amu) corresponds to SP were methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. Peaks at 1185.567 and 1207.55 are hHK-1 and the Na^+ adduct. (**C**) Fraction 27: the peak detected at m/z 1185.567 corresponds to hHK-1. The peak detected at m/z 1201.535 (ΔM +15.999 amu) corresponds to hHK-1 were methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. The peak detected at m/z 1223.53 (ΔM +37.963 amu) is the K^+ adduct of hHK-1. (**D**) Fraction 46: the peak detected at m/z 1210.511 corresponds to NKB. The peaks detected with m/z 1232.51 (ΔM +21.989 amu) and 1248.494 (ΔM +37.963 amu) are the Na^+ and K^+ adducts respectively of the NKB peptide.

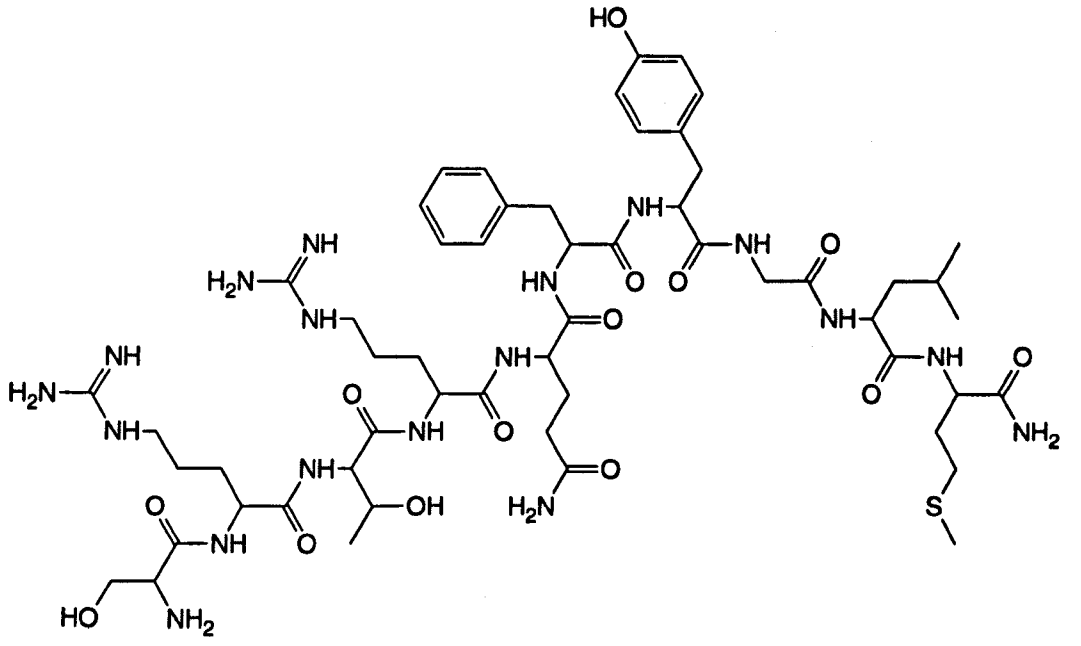


Figure 3.15. The chemical form of mHK-1 (SRTRQFYGLM-NH_2).

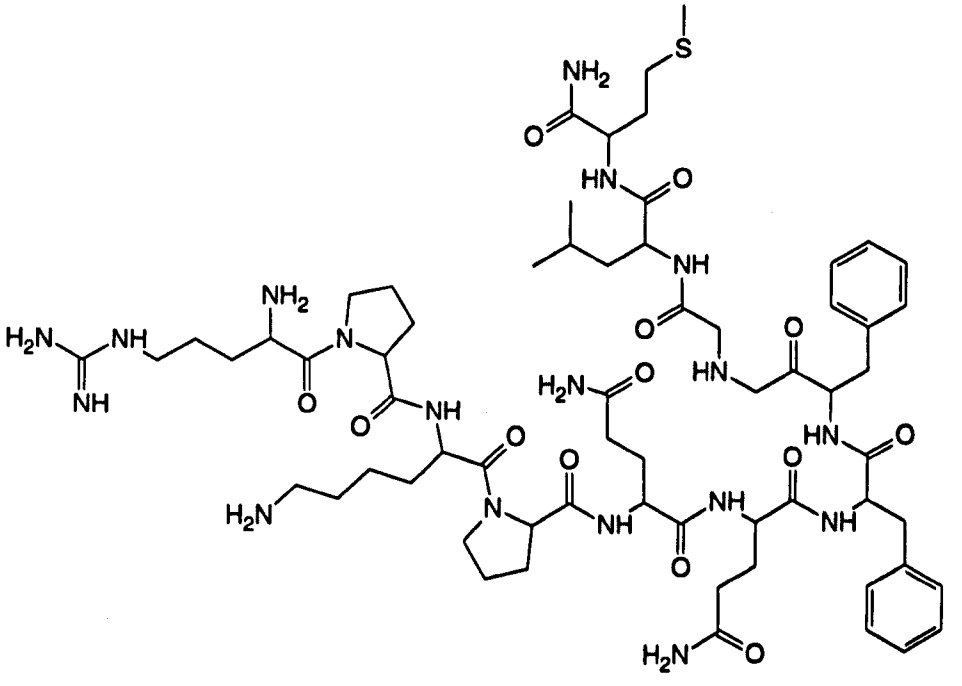


Figure 3.16. The chemical form of SP (RPKPQQFFGLM-NH_2).

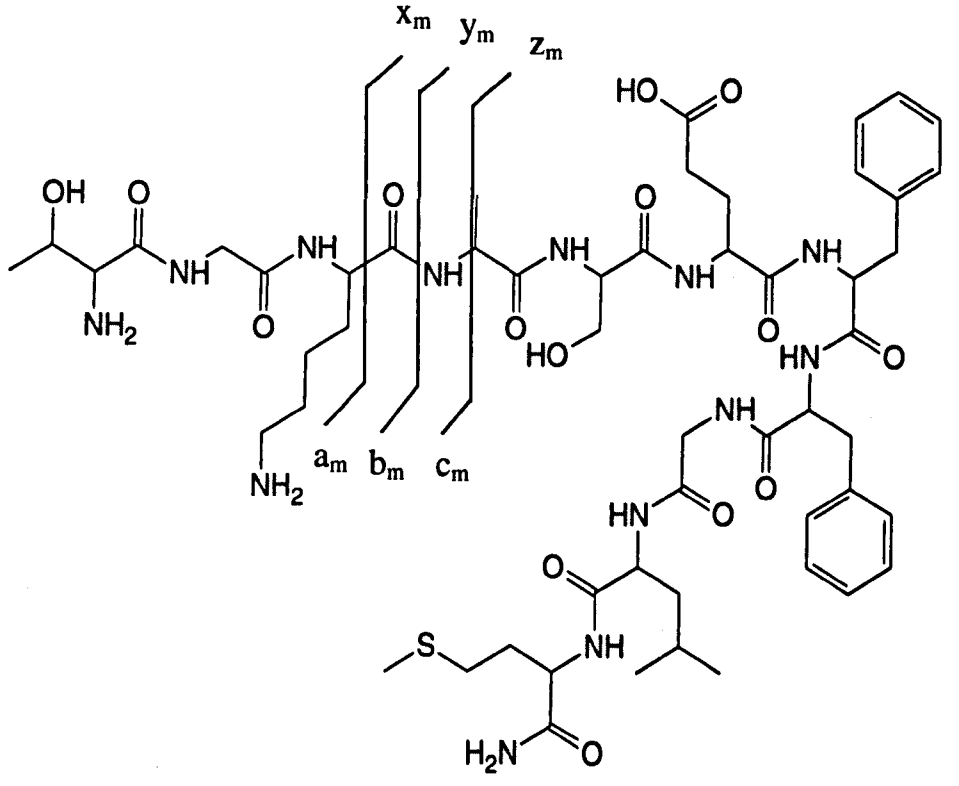


Figure 3.17. The chemical form of hHK-1 (TGKASQFFGLM-NH_2). The a/b/c ions where the charge is retained by the N-terminal fragment and the x/y/z ions where the charge is retained by the C-terminal fragment are shown.

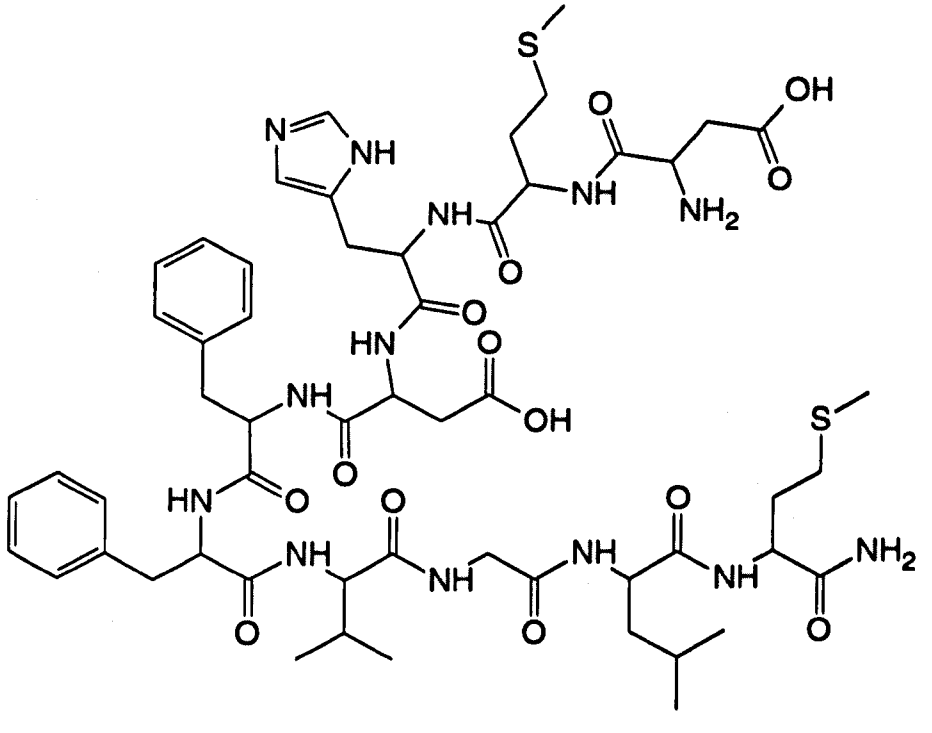
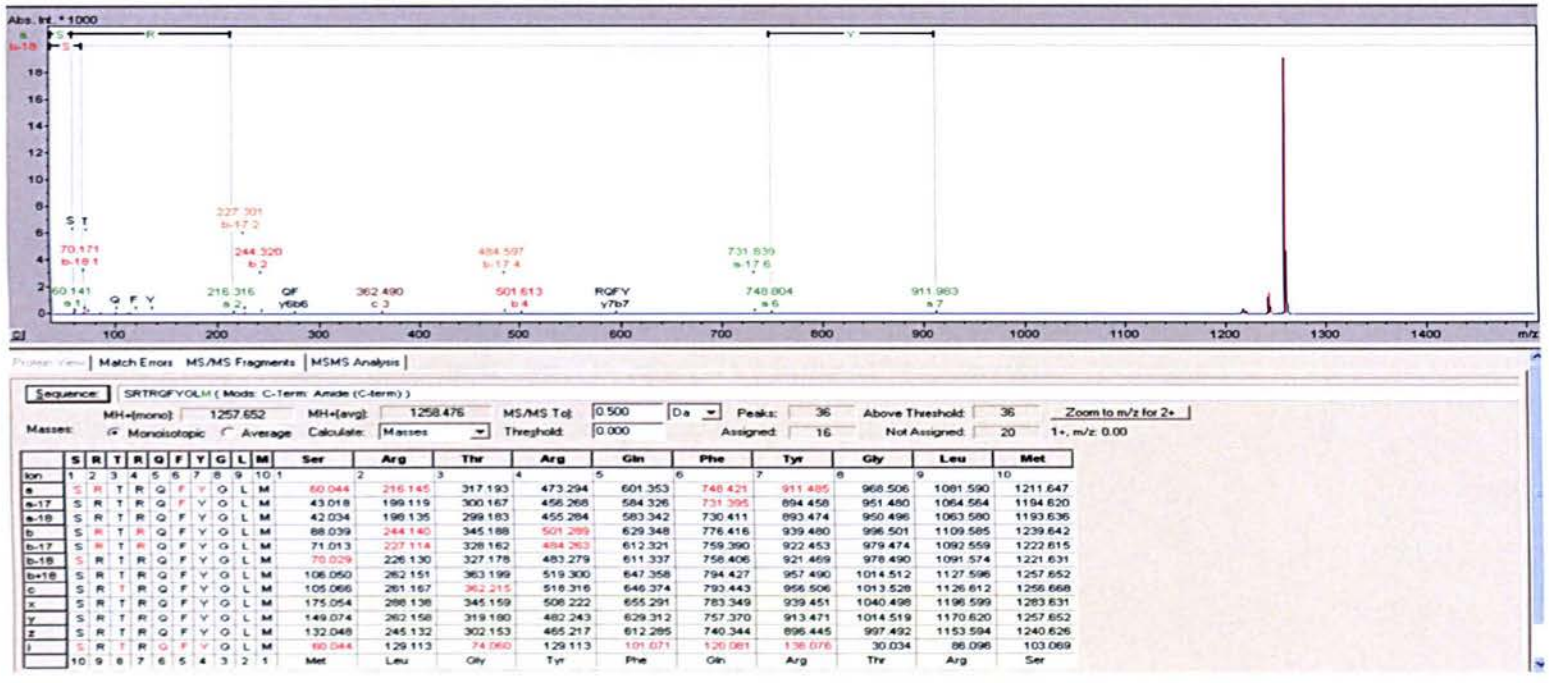


Figure 3.18. The chemical form of NKB (DMHDFFVGLM-NH_2).

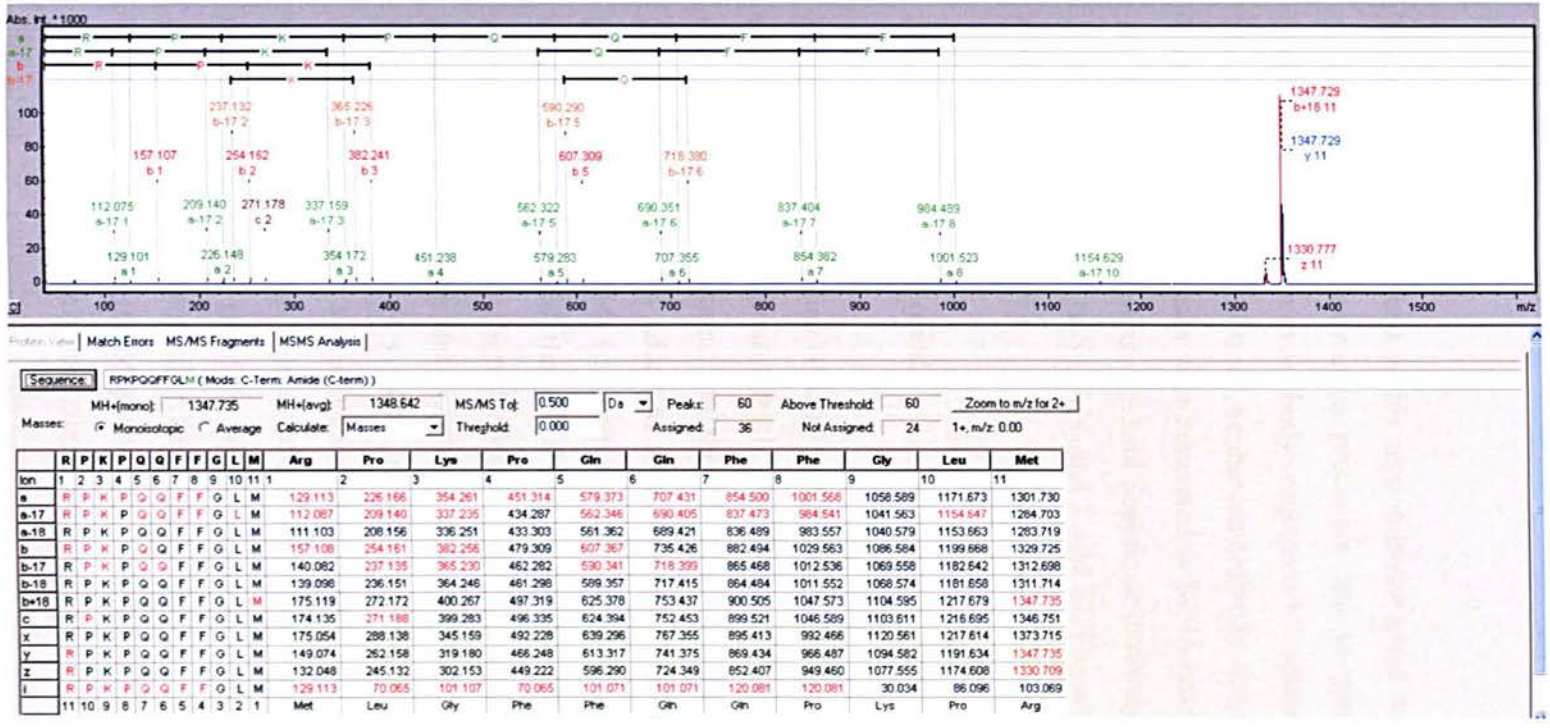
3.4.6) Peptide sequencing with collision induced dissociation.

The synthetic peptides were fragmented into their daughter ions using Collision Induced Dissociation (CID) to investigate the amino acid sequence. The software analysis component of the MALDI-TOF (Bruker Autoflex III) was switched to LIFT.lft thus permitting the passing of only the chosen parent ion through the magnetic field. The mass range was set to 40-1520 m/z, the laser intensity was set to 30% and 500 shots were performed, and the CID gas (argon) switched on. The mass of the singly charged peptide was selected as the parent mass (values listed in Table 3.9). Each peptide was then fragmented; the spectrum of the fragmentation pattern of mHK-1 is shown in Figure 3.19. The masses of the daughter ions were aligned with the theoretical masses of the a/b/c/x/y/z ions (according to the Roepstorff-Fohlmann-Biemann nomenclature; Roepstoff & Fohlman, 1984; Biemann, 1992) or the a-17 (loss of ammonia), a-18 (loss of water), b-17 and b-18 ions (Dancik *et al.*, 1999) using the Biotools software. Mass tolerance MS/MS was set to 0.5 Da (default setting). The peptide sequences were deduced from the mass differences between consecutive a-type ions. However as shown in Figure 3.19 only the first two amino acids of the mHK-1 peptide (SRTRQFYGLM-NH_2) *i.e.* serine (S) and arginine (R) followed by tyrosine (Y) were deduced. Also from the b-18 ion series (b ions with the loss of water) only serine (S) was deduced. A few more daughter ions were detected as well such as the RQFY fragment (y7b7 ion) and the QF (y6b6) fragment. The sequencing of synthetic mHK-1 was not complete as the whole series of the a/b/c/x/y/z types of ions was not detected. Figure 3.20 shows the fragmentation of SP, where sequencing of the synthetic peptide was more successful as most of the amino acids of the sequence were deduced. Sixty ion peaks were detected (compared to 36 ion peaks detected from the dissociation of mHK-1) with the a-ion types being more predominant. The mHK-1 sequence was deduced in the amino-to-carboxy-terminus direction. SP is the "gold standard" to verify instrument and method performance due not only to the number of basic residues *i.e.* arginine and lysine but also proline residues it comprises which ionise efficiently (Keller & Li, 2001). Hence, the sequencing of the SP peptide was more efficient (than the other three of this study's interest) however still the last three C-terminal amino acids, glycine, leucine, methionine of the peptide were not deduced.



	S	R	T	R	Q	F	Y	G	L	M	Ser	Arg	Thr	Arg	Gln	Phe	Tyr	Gly	Leu	Met
Ion	1	2	3	4	5	6	7	8	9	10	1		2		3		4		5	
a	S	R	T	R	Q	F	Y	G	L	M	60.044	216.145	317.193	473.294	601.353	748.421	911.485	968.506	1081.590	1211.647
a-17	S	R	T	R	Q	F	Y	G	L	M	43.018	199.119	300.167	456.268	584.326	731.395	894.458	951.480	1064.564	1194.620
a-18	S	R	T	R	Q	F	Y	G	L	M	42.034	198.135	299.183	455.284	583.342	730.411	893.474	950.496	1063.580	1193.636
b	S	R	T	R	Q	F	Y	G	L	M	88.039	244.140	345.188	501.289	629.348	776.416	939.480	996.501	1109.585	1239.642
b-17	S	R	T	R	Q	F	Y	G	L	M	71.013	227.114	328.162	484.263	612.321	759.390	922.453	979.474	1092.559	1222.615
b-18	S	R	T	R	Q	F	Y	G	L	M	70.029	226.130	327.178	483.279	611.337	758.406	921.469	978.490	1091.574	1221.631
b+18	S	R	T	R	Q	F	Y	G	L	M	106.050	262.151	363.199	519.300	647.358	794.427	957.490	1014.512	1127.596	1257.652
c	S	R	T	R	Q	F	Y	G	L	M	105.066	261.167	362.215	518.316	646.374	793.443	956.506	1013.528	1126.612	1256.668
x	S	R	T	R	Q	F	Y	G	L	M	175.054	288.138	345.159	508.222	655.291	783.349	939.451	1040.498	1196.599	1283.631
y	S	R	T	R	Q	F	Y	G	L	M	149.074	262.158	319.180	482.243	629.312	757.370	913.471	1014.519	1170.620	1257.652
z	S	R	T	R	Q	F	Y	G	L	M	132.048	245.132	302.153	465.217	612.285	740.344	896.445	997.492	1153.594	1240.626
i	S	R	T	R	Q	F	Y	G	L	M	60.044	129.113	74.060	129.113	101.071	120.081	136.076	30.034	86.096	103.069
	10	9	8	7	6	5	4	3	2	1	Met	Leu	Gly	Tyr	Phe	Gln	Arg	Thr	Arg	Ser

Figure 3.19. The collision-induced dissociation spectrum of synthetic mHK-1. The software analysis of the MALDI-TOF (Bruker Autoflex III) was switched to LIFT.lft, with the parent ion peak 1257.652 Da was dissociated into daughter ions. The laser intensity was set at 30% and 500 shots were performed, also CID gas (argon) was switched on. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. In the top panel the ion peaks of the daughter ions produced in the mass to charge (m/z) ranging from 40 to 1520 are shown. In the bottom panel the sequence of mHK-1 (SRTRQFYGLM-NH_2) with C-terminal amide set as a post-translational modification was inserted into the Biotools software and the theoretical masses of the a/b/c/x/y/z ions were automatically calculated. MS/MS tolerance was set to 0.5 Da (default setting). The masses of the daughter ion peaks detected were aligned against the theoretical masses of the a/b/c/x/y/z ion type series. The mass differences between the a-ions deduced the amino acids, serine (S), arginine (R) and tyrosine (Y). The a-ion types (and b-18 *i.e.* b ions with the loss of water) were predominant, and hence the sequence is written in the amino-to-carboxy-terminus direction going from left to right.



	R	P	K	P	Q	Q	F	F	G	L	M	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	R	P	K	P	Q	Q	F	F	G	L	M	129.113	226.166	354.261	451.314	579.373	707.431	854.500	1001.568	1058.589	1171.673	1301.730
a-17	R	P	K	P	Q	Q	F	F	G	L	M	112.087	209.140	337.235	434.287	562.346	690.405	837.473	984.541	1041.563	1154.647	1284.703
a-18	R	P	K	P	Q	Q	F	F	G	L	M	111.103	208.156	336.251	433.303	561.362	689.421	836.489	983.557	1040.579	1153.663	1283.719
b	R	P	K	P	Q	Q	F	F	G	L	M	157.108	254.161	382.256	479.309	607.367	735.426	882.494	1029.563	1086.584	1199.668	1329.725
b-17	R	P	K	P	Q	Q	F	F	G	L	M	140.082	237.135	365.230	462.282	590.341	718.399	865.468	1012.536	1069.558	1182.642	1312.698
b-18	R	P	K	P	Q	Q	F	F	G	L	M	139.098	236.151	364.246	461.298	589.357	717.415	864.484	1011.552	1068.574	1181.658	1311.714
b+18	R	P	K	P	Q	Q	F	F	G	L	M	175.119	272.172	400.267	497.319	625.378	753.437	900.505	1047.573	1104.595	1217.679	1347.735
c	R	P	K	P	Q	Q	F	F	G	L	M	174.135	271.188	399.283	496.335	624.394	752.453	899.521	1046.589	1103.611	1216.695	1346.751
x	R	P	K	P	Q	Q	F	F	G	L	M	175.054	288.138	345.159	492.228	639.296	767.355	895.413	992.466	1120.561	1217.614	1373.715
y	R	P	K	P	Q	Q	F	F	G	L	M	149.074	262.158	319.180	466.248	613.317	741.375	869.434	966.487	1094.582	1191.634	1347.735
z	R	P	K	P	Q	Q	F	F	G	L	M	132.048	245.132	302.153	449.222	596.290	724.349	852.407	949.460	1077.555	1174.608	1330.709
i	R	P	K	P	Q	Q	F	F	G	L	M	129.113	70.065	101.107	70.065	101.071	101.071	120.081	120.081	30.034	86.096	103.069
	11	10	9	8	7	6	5	4	3	2	1	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg

Figure 3.20. The collision-induced dissociation spectrum of synthetic SP. The software method of the MALDI-TOF (Bruker Autoflex III) was switched to LIFT.lft, the parent ion peak 1347.735 Da was dissociated into daughter ions. The laser intensity was set at 30% and 500 shots were performed, also CID gas (argon) was switched on. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. In the top panel the ion peaks of the daughter ions produced in the mass to charge (m/z) range from 40 to 1600 are shown. In the bottom panel the sequence of SP (RPKPQQFFGLM-NH_2) with C-terminal amide set as a post-translational modification was inserted into the Biotools software and the theoretical masses of the a/b/c/x/y/z ions were automatically calculated. MS/MS tolerance was set to 0.5 Da (default setting). The masses of the daughter ion peaks detected were aligned against the theoretical masses of the a/b/c/x/y/z ion type series. The mass differences between the a-ions, a-17 ions, b ions and b-17 ions deduce most of the amino acids of the SP peptide. The sequence was deduced in the amino-to-carboxy-terminus direction going from left to right.

3.5) Immunoaffinity purification of the four synthetic peptides and their detection on MALDI-TOF.

The experiment described in this section investigated the recovery of peptide during the methanol/chloroform extraction procedure and in parallel showed the ability of the Sepharose-anti-tachykinin antibody conjugates to capture and enrich tachykinin peptides. The development of the three Sepharose-antibody conjugates was described in Section 2.3.6; the three conjugates were annotated as BO16 mixture, BO16 1E1 EXP1 and BO16 1E1 EXP2 (each consisting of ~3 ml Sepharose-antibody complex in 5 ml storage buffer, 1 M NaCl with 0.02% (w/v) NaN_3 and 1 mM EDTA) and stored in 50 ml Falcon tubes at 4 °C.

3.5.1) Materials and methods.

Phosphate buffered saline (PBS) 1x with 0.02% (w/v) NaN_3 was prepared, by dissolving one tablet of PBS (Sigma-Aldrich, P4417) into 200 ml of distilled H_2O following the manufacturer's instructions. Into the 200 ml of 1x PBS solution, 2 ml of 2% (w/v) NaN_3 (BDH, #0232540) was added as a bacteriostat. A solution of 0.1 M disodium EDTA (Sigma-Aldrich, E-1644) was prepared (3.72 g of the disodium EDTA dissolved in 100 ml of distilled H_2O with the pH of the solution adjusted to pH ~7). The detergent octyl glucoside (1 g) (O.G.; Sigma-Aldrich, O8001) was reconstituted to 10% (w/v) octyl glucoside (by pipetting and dissolving in 1 g of octyl glucoside into 10 ml of distilled H_2O). Binding buffer (1 x PBS, 10 mM EDTA, 0.01% (w/v) octyl glucoside, 0.02% (w/v) NaN_3 and serine and cysteine proteases inhibitors) was prepared. This was done by measuring into a 50 ml Falcon tube (Greiner, Bio-one), 20 ml of 1 x PBS with 0.02% (w/v) NaN_3 and then 2 ml of 0.1 M EDTA and 20 µl of 10% (w/v) O.G. followed by two protease inhibitor tablets (Roche, #11836170001). The Sepharose-antibody complex wash buffer (50 ml of 0.1 M glycine pH ~2.5) was prepared by transferring into a 50 ml Falcon tube 0.38 g of glycine (Sigma- Aldrich, G7126) and 30 ml of distilled H_2O. Then 500 µl of 2% (w/v) NaN_3 were pipetted into the solution and the pH of the solution adjusted to 2.5 using 1 M HCl. The final volume of the solution was brought to 50 ml

with distilled H_2O. The peptide extraction buffer (90% (v/v) methanol with 9% (v/v) acetic acid) was prepared as described in Section 4.3.1. The captured antibody was eluted with elution buffer, 0.5% (v/v) TFA. Samples were neutralised with 1 M TRIS-BASE pH ~10 prepared by dissolving 1.25 g of TRIS-BASE (Sigma-Aldrich, Cat. #25,285-9) into 10 ml of distilled H_2O.

3.5.2) Experimental procedure to test the Sepharose-antibody complex.

A preliminary experiment was performed for the investigation of the recovery of synthetic peptide during each stage of the methanol/chloroform extraction or during the concentration of samples with the removal of organic solvents (under N_2 flow). The methanol/chloroform extraction method comprises three stages *i.e.* the homogenisation of tissue and incubation with acidified methanol; the centrifugation and collection of supernatant; the removal of proteins and lipids with methanol/chloroform extraction and the collection of upper phase (from the three phases); and also the removal of organic solvents from the upper phase. Hence, the recovery of peptide during the procedure (or at a particular step of the procedure) was investigated. Each step of the experiment was spiked with an amount of synthetic peptide considering the relative recovery of peptide from the previous stage (the procedure is summarised in Figure 3.21). A sample 50 µg/ml mixture of the four synthetic peptides in methanol was prepared transferring 2 µl of a 5 mg/ml stock mixture of the four synthetic peptides in DMSO into 200 µl of methanol. The tube containing the sample in methanol was placed at -20 °C overnight. As part of this preliminary experiment, readily available frozen sheep liver (~4 g) was homogenised with a mortar and a pestle while maintaining the tissue frozen with liquid nitrogen. The homogenised tissue was split equally *i.e.* ~1 g into four 2 ml Eppendorf tubes. Into each tube with homogenised tissue, 1 ml of peptide extraction buffer, 90% (v/v) methanol with 9% (v/v) acetic acid was added. The tissue and buffer was blended into a homogenous mixture by fitting a scalpel into the 2 ml tubes. Into the **"first"** of the four tubes with homogenised tissue 40 µl of the 50 µg/ml synthetic peptide mixture in methanol was pipetted. The four 2 ml Eppendorf tubes were placed at 4°C for 3 hours.

The four tubes were centrifuged at 7558 x g (13,000 RPM) for 15 min at room temperature. The supernatant from each tube (800 µl) was collected and transferred into respective 1.5 ml Eppendorf tubes (favoured for the precipitation of tissue debris). The four fresh tubes containing the supernatant were re-centrifuged at 7558 x g (13,000 RPM) for 15 min for tissue debris still present in the supernatant to precipitate; from the supernatant 700 µl was again collected and transferred into four clean 2 ml tubes. Into the "**second**" of the four tubes containing the supernatant collected, 14 µl of the 50 µg/ml of synthetic peptide mixture in methanol (*i.e.* 700 ng of the synthetic peptide mixture; estimating that an amount of peptide potentially is not recovered) was added.

An equal volume of chloroform (700 µl) was added into the 700 µl of supernatant and the four 2 ml tubes were vortexed for 15 min and centrifuged at 7558 x g (13,000 RPM) for 10 min. Three phases were formed in each tube, an upper phase (aqueous methanol) containing peptides and sugars, an interphase with denatured proteins and a lower phase (chloroform) with lipids. The upper phase was collected (500 µl) from the four tubes and transferred into four clean 1.5 ml Eppendorf tubes, previously rinsed with methanol and left to air-dry. Into the "**third**" of the four tubes containing the collected "upper phase", 10 µl of the 50 µg/ml synthetic peptide mixture in methanol was added (500 ng of the synthetic peptide mixture) estimating a better recovery than the previous stages. A 50 µl mark was made on the first three tubes and a 40 µl mark on the "**fourth**" tube. The four samples were concentrated by blowing a "slow" stream of nitrogen gas over the sample at room temperature to reduce to the respective volume. Into the "40 µl" sample 10 µl of the 50 µg/ml synthetic peptide mixture in methanol (500 ng of the synthetic peptide mixture) was added. Into a separate tube "**positive control**" 40 µl of methanol was transferred and 10 µl of the 50 µg/ml synthetic peptide mixture in methanol were added.

Table 3.10. The steps of the experimental procedure and the amount of synthetic peptide "spiked" into the sample.

Tube (step)	Sample	Spiked Amount
First	Homogenised tissue	2 µg
Second	Supernatant	700 ng
Third	Upper phase	500 ng
Fourth	Concentrated upper phase	500 ng

3.5.3) Immunoaffinity purification.

The synthetic peptides recovered in the methanol extracts were enriched using the Sepharose-antibody conjugates developed in Section 2.3.6. The three 50 ml Falcon tubes containing the Sepharose-antibody complexes BO16 mixture, BO16 1E1 EXP1, BO16 1E1 EXP2 in storage buffer (1 M NaCl with 0.02% (w/v) NaN_3 and 1 mM EDTA) were taken out of storage at 4°C. The contents of the three Falcon tubes were mixed into homogenous gel. A mixture of the three Sepharose-antibody complexes was prepared by transferring 120 µl of BO16 mixture, 120 µl of BO16 1E1 EXP2 and 120 µl of BO16 1E1 EXP1 slurry (using a yellow tip with the end cut-off to allow the removal of both Sepharose beads and buffer) into a 1.5 ml Eppendorf tube. Into this tube (containing the 360 µl of Sepharose-antibody in buffer, ~250 µl Sepharose-antibody complex), 1 ml Sepharose wash buffer (0.1 M glycine pH ~2.5 with 0.02% (w/v) NaN_3) was added. The tube was mixed with gentle inversion and the mixture was incubated for 3 min on the bench. Next, the tube was centrifuged at 716 x g (4000 RPM) for 2 min for the Sepharose-antibody complex to form a pellet. The Sepharose wash buffer was aspirated and dispensed to waste. The washing step was repeated three times in total. The Sepharose-antibody complex was brought to "binding conditions" i.e. pH 8.4. 1 ml working buffer (1 x PBS containing 10 mM EDTA with 0.01% (w/v) O.G., 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors) was added to the 1.5 ml Eppendorf tube, which was inverted several times and left on the bench for 3 min. The tube was centrifuged at 716 x g (4000 RPM) for 2 mins for the Sepharose-antibody complex to

form a pellet. The buffer was then aspirated and dispensed with the procedure repeated three times in total.

Into each of the five 50 µl methanol extract samples (Section 3.5.2), 150 µl of binding buffer (1x PBS containing 10 mM EDTA with 0.01% (w/v) O.G., 0.02% (w/v) NaN_3 and serine and cysteine proteases inhibitors) was added and the pH was adjusted to ~7.5 with 2 µl of 1 M TRIS-BASE pH ~10 before the final volume of the samples was brought to 400 µl with the same buffer. The "washed" ~250 µl of Sepharose-antibody complex was mixed with 250 µl of binding buffer. Into each of the five samples ~100 µl (~50 µl) of Sepharose-antibody in binding buffer mixture was transferred, using a yellow tip with the end cut-off. The five 1.5 ml Eppendorf tubes (each with a final reaction volume of 500 µl) were placed on a rotating platform overnight at room temperature.

The following day, the five 1.5 ml Eppendorf tubes were spun at 716 x g (4000 RPM) for 1 min. The Sepharose-antibody-peptide complex formed a pellet from which the supernatant was aspirated and discarded. 1 ml of working buffer (1x PBS with 10 mM EDTA and 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 with serine and cysteine proteases inhibitors) was added to every tube. The Sepharose-antibody-peptide complex was incubated on the bench for 2 min. The tubes were inverted and spun at 716 x g (4000 RPM) for 1 min. This step was repeated three times in total. After the last wash, 500 µl working buffer was added into each tube and used to transfer each Sepharose-antibody-peptide complex into a respective mini-column set over a 2 ml tube. The buffer was left to drip through. Then, into each mini-column 500 µl of distilled H_2O were added and dripped through removing salt-containing buffer. Following the last wash the five mini-columns over a 2 ml tube were spun at 45 x g (1,000 RPM) for 1 min and residual buffer and salt or distilled H_2O was removed.

The captured peptide was eluted by adding into the Sepharose-antibody-peptide complexes 50 µl of 0.5% (v/v) TFA with slight stirring with a pipette tip followed by incubation for 5 min. The mini-columns were "uncapped" and placed over 1.5 ml Eppendorf tubes previously rinsed with methanol and left to air-dry. The mini-columns

and tube set ups were spun at 716 x g (4000 RPM) for 2 min. The eluent (containing the captured peptide) was collected into the respective 1.5 ml tube.

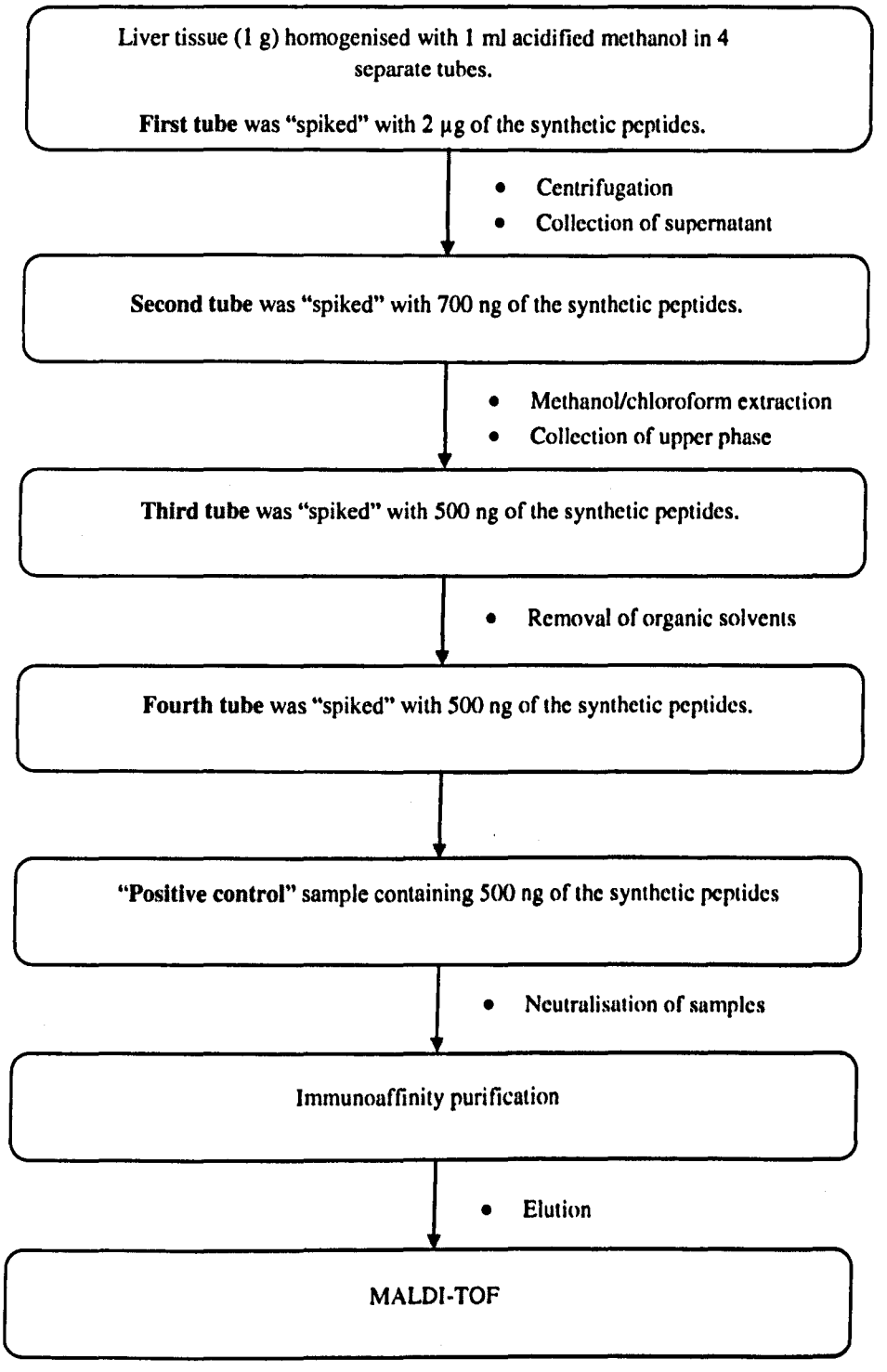


Figure 3.21. Experimental procedure. The schematic overview of the experimental procedure followed to investigate the recovery of the acidified methanol/ chloroform extraction procedure and the Sepharose-antibody complex for ability for the ability to capture the four synthetic tachykinin peptides.

3.5.4) Detection of peptides in the immunoaffinity purification enrichments using MALDI-TOF.

The α-HCCA matrix was diluted (2:1) in ethanol with 1% (v/v) TFA and 1 µl of each of the five immunoaffinity purification enrichments (derived from the four "spiked" methanol extracts and the "positive control" experiment) was mixed with 4 µl of the matrix in ethanol with TFA for the crystallisation of the peptides present in the eluents (as described in Section 3.3.4). 1 µl of this mixture of eluent in matrix with ethanol and TFA was used to spot a MALDI-TOF plate in triplicate. The MALDI-TOF was calibrated with the peptides yielded from the trypsinisation of BSA. The laser intensity was set to 50% and 200 shots were made on each spot. Lower laser intensities in the range of 40% to 50% were investigated as well as more shots (up to 500). The lower laser intensities did not ionise the peptides effectively while more laser shots "burned" the crystals. The peptides in the five eluents after enrichment with immunoaffinity purification were detected with MALDI-TOF.

Figure 3.22 shows the spectrum of the eluent after enrichment with immunoaffinity purification from the "first" tube (sample spiked with synthetic peptide at the homogenised tissue in peptide extraction buffer stage). The four peptides mHK-1, SP, hHK-1 and NKB and the Na^+ and K^+ adduct ions were detected in high abundance. In the interim spectrum the mass range 1180-1260 is highlighted. The arrows highlight peaks corresponding to $[NKB + H]^+$ (1210.470 amu), $[NKB + Na]^+$ (1232.401 amu) and $[NKB + K]^+$ (1248.432 amu). The calculated masses of the monoisotopic peaks of the four peptides and adducts have been summarised in Table 3.9.

The MALDI-TOF spectrum of the eluent after enrichment with immunoaffinity purification from the "second" tube (in which the supernatant collected after centrifugation of the mixture of the homogenised tissue in acidified methanol buffer was spiked with synthetic peptide) is shown in Figure 3.23, A. The peak at 1257.552 corresponds to mHK-1. The peak at 1273.536 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.582 (ΔM

-36 amu) is mHK-1 with water loss of serine and threonine residues. The peak at m/z 1347.615 is SP, the peak at m/z 1369.605 is the Na^+ adduct (ΔM +21.989 amu) and the peak with m/z 1311.554 (ΔM -36 amu) is the peptide with the loss of two water molecules. In Figure 3.23, B the MALDI-TOF spectrum of the eluent after enrichment with immunoaffinity purification from the "third" tube in which the upper phase (from the three phases) after methanol/chloroform extraction was "spiked" with synthetic peptide is shown. The peaks of the four peptides and their Na^+ and K^+ adducts were detected at high abundance. In Figure 3.23, C the MALDI-TOF spectrum of the eluent after enrichment with immunoaffinity purification from the "fourth tube" is shown, in which the sample of upper phase after removal of organic solvents was "spiked" with synthetic peptide. The same peaks of the four peptides and Na^+ and K^+ adducts were detected. In Figure 3.23, D the spectrum of the eluent from the "positive control" (a 50 µl sample of 10 ng/µl peptide in methanol diluted 10 times in binding buffer and enriched with immunoaffinity purification) is shown. The relative abundance of the ion peaks of the four peptides between the five spectra is in the same range; hence no significant loss of peptide was recorded throughout the procedure. The discrepancy between the values of the ion peaks could be attributed to the calibration of the instrument; and also to inaccuracy in the annotation of peaks with the FlexAnalysis software.

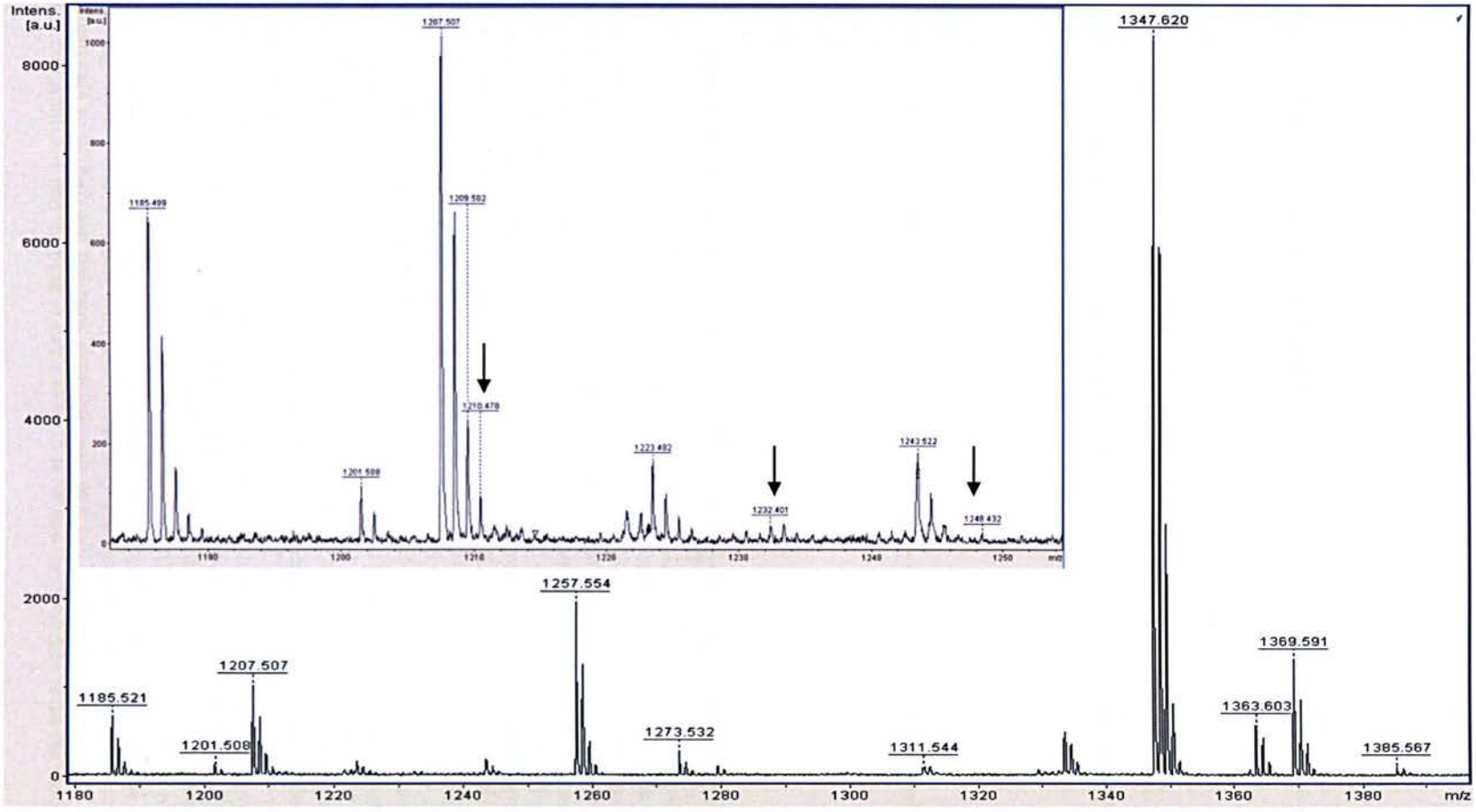


Figure 3.22. Ion spectrum showing peaks detected in the eluent after enrichment with immunoaffinity purification of the sample spiked with synthetic peptide(s) at the homogenised tissue in peptide extraction buffer stage. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1180 to 1390 is shown. The peptides hHK-1 (1185.521 amu), mHK-1(1257.554 amu), SP (1347.62 amu) are detected with high abundance. In the interim spectrum the mass to charge range 1180-1260 is highlighted. The arrows highlight peaks corresponding to $[NKB + H]^+$ (1210.470 amu), $[NKB + Na]^+$ (1232.401 amu), $[NKB + K]^+$ (1248.432 amu).

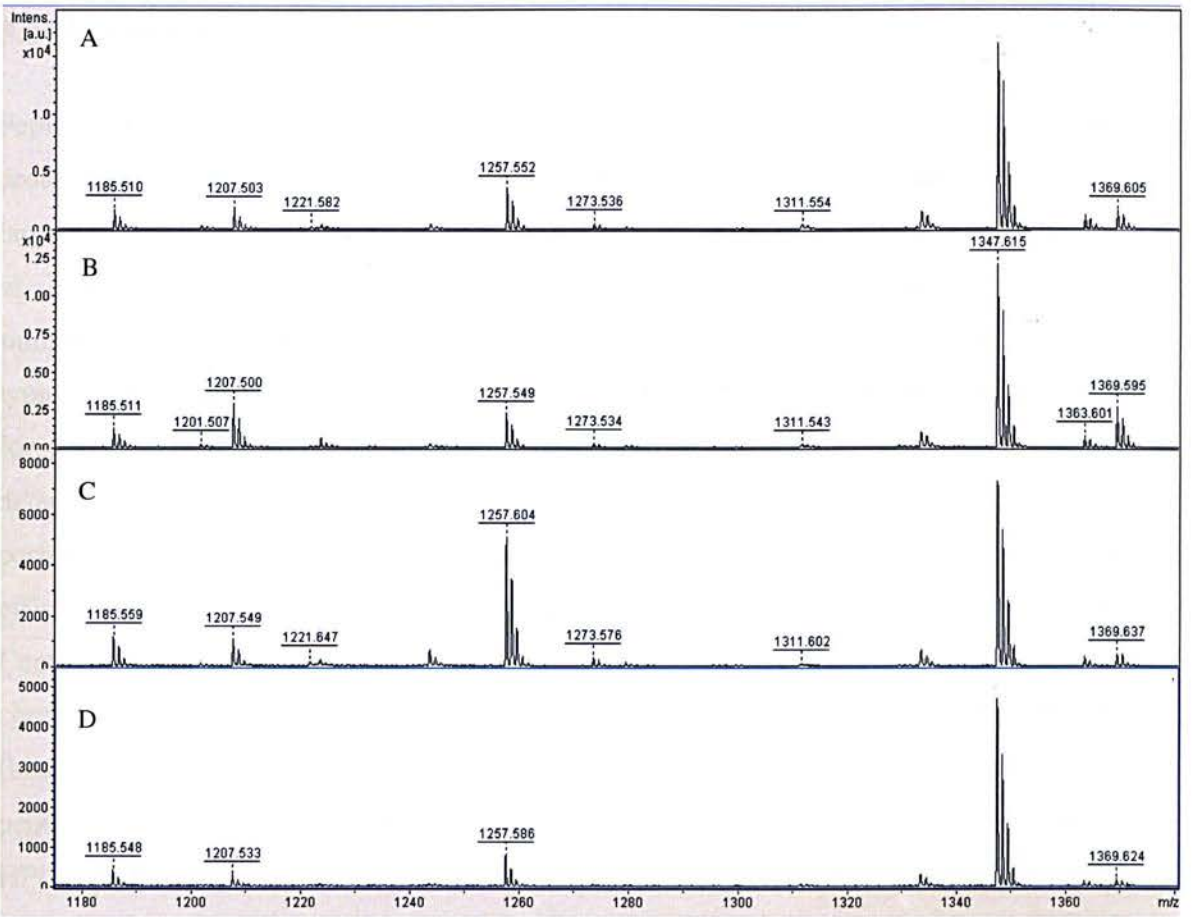


Figure 3.23. Ion spectra of the synthetic peptides collected in the eluents after immunoaffinity purification. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1170 to 1380 is shown. (**A**) Ion peaks detected in the "second" tube, in which the sample was spiked at the stage of the supernatant collected after centrifugation of the mixture of the homogenised tissue in acidified methanol buffer. The peak with m/z at m/z 1185.51 is hHK-1 and the peak at m/z 1207.503 is the Na^+ adduct (ΔM +21.989 amu). The peak at 1257.552 corresponds to mHK-1. The peak 1273.536 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.582 (ΔM -36 amu) is mHK-1 with water loss of serine and threonine residues. The peak at m/z 1347.615 is SP, the peak at m/z 1369.605 is the Na^+ adduct (ΔM +21.989 amu) and the peak with m/z 1311.554 (ΔM -36 amu) is the peptide with the loss of two water molecules. (**B**) Ion peaks detected in the "third" tube in which the upper phase (from the three phases) after methanol/chloroform extraction was "spiked" with synthetic peptide are shown. The peak detected at m/z 1185.511 is hHK-1 and the peak at 1207.5 is the Na^+ adduct (ΔM +21.989 amu). The peak at 1257.549 is mHK-1 and at 1273.534 is the oxidised form of the peptide. The peak at 1347.615 corresponds to SP and the peak detected at m/z 1363.595 (ΔM +15.999 amu) corresponds to SP were methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. (**C**) Ion peaks detected in the "fourth" tube in which the sample of upper phase after removal of organic solvents was "spiked" with synthetic peptide; the peak detected at m/z 1185.559 corresponds to hHK-1. The peak detected at m/z 1207.549 is the Na^+ adduct of hHK-1. The peak at m/z 1257.604 is mHK-1 and the peak at m/z 1273.576 is the oxidised form of the peptide. The peak at 1347.615 is SP and the peak at 1369.637 is the oxidised form of SP. (**D**) Ion peaks detected in the "positive control" (a 50 µl sample of 10 ng/µl peptide in methanol diluted 10 times in "binding buffer" and enriched with immunoaffinity purification). The peak detected at m/z 1185.548 corresponds to hHK-1 and the peak detected with m/z 1207.533 is the Na^+ adduct. The peak with m/z 1257.586 is mHK-1 and the peak at m/z 1347.615 is SP and the 1369.624 is the Na^+ adduct.

3.6) Discussion.

Peptides as a set of molecules that are produced by enzymatic cleavage from longer protein precursor molecules by intracellular prohormone convertases are expressed in large numbers in organ regions or even in cell cultures (Clynen *et al.*, 2003; Hummon *et al.*, 2006). Such variety in peptide or neuropeptide availability demands a range of sample handling and analysis methods (Desiderio *et al.*, 1997; Hummon *et al.*, 2006). A typical strategy for the identification of novel peptides involves an efficient methodology for peptide extraction, purification and separation of the repertoire of peptides and a detection system of high sensitivity (Boonen *et al.*, 2008). Tissue homogenisation is performed in the presence of a variety of solvent mixtures the more established (due to efficiency, ease and low cost) being acidified methanol and Bennett's solution. Centrifugation is performed to eliminate unwanted cellular debris prior to chromatographic fractionation. Moreover, peptide concentration using C18 cartridges (binding all hydrophobic molecules) also provides another clean up stage of the targeted peptides from the proteins not eluting at the specific composition of the elution buffer. HPLC performed with a reversed phase column and a gradient of acetonitrile/ water offers the advantage of separating peptides into fractions then screened on MALDI-TOF (Hummon *et al.*, 2006).

In the present study, two extraction methods were described, one with an acidified methanol and a second using Bennett's solution to extract neuropeptides from two rat brains. A RP-HPLC method was used to fractionate and separate the repertoire of neuropeptides present in the extract. The combination of RP-HPLC with immunoassay quantitatively compared the efficiency of the two peptide extraction buffers (aqueous acidic buffer and acidified methanol). A HPLC method was also developed on a reverse phase C18 column calibrating in the first instance with the four synthetic peptides mHK-1, SP, hHK-1, and NKB and spotting fractions on MALDI-TOF. Finally, a preliminary experiment was performed (Figure 3.21) to investigate the recovery of synthetic peptide during each stage of the methanol/chloroform extraction and during the removal of

organic solvents (under N_2 flow). The same experiment also proved the anti-tachykinin Sepharose-antibody conjugate's ability to capture tachykinins.

In Section 3.3.1, the two peptide extraction buffers, acidified methanol buffer (Sturm *et al.*, 2010, Dowell *et al.*, 2006) and Bennett's solution (Bennett, 1981; Nassel *et al.*, 2000) were compared; the rat brain content of peptides was extracted and fractions were checked for specific anti-tachykinin immunoreactivity with an ELISA assay with immunopurified anti-tachykinin antibodies (Figure 3.2). Both extraction buffers had the ability to extract peptides as revealed in the chromatogram (Figure 3.3.A and Figure 3.4.A). The application of Bennett's solution offers efficient extraction of peptides as also shown in the "*immuno-gram*" (Figure 3.3.B) but also possibly extracts other proteins and impurities. The advantage of the combination of acidified methanol with chloroform extraction was the elimination of bulk contaminant proteins and lipids, hence, more distinct peaks were revealed in the "*immuno-gram*" (Figure 3.4.B). However, the application of the acidified methanol with chloroform extraction requires gentle handling, due to the forming of the denatured protein interphase where peptides can potentially be caught. Contaminant proteins or other agents can interfere with the immobilised antibody and peptide interactions (binding interference) and reduce the efficiency of the immunoaffinity purification procedure.

The HPLC method using the reverse phase C12 column appeared efficient in separating the three tachykinin peptides found in the rat brain *i.e.* mHK-1, SP and NKB (retention times ~18 min, ~22 min and ~28 min, respectively). However, the HPLC instrument controlling the flow rate through the reverse phase C12 column had the shortcoming of not having an efficient way to collect the eluent after separation. It was observed that the peaks corresponding to mHK-1, SP and NKB on the chromatogram (Figure 3.2, A) compared to the peaks of the three peptides on the "*immuno-gram*" showing the immunoreactivity of the fractions (Figure 3.2, B) were out of synchronisation by 1.5 min. This was attributed to the tubing connecting the HPLC instrument to the fraction collector. Therefore, another analytical method was developed on a C18 column on a different HPLC system (Waters 600E system controller with a Waters 600E pump;

Millipore) with more efficient tubing for the collection of fractions (described in Section 3.3) having the advantages of maintaining a constant back-pressure and having an outlet with narrower tubing. Therefore, the collected fractions were not being merged. The steps taken to develop a method on the reversed phase C18 column for the separation of the four peptides was described in Section 3.4. Fractions were again collected and the elution fraction of each peptide was confirmed with MALDI-TOF. The peptides SP and hHK-1 have similar polarities hence were found in almost the same fractions; modification of the gradient (by reducing the percentage of acetonitrile change per minute) would make the method more resolving and hence would resolve the two peptides. The four tachykinin peptides of this study's interest have very similar molecular masses and hence they were separated based on their polarity on a reversed phase column rather than other analytical methods such as size-exclusion chromatography, which would not provide as "analytical" separation (resolution) between the peptides (Page *et al.*, 2009). We investigated the use of cation exchange chromatography (using a cation-exchange resin) as another method of separating the tachykinin peptides based on their isoelectric point (pI) values from a mixture of peptides. We separated synthetic NKB from the mixture of the other three peptides (mHK-1, SP, hHK-1). This method could be applied to separate the four peptides; however, this type of chromatography provided a "rigid" method of eliminating some peptides from a mixture rather than "fractionating" the repertoire into fractions. Recently, a combination of reversed-phase silica beads and cation exchange beads have been developed as a stationary phase for the separation of peptides; providing better selectivity values and improving the purification efficiency compared to classical materials (Bernardi *et al.*, 2013).

The ability of the anti-tachykinin Sepharose-antibody conjugate to capture and concentrate tachykinin peptides, was investigated (Section 3.5). In parallel, an estimation of recovery of synthetic peptide in each stage of the procedure of homogenisation, centrifugation, methanol/chloroform extraction, concentration of sample with the removal of organic solvents under N_2 flow and immunoaffinity purification was attempted (Section 3.5). We observed that the procedure was efficient in recovering peptide in every step of the work flow and that no major peptide losses occurred at any step. As part

of this experiment we practiced spotting on MALDI-TOF the methanol extracts (containing the synthetic peptides) without immunoaffinity enrichment, which resulted in no peaks detected, due to high background noise. From our experience, we report immunoaffinity purification as a powerful tool for the capture and enrichment of tachykinins from a large repertoire of peptides and other molecules, by lowering the detection limits by the removal or minimising of background matrix components (noise) increasing the overall sensitivity of the methodology and providing a purer analyte. Previous reports confirm, interferences from endogenous compounds extracted from the initial tissue result in peak suppression of the analyte of interest and removal of contaminant proteins with the application of immunoaffinity purification is required (Oe *et al.*, 2006). The term "targeted analyte enrichment" has been coined to describe the purification of analyte with antibodies, reducing the noise and thus increasing the detection limits of the method (Anderson *et al.*, 2004, Kawashima *et al.*, 2009; Whiteaker *et al.*, 2010). The amounts of synthetic peptides used in our experiment were of higher levels than the peptides found in rat brains. Therefore, further separation for the removal of more agents by means of RP-HPLC and concentration of endogenous peptides by means of C18 tips *i.e.* ZipTips was required in the case of the capture of naturally occurring tachykinins in tissues.

The advantages of using a combination of off-line sample separation on RP-HPLC and detection on MALDI-TOF are the non-destructive method as most of the sample (*e.g.* the immunoaffinity eluent) and fractions are reserved for repeat analysis and the intrinsic ability of MALDI-TOF to generate predominantly singly-charged ions which simplify data analysis (Pan *et al.*, 2009). The synthetic peptides (mHK-1, SP, hHK-1 and NKB) were fragmented into their daughter ions using Collision Induced Dissociation (CID) to investigate their sequencing. The mass of the singly charged peptide was selected as the "parent" mass and was fragmented with laser and CID gas (argon). The masses of the "daughter" ions were aligned with the theoretical masses of the a/b/c/x/y/z ions or the "satellite" ions, a-17 (loss of ammonia from lysine or arginine), a-18 (loss of water from serine or threonine) or b-17 and b-18 ions *i.e.* b ions with loss of ammonia or water from the above amino acid side chains. The peptide sequences were deduced from the mass

differences between consecutive ions. The advantages of sequencing by means of mass spectrometry (*e.g.* compared to Edman degradation) are (i) the ability to perform sequencing of a peptide within a mixture of peptides allowing only one parent mass through the magnetic field and thus reducing detection limits and (ii) the smaller sample amount required for sequencing. A third advantage of sequencing by mass spectrometry is the efficient fragmentation even in the presence of a post translational modification *e.g.* in the N-terminus. The limitation of sequencing by mass spectrometry is the complex fragment ion spectra deduced (reviewed in Chaurand *et al.*, 1999). Due to the complexity of the deduced ion spectra interpretation can be ambiguous. An efficient way to deduce the sequence of the peptide is to interpret the fragmentation pattern of only one type of fragment ions (from the a/b/c/x/y/z types) especially the b or y which derive from the fragmentation of the peptide bond. In this study, the sequencing of synthetic SP was investigated and fragmentation pattern of SP produced a meaningful spectrum as most of the amino acids of the sequence were deduced (Figure 4.20).

Proteases play a role in the degradation of neuropeptides and of proteins to peptide fragments (Skold *et al.*, 2002; Zhu & Desiderio, 1993; Theodorsson *et al.*, 1990) and hence these processing fragments and degradation products of proteins or polypeptides may cause peak suppression of the signals of low abundance neuropeptides on MALDI-TOF (Che *et al.*, 2005). Hence, separation by RP-HPLC is imperative following immunoaffinity enrichment of tachykinins in order to detect peaks on MS (Dowell *et al.*, 2006; Svensson *et al*, 2003). The peptide separation by RP-HPLC increases the signal to noise ratio and increases the number of ion signals rather than spotting an immunoaffinity enriched eluent directly on MALDI-TOF (Aristoteli *et al.*, 2006). Furthermore, concentration with ZipTips offers a higher signal to noise ratio for two reasons. The procedure lowers the Na^+ or K^+ adducts of the peptides, removing salt during the washing step. The small tip bed volume allows sample elution in a small volume of matrix solution (Gobom *et al.*, 1999). This yields small sample spots highly enriched in analyte molecules. In conclusion, the combination of enrichment with immunoaffinity purification and further purification with RP-HPLC and concentration of analyte with ZipTips extended the limits of detection. LC-ESI-MS is a commonly used method for the

identification of novel peptides and also quantification. For example, NKB has been detected and quantified in various brain regions, in response to cocaine administration with separation and identification with LC-ESI-MS (Che *et al.*, 2006). Furthermore, with the application of LC-ESI-MS tachykinins have been detected in brain regions such as the hypothalamus (Tenorio-Laranga *et al.*, 2009). Moreover, quantification of the levels of SP in the spinal cord was achieved with LC-ESI-MS (Beaudry & Vachon, 2006; Beaudry *et al.*, 2008). In other brain tissues such as the pituitary, LC-ESI-MS has been applied for the quantification of neuropeptides such as vasopressin and oxytocin or the discovery of novel potential endogenous peptides and the comparison of the efficiency of two extraction buffers *i.e.* a more established aqueous acetic acid and a urea buffer (Altelaar *et al.*, 2008).

In summary, in this Chapter the development of a RP-HPLC method on a C18 column for the separation of the four peptides of this study's predominant interest was described and also their detection using MALDI-TOF. Moreover, two peptide extraction methods were described and the investigation of the ability of the Sepharose-antibody conjugates to purify peptides as well. In our experiments we used synthetic peptides made according to the published sequences for SP, NKB and hHK-1. The sequence of hHK-1 was derived from the molecular cloning experiments of the human *TAC4* gene. On the other hand, the sequence of mHK-1 was synthesised as an amidated decapeptide with the aim of generating control spectra on MS and chromatograms on HPLC to compare to the results from the endogenous mHK-1 and see how processing occurs and whether there are any post-translational modifications.

Chapter 4. Identification of a novel post-translational modification on the mHK-1 decapeptide found in the rat brain, by means of immunoaffinity purification, RP-HPLC and MALDI-TOF.

4.1) Introduction.

Tachykinins are expressed as preprotachykinin precursors, released upon processing by prohormone convertases at cleavage sites, acquiring bioactivity, firstly, with the action of the enzymes carboxypeptidases that remove the dibasic residues from the carboxyl terminal of the released peptide and secondly, the glycine converting enzyme PAM (reviewed in Nelson & Bost, 2004). Further tissue-specific post-translational modifications might also occur (Lovell *et al.*, 2007). The well-studied tachykinins SP and NKA are encoded on the TAC1 precursor. Processing of the TAC1 precursor at both the dibasic cleavage sites (KR) that flank these peptides releases their mature forms, while the incomplete processing of the precursor at the N-terminus dibasic cleavage sites (KR) releases the extended forms of NKA, namely NPK and NPγ (Page, 2005). The TAC3 preprotachykinin precursor comprises the tachykinin NKB which is a conserved sequence in mammalian species, flanked by dibasic KR cleavage sites, where complete processing releases the NKB decapeptide while partial processing at the N-terminus releases the extended form of NKB (Page, 2005). The TAC3 preprotachykinin precursor also comprises an additional dibasic cleavage site at its N-terminus where processing releases residues 50-79, a non-tachykinin peptide, namely peptide 2 (Lang & Sperk, 1995). The human TAC4 preprotachykinin precursor was found to comprise the hHK-1 undecapeptide and its extended forms EKA/B. The tachykinin hHK-1 is flanked by a dibasic cleavage site at its C-terminus capable of releasing the amidated hHK-1 undecapeptide, however at its N-terminus a single base substitution was observed that changes the R residue to T, hence destroying the N-terminal dibasic cleavage site (Page *et al.*, 2003). The human TAC4 preprotachykinin precursor processing at the signal peptide cleavage site releases the extended forms of hHK-1, namely EKA/B comprising 47 or 41 residues (Page *et al.*, 2003). The alternative processing of the human TAC3 and TAC4 precursors is of particular interest in pathological conditions such as pre-eclampsia

(Page, 2006; Page, 2009). Moreover, the presence of a potential post-translational modification such as phosphocholine on the mature NKB peptide in pre-eclampsia is of interest for its biological significance, potentially, in attenuating the immune system of the mother (Lovell *et al.*, 2007). The discovery of mHK-1 introduced the group of mammalian tachykinins called endokinins, characterised by their peripheral expression and endocrinological roles (Page, 2004). Zhang and colleagues (2000) first discovered and isolated the *Tac4* gene encoding the pre-protachykinin C (PPT-C, or TAC4) precursor in mice that was differentially expressed during differentiation of pro-B cells. The mouse TAC4 precursor contained a peptide that comprised the FYGLMG tachykinin signature motif flanked by dibasic lysine-arginine (KR) cleavage sites, inferring the existence of the novel tachykinin peptide HK-1. The sequence of the mHK-1 peptide was deduced from the nucleotide sequence of the TAC4 precursor gene and Zhang *et al.*, (2000) predicted the cleavage of the precursor released an amidated undecapeptide with the sequence RSRTRQFYGLM-NH_2 (Zhang *et al.*, 2000). However, the processing of the mouse TAC4 precursor is unlikely to be the same as TAC1 precursor processing for the release of the SP undecapeptide, where in the presence of a proline, cleavage occurs between the dibasic RR cleavage site.

Further studies showed that the cloned rat (*Tac4*) and human (*TAC4*) orthologs had significant sequence homology to the mouse *Tac4* (Kurtz *et al.*, 2002; Page *et al.*, 2003). The mouse and rat TAC4 orthologs are highly conserved and the sequence of mHK-1 is identical between the two species; both mouse and rat peptides have been named as rat/mouse HK-1 and the sequence for r/m HK-1 was reported as RSRTRQFYGLM-NH_2 (Zhang *et al.*, 2000, Kurtz *et al.*, 2002). The mHK-1 tachykinin was reported to resemble most closely SP in sequence and function compared to the other known mammalian tachykinins. Binding studies on the NK-1 receptor showed r/m HK-1 to have equipotent activity on this receptor as SP (Morteau *et al.*, 2001; Bellucci *et al.*, 2002; Camarda *et al.*, 2002) and to show the similar affinity for each of the mouse, rat and human NK-1 receptors (Kurtz *et al.*, 2002; Duffy *et al.*, 2003; Page *et al.*, 2003). However, later studies have suggested that potentially the mHK-1 and SP could bind to different subtypes of NK-1 receptor (Page, 2006; Sunakawa *et al.*, 2010).

Page (2004) reported the expression of the *Tac1* and *Tac4* genes in both rat spleen and rat brain tissues. In accordance with this study the expression of the *Tac4* gene in the rat brain has also been shown by RT-PCR (Kurtz *et al.*, 2002, Nelson & Bost, 2005). Duffy and colleagues (2003) performed expression analysis by quantitative PCR of the *Tac4* and *Tac1* (encoding SP) genes on a wide range of mouse tissues and specific brain regions. Their study showed the *Tac4* mRNA was detected in the mouse brain and spleen. In the brain regions examined, both mouse *Tac4* and *Tac1* were detected, although *Tac4* expression levels were considerably lower than *Tac1*. However, the detection of *Tac1* was not reported in the mouse spleen (Duffy *et al.*, 2003). The *Tac4* gene has also been reported to be expressed in hematopoietic and immune cells and not detected in brain (neuronal) tissue as shown by Northern blot analysis (Zhang *et al.*, 2003; Tran *et al.*, 2009).

The mouse *Tac4* gene cDNA sequence comprises 1248 base pairs encoding a 128 amino acid PPT-C (Zhang *et al.*, 2000). Cleavage of the precursor occurs by the enzymes prohormone convertases also referred to as proprotein convertases (Rholam & Fahy, 2009). The prohormone convertases (*e.g.* PC1/3 and PC2) cleave the preprotachykinin at the carboxyl terminus of the lysine-arginine (KR) or arginine-arginine (RR) cleavage sites (Douglas Lu *et al.*, 2012). The remaining KR residues on the carboxyl terminus of the cleaved peptide are removed by carboxypeptidase E (Douglas Lu *et al.*, 2012). In the presence of a proline adjacent to a dibasic cleavage site, as is found at the N-terminus of the SP sequence, there is resistance to enzymatic cleavage by prohormone convertases C-terminal to the RR (Gainer *et al.*, 1984). Instead cleavage with a proline adjacent to the RR causes processing between the two arginines. We suggest that in the absence of a proline residue it is unlikely processing occurs between the two K/R residues of the N-terminal cleavage site flanking mHK-1. Hence, the released peptide from the cleavage of the TAC4 precursor is most probably an amidated decapeptide with molecular weight 1256.6 Da.

4.2) Identification of the molecular weight of tachykinin peptides and investigation of post-translational modifications

In the previous Chapters 2 and 3 we described the methodology of developing a Sepharose-anti-tachykinin antibody conjugate efficient to capture and enrich tachykinin peptides. We have also described two peptide extraction methods and a RP-HPLC method using a C18 column efficient in separating a mixture of tachykinins into specific fractions. The scope of the procedures described in this Chapter, is the application of this methodology to capture, enrich and separate tachykinins extracted from rat brain, spleen, and placentas as well as human placenta and their detection by MALDI-TOF.

Most (if not all) commercial sources of mHK-1, provide the peptide chemically synthesised by a solid phase peptide synthesiser as the predicted undecapeptide (RSRTRQFYGLM-NH_2) proposed initially by Zhang and colleagues (2000). Nonetheless, no reports presently exist on the precise sequence of the naturally occurring peptide or have investigated the possibility of any post-translational modifications. Moreover, existing data on the expression of the *Tac4* gene in the rat/mouse brain are contradictive. Therefore, the scope of the research covered in this Chapter was the capture of mHK-1 from rat brains and spleens (an example peripheral tissue), the confirmation of its molecular weight and the identity of any potential post-translational modifications. We suggest that unlike SP, which in the presence of a proline residue is cleaved at the carboxyl terminus of the first R residue (of the dibasic RR site), mHK-1 is cleaved at the carboxyl terminus of the KR dibasic cleavage site. Thus, the cleavage product is an amidated decapeptide (and not an undecapeptide) with the sequence SRTRQFYGLM-NH_2 and molecular mass 1256.6 Da.

Moreover, the above methodology was applied on rat and human pre-eclamptic placentas with the scope of investigating the alternative processing of the TAC3 and TAC4 precursors and comparing the processing pattern with the rat brain. According to previous findings processing of the TAC3 and TAC4 precursors in the human placenta will produce the extended forms of NKB and hHK-1. Processing of the TAC3 precursor in the

pre-eclamptic placenta releases the amidated NKB decapeptide (same as in the rat brain), while processing of the human TAC4 precursor in the pre-eclamptic placenta releases the amidated hHK-1 peptide.

4.3) Materials and Methods.

4.3.1) Rat spleen homogenisation and peptide extraction.

Rat spleen tissue (1 g) kept frozen (at -70°C) was ground using a pestle and mortar while keeping frozen (at -160°C) with the addition of liquid nitrogen. The ground tissue was transferred into a 2 ml Eppendorf tube and 1 ml of extraction buffer (90% (v/v) methanol with 9% (v/v) acetic acid, stored at 4 °C, Section 4.3.1) was added. The ground tissue was mixed well with the acidified methanol buffer using a scalpel fitted into the 2 ml tube. The homogenous mixture was left at 4 °C overnight. The following day the mixture was spun at 7558 x g (13,000 RPM) for 15 min (at room temperature) for the tissue to form a pellet. The supernatant (~1 ml) was collected and transferred into a clean 2 ml tube. The tissue was re-homogenised using 1 ml of 90% (v/v) methanol with 9% (v/v) acetic acid and placed at 4 °C for 1 hour. The mixture was centrifuged again at 7558 x g (13,000 RPM) for 15 min and the supernatant was collected and transferred into a clean 2 ml tube.

Into the two 2 ml tubes (each containing 1 ml of the supernatant from the two extractions), 1 ml of chloroform was added. The two tubes were vortexed for 15 min until a homogenous mixture was formed. The mixtures were immediately spun at 7558 x g (13,000 RPM) for 10 min at room temperature. Three phases were visible in each 2 ml tube, a lower clear phase (containing lipids ~1 ml), an intermediate "pink" phase (denatured proteins) and an upper phase (containing peptides and sugars ~1 ml). From each tube ~0.8 ml of the upper phase containing the peptide was carefully collected (the interphase was not disrupted) and transferred into two clean 2 ml tubes. The chloroform/methanol extraction was repeated for the removal of further protein and lipids; another 1 ml of chloroform was added into each sample, which was vortexed for 15 min and centrifuged at 7558 x g (13,000 RPM) for 10 min at room temperature. Three phases were formed, an upper phase, a "faint" interphase with denatured proteins and a lower phase. The upper phase (~0.5 ml) was collected and transferred into a clean tube.

The upper phase sample (~1 ml in total; approximate composition: 45% methanol, 5% acetic acid, 50% water) appeared clear.

The 1 ml of peptide extract sample was diluted with 8 ml of 0.1% (v/v) TFA. A C18 Sep Pak cartridge was primed with 10 ml of ~100% methanol and equilibrated with 5 ml of 0.1% (v/v) TFA. The peptide was concentrated using a C18 cartridge; the 9 ml of diluted peptide extract were passed through the C18 cartridge at a flow rate of 1 ml/ min, which was then "washed" by pushing through 5 ml of 0.1% (v/v) TFA followed by 5 ml of distilled H_2O (to remove any residual TFA). The bound peptide was eluted from the C18 cartridge with 1 ml of ~100% methanol. Binding buffer, 100 µl of 1x PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors (prepared as described in Section 4.5.1), was added to the C18 eluent. The sample was concentrated to 100 µl by removing residue methanol by blowing a "slow" stream of nitrogen gas over the sample at room temperature. The procedure is illustrated in Figure 4.1.

4.3.2) Sepharose-antibody washing procedure.

Immunoaffinity purification was performed using the three Sepharose-antibody conjugates *i.e.* BO16 mixture, BO16 1E1 EXP1, BO16 1E1 EXP2 (described in Section 2.3.6). The three Sepharose-antibody conjugates (~3 ml each) were stored in storage buffer, 1 M NaCl with 10 mM EDTA and 0.02% (w/v) NaN_3 (total volume ~5 ml) in three 50 ml Falcon tubes at 4°C. The three Sepharose-antibody affinity media (BO16 mixture, BO16 1E1 EXP1, BO16 1E1 EXP2 were mixed into homogenous slurry. An aliquot (50 µl of mixture or ~30 µl of Sepharose-antibody complex) of each Sepharose-antibody in storage buffer mixture was removed and transferred into a clean 1.5 ml tube (using a yellow tip with the end cut off, taking up equal amount of beads and buffer).

The mixture (aliquots) of the three Sepharose-antibody complexes was incubated with acidic buffer of high ionic strength (0.1 M glycine pH ~2.5) for the removal of potential impurities weakly bound onto the Sepharose and the antibodies. Into the 1.5 ml tube

containing the ~90 µl mixture of Sepharose-antibody complex, 1 ml of "resin wash" buffer (0.1 M glycine pH ~2.5 with 0.02% (w/v) NaN_3 prepared as described in Section 3.5.1) was added. The 1.5 ml tube of the Sepharose-antibody complex in "resin wash" buffer was placed for 3 min on the bench, then inverted twice before spinning at 716 x g (4000 RPM) for 1 min for the Sepharose-antibody beads to make a pellet and the supernatant was discarded. This acidic "washing" procedure was repeated 3 times. The clean Sepharose-antibody pellet was brought to "binding conditions" by adding 1 ml of the binding buffer (1 x PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitor; prepared as in Section 4.5.1) and left for 3 min at room temperature, before inverting twice. The 1.5 ml tube was spun at 716 x g (4000 RPM) for 1 min, the Sepharose-antibody beads made a pellet and the buffer was discarded. The procedure was repeated three times in total. Following the last wash the pH of the Sepharose-antibody was checked as pH ~7.5 with a final volume of ~100 µl.

4.3.3) Immunoaffinity purification.

The pH of the 100 µl of the peptide extract (Section 4.3.1) was adjusted to pH ~7.5 (using 1 M TRIS-BASE, pH10). The ~100 µl of the "washed" Sepharose-antibody affinity medium (see Section 4.3.2) was transferred into a 1.5 ml tube containing the peptide extract and the final volume of the reaction was brought to 500 µl in "binding" buffer (1 x PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors). The 1.5 ml reaction tube was incubated overnight at room temperature on a rotating platform to allow antibody-antigen interactions. The following day the 1.5 ml reaction tube was spun at 548 x g (4,000 RPM) for 1 min. The Sepharose-antibody-peptide complex formed a pellet from which the supernatant was aspirated and discarded. The Sepharose-antibody-peptide conjugate was washed by adding 1 ml working buffer (1 x PBS with 10 mM EDTA and 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors) to remove non-specifically bound peptide. The Sepharose-antibody-peptide complex was incubated in the buffer on the bench for 2 min, inverting twice. The 1.5 ml tube was spun at 548 x g

(4,000 RPM) for 1 min. This step was repeated three times in total. After the last wash, 500 µl of working buffer was added into the 1.5 ml tube and used to transfer the Sepharose-antibody-peptide complex into a mini-column set over a 2 ml tube. The buffer was left to drip through. Then, into the mini-column 500 µl of distilled H_2O were added and left to drip through removing salt-containing buffer. The mini-column and tube set-up was spun at 45 x g (1,000 RPM) for 1 min to remove residual buffer and salt or excess distilled H_2O.

Elution buffer (90 µl of 0.5% (w/v) TFA) was added to the Sepharose-antibody-peptide conjugate. The Sepharose-antibody-peptide was incubated with the buffer for 5 min on the bench (mixed slightly with the pipette tip). After 5 min incubation the mini-column was uncapped and spun at 716 x g (4000 RPM) for 5 min. The elution procedure (of adding elution buffer, incubation and centrifugation to collect the eluted peptide) was repeated three times. The eluent (~ 400 µl) containing the captured tachykinin peptides was collected into a 1.5 ml tube and stored at -20°C.

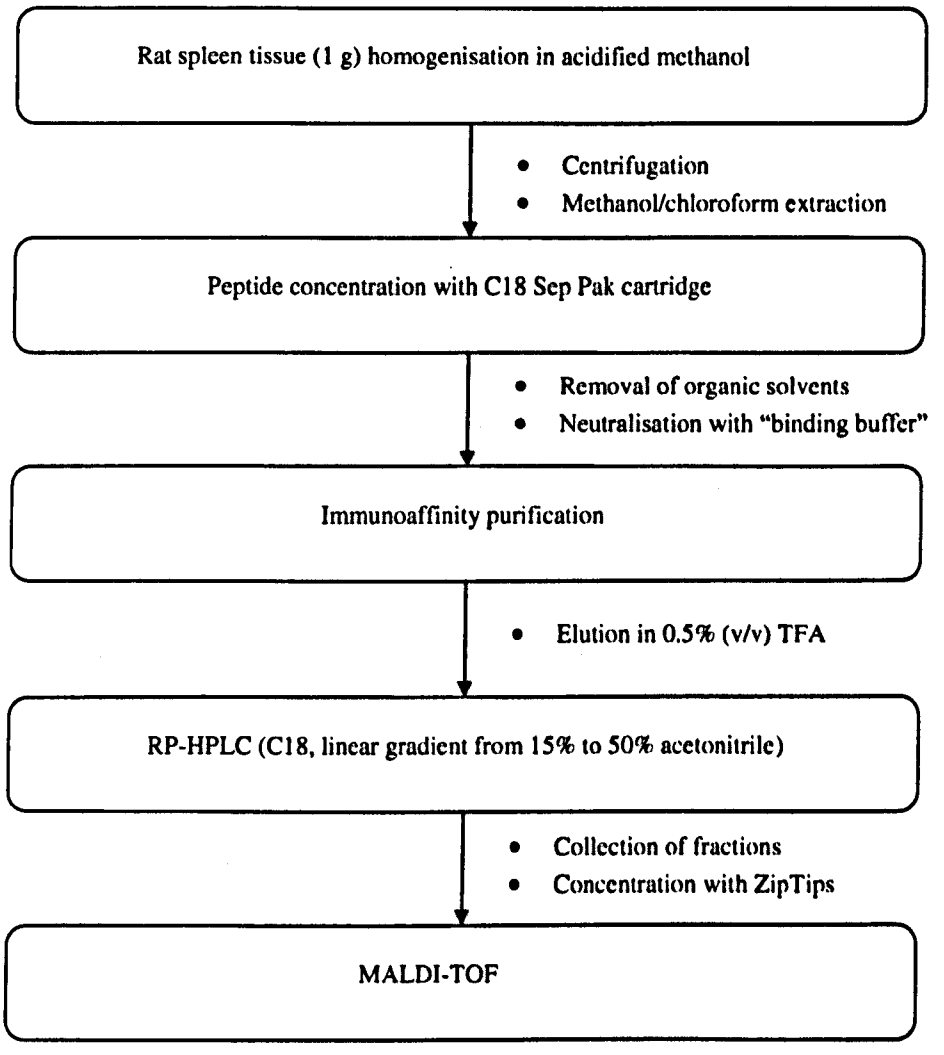


Figure 4.1. The schematic overview of the experimental procedure followed to capture and characterise the tachykinins expressed in the rat spleen.

4.3.4) Rat brain homogenisation and peptide extraction.

One rat brain ~1 g weight (kept at -70°C) was homogenised with a 10 ml syringe (without a needle) into a 2 ml Eppendorf tube and 1 ml of either extraction buffer *i.e.* Bennett's solution consisting of 1 M HCl containing 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA, or 90% (v/v) methanol with 9% (v/v) acetic acid was added. The mixture of tissue in either extraction buffer was placed at 4 °C overnight. The next day, the mixture was spun at 7,558 x g (13,000 RPM) on a table-top centrifuge for 15 min at room temperature. The homogenised tissue formed a pellet and the supernatant containing the peptide extract was recovered (around 0.9 ml), transferred into a clean 2 ml tube and placed on ice. An equivalent volume (0.9 ml) of chloroform was added into the 2 ml tube containing the supernatant from the methanol extract. The methanol/chloroform extraction was performed as described for the spleen tissue (Section 4.3.1). In total 0.8 ml of peptide extract was collected from this methanol/chloroform extraction. The mixture of the homogenised brain in 1 M HCl containing 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA was centrifuged as described above. A peptide extract of 0.9 ml was collected from this second extraction.

The two peptide extracts (using either acidified methanol or Bennett's solution) were diluted to 8 ml in 0.1% (v/v) aqueous TFA. Two C18 Sep Pak cartridges (Waters, WAT051910) were primed with ~ 100% methanol (10 ml) and equilibrated with 0.1% (v/v) TFA (5 ml). A 10 ml syringe was attached to each cartridge as a funnel and ~5 cm of silicon tubing was attached to establish a ~1 ml/min flow rate. The diluted peptide extracts were passed through the two cartridges. Each cartridge was washed by pushing through 10 ml of 0.1% (v/v) TFA followed by 10 ml of distilled H_2O. The bound peptide was eluted from the C18 cartridge retaining the peptide extracted using 1 M HCl containing 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA by pushing through 1 ml of 60% (v/v) acetonitrile with 0.1% (v/v) TFA. The retained peptide on the C18 cartridge extracted using the acidified methanol buffer was eluted by pushing through 1 ml of methanol. The eluents were collected into two 1.5 ml Eppendorf tubes. Binding buffer (100 µl of PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v)

NaN_3 plus serine and cysteine proteases inhibitors) was added into the two tubes containing the concentrated peptide extracts. The samples were concentrated to 100 µl by removing the organic solvents under N_2 flow at room temperature. The outline of the procedure followed is illustrated in Figure 4.2.

4.3.5) Immunoaffinity purification.

The peptides contained in each of the two 100 µl extractions from acidified methanol or Bennett's solution, were enriched using immunoaffinity purification. The three Sepharose-antibody affinity matrixes BO16 mixture, BO16 1E1 EXP1 and BO16 1E1 EXP2 (stored in 1 M NaCl with 0.02% (w/v) NaN_3 and 10 mM EDTA at 4°C, section 3.2.6) were taken out of 4°C and mixed into homogenous slurry of Sepharose-antibody complex in storage buffer. A 60 µl aliquot of Sepharose-antibody complex was removed and transferred into a 1.5 ml tube. The Sepharose-antibody complex was "washed" with low pH buffer (0.1 M glycine pH ~2.5 with 0.02% (w/v) NaN_3) and brought to "binding conditions" with binding buffer (1 ml of 1 x PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors) following the same procedure as described in Section 4.3.2.

The pH of the two 100 µl peptide extracts (Section 4.3.4) was adjusted to pH ~7.5 using 1 M TRIS-BASE pH 10. Each extract was mixed with 90 µl of "washed" Sepharose-antibody affinity conjugate and the final volume of the two reactions was brought to 500 µl in binding buffer. The two reactions were incubated overnight at room temperature on a gentle rotating platform. The washing and elution procedures are described in Section 4.3.3. Briefly, non-specifically bound peptide was washed off three times with 1 ml of working buffer (1 x PBS containing 10 mM EDTA with 0.01% (w/v) octyl glycoside and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors). After the last wash, 500 µl working buffer was added into each of the two 1.5 ml tubes and used to transfer the Sepharose-antibody-peptide complexes into two mini-columns set over a 2 ml tube; then also washed with distilled H_2O. Elution buffer (90 µl of 0.5% (w/v) TFA) was added into the Sepharose-antibody-peptide conjugates, incubated with the buffer for 5 min and spun

to collect the eluted peptide. The elution procedure was performed four times and then the four aliquots were combined into a 400 μl sample.

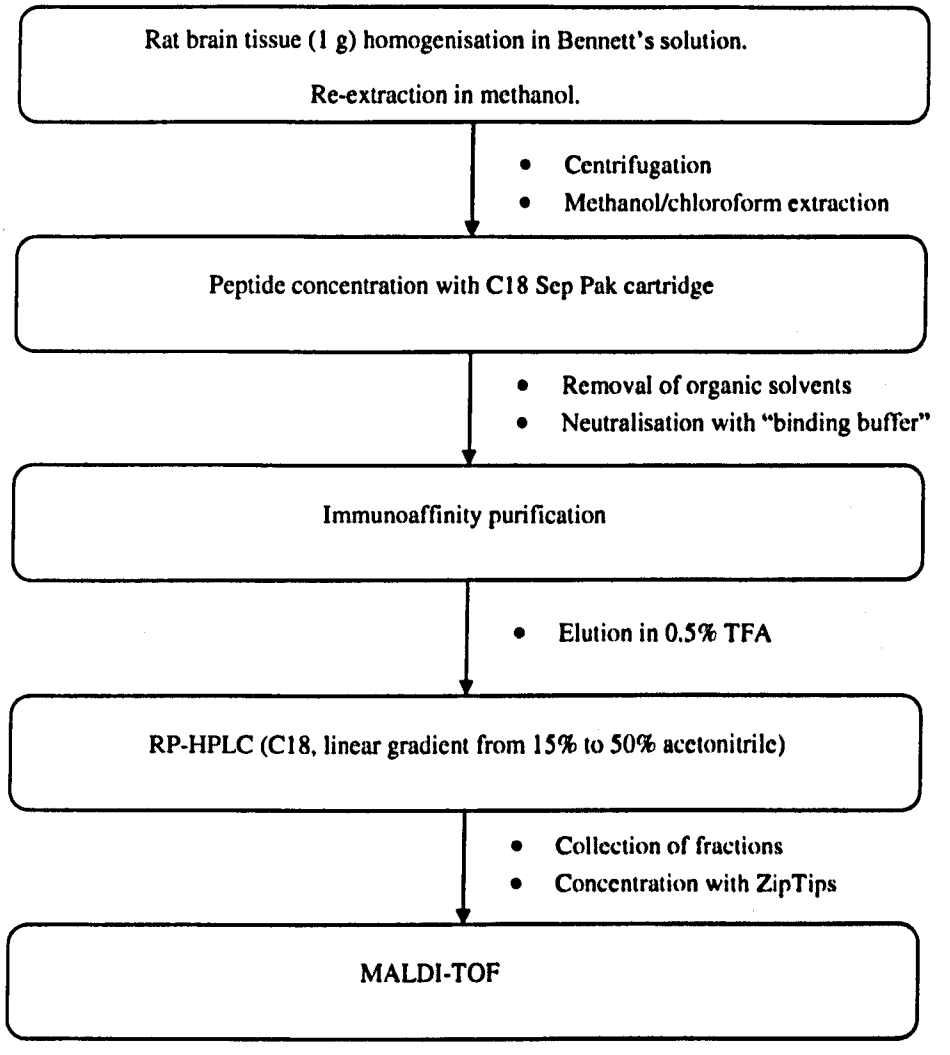


Figure 4.2. The schematic overview of the experimental procedure followed to capture and characterise the tachykinins expressed in the rat brain. Two extractions were performed using 1 rat brain. The same experimental procedure as for the rat spleen was followed.

4.3.6) Rat placenta homogenisation, peptide extraction and enrichment of peptide.

Rat placentae ~1 g weight (kept at -70°C) were ground to cellular level using a pestle and mortar while keeping frozen (at -160 °C) with the addition of liquid nitrogen. The tissue was transferred into a 2 ml Eppendorf tube and 1 ml of extraction buffer either Bennett's solution or 90% (v/v) methanol with 9% (v/v) acetic acid was added. The mixture of tissue in either extraction buffer was placed at 4 °C overnight. The next day, the mixture was spun at 7,558 x g (13,000 RPM) on a table-top centrifuge for 15 min at room temperature. The homogenised tissue formed a pellet and the supernatant containing the peptide extract in acidified methanol buffer was recovered (around 0.9 ml), transferred into a clean 2 ml tube and placed on ice. An equivalent volume (0.9 ml) of chloroform was added into the 2 ml tube containing the supernatant from the acidified methanol extract. The methanol/chloroform extraction was performed as described for the spleen tissue (Section 4.3.1). In total 0.8 ml of peptide extract was collected from this methanol/chloroform extraction. The mixture of the homogenised rat placental tissue in Bennett's solution was centrifuged as described above. A peptide extract of 0.9 ml was collected from this second extraction. The peptide extracts from the two extractions were concentrated using a C18 cartridge as described above. Organic solvents were removed under N_2 flow at room temperature. The peptides contained in each of the two 100 µl extractions from acidified methanol or Bennett's solution, were enriched using immunoaffinity purification. The three Sepharose-antibody affinity matrixes BO16 mixture, BO16 1E1 EXP1 and BO16 1E1 EXP2 (stored in 1 M NaCl with 0.02% (w/v) NaN_3 and 10 mM EDTA at 4°C) were taken out of 4°C and mixed into homogenous slurry of Sepharose-antibody complex in storage buffer. A 60 µl aliquot of each Sepharose-antibody complex was removed and transferred into a 1.5 ml tube. The Sepharose-antibody complex was "washed" and brought to "binding conditions" following the same procedure as described in Section 4.3.2. The pH of the two 100 µl peptide extracts was adjusted to pH ~7.5 using 1 M TRIS-BASE pH 10. Each extract was mixed with 90 µl of "washed" Sepharose-antibody affinity conjugate and the final volume of the two reactions was brought to 500 µl in binding buffer. The two reactions were incubated overnight at room temperature on a gentle rotating platform. The

washing-off of non-specifically bound peptide with 1 ml of working buffer (1 x PBS containing 10 mM EDTA with 0.01% (w/v) octyl glycoside and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors) was performed as described before. Elution buffer (90 µl of 0.5% (w/v) TFA) was added into the Sepharose-antibody-peptide conjugates, incubated with the buffer for 5 min and spun to collect the eluted peptide. The elution procedure was performed four times and then the four aliquots were combined into a 400 µl sample.

4.3.7) Human placenta homogenisation, peptide extraction and enrichment of peptide.

Human placental samples were received from a pre-eclamptic patient recruited at Kingston Hospital after the patient's written consent and COREC approval. The homogenisation procedure was conducted in a Class II Microbiological Safety Cabinet (LABCAIRE). The placental tissue ~40 g (kept at -80 °C) was homogenised using a blender with ~85 ml of cold (4 °C) extraction buffer, 90% (v/v) methanol with 9% (v/v) acetic acid. The homogenised tissue in extraction buffer (making a total volume of ~180 ml) was split into four 50 ml Falcon tubes. The four 50 ml Falcon tubes were left at 4 °C overnight.

The following day the four 50 ml Falcon tubes containing the homogenised placenta in the extraction buffer were removed from 4°C and centrifuged at 1509 x g (3,000 RPM) for 15 min. The unbroken cells and tissue debris formed a pellet. From the four 50 ml Falcon tubes ~25 ml from the supernatant was aspirated and transferred into a clean 50 ml Falcon tube. Into the ~25 ml of supernatant were added ~25 ml of chloroform. The four 50 ml Falcon tubes were vortexed for 15 min until a homogenous mixture was formed and then centrifuged at 1509 x g (3,000 RPM) for 10 min. Three phases were visible in every 50 ml Falcon tube, a lower phase containing lipids, an interphase with denatured proteins and an upper phase with peptides and sugars. The upper phase containing the peptides was aspirated from each 50 ml Falcon and transferred into two 50 ml Falcon tubes. The collected upper phase was ~70 ml (approximate constitution, 45% methanol, 50% water, 5% acetic acid).

The extracted peptides were concentrated using a C18 Sep Pak cartridge. The upper phase (pale coloured) approximately 70 ml was diluted nine times with 0.1% (v/v) TFA. A C18 Sep Pak cartridge was primed with 5 ml methanol and equilibrated with 10 ml of 0.1% (v/v) TFA. The 650 ml of diluted peptide extract in 0.1% (v/v) TFA was passed through the C18 Sep Pak cartridge for ~3 hr using a 60 ml syringe as a funnel and silicon tubing. Subsequently, the C18 Sep Pak cartridge was washed with 5 ml 0.1% (v/v) TFA followed by 5 ml distilled H_2O. The bound peptide was eluted with 2 ml methanol into a 2 ml Eppendorf tube. Binding buffer, 100 µl of 1x PBS containing 10 mM EDTA with 0.01% (w/v) O.G. and 0.02% (w/v) NaN_3 plus serine and cysteine proteases inhibitors was added to the 2 ml C18 eluent. The sample was concentrated to 100 µl removing methanol by blowing a "slow" stream of nitrogen gas over the sample at room temperature.

The pH of the 100 µl peptide extract was adjusted to pH ~7.5 using 1 M TRIS-BASE pH 10. The extract was mixed with 90 µl of "washed" Sepharose-antibody affinity conjugate and the final volume of the reactions was brought to 500 µl in binding buffer. The immunoaffinity purification reaction was placed overnight at room temperature on a gentle rotating platform. The washing and elution procedures are described in Section 4.3.3. Elution buffer (90 µl of 0.5% (v/v) TFA) was added into the Sepharose-antibody-peptide conjugate, incubated with the buffer for 5 min and spun to collect the eluted peptide.

A trypsinisation reaction was performed using an aliquot of the placental immunoaffinity purification eluent to spot on MALDI-TOF for peptide mass fingerprinting. An aliquot (10 µl) of the immunoaffinity purification eluent was transferred into a 1.5 ml Eppendorf tube and neutralised with 1 µl of 1 M TRIS-BASE pH 10. Into the mixture 1 µl of 1 mg/ml trypsin and 1.3 µl of 10 mM $CaCl_2$ were added. The final volume of the reaction was ~13 µl and pH was adjusted to pH ~8.5 -9. The trypsinisation was performed at 37 °C for 3 hours. An aliquot of the placental pull-down was trypsinised and the resultant peaks were detected on MALDI-TOF. A "mass" list of peptide fragments was exported from the instrument and the detected masses were inserted onto the on-line database

MASCOT. The "Swiss Prot" database was chosen and parameters of the algorithm were set as, mass tolerance ±0.1 Da, enzyme: trypsin, allow up to 1 missed cleavages.

4.3.8) Separation of enriched peptides on RP-HPLC and the collection of fractions.

The RP-HPLC method that was applied for the separation of the mixture of tachykinin peptides consisted of a reversed phase C18 column (Vydac 218TP54, 5 mm, 4.6 mm x 250 mm) and a mobile phase A that consisted of 50% (v/v) acetonitrile with 0.04% (v/v) TFA and a mobile phase B that consisted of distilled H_2O with 0.04% (v/v) TFA prepared as described in Section 3.4.4. The gradient method to allow separation of the four peptides was set up as presented in Table 3.8 with the absorbance set at 205 nm. Into the system, 20 µl of a 20 µg/ml sample of mHK-1, SP, hHK-1 and NKB were injected; then the samples of rat spleen pull-down (acidified methanol) and rat brain (acid extract) pull-down were injected. Eighty fractions were collected from 5 min after the beginning of the run to 45 min (1 every 30 s) with a final volume of 250 µl each. Each of the eighty fractions collected from the separation of the rat spleen pull-down and rat brain (acid extract) pull-down was concentrated to 20 µl by blowing a "slow" stream of nitrogen gas over the sample.

4.3.9) Concentration of fractions with ZipTip$_{\mu\text{-}C18}$ and detection on MALDI-TOF.

Peptides were detected using MALDI-TOF (Bruker Autoflex). The materials used for the detection of the peptides are described in Section 3.3.4. The 0.6 ml poly-propylene tubes and yellow tips used and MALDI-TOF ground stainless steel plate were rinsed with methanol and left to air dry. A fresh solution of α-HCCA matrix was prepared by transferring into a clean micro-centrifuge the appropriate amount of the α-HCCA matrix (6.2 mg/ml) and diluting 2:1 in 100% (v/v) ethanol with 1% (v/v) TFA. Each 20 µl fraction was further concentrated with a ZipTip$_{\mu\text{-}C18}$ (Millipore); each ZipTip$_{\mu\text{-}C18}$ was primed by taking up and releasing 5 x 20 µl of 50% (v/v) methanol with 0.1% (v/v) TFA and equilibrated by taking up and releasing five times 20 µl of 0.1% (v/v) TFA. The peptide present in each 20 µl fraction was bound to the C18 ZipTip$_{\mu\text{-}C18}$ by taking up and

releasing the sample five times and washed by taking up and dispensing five times 20 µl of 0.1% (v/v) TFA. The bound peptide was eluted by taking up and releasing 2 µl of the α-HCCA matrix in 70% (v/v) ethanol with 0.7% (v/v) TFA five times and spotting immediately on a MALDI-TOF plate. Moreover, 1 µl of a 5 µg/ml of trypsinised BSA stock (as described in Section 5.3.6) was pipetted into a 4 µl aliquot of α-HCCA matrix in 70% (v/v) ethanol with 0.7% (v/v) TFA. 1 µl of the trypsinised BSA and matrix mixture was taken up and used to make three spots on the MALDI-TOF plate. The MALDI-TOF instrument was calibrated with the trypsinised BSA fragments.

4.4) Results.

4.4.1) Tachykinins SP and mHK-1 detected in rat spleens.

A mixture of the four synthetic peptides, mHK-1, SP, hHK-1and NKB, was run on the RP-HPLC system (chromatogram shown in Figure 4.3, A) and 80 fractions were collected and spotted on a stainless steel plate. Each spot was investigated using the MALDI-TOF for the detection of peaks of the four peptides and the elution fraction of each peptide was found. The synthetic mHK-1 decapeptide (comprising the sequence SRTRQFYGLM-NH_2) has a theoretical singly charged mass $[M+H]^+$ 1257.652 and was found to elute in fraction 9 (retention time 834 s) as proven by the mass spectrum shown in Figure 4.4, A. The singly charged ion peak with mass-to-charge ratio (m/z) 1257.629 detected in the spectrum corresponds to the synthetic mHK-1; also the peak detected at m/z 1273.632 is the oxidised form of the peptide (the product of oxidised methionine, sulphur to sulphoxide). The predicted $[M+H]^+$ of SP is 1347.735; in fraction 25 (retention time 1635 s) from the separation of the synthetic peptide mixture, two ion peaks were detected in the spectrum corresponding to SP with m/z 1347.682 and the oxidised form (the product of oxidised methionine, sulphur to sulphoxide) with m/z 1363.657 (Figure 4.5, A); the peak of the un-oxidised form m/z 1347.682 was detected with greater intensity than the oxidised form m/z 1363.657.

Rat spleen was chosen as a peripheral tissue to isolate the mHK-1 peptide and identify the M.W. of the mature sequence of the peptide as a result of the high expression of *Tac4* in the tissue as reported by Page (2004). The rat spleen tissues were homogenised in acidified methanol and the extracted tachykinins were enriched with immunoaffinity purification. The eluent of the captured tachykinins (pull-down) was injected into a reversed-phase C18 HPLC column (Figure 4.3, B). The eighty fractions collected from the separation of the rat spleen pull-down were concentrated with ZipTips and spotted on a stainless steel MALDI-TOF plate. It was found that the two tachykinins detected in the rat spleen were mHK-1 and SP, while NKB was not found in the tissue as the *Tac2* gene in spleen is not present either (Page, 2004). The peaks of mHK-1 and SP (and their

derivatives) are summarised in Table 4.1. In fraction 9 from the rat spleen pull-down a peak with m/z 1257.701 was detected corresponding to the singly-charged $[M+H]^+$ endogenous mHK-1 (Figure 4.4, B). In the same spectrum were also detected a peak with m/z 1273.697 (ΔM +15.999 amu), the product of oxidised methionine (sulphur to sulphoxide); a peak with m/z 1221.523 (ΔM -36 amu) the product of water loss of serine (S) and threonine (T) residues; and a peak with m/z 1209.698 (ΔM -48 amu), the product of decomposed carboxymethylated methionine and/or the elimination of methanesulphenic acid (CH_3SOH, ΔM -64 amu) from methionine sulphoxide (Figure 4.6). The m/z 1209.698 peak is most likely the result of post-source decay which occurred during ionisation.

In the spectrum obtained from fraction 25 of the rat spleen pull-down three peaks were detected corresponding to singly charged SP, $[M+H]^+$ 1347.607, the oxidised form of SP (O) 1363.657 (Figure 4.5, B) and a peak with m/z 1311.568; the product of SP with the neutral loss of two water molecules. The peak of the oxidised form of SP (O) 1363.657 was detected with greater intensity than the normal form of SP $[M+H]^+$ 1347.607. Moreover, in Figure 4.5, C the spectrum of fraction 37 from the rat spleen pull-down is shown; the ions corresponding to SP, $[M+H]^+$ 1347.894, its oxidised form SP (O) 1363.896 and SP with the loss of two water molecules (m/z 1311.662) were detected in high abundance.

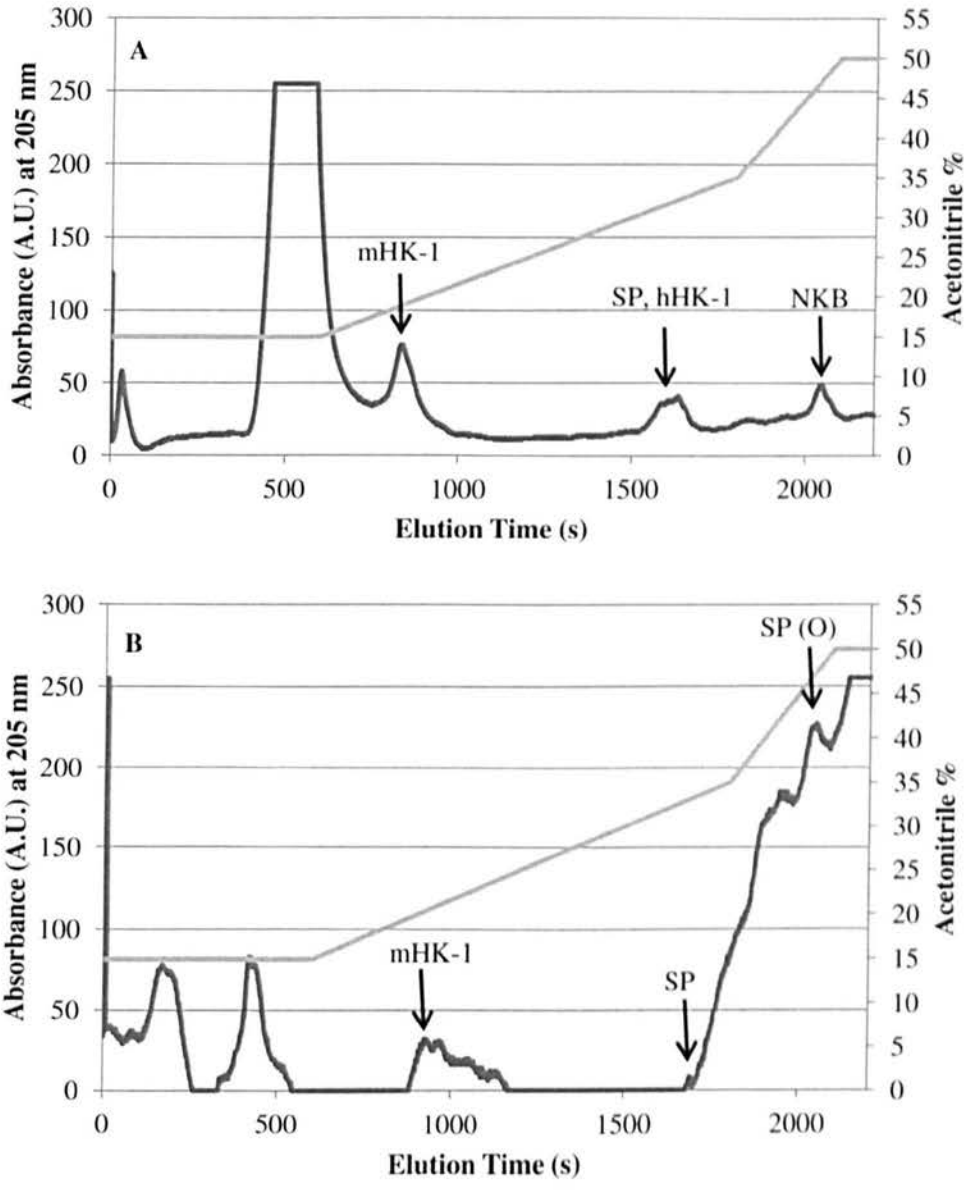


Figure 4.3. **(A)** Chromatogram of the separation of the synthetic peptides mHK-1, SP, hHK-1 and NKB. Synthetic peptide mixture (20 µl) was injected into the system. The peptides were retained on a C18 column and eluted with a linear gradient of 15% (v/v) acetonitrile to 35% (v/v) acetonitrile with 0.04% (v/v) TFA over 20 min (right ordinate, gradient shown with a grey solid line). The absorbance was measured at 205 nm (in arbitrary units, left ordinate). The retention time of the peptides is recorded in seconds. The peaks of the four peptides are indicated with an arrow. **(B)** Chromatogram of the separation of the rat spleen immunoaffinity purification eluent (pull-down) using acidifed methanol extraction. 20 µl of the sample were injected into the RP C18 column and eluted under the same conditions as the synthetic sample. The peaks from the elution of mHK-1, SP and its oxidised form SP (O) are indicated with an arrow.

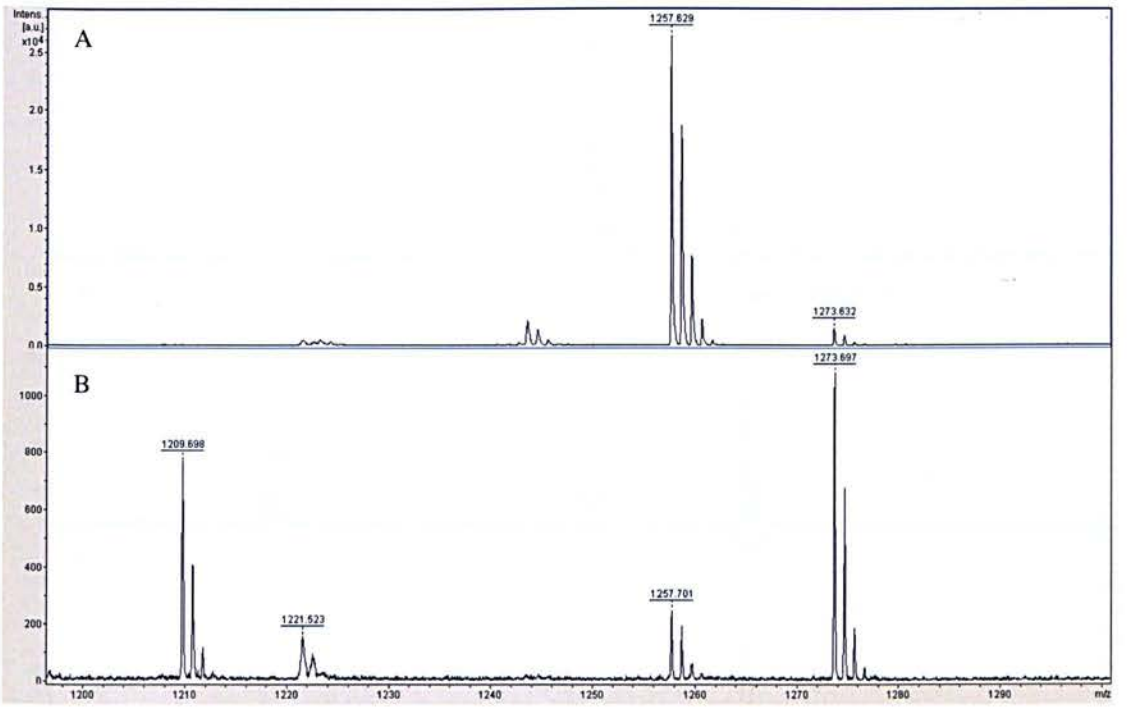


Figure 4.4. MALDI-TOF ion spectra from fraction 9. **(A, B)** The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1200 to 1300 is shown. **(A)** MALDI-TOF spectrum of peaks detected in Fraction 9 collected from the separation of synthetic mHK-1 on RP-HPLC. The annotated peak m/z 1257.629 corresponds to the mHK-1 sequence SRTRQFYGLM-NH_2. The annotated peak at m/z 1273.632 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). **(B)** MALDI-TOF spectrum of the peaks detected in fraction 9 from the separation of the rat spleen pull-down (using acidified methanol as an extraction buffer) on RP-HPLC. The annotated peak with m/z 1257.701 corresponds to the sequence SRTRQFYGLM-NH_2. The peak at m/z 1273.697 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.523 (ΔM -36 amu) is mHK-1 with water loss of serine (S) and threonine (T) residues. The peak at 1209.698 (ΔM -48 amu) is another form of mHK-1 comprising decomposed carboxymethylated methionine and/or the elimination of mathanesulphenic acid (CH_3SOH, ΔM -64 amu) from the methionine sulphoxide.

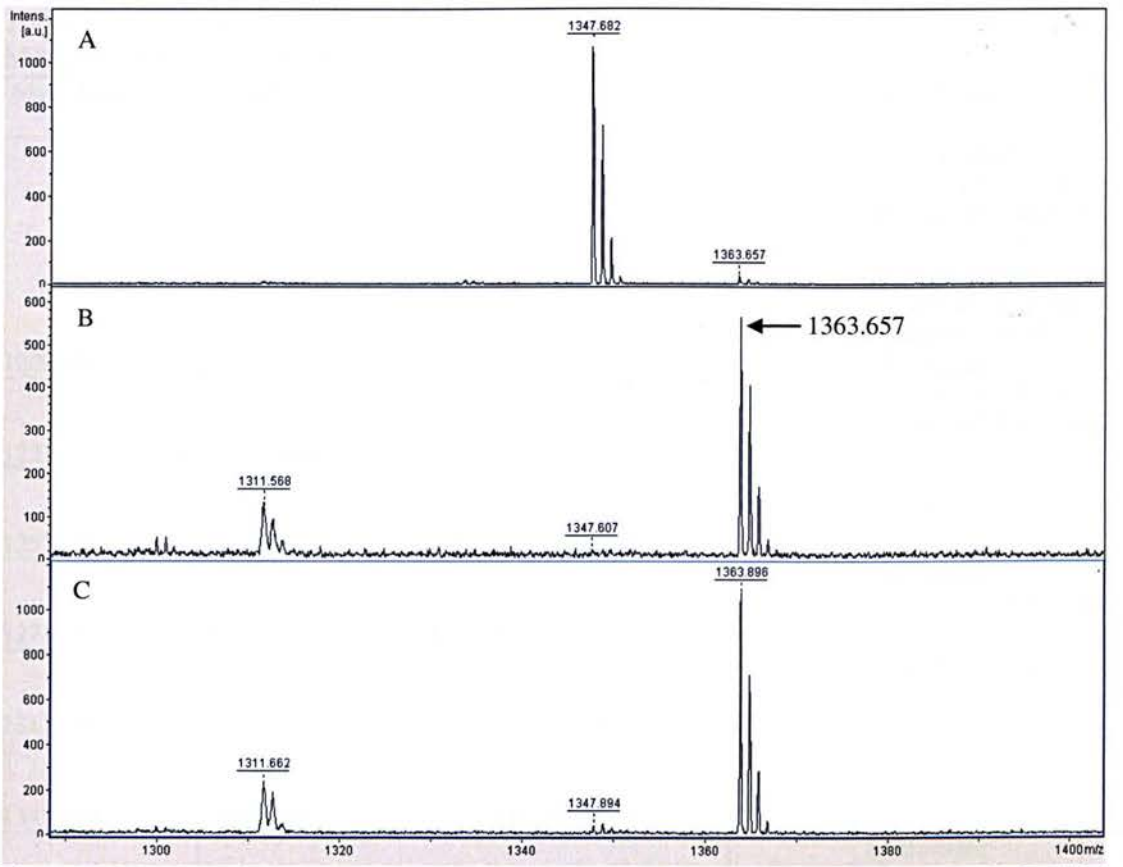


Figure 4.5. MALDI-TOF ion spectra showing peaks detected in fraction 25 and fraction 37. **(A, B, C)** The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1290 to 1400 is shown. **(A)** MALDI-TOF spectrum of the peaks detected in fraction 25 from the separation of the synthetic peptide mixture on reversed phase HPLC. The peak detected at m/z 1347.682 corresponds to SP. The peak detected at m/z 1363.657 (ΔM +15.999) corresponds to SP where methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. **(B)** MALDI-TOF spectrum of the peaks detected in fraction 25 from the separation of the rat spleen pull-down (using acidified methanol) on RP-HPLC. The peak detected at m/z 1311.568 corresponds to SP with loss of two water molecules. The peak at m/z 1347.607 corresponds to SP. The peak detected at m/z 1363.657 (ΔM +15.999 amu) corresponds to SP were methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure. **(C)** MALDI-TOF spectrum of the peaks detected in fraction 37 from the separation of the rat spleen pull-down (using acidified methanol) on RP-HPLC. The peak detected at m/z 1311.662 corresponds to SP with loss of two water molecules. The peak at m/z 1347.894 corresponds to SP. The peak detected at m/z 1363.896 (ΔM +15.999 amu) corresponds to SP comprising the oxidised form of methionine (sulphur to sulphoxide). In fraction 37 from the synthetic peptide separation no peptides were detected (data not shown). It is likely, in the rat spleen pull-down SP existed in the oxidised form before separation on RP-HPLC, hence also eluting in fraction 37.

Table 4.1. Tachykinin peptides identified in fraction 9 (mHK-1) and fraction 37 (SP) by MALDI-TOF in rat spleen and their modifications.

[1]Mass detected	Peptide	Sequence	[3]ΔM (a.m.u.)	Modification
1209.698	Mouse hemokinin-1	SRTRQFYGLM-NH_2	-48 and/ or -64	Decomposed carboxymethylated Methionine[4,5] and/ or elimination of methanesulphenic acid (CH_3SOH) from the oxidised form of Methionine[6]
1221.523	Mouse hemokinin-1	SRTRQFYGLM-NH_2	-36	Loss of water from Serine and Threonine
1257.701	Mouse hemokinin-1	SRTRQFYGLM-NH_2		
1273.697	Mouse hemokinin-1	SRTRQFYGLM-NH_2 $(O)^2$	16	Methionine (sulphur to sulphoxide)
1311.568	Substance P	RPKPQQFFGLM-NH_2	-36	Loss of two water molecules
1347.894	Substance P	RPKPQQFFGLM-NH_2		
1363.896	Substance P	RPKPQQFFGLM-NH_2 $(O)^2$	16	Methionine (sulphur to sulphoxide)

[1]Mass indicates $[M+H]^+$. [2](O) indicates oxidation. [3]ΔM indicates the mass difference from the naturally occurring form, measured in amu (atomic mass units).[4]Hammel *et al.*, 1997; [5]Jones *et al.*, 1993; [6]Lagerwerf *et al.*, 1996.

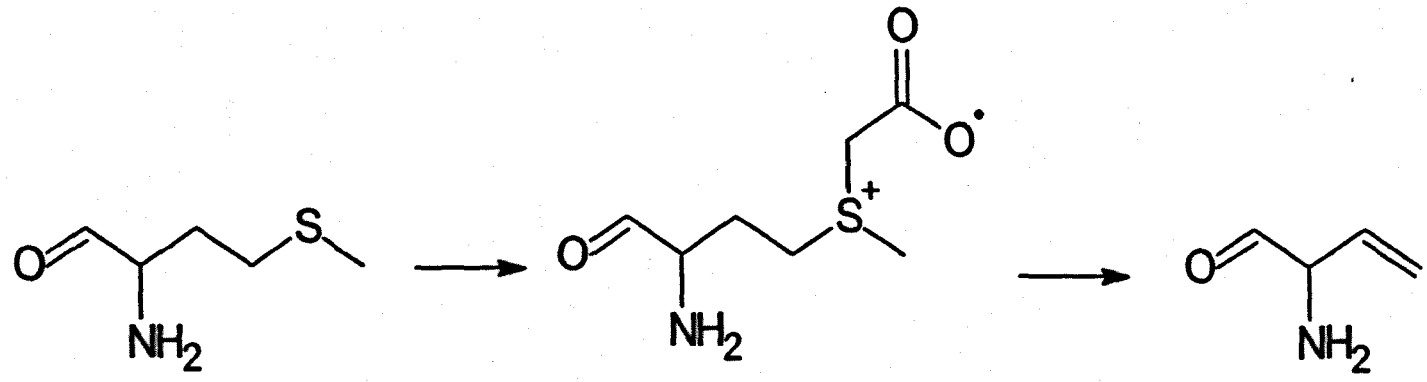


Figure 4.6. The stages in the decomposing of carboxymethylated methionine. The above modification was observed in mHK-1. The structure of methionine (1), the structure of the S-carboxymethylmethionine (2) with additional mass ΔM +58 amu and decomposed carboxymethylated methionine (3) with the elimination of methanethiol (CH_3-S-H) ΔM -48 amu and/ or elimination of methanesulphenic acid (CH_3SOH) ΔM -64 amu from the oxidised form of methionine. Created using ChemSketch.

4.4.2) Post-translational modifications of tachykinin peptides detected in rat brain.

One rat brain was homogenised in Bennett's solution and re-extracted acidified methanol and tachykinins were enriched with immunoaffinity purification. Because of its high concentration in the rat brain SP was abundantly detected in the pull-down eluent without further separation on RP-HPLC and ZipTip concentration. In Figure 4.7 is shown the MALDI-TOF spectrum of the peaks detected in the rat brain pull-down (using acidified methanol) without separation of the eluent on RP-HPLC. The peak detected at m/z 1347.716 corresponds to SP. The peak detected at m/z 1363.699 (ΔM +15.999) corresponds to SP where methionine was oxidised (sulphur to sulphoxide) possibly during the ionisation procedure (or due to the harsh extraction procedure). In Figure 4.8 is shown the peak corresponding to SP sequenced by Collision Induced Dissociation (CID). The software method of the MALDI-TOF was switched to LIFT.lft thus permitting the passing of only the chosen parent ion through the magnetic field. The parent ion peak 1347.716 was dissociated to a/b/c/x/y/z ions. The peaks detected in the product ion spectrum were aligned with the theoretical masses of the daughter ions (using the algorithm Mascot). Predominantly, the a-ions were detected (produced from a loss of a C=O group yielding a mass difference of 27.9949 Da relative to the b-ion). The b-ions (produced from the fragmentation of the peptide bond, where the charge is retained by the amino-terminal fragment) were fewer. The mass differences between the a-ions, a-17 (loss of ammonia), b-ions and b-17-ions series indicate the sequence of the peptide. As the a-ion series was predominant, the amino acid sequence is written in the amino-to-carboxy-terminus direction going from left to right.

An aliquot of the pull-down of the captured tachykinins using Bennett's solution was injected into a reversed phase C18 HPLC column and the retained peptides were eluted and collected in eighty fractions (applying the method described in Section 4.3.7). The eighty fractions were concentrated with ZipTips and spotted on a MALDI-TOF plate. In Figure 4.9 the chromatogram of the separation of the rat brain pull-down on the C18 column is shown. The peaks of the retention time of mHK-1 and SP are indicated with an arrow and their retention times were found to be the same as those of the synthetic peptides shown in Figure 4.3.A. The less abundant peptide mHK-1 was separated from

the abundantly expressed SP (in the brain) using RP-HPLC. The processing of mHK-1 in the rat brain was proven by capturing (by means of immunoaffinity purification), and separating the peptide from the repertoire of captured peptides with subsequent confirmation of its molecular weight on MALDI-TOF. The spectrum of peaks detected in fraction 8 collected from the separation of the rat brain pull-down on RP-HPLC is shown in Figure 4.10, B. It is shown that the same peaks as in fraction 9 from the rat spleen pull-down were detected (Figure 4.10, A). The annotated peak m/z 1257.661 corresponds to the mHK-1 sequence. The annotated peak at m/z 1273.697 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.577 (ΔM -36 amu) is mHK-1 with water loss of serine and threonine residues. The peak at m/z 1299.710 (ΔM +42 amu) is mHK-1 with a potential acetylation of the oxygen of the serine[1] residue (Floyd *et al.*, 1999; Polevoda & Sherman, 2002). The schematic diagram of the acetylation of serine reaction is shown in Figure 4.13. Moreover, SP was detected in fraction 37 from the separation of the rat brain pull-down (using Bennett's solution) on RP-HPLC. In Figure 4.11, B the peaks detected in the rat brain pull-down are shown. The spectrum (Figure 4.11, A) from the peaks detected in fraction 38 from the rat spleen pull-down are also shown for comparison; SP was detected abundantly in the rat brain with m/z 1347.971. The peak detected at m/z 1363.894 (ΔM +16) corresponds to SP comprising the oxidised form of methionine (sulphur to sulphoxide); the peak at m/z 1311.738 is SP (with the loss of two water molecules). The peaks of the tachykinin peptides identified by MALDI-TOF in rat brains and their modifications are summarised in Table 4.2.

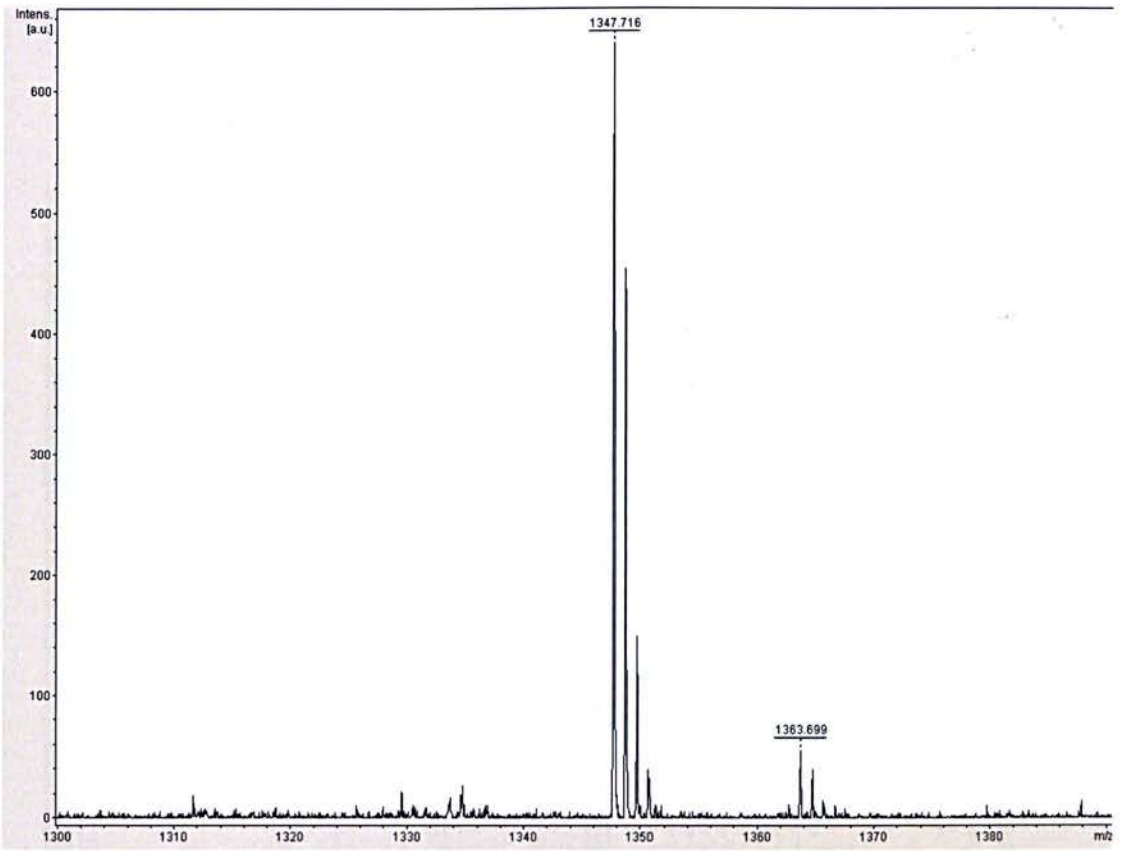
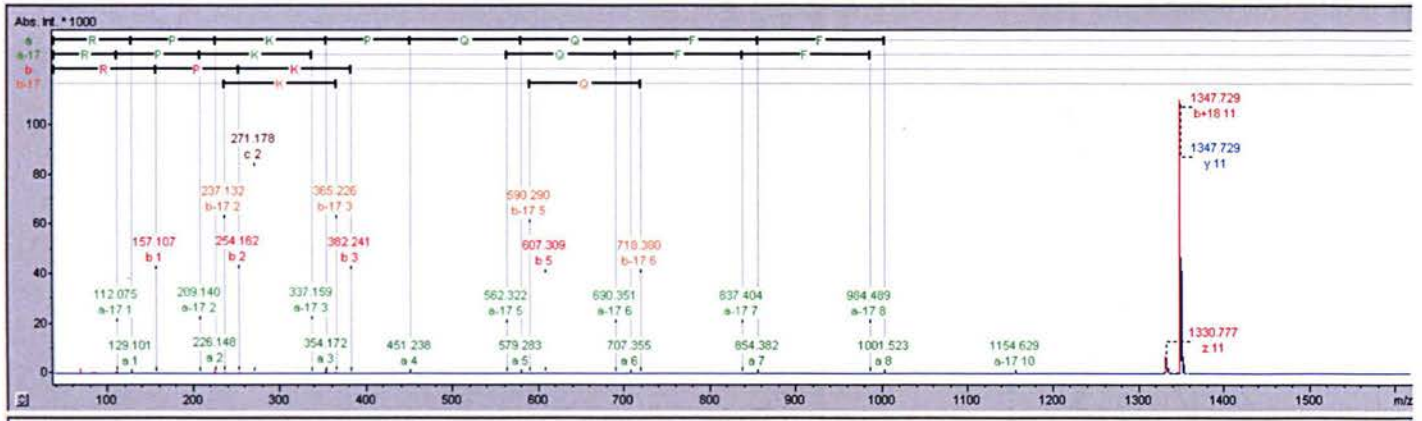


Figure 4.7. MALDI-TOF spectrum of the peaks detected in the rat brain pull-down using acidified methanol and extraction procedure. The mass/charge (m/z) range 1300 to 1390 is shown. Peak intensity is shown in arbitrary units. The peak detected at m/z 1347.716 corresponds to SP. The peak detected at m/z 1363.699 (ΔM +16 amu) corresponds to SP with methionine oxidised (sulphur to sulphoxide).



Protein View | Match Errors | MS/MS Fragments | MSMS Analysis

Sequence: RPKPQQFFGLM (Mods: C-Term: Amide (C-term))

MH+(mono): 1347.735 MH+(avg): 1348.642 MS/MS Tol: 0.500 Da Peaks: 60 Above Threshold: 60 Zoom to m/z for 2+

Masses: Monoisotopic Average Calculate: Masses Threshold: 0.000 Assigned: 36 Not Assigned: 24 1+, m/z: 0.00

	R	P	K	P	Q	Q	F	F	G	L	M	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	R	P	K	P	Q	Q	F	F	G	L	M	129.113	226.166	354.261	451.314	579.373	707.431	854.500	1001.568	1058.589	1171.673	1301.730
a-17	R	P	K	P	Q	Q	F	F	G	L	M	112.087	209.140	337.235	434.287	562.346	690.405	837.473	984.541	1041.563	1154.647	1284.703
a-18	R	P	K	P	Q	Q	F	F	G	L	M	111.103	208.156	336.251	433.303	561.362	689.421	836.489	983.557	1040.579	1153.663	1283.719
b	R	P	K	P	Q	Q	F	F	G	L	M	157.108	254.161	382.256	479.309	607.367	735.426	882.494	1029.563	1086.584	1199.668	1329.725
b-17	R	P	K	P	Q	Q	F	F	G	L	M	140.082	237.135	365.230	462.282	590.341	718.399	865.468	1012.536	1069.558	1182.642	1312.698
b-18	R	P	K	P	Q	Q	F	F	G	L	M	139.098	236.151	364.246	461.298	589.357	717.415	864.484	1011.552	1068.574	1181.658	1311.714
b+18	R	P	K	P	Q	Q	F	F	G	L	M	175.119	272.172	400.267	497.319	625.378	753.437	900.505	1047.573	1104.595	1217.679	1347.735
c	R	P	K	P	Q	Q	F	F	G	L	M	174.135	271.188	399.283	496.335	624.394	752.453	899.521	1046.589	1103.611	1216.695	1346.751
x	R	P	K	P	Q	Q	F	F	G	L	M	175.054	288.138	345.159	492.228	639.296	767.355	895.413	992.466	1120.561	1217.614	1373.715
y	R	P	K	P	Q	Q	F	F	G	L	M	149.074	262.158	319.180	466.248	613.317	741.375	869.434	966.487	1094.582	1191.634	1347.735
z	R	P	K	P	Q	Q	F	F	G	L	M	132.048	245.132	302.153	449.222	596.290	724.349	852.407	949.460	1077.555	1174.608	1330.709
i	R	P	K	P	Q	Q	F	F	G	L	M	129.113	70.065	101.107	70.065	101.071	101.071	120.081	120.081	30.034	86.096	103.069
	11	10	9	8	7	6	5	4	3	2	1	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg

Figure 4.8. The collision-induced dissociation spectrum of endogenous SP. The parent ion peak 1347.729 was dissociated into a/b/c/x/y/z ions. The peaks of the product ions were aligned against the theoretical masses of the above ion series. The mass differences between the a-ion, a-17 (loss of ammonia), b and b-17 series indicate the amino-acid series, which is shown in the above spectrum. As the a-ion series is predominant, the sequence is written in the amino-to-carboxy-terminus direction going from left to right. Mass tolerance was set to 0.5 Da (default setting).

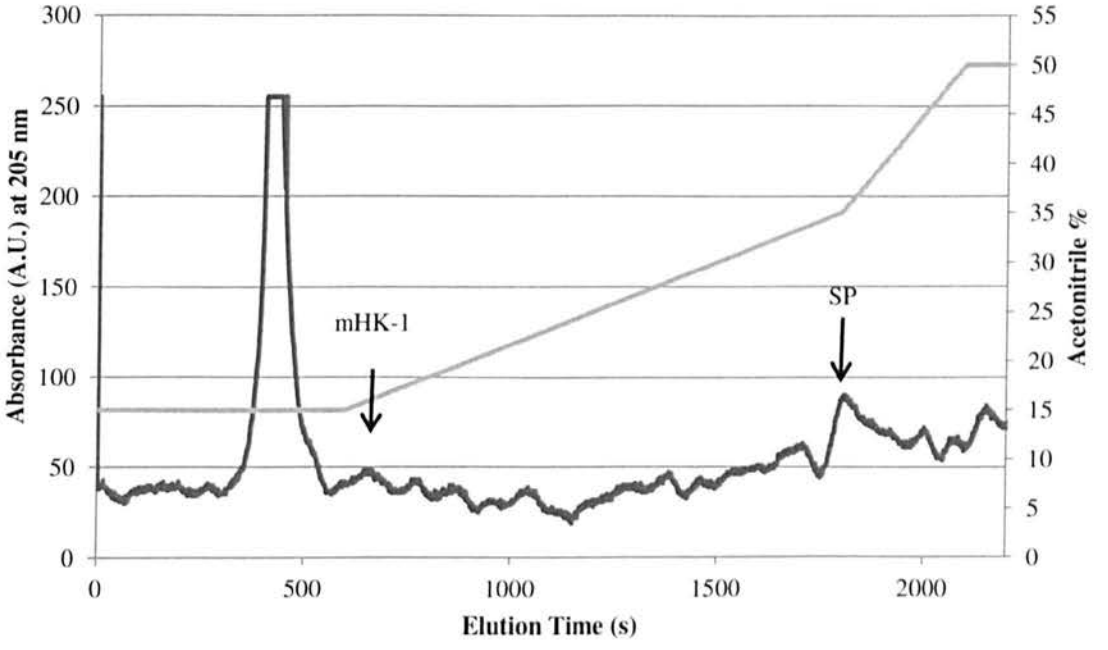


Figure 4.9. The chromatogram of the rat brain pull-down. 20 µl of the rat brain pull-down (using Bennett's solution) was injected into the RP-HPLC system. The sample was separated using a reversed phase C18 column and eluted with a linear gradient of acetonitrile starting a 15% and taken to 50% (shown with a line, right ordinate). The absorbance was set at 205 nm (measured in arbitrary units, left ordinate). The retention time of the peptides is shown in seconds. The peaks from the elution of mHK-1 and SP are indicated with an arrow.

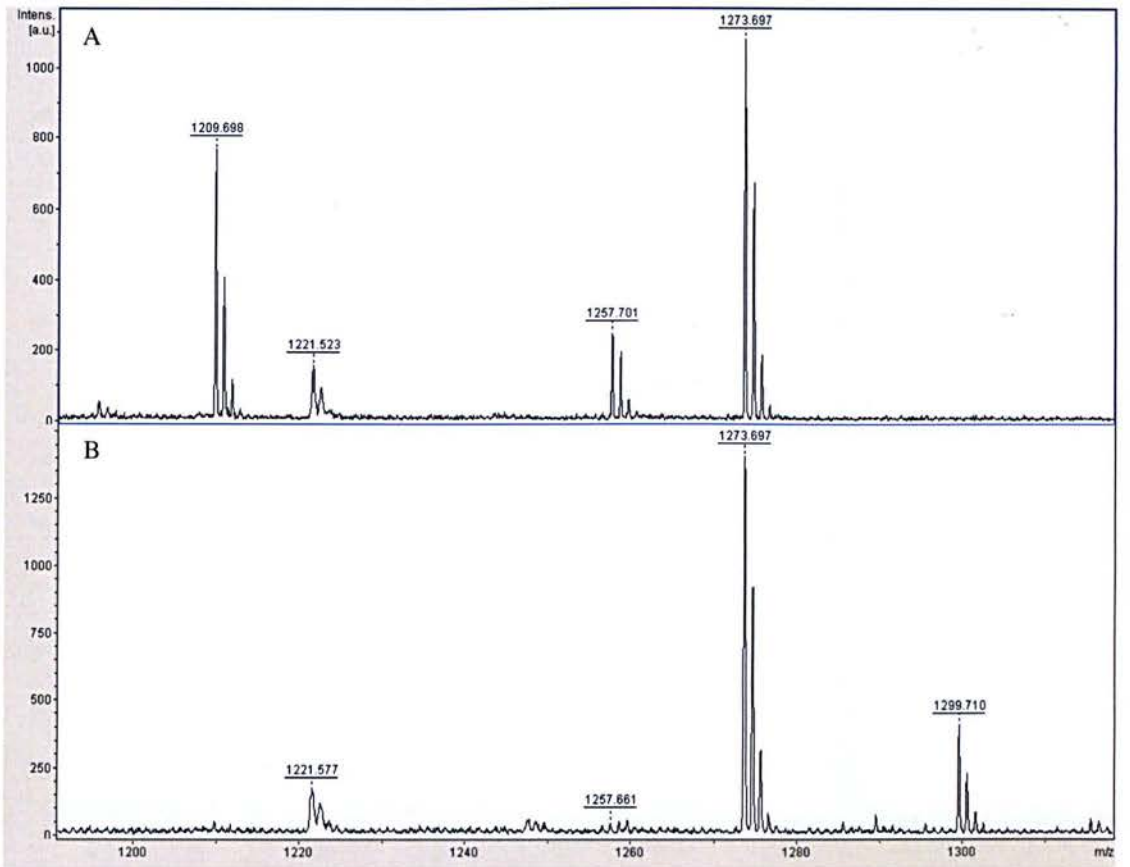


Figure 4.10. MALDI-TOF ion spectra showing peaks detected in the spleen and rat brain pull-down separated into fractions. The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1200 to 1320 is shown. **(A)** MALDI-TOF spectrum of the peaks detected in fraction 9 from the separation of the rat spleen pull-down (using acidified methanol) on RP-HPLC. The annotated peak with m/z 1257.701 corresponds to the sequence SRTRQFYGLM-NH_2. The peak at m/z 1273.697 (ΔM +16 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.523 (ΔM -36 amu) is mHK-1 with water loss of serine and threonine residues. The peak at m/z 1209.698 (ΔM -48.003 amu) is another form of mHK-1 comprising decomposed carboxymethylated methionine. **(B)** MALDI-TOF spectrum of peaks detected in fraction 8 collected from the separation of the rat brain pull-down (using Bennett's solution) on RP-HPLC. The annotated peak m/z 1257.661 corresponds to the mHK-1 sequence SRTRQFYGLM-NH_2. The annotated peak at m/z 1273.697 (ΔM +15.999 amu) is mHK-1 comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1221.577 (ΔM -36 amu) is mHK-1 with water loss of serine and threonine residues. The peak at m/z 1299.710 (ΔM +42 amu) is mHK-1 with potential acetylation of the oxygen of the serine residue.

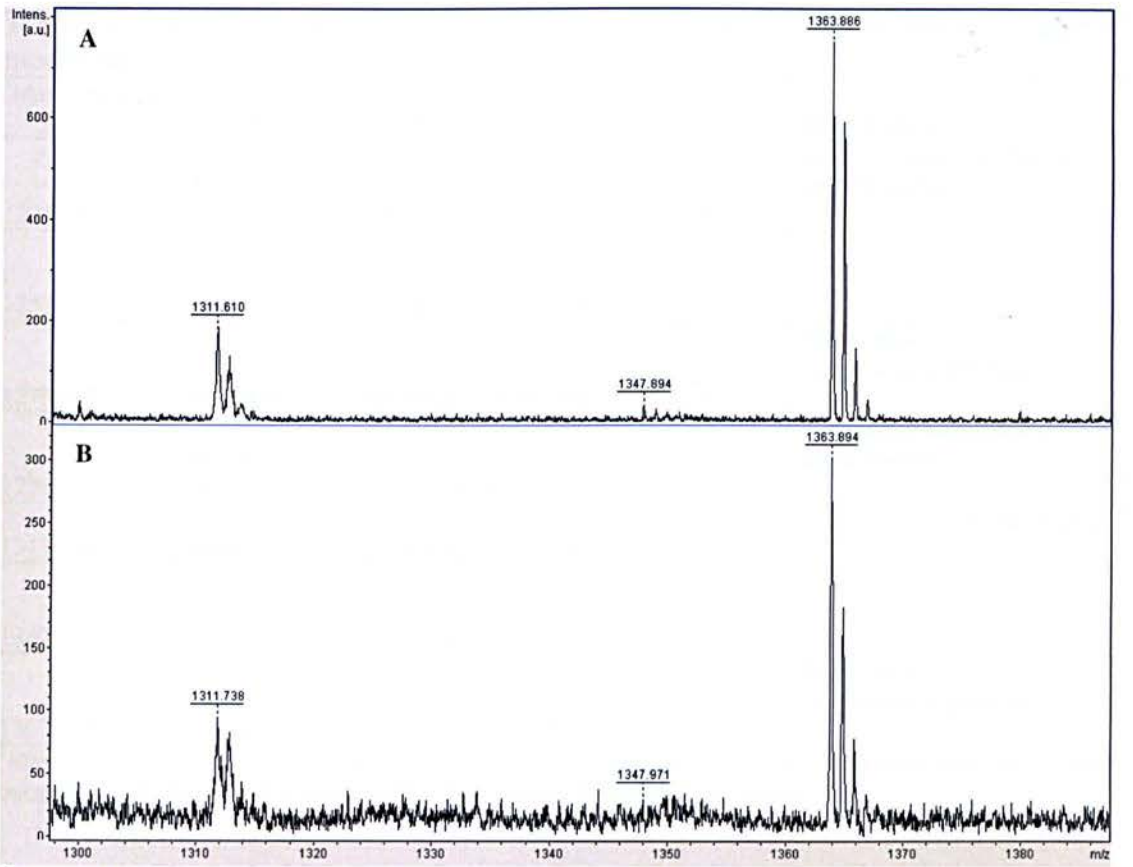
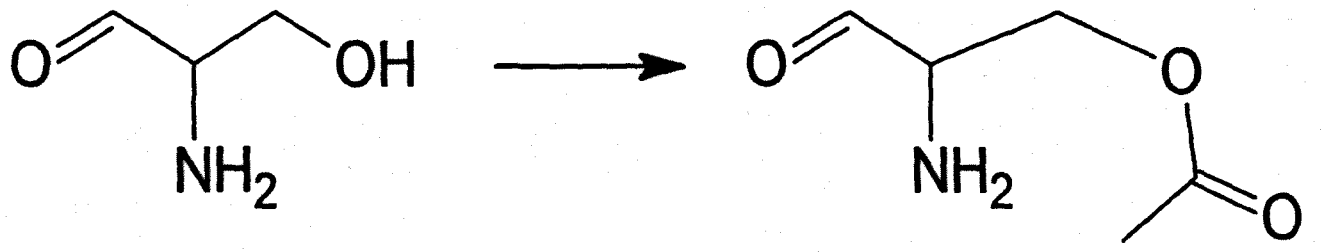


Figure 4.11. MALDI-TOF ion spectra showing peaks detected in fraction 37. (A, B). The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity (arbitrary units, A.U.) of the molecular ions. The mass/charge (m/z) range 1300 to 1400 is shown. **(A)** MALDI-TOF spectrum of the peaks detected in fraction 37 from the separation of the rat spleen pull-down on HPLC (acidified methanol extraction). The peak detected at m/z 1347.894 corresponds to SP. The peak detected at m/z 1363.885 (ΔM +15.999 amu) corresponds to SP were methionine was oxidised (sulphur to sulphoxide) during the ionisation procedure, while the peak at m/z 1311.738 is SP with the loss of two water molecules. **(B)** MALDI-TOF spectrum of the peaks detected in Fraction 37 from the separation of the rat brain pull-down on RP-HPLC (Bennett's solution). The peak detected at m/z 1347.971 corresponds to SP. The peak detected at m/z 1363.894 (ΔM +15.999 amu) corresponds to SP comprising the oxidised form of methionine (sulphur to sulphoxide). The peak at m/z 1311.738 is potentially SP with loss of two water molecules.

Table 4.2. Tachykinin peptides identified by MALDI-TOF in rat brain and their potential modifications.

[1]Mass detected	Peptide	Sequence	[3]ΔM (a.m.u.)	Modification
1221.577	Mouse hemokinin-1	SRTRQFYGLM-NH_2	-36	Loss of water from Serine and Threonine
1257.661	Mouse hemokinin-1	SRTRQFYGLM-NH_2		
1273.697	Mouse hemokinin-1	SRTRQFYGLM-NH_2 $(O)^2$	16	Methionine (sulphur to sulphoxide)
1299.71	Mouse hemokinin-1	Ac-SRTRQFYGLM-NH_2	42	N-terminal acetylation of serine residue[4]
1311.738	Substance P	RPKPQQFFGLM-NH_2	-36	Loss of two water molecules
1347.971	Substance P	RPKPQQFFGLM-NH_2		
1363.694	Substance P	RPKPQQFFGLM-NH_2 $(O)^2$	16	Methionine (sulphur to sulphoxide)

[1]Mass indicates $[M+H]^+$. [2](O) indicates oxidation. [3]ΔM indicates the mass difference from the naturally occurring form, measured in amu (atomic mass units). [4]Polevoda & Sherman, 2002.



1

2

(ΔM +42 amu)

Figure 4.12. The schematic diagram of the acetylation of serine. The structure of serine (1), the structure of the acetyl-serine (2) with additional mass ΔM +42 amu. Acetyl-serine is potentially biosynthesised by acetylation of the serine by the brain enzyme serine transacetylase.

4.4.3) Rat and human placenta homogenisation, peptide extraction and enrichment of peptide.

Eight rat placentas weighing ~1g were homogenised in Bennett's solution. Following overnight incubation, the mixture of homogenised tissue in extraction buffer was centrifuged and the peptide extract was collected. The homogenised tissue pellet was re-suspended in acidified methanol and the methanol/ chloroform extraction procedure was applied. Tachykinins in the two repertoires of peptide extracts were enriched with immunoaffinity purification. Two pull-downs (from the two peptide extractions) were yielded and injected into the RP-HPLC system for the separation of the captured peptides into fractions. Eighty fractions from each pull-down were collected; organic solvents were removed under N2 flow and the fractions were further concentrated with ZipTips and spotted on the MALDI-TOF ground stainless steel plate. However, no peaks of the tachykinins were identified in any of the collected fractions. SP is not expressed in the placenta, as the *Tac1* gene is not expressed. The *Tac4* and *Tac2* genes are found expressed in rat placenta that express the TAC4 and TAC3 precursors. It is possible that incomplete processing of the precursors does not release the fully processed forms of mHK-1 and NKB peptides. This is described in further detail later on.

Human placental samples were received from a pre-eclamptic patient recruited at Kingston Hospital after the patient's written consent and COREC approval. The placental tissue ~40 g (kept at -80°C) was homogenised using a blender with ~85 ml of cold acidified methanol. The extracted peptides were concentrated using a C18 Sep Pak cartridge. The sample was concentrated removing methanol by blowing a "slow" stream of nitrogen gas over the sample at room temperature. After neutralisation, the extract was mixed with Sepharose-antibody affinity conjugate. The immunoaffinity purification reaction pull-down was spotted on MALDI-TOF; however no peaks were detected in the sample. A trypsinisation reaction was performed using an aliquot of the placental immunoaffinity purification eluent to spot on MALDI-TOF for peptide mass fingerprinting. A "mass" list of peptide fragments was exported from the instrument and the detected masses were inserted onto the on-line database MASCOT. The "Swiss Prot"

database was chosen and parameters of the algorithm were set as, mass tolerance ±0.1 Da, enzyme: trypsin, allow up to 1 missed cleavages. Only two peptides were identified from the mass list (with MW 1529.676 and 1832.8846 amu) belonging to the α chain of hemoglobin. This result indicates the apparent abundancy of hemoglobin in placental tissue (an organ abundant with blood vessels). The elimination of hemoglobin was not sufficient and thus interefered with the detection of tachykinins.

4.5) Discussion.

The well-studied mammalian tachykinins SP, NKA and its extended forms NPK and NPγ were originally identified with biochemical purification and sequencing methods (Severini *et al.*, 2002). In the molecular biology era, the field of hemokinins/endokinins was introduced with the identification of mHK-1, discovered as a tachykinin peptide with an autocrine/paracrine role in B-cell development (Zhang *et al.*, 2000). The advancement of the bioinformatics tools in combination with molecular cloning contributed to the identification of hHK-1 and the endokinins (Page, 2003). The sequence of the mHK-1 was deduced from the nucleotide sequence of the novel cDNA, initially named as PPT-C and then named as *Tac4* gene, using the updated standardised nomenclature (Patacchini *et al.*, 2004). The mHK-1 was predicted as an amidated undecapeptide RSRTRQFYGLM-NH_2 (Zhang *et al.*, 2000). Unlike the other mammalian peptides such as SP, NKA (NPK, NPγ) and NKB whose sequences have been identified with sequencing (*e.g.* Edman degradation), the peptide sequence of mHK-1, hHK-1 and the endokinins have only been deduced from cDNA studies. Page and Weston-Bell (2010) have reported specific methodologies of anti-tachykinin antibody development, peptide extraction and immunoaffinity purification for the capture and purification of tachykinin peptides. Therefore, a more definite characterisation of the mHK-1 was required for the identification of its MW and therefore its sequence, as well as any post-translational modifications apart from the well-established C-terminal amidation.

4.5.1) Identification of mHK-1 using MALDI-TOF.

The mature mHK-1 peptide is released from cleavage of the TAC4 precursor by the enzymes pro-hormone convertases (or pro-protein convertases) at the carboxyl terminus of the lysine-arginine (KR) cleavage sites and the remaining KR residues are removed by carboxypeptidase E (Douglas Lu *et al.*, 2012). However, in the presence of a proline (like in the case of SP processing) because of arginine-proline bonds, which resist enzymatic cleavage (Gainer *et al.*, 1984) processing occurs between the two RR residues on the N-terminal releasing the SP undecapeptide. The alignment of the rat TAC1 precursor

isoform 3 (containing only SP) with the rat TAC4 precursor using ClustalW is shown in Figure 4.13. The two tachykinins are similar at their C-terminus but divergent in sequence at their N-terminus. Both comprise F or Y in the tachykinin motif, conserved aromatic amino acids of closely similar properties. Both sequences comprise Q (glutamine) in position 5 (for mHK-1) or position 6 (for SP) a highly conserved residue. In position 2 and 4 (for mHK-1) or 3 and 5 (for SP) the peptides contain conserved basic residues of highly similar properties *i.e.* Q, R or R, K. The two peptides bind to, potentially different, subtypes of the NK1 receptor and no significant differences in biological activity have been reported when using Ca^{2+} mobilisation in astrocytoma cells as a functional parameter (Berger & Paige, 2005).

This study confirms the translation of the TAC4 precursor in the rat brain and spleen and its full processing to release mHK-1. It also clarifies the molecular weight of the mature mHK-1 peptide, and also suggests the presence of a post-translational modification *i.e.* the acetylation of Ser^1 in the brain as compared to the periphery (spleen). Kurtz and colleagues (2002) have reported significant levels of mRNA expression of the *Tac4* gene at various rat tissues *e.g.* spleen, brain, heart, kidney, thymus, stomach, and lung, skin. In this study spleen was chosen as the tissue to capture the mHK-1 peptide, also because of the predominant role of mHK-1 in the function of the immune system. Two extraction buffers were applied, acidified methanol for the extraction of the spleen peptidome and Bennett's solution for the extraction of the neuropeptidome. The presence of methanol in the spleen extraction buffer, gives a semi-quantitative yield of peptides, however, gives a better qualitative extraction of polar and less polar peptides with respect to an acid buffer (Figure 3.3 and Figure 3.4). Subsequently, as a further control, the quantitative/qualitative comparison of profile of post-translational modifications between neuronal tissue and the periphery using the same buffer should be performed. A different profile of post-translational modifications (*i.e.* acetylation) was observed between neuronal tissue and the periphery. In the spectra received from synthetic peptide analysis, we have not observed acetylation as an *in vitro* modification (compared to oxidation, or Na^+ or K^+ adducts). We have disproved the predicted sequence of mHK-1 as RSRTRQFYGLM-NH_2 (mass 1412.75 Da). The mature mHK-1 tachykinin peptide was captured from rat

spleen and brain and was proven to be an amidated decapeptide with the sequence and MW corresponding to SRTRQFYGLM-NH_2 identified by means of immunoaffinity purification, RP-HPLC and MALDI-TOF. We have confirmed the mass of the mHK-1 as $[M+H]^+$ 1257.701 Da same as the theoretical (1257.652 Da). The ion peak detected at 1209.698 (ΔM -48.003 amu) is another form of mHK-1 with decomposed carboxymethylated methionine. This species was most likely generated *in vitro*, due to harsh extraction conditions. The expression of the mHK-1 in brain was not clear. Although, Zhang and colleagues (2000) reported the *Tac4* gene not to be expressed in mouse brain and spleen but rather in lymphopoietic cells of the periphery, using a rather insensitive Northen blot analysis, further reports have showed the expression of *Tac4* in the rat brain (Kurtz *et al.*, 2002; Duffy *et al.*, 2003). We attempted to interrogate the UniGene database (http://www.ncbi.nlm.nih.gov/unigene) to investigate the *Tac4* gene expression levels in different tissues, deduced from the analysis of EST counts. However, such a gene expression profile does not exist for the rat *Tac4* but only for the mouse *Tac4*, where an EST gene expression profile shows the expression of mouse *Tac4* in the pineal gland and also only in the developmental stage of the juvenile mouse.

The "pull-down" experiment *i.e.* the homogenisation and immunoaffinity purification enrichment; followed by separation of the "pull down" into fractions with MALDI-TOF detection, was performed using rat placentae as well. In the rat placenta both the *Tac3* and *Tac4* genes have been reported to be expressed, but not the *Tac1* gene (Page *et al*, 2004). The scope of applying the above methodology on rat placentae was to investigate not only the processing of TAC4 precursor but also the TAC3 precursor comprising NKB and compare the peptide species detected among the three tissues investigated (brain, spleen and placenta). However, in the fractions collected no peaks were detected from the placenta. Potentially, the N-terminally extended forms of the two peptides are expressed in the tissue such as the N-terminal extended form of NKB (M.W. 4701.526 Da), LYDSRPISLEGLLKVLSKASVGPKETSLPQKRDMHDFFVGLM-NH_2 (also found in the brain; Lang *et al.*, 1995). Moreover, the N-terminal extended form of mHK-1 could potentially be expressed. As shown in Figure 4.14 the cleavage of the TAC4 precursor by

signal peptidases would produce the extended form of mHK-1 with MW (5778.068 Da; TTTRDREDLTFGAEAESWVTVNLKGIPVPSIELKLQELKRSRTRQFYGLM-NH_2).

The mHK-1 peptide (SRTRQFYGLM-NH_2) comprises two potential monobasic cleavage sites at the R residues, theoretically releasing the truncated peptides TRQFYGLM-NH_2 (1014.519 Da) and QFYGLM-NH_2 (757.37 Da). However, the above species were not detected in either of the tissues investigated. Both truncated peptides would show affinity for the NK-1 receptor and elicit a biological response as the minimum peptide fragment with the ability to show a similar biological response on the NK receptors as the mature form is the C-terminal hexapeptide (Almeida *et al.*, 2005).

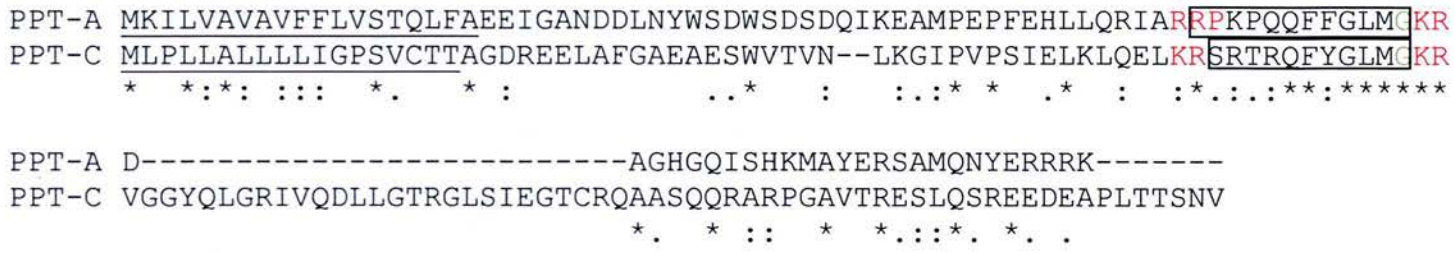


Figure 4.13. The alignment of the rat preprotachykinin A isoform 3 (Accession number: CAA39752.1) and preprotachykinin C precursors (Accession number: AAP30873.1) using ClustalW. An (asterisk, *) indicates positions, which have a single, fully conserved residue; a : (colon) indicates conservation between groups of strongly similar properties; a . (period) indicates conservation between groups of weakly similar properties. The signal peptide as predicted by SignalP is underlined. The dibasic KR, RR cleavage sites are highlighted and the adjacent G residue which provides the amidation as well as the presence of the proline residue in SP. The mature tachykinins released from the processing of the preprotachykinin A (SP: RPKPQQFFGLM-NH_2) and preprotachykinin C (mHK-1: SRTRQFYGLM-NH_2) precursors are boxed.

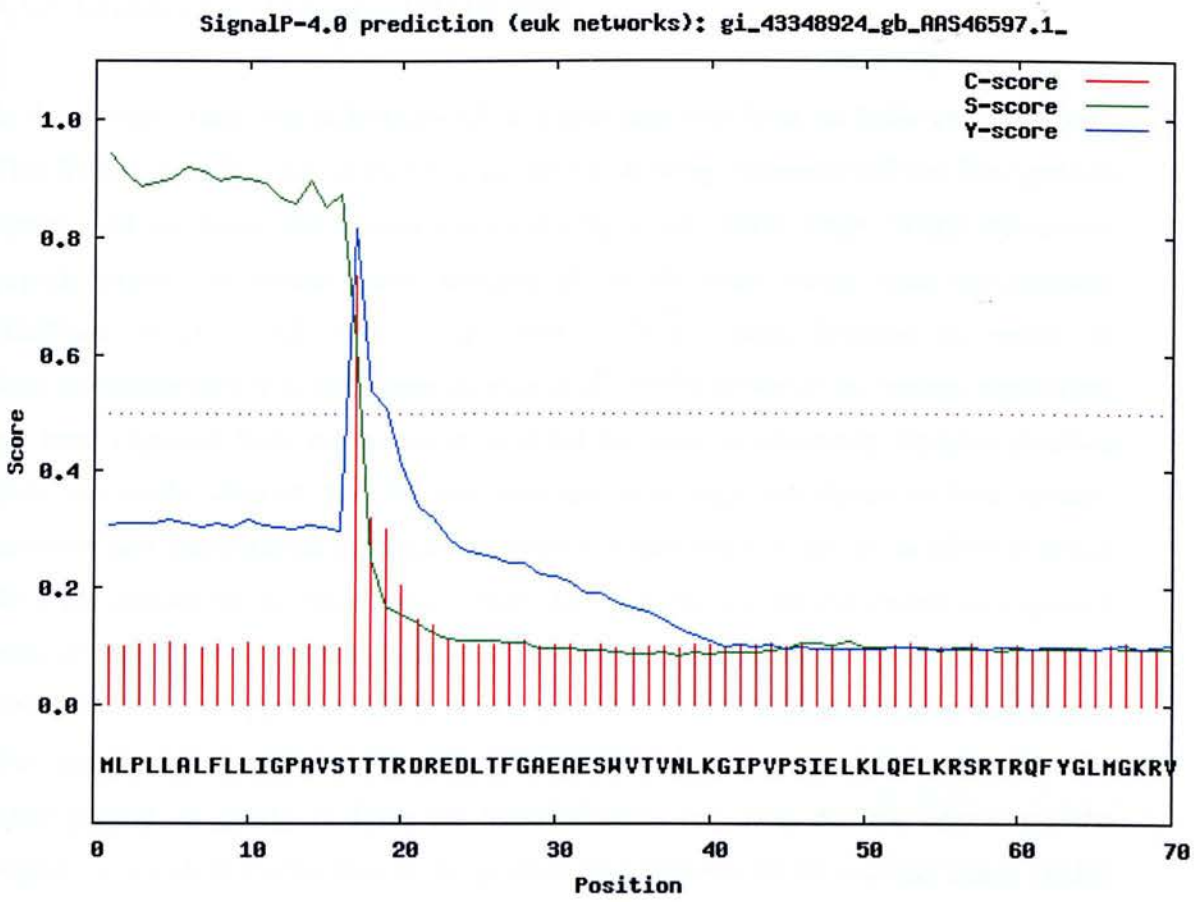


Figure 4.14. Predictive location of signal peptide cleavage site in the rat TAC4 precursor. The TAC4 sequence (GenBank$_{TM}$ accession number AAS46597.1) was uploaded into the SignalP server. The graphical output from SignalP server predicts cleavage between the AVS-TT residues releasing a proteolytic product from the PPT-C precursor of 50 residues, potentially an extended form of the mHK-1 tachykinin.

4.5.2) Identification of SP using MALDI-TOF.

In the present study, the tachykinin SP was also captured from rat brain and rat spleen. This finding is well in line with previous reports showing expression of the *Tac1* gene in human and rat brain and human spleen (Duffy *et al.*, 2003; Page, 2006). Moreover, peptide expression studies have detected SP in the brain using mass spectrometry (Kallback *et al.*, 2012; Wei *et al.*, 2006). SP has been detected by means of immunocytochemistry in the spleen (Lorton *et al.*, 1991); however the mature peptide has not been captured from the tissue or detected by mass spectrometry. Spectra obtained from our study showed that SP was detected with high abundance in both tissues; however post-translational modifications were not identified on SP. In an effort to detect the NKB peptide in the mass spectra from the rat brain, we did not detect the expected peak at m/z 1210.511 although the *Tac2* gene is expressed in the brain (Kako *et al.*, 1993). From our experience NKB shows the lowest relative peak intensity among the four peptides. To our knowledge solely Hatcher and colleagues (2008) identified the NKB peptide in the brain from the hypothalamus, the main nucleus expressing the peptide, using direct collection of the peptidome expressed in the nucleus using ZipTip and spotting on the MALDI-TOF plate. Our approach of using a whole brain and not the hypothalamus or spinal cord to capture the peptide may have in fact "diluted" the tachykinin.

4.5.3) Peptide extraction and immunoaffinity purification from human placentas.

Furthermore, human pre-eclamptic placental samples were processed with the aim of capturing an array of potential human tachykinin peptides in particular the mature forms of NKB and hHK-1. The high abundance of haemoglobin in the placental tissue may have interfered with binding of the tachykinin peptides to the Sepharose-antibody conjugate (binding interference). Further development of the methodology is required for the elimination of high abundance proteins (*e.g.* size-exclusion chromatography).

4.5.4) Identification of a post-translational modification on mHK-1. Acetylases and de-acetylases and their function in rat brain.

In this study we identified mHK-1 in the rat brain comprising as a potential post-translational modification acetylation of the oxygen of the serine residue (Floyd *et al.*, 1999). Acetylation occurs by the enzymes N-acetyl-transferases most probably in the Golgi apparatus (Helbig *et al.*, 2010; Walsh *et al.*, 2005). The peak of the mHK-1 $[M+H]^+$ (1257.661 amu) and the acetylated form of mHK-1 (1299.710 amu) potentially exist *in vivo* in a ratio suggesting the existence of an acetylation and de-acetylation system controlled by specific enzymes *i.e.* serine N-acetyl-transferases and de-acetyl-transferases. Acetylation is one of the signature post-translational modifications of neuropeptides and the consensus for the biological role of acetylation is greater stability for the peptide and enhancement of binding interactions of the peptide with its receptor (Wilkinson, 2006). Acetylation inhibits ubiqitylation and therefore prolongs the half life of the peptide, or protects the peptide from degrading enzymes *e.g.* neprilysin and also the biological role of acetylation is the enhancement of the activity of the peptide-receptor interactions (Van Dijk *et al.*, 2011). The TAC4 precursor comprises a signal peptide hence it is directed to Golgi apparatus and stored in vesicles (van den Pol, 2012). We suggest the acetylation of the peptide occurs in the Golgi apparatus and hence the peptide is stored acetylated in the vesicles. Upon release the acetylated mHK-1 activates the NK-1 receptor located on the metasynaptic membrane. De-acetylases that may be present on the metasynaptic membrane to de-acetylate the peptide acting as a potential on/off switch of the NK1 receptor (molecular switch). In conclusion the molecular weight and sequence of mHK-1 was identified as an amidated decapeptide. Moreover, the translation of the mHK-1 in rat brain tissue was confirmed and a potential post-translational modification *i.e.* acetylation of the N-terminus of the peptide is suggested.

Chapter 5. The identification of novel tachykinin sequences by means of *in silico* searches of bioinformatic databases.

5.1) Introduction.

The tachykinins and the structurally related tachykinin-like peptides are a phylogenetically related ancient peptide family (Van Loy *et al.*, 2010; Satake *et al.*, 2013). Tachykinins have been isolated from a wide range of sources including viruses (virokinin; Zimmer *et al.*, 2003) and invertebrates such as the ascidian *Ciona intestinalis* (Ci-tachykinin I, II; Satake, *et al.*, 2004), insects like the mosquito, *Aedes aegypti*, (sialokinin I, II; Champagne *et al.*, 1994), and the octopus, *Octapus vulgaris* (OctTK-I, OctTK-II; Kanda *et al.*, 2003). In vertebrates, they have been found in fish (dogfish, *Scyliorhinus canicula*; scyliorhinin I and II; Conlon *et al.*, 1986; Conlon & Thim, 1988), amphibians (frog, *Rana margaretae*, *Kassina senegalensis*, *Bufo marinus*; kassinin, bufokinin, ranakinin; Liu *et al.*, 1999; Li *et al.*, 2006; Wang *et al.*, 2009), reptiles (SP, alligator, *Alligator mississipiensis*; Wang *et al.*, 1992), birds (SP; *Gallus domesticus*; Conlon *et al.*, 1988) and mammals (NKA, SP, NKB, EKA/B; Anastasi *et al.*, 1964; Chang *et al.*, 1971; Kangawa *et al.*, 1983; Page *et al.*, 2003). Tachykinins comprise the characteristic FXGLM-NH_2 motif where X was initially thought to be a bulky aromatic (F, Y) or β-branched aliphatic (V, I) residue (Severini *et al.*, 2002). Later, the discovery of the tachykinin peptide Ci-TK-II expressed in *Ciona intestinalis* introduced a novel motif FTGLM-NH_2 holding a bulky polar amino acid (Satake *et al.*, 2004). Tachykinins are expressed as part of a large preprotachykinin precursor that undergoes proteolytic cleavage at dibasic or monobasic (and sometimes multibasic) K, R cleavage sites, which flank each tachykinin peptide to release the expressed tachykinins (Page, 2005). The tachykinin-related peptides comprise a more variable characteristic C-terminal consensus sequence FX_1G/AX_2R-NH_2 flanked by K, R cleavage sites (Nassel, 1999; Veenstra, 2000). The structure of the tachykinin-like preprotachykinin precursor comprises multiple copies of one or various tachykinin-like peptides separated by spacer sequences and a signal peptide sequence, guiding the preprotachykinin into the endoplasmic reticulum (Veenstra, 2000).

5.1.1) Theory of the evolution of the tachykinin genes according to the 2R hypothesis.

The mammalian *TAC1*, *TAC3* (or *Tac2*) and *TAC4* genes have similar organisation of their exons suggesting homology (Page, 2006). The tachykinin genes vary in the number of peptides they express; the *TAC1* gene (βTAC1 transcript) expresses NKA and SP while the *TAC3* gene (α/βTAC3 transcripts) comprises only NKB. A second tachykinin on *TAC*3 may have been made redundant through evolutionary pressure while on the γTAC3 transcript no peptides are translated (Page *et al.*, 2009). The *TAC4* gene in humans comprises EKA/B and the tachykinin-gene related peptides EKC/D, the latter having diverged from the classical tachykinins. In mouse, the *Tac4* gene encodes only one transcript expressing only mHK-1 (Kurtz *et al.*, 2002). In rabbit, the *TAC4* gene (αTAC4 transcript) encodes EK-1, showing 100% homology with human HK-1 while the rabbit EK-2 tachykinin-gene related peptide comprises a common C-terminal motif (GLL-NH_2) with the human gene-related peptides, EKC/D (Page, 2004).

The tachykinin genes are located on the same chromosomes as the *HOX* gene clusters that have evolved according to the 2R hypothesis; a concept suggesting the entire genome was duplicated twice in early vertebrate evolution. This is because certain chromosome regions in the human genome exist in four similar copies (Larhammar, 2002; Conlon & Larhammar, 2005). The chromosomal location of the *TAC* genes and their structure, have led to the hypothesis that a precursor tachykinin gene comprising two tachykinin peptides gave rise through two successive rounds of gene duplications to the current known mammalian *TAC1*, *TAC3* (*Tac2*), *and TAC4* genes (Conlon & Larhammar, 2005). The discovery of a fourth tachykinin gene has been anticipated; however the option that accumulated mutations have rendered it inactive has not been excluded (Conlon, 2004). The alternative hypothesis that the three tachykinin genes duplicated independently at different occurrences during the course of evolution and underwent translocation events cannot be excluded either (Conlon, 2004).

5.1.2) Bioinformatic tools and existing studies for the identification of tachykinin or tachykinin-related peptides by means of in silico analysis.

BLAST (Altschul *et al.*, 1990) is a heuristic local alignment algorithm tool that uses a single query sequence to reveal sequences displaying homology. Sequences are presented in the FASTA format (Pearson & Lipman, 1988; Pearson, 2000) in a text-based form for representing nucleotide or amino acid sequences annotated by single-letter codes. The *in silico* analyses of publically accessible bioinformatics databases have allowed new approaches for the study of tachykinin and tachykinin-related occurrence and distribution in previously unreported species (Christie, 2008; Christie *et al.*, 2008). Page and colleagues (2009) investigated the distribution of the *TAC3* (or *Tac2*) gene in mammalian and non-mammalian vertebrate species, interrogating the nucleotide and protein NCBI databases (www.ncbi.nlm.nih.gov/nuccore and www.ncbi.nlm.nih.gov/protein) and the Ensembl database (www.ensembl.org) using "tachykinin 3", "TAC3" and "Tac2" as keywords. Furthermore, the human βTAC3 transcript was used as a "probe" for blastn and tblastn algorithms searches to query the nucleotide collection (nr/nt), the non-human, non-mouse expressed sequenced tags (EST_others) databases and the blastp algorithm to query the non-redundant protein sequences (nr) database. Protein sequences were obtained directly in FASTA format or nucleotide EST sequences were first translated using the ORF finder tool (www.ncbi.nlm.nih.gov/gorf/gorf.html) in the six frames and the sequence with the tachykinin motif selected. The collected sequences were aligned using the Clustal algorithm (Thompson *et al.*, 1994) and phylogenetic analysis was performed using the "Phylip" algorithm. This study extended the number of species NKB is known to be expressed in (such as in birds and reptiles), having only previously been identified in mammals. The study also showed a 100% homology of NKB across the identified species, indicating a strong evolutionary pressure to conserve this peptide.

Jiang and colleagues (2002) discovered by means of database searching a novel tachykinin-like peptide that provokes a pharmacological response on the NK1 receptor. A custom-built program PepPat was used for the mining of a human EST database (generated by Incyte Genomics, Palo Alto, California). NKA was used as a query

sequence holding the motif FVGLMGKR while the novel program recognised the sequence "[4 variable a.a.]-MG-[K/R][K/R]" as a pattern. An EST sequence was identified containing a novel peptide namely C14TKL-1 (human Chromosome 14 tachykinin-like peptide 1) comprising the sequence RHRTPMFYGLM-NH_2. This novel peptide was hypothesised as potentially encoded on the anticipated *TAC5* gene (Page, 2005) however the hypothesis has not been confirmed to date.

Previous studies have also identified tachykinin-like peptides in particular species of interest such as the *aphids* and *crustaceans* using *in silico* approaches (Christie *et al.*, 2008; Christie, 2008). The tblastn algorithm was used to mine for ESTs encoding putative aphid neuropeptide precursors. The known sequence of a *Drosophila melanogaster* tachykinin-related peptide precursor was aligned and the database was set to non-human, non-mouse ESTs (EST_others) and restricted to the Aphidoidea. All hits were translated using the "Translate" tool of EXPASY (www.expasy.ch/tools/dna.html). Signal peptide prediction was performed via the online program SignalP 3.0 (www.cbs.dtu.dk/services/SignalP) (Bendtsen *et al.*, 2004). Sequences were then checked manually for the presence of the FX_1GX_2RG motif, as well as for the typical peptide precursor features, such as the presence of a signal peptide sequence and pro-hormone convertase processing sites (K/R/KK/RR). An *Aphis gossipi* EST was identified encoding a putative partial prepro-TRP comprising 61 amino acids. The identified partial precursor contained four dibasic pro-hormone convertase processing sites (one KR and three KK), putatively liberating four peptides. Following the action of carboxypeptidase and C-terminal α-amidation, the four peptides ASVYGVGPAEGF, ASMGFMGMR-NH_2, DYYSNNKGSAAGFFGMR-NH_2 and APSADAFYGVR-NH_2, were predicted the latter three possessing the -FX_1GX_2R-NH_2 motif characteristic of TRPs. The small size of these insects have rendered them as impractical organisms for biochemical studies as a vast number of organisms were needed to conduct any comprehensive biochemical investigation of their peptidome. This approach expanded the existing knowledge on the peptidergic system of the organism.

Tachykinin-related peptides have been revealed in marine crustaceans as well, such as the barnacle *Balanus amphitrite,* using *in silico* data mining of the UniProt (www.uniprot.org) and the NCBI databases using "tBLASTn" algorithm (Yan *et al.,* 2012). This study identified TRPs in the organism using as search keywords "neuropeptide", "hormone" and "peptide" without "receptor", "signal anchor", or "transmembrane" on the UniProt database. Moreover the NCBI database was mined through the tBLASTn algorithm using known arthropod neuropeptide sequences as a query sequence. The potential neuropeptide sequences (of not only TRPs) revealed by database mining were translated using the EXPASY translate tool. The revealed sequences were evaluated for the identification of neuropeptides based on three typical neuropeptide precursor criteria; the presence of a signal sequence, K/R prohormone processing sites and the peptide precursor length being shorter than 300 amino acids. As a result, 16 neuropeptide families were predicted, and 14 of them were confirmed by gene cloning by RACE-PCR as secondary proof of the *in silico* predictions and provided coverage of peptide isoforms. The neuropeptides identified in this study included tachykinin-related peptides (TRPs). The results significantly expanded the knowledge of the peptide neuro-endocrinology of barnacle species. The *in silico* discovery of peptides overrides the predicament of collecting and sectioning sufficient amounts of nervous tissues from the barnacle *Balanus amphitrite* for mass spectrometry analysis.

5.2) A strategy for the identification of novel tachykinin and tachykinin-related peptides by means of in silico approaches.

Hitherto, identification of **tachykinin** peptides using *in silico* analyses of publicly accessible databases has been conducted only using name searches (in contrast to tachykinin-related peptide searches, which have used sequenced alignments) (Page *et al.,* 2009). This strategy has expanded our knowledge on the number of species tachykinins are found to be expressed in, the divergence of the tachykinin sequences and also gives clues to the prohormone processing sites among species. However, this approach brings the limitation of retrieving only annotated tachykinin sequences either cloned or from computer-generated translation of predicted genes (*e.g.* Ensembl database). While studies

have mined databases (*e.g.* ESTs) via alignment of a known tachykinin-related peptide precursor for the identification of novel non-annotated tachykinin-related peptides, no studies exist on the identification of novel tachykinin peptides using the strategy of sequence alignment.

The focus and aims of the research covered in this Chapter is the *in silico* identification of novel tachykinin peptides by means of the alignment of the tachykinin motif, as part of a thorough tachykinin-identification strategy, and the selection of tachykinins from other entries using typical tachykinin precursor criteria. The NCBI nucleotide collection (nr/nt) and expressed sequence tags (EST) databases were interrogated aligning the tachykinin C-terminal motif FXGLMG, replacing X with the 20 amino acids. The alignment of each six amino acid motif released numerous "hits", and only 100% homologous entries were collected for further analysis. The collected nucleotide sequences were translated into amino acid sequences using a program (recorded by M. Soloviev) on Excel Microsoft capable of translating the input sequences in the six frames and revealing the frame that comprises the tachykinin motif. A "scoring system" based on typical tachykinin precursor characteristics was generated to identify tachykinins from non-tachykinin entries. Subsequently, this "scoring system" (explained in Section 5.3.4) was applied to the collected data from the nucleotide (nr/nt) and EST databases searches. Moreover, in this study the NCBI "nucleotide" and the Ensembl databases were interrogated using specific keywords (Section 5.3.1) retrieving annotated tachykinin sequences derived either from gene cloning or as computer-predicted tachykinin sequences. A vast number of nucleotide and peptide sequences were collected from various species encoded on the three tachykinin genes (or potentially orphan, not falling under the three known tachykinin genes). The nucleotide sequences were translated using the custom-built program. The sequences were tabulated according to gene and then investigated for putative peptides encoded, divergence of tachykinins, and their proteolytic processing sites. Moreover, sequence alignment was performed using the Clustal omega algorithm and protein phylogenetic analysis was conducted with the Jalview version 2.7 algorithm (Waterhouse *et al.*, 2009).

5.3) Methodology

5.3.1) Identification of known tachykinins interrogating the NCBI and Ensembl nucleotide databases.

The NCBI nucleotide database was interrogated using the keywords "tachykinin OR protachykinin OR neurokinin OR substance P NOT receptor or patent". The last database search was performed on 09/01/2013. The mRNA sequences were chosen and exported in the FASTA format in an Excel worksheet. The genomic sequences were manually investigated for the tachykinin coding region; the "Change region shown" tool was used to select the part of the genomic sequence encoding the tachykinin sequence, and then exported in the FASTA format. The Ensembl database was interrogated using "tachykinin" as a keyword search. The last search was performed on 30/01/2013. The computer-generated peptide sequence for each tachykinin gene of each species was collected and stored in an Excel file.

5.3.2) Alignment of the tachykinin motif.

The online algorithm tblastn was used to mine the NCBI database nucleotide collection (nr/nt) via queries with the FXGLMG sequence where X was replaced with the 20 amino acids. The tblastn algorithm parameters were standard and set as: Max target sequences: 1000; Expect threshold: 1,000,000; Word size: 2; Matrix: BLOSUM 62; Gap costs: Existence 12: Extension 1; Compositional adjustments: No adjustment. A high "Expect threshold" parameter value was chosen as sequences with 100% homology were revealed. The complete sequence (up to 4000 bases) of the "hits" 100% homologous to the six residue aligned motif was stored in the FASTA format in an Excel file allocated to each motif. For the genomic sequences a partial sequence (2010 bases) was chosen using the "Change region shown" tool; into the "From" window the nucleotide number minus 1000 bases downstream of the aligned sequence was entered and into the "To" window the nucleotide number 1000 bases upstream of the aligned sequence was also entered.

Also the accession numbers of all 100% homologous "hits" to the six residue query sequence were stored in a Word file.

5.3.3) EST searches.

The database Expressed sequence tags (EST) was interrogated aligning the FXGLMG motif and replacing X with the 20 amino acids. The tblastn algorithm parameters were set as: Max target sequences: 1000; Expect threshold: 1,000,000; Word size: 2; Matrix: BLOSUM 62 (default); Gap costs: Existence 11: Extension 1; Compositional adjustments: No adjustment. The EST nucleotide sequences of all 100% matches to the six residue query sequence were retrieved in the FASTA (text) format and stored in an Excel file. The last interrogation of the EST and nr/nt databases was performed on 08/02/11.

5.3.4) The development of a program for the translation of nucleotide sequences and the identification of tachykinins.

The nucleotide sequences collected from the interrogation of the nr/nt and EST databases were translated using a custom-built program on Excel (recorded by M. Soloviev), translating the nucleotide sequences in the 6 frames and revealing the frame with the tachykinin motif. The program automatically removed duplicated sequences. As intron/exon boundaries cannot easily be defined, genomic sequences were excluded from the list of collected tachykinin sequences. Manually genomic sequences (potentially containing contaminating introns and frameshifts) were separated from mRNA sequences. Sequences annotated as genomic/ synthetic/ chromosome/ genome/ mitochondrial/ mitochondrion/ BAC clone/ PAC clone/ CpG clone/ cosmid were stored in a separate file from sequences annotated as mRNA/ cDNA/ cds protein. The remaining sequences from the nr/nt and EST databases were investigated for the presence of novel motifs. The following typical tachykinin precursor criteria were applied to evaluate the candidate sequences; the presence of prohormone processing sites, monobasic and dibasic K/R, upstream and immediately downstream from the motif, the absence of a

proline immediately next to the monobasic K/R processing site, the absence of a stop codon upstream from the motif and before the initiation of translation methionine.

5.3.5) Signal peptide prediction using SignalP, alignment of sequences using Clustal omega and phylogenetic analysis using Jalview.

Signal peptide prediction was performed via the online program SignalP 4.1 (www.cbs.dtu.dk/services/SignalP). Multiple sequence alignments of amino acids were performed with the Clustal Omega program (www.ebi.ac.uk/Tools/msa/clustalo/). The peptide sequences of the tachykinin peptides revealed were inputted to the Clustal omega tool in FASTA format. Protein phylogenetic analysis was conducted with Jalview version 2.7 (Waterhouse *et al.*, 2009). The aligned sequences were inputted to the Jalview algorithm and phylogenetic analysis was performed using the average distance method (http://www.jalview.org/development/release-history/version-27).

5.4) Results.

5.4.1) Tachykinin peptides found from the interrogation of the nucleotide collection and EST databases using the tachykinin FXGLMG motif as a query sequence.

In an attempt to identify novel non-annotated tachykinin peptides and tachykinin motifs the publicly accessible databases nr/nt (nucleotide collection) and EST were mined using the tBLASTn algorithm, aligning the FXGLMG sequence and replacing X with the 20 amino acids. The tblastn algorithm parameters were set as described in Section 5.3.2 and 5.3.3. The "Expect threshold" was set at a very high value (*i.e.* 1.000.000) for retrieval of only 100% similarity matches to the six amino acids of the query sequence; the "Word size" was set to 2 to increase sensitivity and the "Matrix" was set as the default. The nucleotide sequences of all 100% homologous "hits" to the six amino acid motif were stored in an Excel file in FASTA format for further analysis of tachykinin from non-tachykinin "hits". The collected nucleotide sequences were translated into amino acid sequences using a program (recorded by M. Soloviev) on EXCEL that translated the input sequences in the six frames and revealed the frame that comprises the tachykinin motif. In total 1307 individual sequences were found from the nr/nt database searches and 2459 sequences from the EST database searches. The collected sequences were evaluated to identify tachykinins from randomly retrieved sequences containing identical six residues to the aligned motif. The evaluation was based on preprotachykinin precursor criteria which formed a "scoring system". These criteria were the presence of prohormone processing sites, monobasic and dibasic K/R, upstream and immediately downstream from the motif with the absence of a proline immediately next to a monobasic K/R processing site as a proline residue would prevent proteolytic cleavage. Also, the presence of a stop codon between the motif and the first methionine of the preprotachykinin precursor (initiation of translation) has no biological significance hence such sequences were not scored as tachykinins. Subsequently this scoring system was applied onto the data from the EST searches.

A novel motif FHGLMG motif was identified expressed in five fish species comprising a polar, positively charged residue. The sequences with accession number DQ290174 from the species *Lates calcarifer* (Asian seabass) and XM_001923424 from the species *Danio rerio* (Zebrafish) were identified from the nr/nt database mining while the sequences with accession number GR699402 from the species *Oreochromis niloticu* (Nile tilapia), DK025687 from the species *Oryzias latipes* (Medaka) and DV007667 from the species *Gasterosteus aculeatus* (Three-spined stickleback) were identified from the EST database mining. In Figure 5.1 are shown the preprotachykinin peptide sequences, the signal peptide sequence (identified by SignalP 4.1) and the putative peptides released from the prohormone convertase processing of the precursor, which are underlined. Also the dibasic cleavage sites are highlighted in bold. An interesting observation was made in the sequence with accession number XM_001923424, as the putative peptide KRSKSQHFHGLMGSS expressed in *Danio rerio* is flanked by the classic KR and a previously not identified in tachykinins SS (dibasic serine) cleavage site at its' C-terminal end. Moreover, sequences with accession numbers DV007667 expressed in species *Gasterosteus aculeatus*, DK025687 *Oryzias latipes* and GR699402 *Oreochromis niloticu* comprise the putative tachykinins KRSNGQHFHGLMGRS, KRSKSQQFHGLMGRS, KRSKAQQFHGLMGRS flanked by a dibasic KR and a monobasic R cleavage site or a potential dibasic RS cleavage site. The tachykinin precursors presented in Figure 5.1 all comprise two tachykinin peptides the second peptide comprises the classic FVGLMG motif. The sequence identified in *Lates calcarifer* is partial therefore only a single peptide without the signal peptide sequence is shown. Also the sequence identified in *Oryzias latipes* is an EST sequence containing a string of N amino acids showing a loss of part of the sequence. We observed the FHGLMG motif to be specific to fish species. Subsequently, an alignment of the novel sequences with known ones from mammalian and non-mammalian species was performed and phylogenetic analysis.

A. >DK025687 *Oryzias latipes* (Medaka):

MEPVKFALLLLLVAFAHIVCALGSPFSSEDDGDLWTEPTWQGYPIERGVTLRLADLI**KR**SKSQQF

HGLMG**RS**SGARLPVRLGR**KR**NNNNNNKGEMFVGLMG**RR**SSGGGVEDEWNSDSY

B. >DV007667 *Gasterosteus aculeatus* (Three-spined stickleback):

MEALKFAVVLSVVVFVQVFGALGTPISNEEEDADIWTVENWQSYPVERGITIRLADLI**KR**SNGQH

FHGLMG**RS**SGTSQPLRLGK**KR**NKGEMFVGLMG**RR**SLDGDVEEEWNSDS

C. >GR699402 *Oreochromis niloticu* (Nile tilapia):

MWTVENWQGYPVERGITIRLADLI**KR**SKAQQFHGLMG**RS**GVSHAVRLGR**KR**NKGEMFVGLMG**RR**S

LGGDMEEEFKSDSY

D. >DQ290174 *Lates calcarifer* (Asian seabass):

MCVCVCVCIQGYPLERGITIRLADLI**KR**SKAQQFHGLMG**RS**SG

E. >XM_001923424 *Danio rerio* (Zebrafish):

MDIFKLSALAFILYLQLHNAGASPSEEGDIWTVENLEEKPQVTDVFLRIADLM**KR**SKSQHFHGLM

G**SS**AGNTQPLRLGR**RR**NKGEIFVGLMG**RS**DG

Figure 5.1. Tachykinin precursor sequences comprising the FHGLMG motif expressed in five fish species. The tachykinin precursor sequences were retrieved from the interrogation of the nr/nt and EST databases using FHGLMG as a query sequence. The sequences with accession number DQ290174 from the species *Lates calcarifer* (Asian seabass) and XM_001923424 from the species *Danio rerio* (Zebrafish) were identified from the nr/nt database mining while the sequences with accession number GR699402 from the species *Oreochromis niloticu* (Nile tilapia), DK025687 from the species *Oryzias latipes* (Medaka) and DV007667 from the species *Gasterosteus aculeatus* (Three-spined stickleback) were identified from the EST database mining. The signal peptide sequence (identified by SignalP 4.1) and the putative peptides released from the prohormone convertase processing of the precursor are underlined. The dibasic prohormone cleavage sites are highlighted in bold. In (A) SignalP analysis of the sequence suggested the first 23 amino acids function as a signal peptide, with a cleavage locus located between Gly^{23} and Ser^{24}; in (B) also the first 23 residues of the precursor are predicted as a signal peptide with the cleavage site located between Gly^{23} and Thr^{24}. Sequences (C) and (D) are partial therefore the signal peptide is not shown. In (E) two previously not identified in tachykinins SS (double serine) and RS cleavage sites are shown. The first 22 residues putatively form the signal peptide and cleavage occurs between Ala^{22} and Ser^{23}.

5.4.2) The collection of tachykinin sequences from the interrogation of the nucleotide NBCI and Ensembl databases using keyword searches.

In an attempt to search the NCBI nucleotide and Ensembl databases for annotated tachykinin sequences, keyword searches were performed. Specifically, the NCBI nucleotide database was interrogated using the keywords "tachykinin OR protachykinin OR neurokinin OR substance P NOT receptor or patent" for the retrieval of tachykinins with the above typical annotations and fewer non-tachykinin sequence "hits" *i.e.* sequence retrievals of *e.g.* tachykinin receptor sequences. The last keyword search of the nucleotide NCBI database was performed on 09/01/2013. The nucleotide sequences collected from the interrogation of the NCBI database were translated into the six open reading frames using the custom-built program described in Section 5.3.4. The interrogation of the non-redundant NCBI nucleotide databases revealed 349 sequences annotated as tachykinins. The collected sequences were manually investigated for the presence of a tachykinin motif while the falsely annotated tachykinin sequences, not containing the tachykinin motif, or genomic sequences (containing contaminating introns) were exempt. In total 151 sequences comprising two tachykinin peptides and 99 sequences comprising one tachykinin were revealed. The above sequences were manually investigated for duplicates and also for alternative transcripts of the same gene. This research focused on collecting the longest transcript produced from each gene as the investigation of the divergence of both tachykinin peptides on each preprotachykinin precursor was of interest. 103 preprotachykinin precursor sequences belonging to the three tachykinin genes (or orphan, not falling under the three known tachykinin genes) from a range of species were collected. The Ensembl database was interrogated using the keyword "tachykinin" retrieving sequences input until 30/01/2013 when the last search of the database was performed. Tachykinins were found in 29 mammalian and non-mammalian species. In total, 69 preprotachykinins were collected. Also, the most elongated precursor was chosen for further alignment and phylogenetic analysis.

The preprotachykinin sequences were manually investigated for putative peptides yielded from proteolytic cleavage of the preprotachykinin precursors at K/R (dibasic or

multibasic) sites. In Table 5.1 are shown the names of the 68 species the *TAC1* or *Tac1* gene was found expressed in, collected from keyword searches of the NCBI and Ensembl databases. Also, the sequence of the putative peptides comprised in each precursor following removal of C-terminal dibasic residues by carboxypeptidase and amidation, and the accession number of each preprotachykinin sequence are shown. The SP and NPK (or NPγ) homologues are tabulated and divergent residues are highlighted in red. The putative cleavage site comprised in NPK where proteolytic cleavage releases NKA is highlighted in green. SP and NPK are highly conserved among mammals. Apart from the mammal, *Cryptotis parva* (least shrew; RPKSQQFYGLM-NH_2) where the SP homolog has diverged from the typical mammalian SP sequence (Pro^4 to Ser^4 and Phe^7 to Tyr^7) the rest of the sequence remains unknown as only a partial sequence was retrieved. Also SP expressed in the mammal *Procavia capensis* (rock hyrax; RPKLQKFYGLM-NH_2) divergence of the residues Pro^4 to Leu^4, Gln^6 to Lys^6 and Phe^8 to Tyr^8 are reported. In birds a semi-conservative divergence of Lys^3 to Arg^3 was found (RPRPQQFFGLM-NH_2). In the turtle species *Pelodiscus sinensis* (Chinese softshell turtle; RPRPQQFYGLM-NH_2) divergence of the tachykinin motif Phe^8 to Tyr^8 is shown. The SP orthologs expressed in fish show a higher degree of divergence from archetypal human SP (RPKPQQFFGLM-NH_2) in both the tachykinin motif and the N-terminal sequence of the undecapeptide as shown in *Anoplopoma fimbria* (sablefish; KPRPHQFIGLM-NH_2), *Gasterosteus aculeatus* (stickleback; KPRPHQFVGLM-NH_2) *Oncorhynchus mykiss* (rainbow trout; KPRPHQFFGLM-NH_2) and the amphibian *Hyla simplex* (frog; KPRPDQFYGLM-NH_2). The sequence of the amphibian *Hyla simplex* shown in Table 5.1 is also partial as the sequence of NPK was not identified.

In Table 5.2 are shown the names of the 59 species the *TAC3* (or *Tac2* in rodents) or *tac3* gene was found in and the accession numbers of each sequence. The sequences of the putative peptides released from the proteolyic cleavage of the precursor expressed by the *TAC3* homologues (*i.e.* the N-terminal sequence of the preprotachykinin B precursor and the NKB decapeptide) are shown in the second and third columns. Putative dibasic proteolytic cleavage sites are highlighted in green and divergent residues are highlighted in red. This research shows 100% homology of NKB (DMHDFFVGLM-NH_2) in all

mammalian species apart from the species *Callithrix jacchus* (common marmoset; NMQDLFVGLM-NH_2) and *Saimiri boliviensis* (squirrel monkey; NMQDLFVGLM-NH_2) where divergence of the residues Asp^1 to Asn^1, His^3 to Gln^3 and Phe^5 to Leu^5 is shown. In reptiles NKB shows 100% homology to mammalian NKB. In fish species NKB has diverged from the mammalian homologues. In the fish species *Boreogadus saida* (arctic cod; EMHDIFVGLM-NH_2), *Danio rerio* (zebrafish; EMHDIFVGLM-NH_2), *Gadus morhua* (Atlantic cod; EMHDIFVGLM-NH_2), *Ictalurus punctatus* (channel catfish; EMHDIFVGLM-NH_2), *Pimephales promelas* (fathead minnow; EMHDIFVGLM-NH_2), *Osmerus mordax* (rainbow smelt; EMHDIFVGLM-NH_2) and *Sebastes rastrelliger* (grass rockfish; EMHDIFVGLM-NH_2) the sequence of the NKB decapeptide has diverged from Asp^1 to Glu^1 and Phe^5 to Ile^5. Furthermore, in the fish species *Dissostichus mawsoni* (Antarctic toothfish; EMNDIFVGLM-NH_2) the residue His^3 diverged to Asn^3. In the species *Oryzias latipes* (Medaka; DMDDIFVGLM-NH_2) we report the divergence of the residues His^3 to Asp^3 and Phe^5 to Ile^5. In the species *Salmo salar* (Atlantic salmon) two NKB –like peptides were revealed EMDDVFVGLM-NH_2 (Asp^1 to Glu^1, His^3 to Asp^3 and Phe^5 to Val^5) and the peptide DMDDVFVGLL-NH_2 comprising the tachykinin-like motif FVGLL-NH_2. The divergence of NKB in fish *Tetraodon nigroviridis* (ELHDIFVGLM-NH_2) Asp^1 to Glu^1, Met^2 to Leu^2 and Phe^5 to $Ile^5$and *Xenopus tropicalis* (EMNDFFVGLM-NH_2) Asp^1 to Glu^1 and His^3 to Asn^2 are shown.

In Table 5.3 the names of the 34 species the *TAC4* (or *Tac4* or *tac4*) gene was found expressed in, collected from keyword searches of the NCBI and Ensembl databases are shown. The putative peptides released from the proteolytic cleavage of the preprotachykinin precursor and the accession number of each preprotachykinin sequence, are also shown. The 34 preprotachykinin sequences revealed from the above searches were manually investigated for the putative tachykinin and tachykinin-gene related homologues shown in Table 5.3. The putative homologous peptides shown, released from *TAC4* were manually predicted, as proposed yielded from the processing of the precursor at the signal peptide sequence (by signal peptidases) and at the C-terminal dibasic cleavage site. Prediction of cleavage of each precursor at the signal peptide sequence was performed using the SignalP algorithm (results shown in Appendix 2). In mammalian

species, the divergent residues between the HK-1 orthologs are highlighted in red, showing that the sequence of HK-1 is not 100% homologous among all mammalian species. However, rather than the divergence of the HK-1 sequence, of particular interest is the divergence of the dibasic cleavage site, also highlighted in green, located upstream the sequence of the hemokinin-1 homologues. In certain species the sequence of HK-1 has diverged to comprise a monobasic (rather than the commonly occurring dibasic KR cleavage site). As shown in Table 5.3 the species *Dasypus novemcinctus* (armadillo; KTGKASQFFGLM-NH_2), *Gorilla gorilla* (KTGKASQFFGLM-NH_2), *Homo sapiens* (KTGKASQFFGLM-NH_2), *Macaca mulatta* (macaque, KTGKASQFFGLM-NH_2), *Pan paniscus* (pygmy chimpanzee; KMGKASQFFGLM-NH_2), *Pongo abelii* (sumatran orangutan; KTGKPSQFFGLM-NH_2) and *Pan troglodytes* (chimpanzee; KMGKASQFFGLM-NH_2) comprise a monobasic cleavage site compared to the other mammalian species. As discussed further on, this divergence of the dibasic cleavage site to a monobasic cleavage site in these particular species has a biological function. We suggest a linkage between alternative processing of the preprotachykinin precursor at the monobasic cleavage site for the release of the mature form of HK-1 (TGKASQFFGLM-NH_2) such as occurring in pre-eclampsia and normal pregnancies.

Tachykinin gene-related peptides are known to be released from the proteolytic cleavage of the TAC4 preprotachykinin precursor, previously only reported in human and rabbit, possessing the motif GLL-NH_2. Our investigation revealed a number of novel, putative, tachykinin gene-related peptides released from the proteolytic cleavage of the TAC4 preprotachykinin precursor at K/R (dibasic or multibasic) sites. In Table 5.3 are shown the putative tachykinin-gene related peptides released from the proteolytic cleavage of the preprotachykinin precursor at K/R (dibasic or multibasic) sites following removal of C-terminal dibasic residues by carboxypeptidase and amidation. Tachykinin-gene related peptides were expressed in the mammalian eutherian species *Callithrix jacchus* (marmoset; KKCYQLEHTLQGLL-NH_2), *Dasypus novemcinctus* (armadillo; AGGISPIQLVSITGHQTGQRVQGLL-NH_2), Gorilla (KKGYQLEHTFQGLL-NH_2), *Pan paniscus* (pygmy chimpanzee; KKAYQLEHTFQGLL-NH_2), *Pan troglodytes* (chimpanzee; KKAYQLEHTFQGLL-NH_2), *Pongo abelii* (sumatran orangutan; KKVYQLQHTFQGLL-

NH_2), *Procavia capensis* (rock hyrax; VGGISPIQPLRTIYQQVVQGLL-NH_2) and *Saimiri boliviensis* (squirrel monkey; KKVYQLEHTLQGLL-NH_2). The above species comprised tachykinin-gene related peptides with the known –GLL-NH_2 motif. However, the species *Ictidomys tridecemlineatus* (squirrel; QVEGIPPIQPERAAGYKLGQMVQALL-NH_2), *Ochotona princeps (pika;* VRGIHPIPWRTTGYRTGQMVQGSL-NH_2*),* comprise a different C-terminal motif.

In Table 5.4 are shown the names of the 15 species tachykinin orphan genes (not falling under the typical tachykinin gene nomenclature) were found expressed in, collected from keyword searches of the NCBI and Ensembl databases and from the alignment of the tachykinin motif. The preprotachykinin precursors were investigated manually for putative peptides derived from the preprotachykinin precursor processing at the typical proteolytic cleavage sites and are presented following removal of C-terminal residues by carboxypeptidase and C-terminal amidation by amidase. The tachykinin sequences described in Section 5.4.1 were included among these sequences. We further conducted sequence alignment and phylogenetic analysis to investigate the clades these precursors and their peptides fall under.

Table 5.1. Putative tachykinin peptides from *TAC1* gene.

[1]Species	[2]Peptide 1	[3]Peptide 2	[4]Accession number
Ailuropoda melanoleuca (giant panda)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSAMEP00000017243.1
Anoplopoma fimbria (sablefish)	KPRPHQFIGLM-NH_2	SMANAQITRKRHKVNSFVGLM-NH_2	JO691854.1
Bos Taurus (cow)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	BC151422.1
Callithrix jacchus (marmoset)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSCJAP00000001061.1
Canis lupus-familiaris (dog)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_532472.3
Carassius auratus (goldfish)	KPRPHQFIGLM-NH_2	SPANAQITRKRHKINSFVGLM-NH_2	CAU61272
Cavia porcellus (guinea pig)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	NM_001172899.1
Choloepus hoffmanni (sloth)	RPKPQQFFGLM-NH_2	DADSAIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSCHOP00000003135.1
Cryptotis parva (least shrew)	RPKSQQFYGLM-NH_2		FJ696706.1
Danio rerio (zebrafish)	KPRPHQFIGLM-NH_2	SSANAQITRKRHKINSFVGLM-NH_2	BK008124.1
Dasypus novemcinctus (armadillo)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSDNOP00000011010.1
Dipodomys ordii (kangaroo rat)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSDORP00000001980
Echinops telfairi (lesser hedgehog)	RPKPQQFFGLM-NH_2	DADTALENQVALLKALYGRGQVSHKRYKTDPFVGLM-NH_2	ENSETEP00000015772.1
Equus caballus (horse)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSECAP00000002225.1
Erinaceus europaeus (hedgehog)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQVSHKRHRTDAFVGLM-NH_2	ENSEEUP00000002012.1
Felis catus (cat)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_003982791.1
Gadus morhua (Atlantic cod)	KPRPQQFIGLM-NH_2	SAADAQITRKRHKINSFVGLM-NH_2	ENSGMOP00000005761.1
Gallus gallus (chicken)	RPRPQQFFGLM-NH_2	DAGYGQISHKRHKTDSFVGLM-NH_2	BK008126.1

Gasterosteus aculeatus (stickleback)	KPRPHQFVGLM-NH_2	SMANAQITRKRHKVNSFVGLM-NH_2	ENSGACP00000000114.1
Gorilla Gorilla (gorilla)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_004045800.1
Homo sapiens (human)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	HSU37529
Hyla simplex (frog)	KPRPDQFYGLM-NH_2		HM747308.1
Ictalurus punctatus (channel catfish)	KPRPHQFIGLM-NH_2	SSANTQITRKRHKINSFVGLM-NH_2	NM_001200768.1
Ictidomys tridecemlineatus (squirrel)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSSTOP00000004825.2
Loxodonta africana (elephant)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSLAFP00000000757.2
Macaca fascicularis (macaque)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKAPYGHGQISHKRHKTDSFVGLM-NH_2	AB220474.1
Macaca mulatta (rhesus Monkey)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSMMUP00000035078.1
Macropus eugenii (tammar wallaby)	RPKPQQFFGLM-NH_2	DADSSVEKQVGMLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSMEUP00000003075.1
Melanochromis auratus (fish)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	X80662.1
Meleagris gallopavo (turkey)	RPRPQQFFGLM-NH_2	DAATLSFFFLSFLLQPGYGQISHKRHKTDSFVGLM-NH_2	ENSMGAP00000010069.2
Microcebus murinus (mouse lemur)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSMICP00000014896.1
Monodelphis domestica (opossum)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSMODP00000020552.3
Mus musculus (house mouse)	RPKPQQFFGLM-NH_2	DADSSVEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	BC117081.1
Mustela putorius (polecat)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	JP019158.1
Myotis lucifugus (bat)	RPKPQQFFGLM-NH_2	DADSSIAKQVALLKALYGHAHFSNKKRHRTDAFIGLM-NH_2	ENSMLUP00000002112.2
Nomascus leucogenys (gibbon)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSNLEP00000019442.1
Ochotona princeps (pika)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSOPRP00000005183.1

Oncorhynchus mykiss (rainbow trout)	KPRPHQFFGLM-NH_2	SSANPQITRKRHKINSFVGLM-NH_2	BK008119.1
Oryctolagus cuniculus (rabbit)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	X62994.1
Oryzias latipes (medaka)	KPRPHQFIGLM-NH_2	STANAQITRKRHKVNSFVGLM-NH_2	AB441191.1
Osmerus mordax (rainbow smelt)	KPRPHQFFGLM-NH_2	SSANAQITRKRHKLNSFVGLM-NH_2	BT075724.1
Otolemur garnettii (galago)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_003782698.1
Ovies aries (sheep)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	NM_001082596.1
Pan paniscus (pygmy chimpanzee)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_003809689.1
Pan troglodytes (chimpanzee)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_001171112.2
Papio anubis (olive baboon)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_003896287.1
Pelodiscus sinensis (Chinese softshell turtle)	RPRPQQFYGLM-NH_2	DAGYGQMSHKRHKTDSFVGLM-NH_2	ENSPSIP00000004426.1
Pongo abelii (sumatran orangutan)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_002818228.1
Procavia capensis (rock hyrax)	RPKLQKFYGLM-NH_2	DADSSVEKQVALLKALYGHGQTSHKRHKTDSFVGLM-NH_2	ENSPCAP00000015000.1
Pteropus vampyrus (bat)	RPKSQQFFGLM-NH_2	DADSSTEKQVALLKALYGHGQISHKRYKTDSFVGLM-NH_2	ENSPVAP00000014792.1
Rattus norvegicus (rat)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	NM_012666.2
Saimiri boliviensis (squirrel monkey)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	XM_003921239.1
Salmo salar (atlantic salmon)	KPRPHQFFGLM-NH_2	SSANAQITRKRHKINSFVGLM-NH_2	BT125493.1
Salvelinus fontinalis (brook trout)	KPGPHQFFGLM-NH_2	SSANPQITRKRHKINSFVGLM-NH_2	BK008120.1
Sarcophilus harrisii (tasmanian devil)	RPKPQQFFGLM-NH_2	DADTSVEKQVGMLKALYGHGQMSHKRHKTDSFVGLM-NH_2	XM_003772510.1
Sebastes caurinus (copper rockfish)	KPRPHQFIGLM-NH_2	SMANAQITRKRHKINSFVGLM-NH_2	BK008122.1

Sebastes rastrelliger (rockfish)	KPRPHQFIGLM-NH_2	SMANAQITRKRHKINSFVGLM-NH_2	BK008106.1
Sorex araneus (shrew)	RPKSQQFFGLM-NH_2	DADSSIEKQMALLKALYGHGQISHKRHKTDSFVGLM-NH_2	ENSSARP00000008825.1
Sus scrofa (pig)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYVGHGQISHKRHKTDSFVGLM-NH_2	ENSSSCP00000020239.1
Taeniopygia guttata (zebra finch)	RPRPQQFFGLM-NH_2	DAGYGQISHKRHKTDSFVGLM-NH_2	XM_002197398.1
Takifugu rubripes (pufferfish)	KPRPHQFIGLM-NH_2	SMANAQITHKRHKINSFVGLM-NH_2	ENSTRUP00000014475.1
Tetraodon nigroviridis (Spotted Green Puffer	KPRPQQFIGLM-NH_2	SMGESGGQQAASRRPAGANAQITHKRHKINSFVGLM-NH_2	ENSTNIP00000021330.1
Tupaia belangeri (northern treeshrew)	RPKPQQFFGLM-NH_2	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2	Z50786.1
Tursiops truncatus (dolphin)	RPKPQQFFGLM-NH_2	DADSSVEKQVALLQALYGHGQLSHKRHKTDSFVGLM-NH_2	ENSTTRP00000004482.1
Vicugna pacos (alpaca)	RPKPQQFFGLM-NH_2	DADSSVEKQVALLKALYGLGQISHKRHKTDSFVGLM-NH_2	ENSVPAP00000010638
Xenopus laevis (African clawed frog)	KPRPDQFYGLM-NH_2	NGITDWVKFLANGINQVPFFGLM-NH_2	NM_001096537.1
Xenopus tropicalis (western clawed frog)	KPRPDQFYGLM-NH_2	NNGFGQISRKRYKSGSFFGLM-NH_2	BC155674.1
Xiphophorus maculates (platyfish)	KPRPHQFIGLM-NH_2	SMANPQITRKRHKINSFVGLM-NH_2	ENSXMAP00000016896.1

1. The names of the 68 species the *TAC1* or *Tac1* or *tac1* gene was found expressed in, collected from keyword searches of the NCBI and Ensembl databases. 2. The sequence of the putative peptides SP homologues, divergent residues compared to human are highlighted red. 3. The sequence of putative NPK and/or NPγ homologues, cleavage sites are highlighted green. 4. The accession number of each preprotachykinin sequence.

Table 5.2. Putative tachykinin peptides from *TAC3* gene.

[1]Species	[2]Peptide 1	[3]Peptide 2	[4]Accession number
Ailuropoda melanoleuca (*giant panda*)	DADLYKLPPSLLRKLYDSRSVSLDGLLKMLSKASLDPKESPLPQ	DMHDFFVGLM-NH_2	XM_002916001.1
Alligator mississippiensis (American alligator)	LYQGVSYEALLQLADKAPVGLQALAPPQ	DMHDFFVGLM-NH_2	BK008115.1
Anolis carolinensis (anole lizard)	LYDGQGISYEALLRLSGKEEIGPQTLASSQ	DMHDFFVGLM-NH_2	ENSACAP00000007184.2
Boreogadus saida (arctic cod)	RPLSDPSLVNRRNIVRRFSDLDYDSFVGLM-NH_2	NTEAANELPSANELPSANKREMHDIFVGLM-NH_2	BK008109.1
Bos Taurus (cow)	LYDSRVVSLDGLLKMLSKASVGPKESPLPQ	DMHDFFVGLM-NH_2	NM_181017.1
Callithrix jacchus (marmoset)	NMNLYQLVQRLYEIHSFSLEELLVALSQAILDSRGSETALPR	NMQDLFVGLM-NH_2	XM_002752636.2
Canis lupus familiaris (dog)	DSDLYQLPPSLLRKLYDSGSVSLEGLLKMLSKASVDPKESPLPQ	DMHDFFVGLM-NH_2	XM_843938.2
Carpa hircus (goat)	LYDSRVISLDGLLKMLSKASVGPKESPLPQ	DMHDFFVGLM-NH_2	AB499062.1
Cavia porcellus (guinea pig)	LYDSRSVSLEGLLKVLSKASMDPKESSLPQ	DMHDFFVGLM-NH_2	XM_003475961.1
Cricetulus griseus (Chinese hamster)	LYDSRSVSLEGLLKVLSKASMGPKETSLPQ	DMHDFFVGLM-NH_2	XM_003507519.1
Danio rerio (zebrafish)	YNDIDYDSFVGLM-NH_2	EMHDIFVGLM-NH_2	JN392856.1
Danio rerio (zebrafish)	YDDIDYDSFVGLM-NH_2	STGINREAHLPFRPNMNDIFVGLL-NH_2	JN392857.1
Dasypus novemcinctus (armadillo)	LYDSRSVSLDGLLKMLSKASVDSKELSLPQ	DMHDFFVGLM-NH_2	ENSDNOP00000008709.1
Dipodomys ordii (kangaroo rat)	LYDSRSVSLEELLKVLSKASVDSKESSLPQ	DMHDFFVGLM-NH_2	ENSDORP00000001191.1

Dissostichus mawsoni (Antarctic toothfish)	NILKRYSDLDYDSFVGLM-NH_2	DADDSAVPAPQKREMNDIFVGLM-NH_2	BK008104.1
Echinops telfairi (lesser hedgehog)	LVDSQTVSLEGLLKILSSASVGPKKLSLFQ	DMHDFFVGLM-NH_2	ENSETEP00000012112.1
Erinaceus europaeus (hedgehog)	LYEGRSASLDGLLKVLSKASMGAKEPSLPQ	DMHDFFVGLM-NH_2	ENSEEUP00000014342.1
Equus caballus (horse)	LYDSRSVSLDGLLKMLSQASVDPKESSLPQ	DMHDFFVGLM-NH_2	XM_001488415.3
Felis catus (cat)	DLDLYQLPPSLLRKLYDSRSVSLDGLLKMLSKASVDPKELPLPQ	DMHDFFVGLM-NH_2	XM_003988924.1
Gadus morhua (Atlantic cod)	RPLSDPSLVNRRNIVRRSSDLDYDSFVGLM-NH_2	QIKTGEMHDIFVGLM-NH_2	BK008107.1
Gorilla Gorilla (gorilla)	DPDLYQLLQRLFKSHSSLEGLLKALSQASTDPKESTSPE	DMHDFFVGLM-NH_2	XM_004053407.1
Homo sapiens (Human)	DPDLYQLLQRLFKSHSSLEGLLKALSQASTDPKESTSPE	DMHDFFVGLM-NH_2	AF537113.1
Ictalurus punctatus (channel catfish)	YHDIDYDSFVGLM-NH_2	SADAAAAAEDQSQRKREMHDIFVGLM-NH_2	BK008101.1
Ictidomys tridecemlineatus (squirrel)	LFDSRPVSLEGLLKVLSKASVDPKESSLPQ	DMHDFFVGLM-NH_2	ENSSTOP00000011019.2
Loxodonta africana (elephant)	LLDSRSVYLDGLLKMLSKASLGPKESSLPQ	DMHDFFVGLM-NH_2	XM_003405564.1
Macaca mulatta (macaque)	DLDLYQLLQRLFKSHSSLEGFLKAMSQARTDPKESTSPE	DMHDFFVGLM-NH_2	XM_001115535.1
Macropus eugenii (tammar wallaby)	LYNSRSLSLDGLLRLLSKTSVDPKETMDFQ	DMHDFFVGLM-NH_2	ENSMEUP00000006824.1
Microcebus murinus (mouse lemur)	LYDSRSVSLEGLLKVLSKASVDPKESSLPQ	DMHDFFVGLM-NH_2	ENSMICP00000001966.1
Monodelphis domestica (opossum)	LYNSRSISLDGLLRLLSKTSVDSKETMDYQ	DMHDFFVGLM-NH_2	ENSMODP00000024199.2

Mus musculus (house mouse)	LYDSRPVSLEGLLKVLSKASVGPKETSLPQ	DMHDFFVGLM-NH_2	BC031348.1
Mustela putorius (polecat)	DSDLYHLPSSLLRKLYDSRSVSLDGLLKMLSKASVDPKESSLPQ	DMHDFFVGLM-NH_2	ENSMPUP00000001685.1
Nomascus leucogenys (gibbon)	DPDLYQLLQRLFKSHSSLEGLLKALSQASTDPKESTSPE	DMHDFFVGLM-NH_2	XM_003252783.1
Ochotona princeps (pika)	LHSGRSSLEELLRVLGKASMDPKAVASSPQ	DMHDFFVGLM-NH_2	ENSOPRP00000012136.1
Ornithorhynchus anatinus (platypus)	LYDSRAISLDGLLGLLAQTSADPRELASPQ	DMHDFFVGLM-NH_2	ENSOANP00000028149.2
Oryctolagus cuniculus (rabbit)	LQDSSRRPFSLEELLKVLSKASVDPKAASLPQ	DMHDFFVGLM-NH_2	ENSOCUP00000016272.1
Oryzias latipes (Medaka)	STLGQPISLEEFKRNLLRRYTDLDYDSFVGLM-NH_2	NAEEEAVQSQPKRDMDDIFVGLM-NH_2	BK008114.1
Osmerus mordax (rainbow smelt)	SSSGEIPGLGEMKRNLLKRYSDVDYDSFVGLM-NH_2	ADINGVQSQQKREMHDIFVGLM-NH_2	BK008111.1
Otolemur garnettii	LYDSRSVSLEGLLQVLSKASLDPKESSLPQ	DMHDFFVGLM-NH_2	XM_003790546.1
Ovies aries (sheep)	LYDSRVVSLDGLLKMLSKASVGPKESPLPQ	DMHDFFVGLM-NH_2	XM_004009563.1
Pan paniscus (pygmy chimpanzee)	DPDLYQLLQRLFKSHSSLEGLLKALSQASTDPKESTSPE	DMHDFFVGLM-NH_2	XM_003824897.1
Pan troglodytes (chimpanzee)	DPDLYRLLQRLFKSHSSLEGLLKALSQASTDPKESTSPE	DMHDFFVGLM-NH_2	XM_003313573.1
Papio anubis (olive baboon)	DLDLYQLLQRLFKSHSSLEGLLKAMSQASTDPKESTSPE	DMHDFFVGLM-NH_2	XM_003906628.1
Pimephales promelas (fathead minnow)	YNDIDYDSFVGLM-NH_2	NADTDDFPPQRKREMHDIFVGLM-NH_2	BK008100.1
Pongo abelii (sumatran orangutan)	DLDLYQLLQRLFKSHSSLEGLLKALSQASTDPKESTSPK	DMHDFFVGLM-NH_2	XM_003778069.1
Procavia capensis (rock hyrax)		DMHDFFVGLM-NH_2	ENSPCAP00000011373.1

Pteropus vampyrus (bat)		DMHDFFVGLM-NH_2	ENSPVAP00000003713.1
Rattus norvegicus (rat)	LYDSRPISLEGLLKVLSKASVGPKETSLPQ	DMHDFFVGLM-NH_2	ENSRNOP00000005679.1
Saimiri boliviensis (squirrel monkey)	DVDLYQLVQRLYEIHSFSLEELLIALSQAILDSRGSETPLPR	NMQDLFVGLM-NH_2	XM_003926696.1
Salmo salar (atlantic salmon)	SILKRYNDLDYDSFVGLM-NH_2	GADIYDLPPSPHKREMDDVFVGLM-NH_2	BK008102.1
Salmo salar (atlantic salmon)	YRDIHDDTFVGLM-NH_2	SAGVNDLPSRRSKIRDMDDVFVGLL-NH_2	BK008103.1
Sarcophilus harrisii (tasmanian devil)	LYNSRSLSLDGLLKLLSKTSVDSKESMDFQ	DMHDFFVGLM-NH_2	ENSSHAP00000005749.1
Sebastes rastrelliger (grass rockfish)	NILKRYSDLDYDSFVGLM-NH_2	DADANAVQSPQKREMHDIFVGLM-NH_2	BK008105.1
Sorex araneus (shrew)	LYDSSVSLEGLLRVLSRASVGPKASSLAQ	DMHDFFVGLM-NH_2	ENSSARP00000007866.1
Sus scrofa (pig)	LCDSRSISLDGLLKMLSKASVGAKESSLPQ	DMHDFFVGLM-NH_2	AY758208.1
Tetraodon nigroviridis (Spotted Green Puffer)	YSDLDYDSFVGLM-NH_2	NADAEAAQSPQKRELHDIFVGLM-NH_2	CR713079.2
Tupaia belangeri (northern treeshrew)	LHDSRSVSLEGLLKALSKASVDPKDSSLPQ	DMHDFFVGLM-NH_2	ENSTBEP00000011487.1
Tursiops truncatus (dolphin)	LYDSRSVSLDGLLKMLSKASVGPKESSLPQ	DMHDFFVGLM-NH_2	ENSTTRP00000012378.1
Vicugna pacos (alpaca)	LPDSRLVSLNGLLRVLSRAGTGPKESPLPQ	DMHDFFVGLM-NH_2	ENSVPAP00000008530
Xenopus tropicalis (Western clawed frog)	SSDIYKLPASLLKRFYDDDSFVGLM-NH_2	SDFKEFPSLPLKREMNDFFVGLM-NH_2	BK008110.1

1. The names of the 59 species the *TAC3* or *Tac2* or *tac3* gene was found expressed in, collected from keyword searches of the NCBI and Ensembl databases. 2. The sequence of the putative peptides N-terminal preprotachykinin B homologues where the divergent residues are highlighted in red and the potential cleavage sites are highlighted in green. 3. The sequence of putative NKB homologues. 4. The accession number of each preprotachykinin sequence.

Table 5.3. Putative tachykinin peptides from *TAC4* gene.

[1]Species	[2]Peptide 1	[3]Peptide 2	[4]Accession number
Ailuropoda melanoleuca (giant panda)	DEGLALGAEAGSWITLTLEDGGIHLQLQEVKRGKASQFFGLM-NH_2		ENSAMEP00000012879.1
Callithrix jacchus (marmoset)	GDGGEEQALSTEAETWITVALEEGTVPSIQLQLRKVKRGKASQFFGLM-NH_2	KKCYQLEHTLQGLL-NH_2	XM_002748441.1
Canis lupus-familiaris (dog)	EDLAVGAEAGSWITLTLEDGGIPGIQLQLQLQEVKRGKASQFFGLM-NH_2		ENSCAFP00000031398.1
Choloepus hoffmanni (sloth)	GDGGEELALSAEAESWVTVTLEEGTAPSIQFQLQQVKRGKASQFFGLM-NH_2		ENSCHOP00000003804.1
Dasypus novemcinctus (armadillo)	YPVAGNGGEELALSIEAGPWVTVTLEEGNVPSIQFQLQEVKTGKASQFFGLM-NH_2	AGGISPIQLVSITGHQTGQRVQGLL-NH_2	ENSDNOP00000004237.1
Dipodomys ordii (kangaroo rat)	GGAGPSIQLQLQKAKRSKNRQFFGLM-NH_2		ENSDORP00000013872.1
Echinops telfairi (lesser hedgehog)	EGSVPGIQLQLREEKRGKSSQFFGLM-NH_2		ENSETEP00000010247.1
Equus caballus (horse)	GDEELTLSAEAGSWEGVIPSIQLQLQEVKRGKARQFFGLM-NH_2		ENSECAP00000012476.1
Erinaceus europaeus (hedgehog)	EDKELVLSTESVPWLTLILQEGAIARIQFQIQEAKRSTGKEFYGLM-NH_2		ENSEEUP00000005053.1
Felis catus (cat)	TVDEKLALGAEAGSWVTVTLEEDGVVPHIQLTLQEVKRGKTSQFFGLM-NH_2		ENSFCAP00000002434.2
Gorilla gorilla (Gorilla)	GDGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2	KKGYQLEHTFQGLL-NH_2	XM_004041438.1
Homo sapiens (Human)	GDGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2	KKAYQLEHTFQGLL-NH_2	AF515828.1

Ictidomys tridecemlineatus (ground squirrel)	NTTGDSGEELALSAEAGPWVTLILEEVAVPSIQLQLQEGKRSKANQFFGLM-NH_2	QVEGIPPIQPERAAGYKLGQMVQALL-NH_2	ENSSTOP00000007095.2
Loxodonta africana (elephant)	GDSGEEVALSTEAGLWVTVTLEEGAVPSIQLQVQEEKRGKASQFFGLM-NH_2		ENSLAFP00000012829.3
Macaca mulatta (macaque)	GDSGEEQTSTEAETWITEGAGPSIWLQLQEVKTGKASQFFGLM-NH_2		ENSMMUP00000018485.2
Microcebus murinus (mouse lemur)	AGDGGKERALSTEAEPWVTVTLEEGAPPITQRQLQQVKRGKASQFFGLM-NH_2		ENSMICP00000015034.1
Mus musculus (house mouse)	TTAGDREELAFGAEAESWVTVNLKGIPVPSIELKLQELKRSRTRQFYGLM-NH_2		BC119426.1
Mustela putorius (polecat)	DEELALGAEAGSWITLTLEDGGIHLQLQQVKRGKASQFFGLM-NH_2		JP019159.1
Ochotona princeps (pika)	GDGGEELTLGTEAGSWVTVNLQVGAVASIQLQLHEVKRGKASQFFGLM-NH_2	VRGIHPIPWRTTGYRTGQMVQGSL-NH_2	ENSOPRP00000006609.1
Oryctolagus cuniculus (rabbit)	EDGGEEQTLGAEAGPWVTVTLEAGAVASIQLQLQEVKRGKASQFFGLM-NH_2	VRGYQMGQRGLL-NH_2	AY471576.1
Otolemur garnettii (small-eared galago)	AGDSGEEQTLDPEAESWESPVLRVDLQLQNVKRGKANQFFGLM-NH_2		XM_003786485.1
Ovies aries (sheep)	GDKKLAVDAEVGSWEDVIPSILLQLRDMKKGKASQFFGLM-NH_2		XM_004013353.1
Pan paniscus (pygmy chimpanzee)	GDGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKMGKASQFFGLM-NH_2	KKAYQLEHTFQGLL-NH_2	XM_003818040.1
Pan troglodytes (chimpanzee)	GDGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKMGKASQFFGLM-NH_2	KKAYQLEHTFQGLL-NH_2	XM_001168519.1
Pongo abelii (sumatran orangutan)	GDGGEEQTLSTEAETWITVALEEGAGPSIQLQLQEVKTGKPSQFFGLM-NH_2	KKVYQLQHTFQGLL-NH_2	XM_002834279.1

Procavia capensis (rock hyrax)	RDSREELAIEAGLWVTVTPEEDAGPSIQLQLQEAKRGKARKFFGLM-NH_2	VGGISPIQPLRTIYQQVVQGLL-NH_2	ENSPCAP00000005735.1
Pteropus vampyrus (bat)	GDKELALSTEAGSWVTMTREEDVGPRIHLQIQVKRGKESRFFGLM-NH_2		ENSPVAP00000003545.1
Rattus norvegicus (rat)	TTTRDREDLTFGAEAESWVTVNLKGIPVPSIELKLQELKRSRTRQFYGLM-NH_2		AY471575.1
Sus scrofa (pig)	GDKELTLDAKAGSWVTVTLEEDGIPSIQLQLQEVKRGKNSQFFGLM-NH_2		BK008123.1
Saimiri boliviensis (squirrel monkey)	GDGGEEQTLSTEAETWITVALEGGAVPSVQLQLREVKRGKASQFFGLM-NH_2	KKVYQLEHTLQGLL-NH_2	XM_003931259.1
Tarsius syrichta (Tarsier)	EGSGPSIQLQLREVKRGKASQFFGLM-NH_2		ENSTSYP00000010450.1
Tupaia belangeri (northern treeshrew)	EGAVASVQLQLQGAKRSKPSQFFGLM-NH_2		ENSTBEP00000002423.1
Tursiops truncatus (dolphin)	SKASQFFGLM-NH_2		ENSTTRP00000000445.1
Vicugna pacos (alpaca)	ENAVPSIQLQPREVKRGKASQFFGLM-NH_2		ENSVPAP00000000575.1

1. The names of the 34 species the *TAC4* (or *Tac4* or *tac4*) gene was found expressed in, collected from keyword searches of the NCBI and Ensembl databases. 2. The sequence of the putative peptides the derived from the preprotachykinin C precursor; extended forms of HK-1 homologues. 3. The sequence of putative tachykinin-gene related peptide homologues. 4. The accession number of each preprotachykinin sequence.

Table 5.4. Putative tachykinin peptides from orphan genes.

[1]Species	[2]Peptide 1	[3]Peptide 2	[4]Accession number
Aedes aegypti-sialokininI	NTGDKFYGLM-NH_2		AF108102.1
Boreogadus saida (arctic cod)	SKALRFYGLM-NH_2	SGSRQPIQMNRRRNKGEMFVGLM-NH_2	BK008108.1
Ciona intestinalis	HVRHFYGLM-NH_2	SIGDQPSIFNERASFTGLM-NH_2	ENSCINP00000016566.3
Danio rerio (zebrafish)	SKSQHFHGLM-NH_2	NKGEIFVGLM-NH_2	XM_001923424
Gadus morhua (Atlantic cod)	SRAQQFHGLM-NH_2	STGNKGEMFVGLM-NH_2	ENSGMOP00000003442.1
Gasterosteus aculeatus (Three-spined stickleback)	SNGQHFHGLM-NH_2	NKGEMFVGLM-NH_2	DV007667
Lates calcarifer (Asian seabass)	SKAQQFHGLM-NH_2		DQ290174
Octapus vulgaris-OctTK-I		SEVKPPSSSEFIGLM-NH_2	AB085916.1
Octapus vulgaris-OctTK-II		SEVKPPSSSEFVGLM-NH_2	AB085917.1
Oncorhynchus mykiss (rainbow trout)	SKAHQFYGLM-NH_2	SDDQPQPIGVNRRRDKGEMFVGLM-NH_2	BK008118.1
Oreochromis niloticus (Nile tilapia)	SKAQQFHGLM-NH_2	SSGVSHAVRLGRKRNKGEMFVGLM-NH_2	GR699402
Oryzias latipes (Medaka)	SKSQQFHGLM-NH_2		DK025687
Osmerus mordax (rainbow smelt)	FKSRQFYGLM-NH_2	SDMKQPIKVYRRRNKGDMFVGLM-NH_2	BK008112.1
Rana chensinensis-ranachensinin	DDTSDRSNQFIGLM-NH_2		HE863666.1
Tetraodon nigroviridis	SKALRFYGLM-NH_2	NKGEAFVGLM-NH_2	CR650252.2

1. The names of the 15 species the above orphan genes were found expressed in, collected from keyword searches of the NCBI and Ensembl databases and from the alignment of the tachykinin motif. 2-3. The sequence of the putative peptides the derived from the preprotachykinin precursor processing at the typical proteolytic cleavage sites. 4. The accession number of each preprotachykinin sequence.

5.4.3) Alignment of prepro-tachykinin precursors using Clustal omega and phylogenetic tree analysis using Jalview.

Phylogenetic analysis was undertaken to examine the relationship of the tachykinin homologs and to investigate the relationship of the novel fish tachykinins (described in Section 5.4.1) and the orphan tachykinins (Table 5.4) with respect to the known mammalian tachykinins. A multiple sequence alignment of the 176 tachykinin sequences collected from the above searches using the Clustal omega algorithm was generated (shown in Appendix 3). Subsequently, the aligned sequences were phylogenetically analysed using the Jalview version 2.7 algorithm, calculating phylogeny using the average distance using % method. Figure 5.2 illustrates the phylogenetic tree produced, where the names of each species and the accession number of each sequence included in the analysis are shown. The distance of the branch is also illustrated.

Based on this phylogenetic tree analysis, the evolutionary history of TACs was confirmed as one common ancestral TAC gene, comprising two tachykinin peptides giving rise to four TAC genes after two rounds of genome duplications. The fourth TAC gene grouping was potentially made redundant during the evolutionary process, as a fourth distinct clade of genes was not revealed from this phylogenetic analysis either. As seen in Figure 5.2 the three tachykinin precursors divide phylogenetically into three major groups with the mammalian TAC3 preprotachykinin precursors forming a distinct clade from the TAC1 and TAC4 preprotachykinin precursor homologs. The tachykinin precursors that fall under the *TAC1* gene clade derived from two rounds of gene duplications that gave rise to two sub-clades, the first clade comprises the 46 mammalian species (listed in Table 5.1) and the second sub-clade comprises 16 fish species (also listed in Table 5.1). The three bird's species, *Gallus gallus* (chicken; BK_008126.1), *Meleagris gallopavo* (turkey; ENSMGAP00000010069.2), *Taeniopygia guttata* (zebra finch; XM_002197398.1), diverged from the initial precursor that gave rise to the clade of mammals and gave rise to a separate branch. This analysis suggests the orphan genes for the invertebrate *Octapus vulgaris* (OctTK-I and Oct-TK-II) fall under a branch derived from the TAC1 ancestor and are phylogenetically closer to the mammalian sub-clade rather

than the fish. A second branch derived from the same sub-clade comprises the vertebrate amphibian species *Xenopus laevis*, *Xenopus tropicalis* and *Hyla simplex.*

The mammalian TAC3 preprotachykinin precursor homologs comprise only one tachykinin peptide (NKB, while the other tachykinin was evolutionarily made redundant). Our phylogenetic analysis grouped the 43 mammalian TAC3 orthologs into a separate clade from the TAC1 and TAC4 homologs that encode two tachykinins. Our analysis shows a sub-clade of 11 fish genes annotated as "*tac3*" (comprising two tachykinins), derived from a common TAC ancestor to the mammalian *TAC4* prepro-tachykinin precursor homologs. Therefore, this sub-clade of fish tachykinins was found to be phylogenetically closer to the *TAC4* gene rather than the *TAC3* gene. The orphan genes found in the fish species *Boreogadus saida* (arctic cod; BK008108.1) and *Oncorhynchus mykiss* (rainbow trout; BK008118.1) were also included in the sub-clade. Therefore, they could be annotated as "*tac3*". Moreover, this analysis showed, the *Salmon salar tac3* gene (accession number BK_008103.1) has derived from the same ancestor giving rise to the three clades of tachykinins, however, it evolved separately very early during the evolutionary process, forming a separate branch (Figure 5.2).

The TAC4 prepro-tachykinin precursor homologs (comprising two tachykinins) fall under a clade derived from the same ancestor that gave rise to the clade of the TAC1 tachykinins. The 33 mammalian TAC4 tachykinin precursors diverged into a sub-clade separately from the 11 fish and invertebrate species (*e.g.* the orphan tachykinin genes *Ciona intestinalis* and *Aedes aegypti*) that fall under a separate sub-clade. An interesting observation was that the mouse and rat Tac4 genes diverged into a separate branch earlier in the evolutionary process than the rest of the vertebrate species included in this study. This gives an explanation of the divergence of mouse/rat HK-1 with respect to the rest of the mammalian hemokinins with appear as almost 100% homologous between mammalian species. On the other hand this does not appear to occur with the Tac1 or Tac3 (Tac2) precursors. The orphan genes listed in Table 5.4 (including the genes comprising the FHGLM-NH_2 motif) have fallen under the sub-clade of TAC4 precursors derived from the divergence of a separate branch during the evolutionary process.

Another interesting observation was made on the divergence of the mammalian species that comprised a monobasic cleavage site upstream from the tachykinin sequence. As shown in Table 5.3, the following species expressing HK-1 are flanked by a downstream dibasic K/R cleavage site and an upstream monobasic K cleavage site, *Dasypus novemcinctus* (armadillo; ENSDNOP00000004237.1), *Gorilla gorilla* (XM_004041438.1), *Homo sapiens* (AF515828.1), *Macaca mulatta* (macaque, ENSMMUP00000018485.2), *Pan paniscus* (pygmy chimpanzee; XM_003818040.1), *Pongo abelii* (sumatran orangutan; XM_002834279.1) and *Pan troglodytes* (chimpanzee; XM_001168519.1). These species have diverged from a common ancestor into three branches apart from the armadillo, which diverged earlier in the evolutionary process into a separate branch (Figure 5.2). The above species may be also good models for primate precursor processing and understanding disease states that are unique to this group such as pre-eclampsia compared to other mammalian species where pre-eclampsia does not occur.

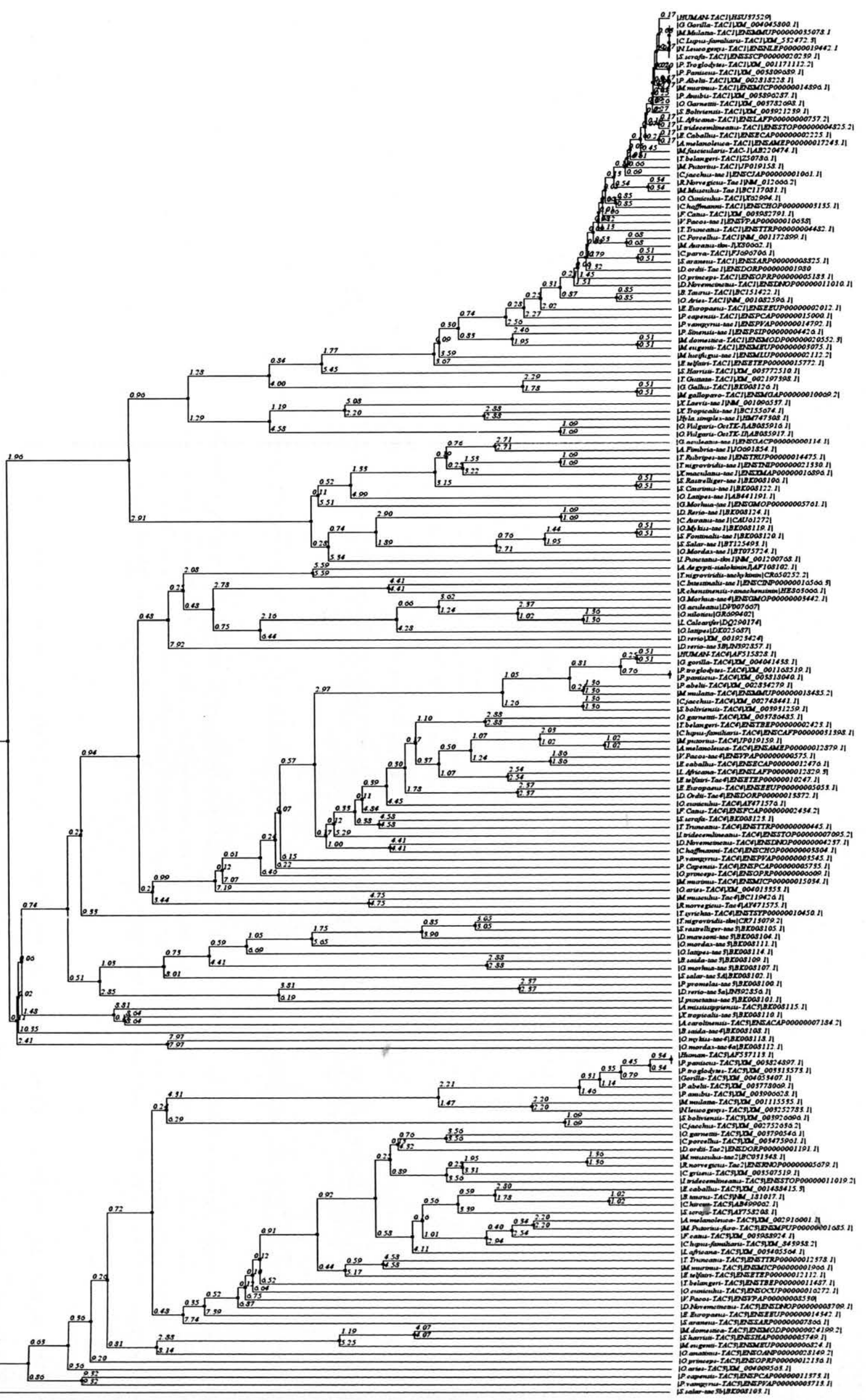

|HUMAN-TAC1|HSU97529|
|G Gorilla-TAC1|XM_004045800.1|
|M.Mulatta-TAC1|ENSMMUP00000035078.1
|C.Lupus-familiaru-TAC1|XM_552472.5|
|N.Leuco gerys-TAC1|ENSNLEP00000019442.1
S.scrofa-TAC1	ENSSSCP00000020259.1
P.Troglodytus-TAC1	XM_001171112.2
P.Panisecus-TAC1	XM_003809689.1
P.Abelis-TAC1	XM_002818228.1
M.murinus-TAC1	ENSMICP00000014896.1
P.Anubis-TAC1	XM_003896287.1
O.Garnettii-TAC1	XM_003782603.1
S.Boliviensis-TAC1	XM_003921259.1
L.Africana-TAC1	ENSLAFP00000000757.2
L.tridecemlineatus-TAC1	ENSSTOP00000004825.2
E.Caballus-TAC1	ENSECAP00000002225.1
A.melanoleuca-TAC1	ENSAMEP00000017243.1
M.fascicularis-TAC-1	AB220474.1
T.belangeri-TAC1	JZ50780.1
M.Putorius-TAC1	JP019158.1
C.jacchus-tae 1	ENSCJAP00000001061.1
R.Norvegicus-Tae 1	NM_012666.2
M.Musculus-Tae 1	BC117081.1
O.Cuniculus-TAC1	X82994.1
C.hoffmanni-TAC1	ENSCHOP00000003155.1
F.Catus-TAC1	XM_003982791.1
V.Pacos-tae 1	ENSVPAP00000010655
T.Truncatus-TAC1	ENSTTRP00000004482.1
C.Porcellus-TAC1	NM_001172899.1
M.Auratus-tbn-1	X80062.1
C.parva-TAC1	FJ690706.1
S.araneus-TAC1	ENSSARP00000005825.1
D.ordii-Tae 1	ENSDORP00000001980
O.princeps-TAC1	ENSOPRP00000005185.1
D.Novemcinctus-TAC1	ENSDNOP00000011010.1
B.Taurus-TAC1	BC151422.1
O.Aries-TAC1	NM_001082596.1
E.Europaeus-TAC1	ENSEEUP00000002012.1
P.capensis-TAC1	ENSPCAP00000015000.1
P.vampyrus-tae 1	ENSPVAP00000014792.1
P.Sinensis-tae 1	ENSPSIP00000004426.1
M.domestica-TAC1	ENSMODP00000020552.3
M.eugenii-TAC1	ENSMEUP00000003075.1
M.hucifugus-tae 1	ENSMLUP00000002112.2
E.telfairi-TAC1	ENSETEP00000015772.1
S.Harrisii-TAC1	XM_003772510.1
T.Guttata-TAC1	XM_002197598.1
G.Gallus-TAC1	BX008126.1
M.gallopavo-TAC1	ENSMGAP00000010069.2
X.Laevis-tae 1	NM_001090537.1
X.Tropicalis-tae 1	BC155074.1
Hyla.simplex-tae 1	BM747508.1
O.Vulgaris-OctTC-1	AB085916.1
O.Vulgaris-OctTC-IN	AB085917.1
G.aculeatus-tae 1	ENSGACP00000000114.1
A.Fimbria-tae 1	JO691854.1
T.Rubripes-tae 1	ENSTRUP00000014475.1
T.nigroviridis-tae 1	ENSTNIP00000021930.1
X.maculatus-tae 1	ENSXMAP00000016896.1
S.Rastrelliger-tae 1	BX008106.1
S.Caurinus-tae 1	BX008122.1
O.Latipes-tae 1	AB441191.1
G.Morhua-tae 1	ENSGMOP00000005761.1
D.Rerio-tae 1	BX008124.1
C.Auratus-tae 1	CAU61272
O.Mykiss-tae 1	BX008119.1
S.Fontinalis-tae 1	BX008120.1
S.Salar-tae 1	BT125495.1
O.Mordax-tae 1	BT075724.1
T.Punctatus-tbn 1	NM_001200768.1
A.Aegypti-sialokinin	AF108102.1
T.nigroviridis-tachykinin	CR650252.2
C.Intestinalis-tae 1	ENSCINP00000010566.5
R.chensinensis-ranachensinin	HE805000.1
G.Morhua-tae4	ENSGMOP00000005442.1
G.aculeatus	DV807667
O.niloticus	GR699402
L.Calcarifer	DQ290174
O.latipes	DK025087
D.rerio	XM_001923424
D.rerio-tae 5B	AL592857.1
HUMAN-TAC4	AF515528.1
G.gorilla-TAC4	XM_004041455.1
P.troglodytus-TAC4	XM_001165519.1
P.paniscus-TAC4	XM_003813040.1
P.abelii-TAC4	XM_002834279.1
M.mulatta-TAC4	ENSMMUP00000018485.2
C.jacchus-TAC4	XM_002748441.1
S.boliviensis-TAC4	XM_003931259.1
O.garnettii-TAC4	XM_003780485.1
T.belangeri-TAC4	ENSTBEP00000002425.1
C.lupus-familiaru-TAC4	ENSCAFP00000031598.1
M.putorius-TAC4	JP019150.1
A.melanoleuca-TAC4	ENSAMEP00000012879.1
V.Pacos-tae4	ENSVPAP00000000575.1
E.caballus-TAC4	ENSECAP00000012470.1
L.Africana-TAC4	ENSLAFP00000012829.1
E.telfairi-Tae4	ENSETEP00000010247.1
E.Europaeus-TAC4	ENSEEUP00000005053.1
D.Ordii-Tae4	ENSDORP00000015872.1
O.cuniculus-TAC4	AF471576.1
F.Catus-TAC4	ENSFCAP00000002454.2
S.scrafa-TAC4	BX008125.1
T.Truncatus-TAC4	ENSTTRP00000000445.1
L.tridecemlineatus-TAC4	ENSSTOP00000007095.2
D.Novemcinctus-TAC4	ENSDNOP00000004257.1
C.hoffmanni-TAC4	ENSCHOP00000003504.1
P.vampyrus-TAC4	ENSPVAP00000003545.1
P.Capensis-TAC4	ENSPCAP00000005755.1
O.princeps-TAC4	ENSOPRP00000000609.1
M.murinus-TAC4	ENSMICP00000015054.1
O.aries-TAC4	XM_004019555.1
M.musculus-Tae4	BC119426.1
R.norvegicus-Tae4	AY471575.1
T.gyrichus-TAC4	ENSTSYP00000010450.1
T.nigroviridis-tbn	CR713079.2
S.rastrelliger-tae 5	BX008105.1
D.mawsoni-tae 5	BX008104.1
O.mordax-tae 5	BX008111.1
O.latipes-tae 5	BX008114.1
B.saida-tae 5	BX008109.1
G.morhua-tae 5	BX008107.1
S.salar-tae 5A	BX008102.1
P.promelas-tae 5	BX008100.1
D.rerio-tae 5a	AL592856.1
T.punctatus-tae 5	BX008101.1
A.mississippiensis-TAC5	BX008115.1
X.tropicalis-tae 5	BX008110.1
A.carolinensis-TAC5	ENSACAP00000007184.2
B.saida-tae 4	BX008108.1
O.mykiss-tae 4	BX008118.1
O.mordax-tae 4a	BX008112.1
Human-TAC5	AF537113.1
P.panisicus-TAC5	XM_003824897.1
P.troglodytus-TAC5	XM_003813575.1
Gorilla-TAC5	XM_004035407.1
P.abelii-TAC5	XM_003778069.1
P.anubis-TAC5	XM_003900628.1
M.mulata-TAC5	XM_001115595.1
N.leuco gerys-TAC5	XM_003752785.1
S.boliviensis-TAC5	XM_003926090.1
C.jacchus-TAC5	XM_002752656.2
O.garnettii-TAC5	XM_003790546.1
C.porcellus-TAC5	XM_005475961.1
D.ordii-Tae2	ENSDORP00000001191.1
M.musculus-tae2	BC091948.1
R.norvegicus-Tae2	ENSRNOP00000005079.1
C.griseus-TAC5	XM_003507519.1
L.tridecemlineatus-TAC5	ENSSTOP00000011019.2
E.caballus-TAC5	XM_001488415.5
B.taurus-TAC5	NM_181017.1
C.hircus-TAC5	AB499002.1
S.scrofa-TAC5	AY758708.1
A.melanoleuca-TAC5	XM_002916001.2
M.Putorius-furo-TAC5	ENSMPUP00000001685.1
F.catus-TAC5	XM_003983924.1
C.lupus-familiaru-TAC5	XM_845958.2
L.africana-TAC5	XM_003405504.1
T.Truncatus-TAC5	ENSTTRP00000012578.1
M.murinus-TAC5	ENSMICP00000001966.1
E.telfairi-TAC5	ENSETEP00000012112.1
T.belangeri-TAC5	ENSTBEP00000011437.1
O.cuniculus-TAC5	ENSOCUP00000010272.1
V.Pacos-TAC5	ENSVPAP00000003590
D.Novemcinctus-TAC5	ENSDNOP00000008709.1
E.Europaeus-TAC5	ENSEEUP00000014542.1
S.araneus-TAC5	ENSSARP00000007866.1
M.domestica-TAC5	ENSMODP00000024199.2
S.harrisii-TAC5	ENSSHAP00000005749.1
M.eugenii-TAC5	ENSMEUP00000006824.1
O.anatinus-TAC5	ENSOANP00000028149.2
O.princeps-TAC5	ENSOPRP00000012156.1
O.aries-TAC5	XM_004009565.1
P.capensis-TAC5	ENSPCAP00000011975.1
P.vampyrus-TAC5	ENSPVAP00000005718.1
S.salar-tae 5b	BX008103.1

Figure 5.2. Phylogenetic tree of prepro-tachykinin amino acid sequences using the average distance method. The 176 precursor sequences mined from the interrogation of the NCBI and the Ensembl databases (shown in Tables 5.1 to 5.4) were aligned using the Clustal omega algorithm and phylogenetic analysis was performed with the Jalview version 2.7 algorithm using the average distance method using % identity. The number next to each branch represents the distance between them.

5.5) Discussion

Tachykinins are a vast family of signalling peptides expressed in numerous species and involved in a range of tachykinin receptor-mediated functions, such as neuronal, neuro-endocrinological and gut motility. In 2002, Severini and colleagues reviewed the number of identified tachykinins expressed in a range of species as approximately forty tachykinins. After the discovery of the hemokinins, the tachykinin family was expanded to include fifty members (Page *et al.*, 2009), a number that has grown even bigger since. The divergence among the species found expressed, has named the tachykinins as a large and ancient family expressed from invertebrates (such as ascidian, *Ciona intestinalis*) to mammals, showing substitutions of a conservative character in the sequence of homologous peptides (Almeida *et al.*, 2004). However, an *in silico* investigation and summary of tachykinins in as complete a range of species found, has not been reported so far. In contrast to tachykinin receptors (Almeida *et al.*, 2004), phylogenetic analysis of the evolution of tachykinins expressed from the three known genes or orphans, has not been reported either. Hitherto, identification of tachykinin peptides using *in silico* analyses of publicly accessible databases has been conducted with name searches; a strategy that has expanded the number of species expressing tachykinins and the divergence of the tachykinin sequences. However, this has the limitation of retrieving only annotated tachykinin sequences (Page *et al.*, 2009). The present study investigated the *in silico* identification of novel tachykinin peptides by means of the alignment of the tachykinin motif on the NCBI database, as part of a thorough tachykinin-identification strategy, and the selection of tachykinins from other entries using typical tachykinin precursor criteria. Moreover, in this study the publicly accessible databases were interrogated using keyword searches retrieving annotated tachykinin sequences derived either from gene cloning or as computer-predicted tachykinin sequences. Multiple sequence alignment and protein phylogenetic analysis elucidated the clades the tachykinins fall into.

The motif FHGLMG (the G serving as an amide donor, during post-translational modifications) was identified in fish species, using the above motif as a query sequence to interrogate the nucleotide NCBI database and identifying unannotated tachykinin

sequences. The motif was found expressed in the five fish species listed in Figure 5.1. During the conduct of this research Mi and colleagues (2010) identified the particular motif in a tachykinin decapeptide expressed in *Danio rerio* (zebrafish), isolated from the skin secretions of the fish that was able to cause contractile activity on guinea-pig ileum, a tissue known to express all three mammalian tachykinin receptors and typically used in the assay on the bio-activity of newly-isolated tachykinins (Maggi *et al.*, 1990). Tachykinins are reported as peptides with pleiotropic functions such as neuroendocrine peptides, or gut mobility peptides, or peptides with a peripheral signalling and vasodilatory function also involved in pain transmission. The biological role of this new tachykinin peptide in the fish skin secretion was hypothesised to be part of a defensive mechanism causing algesic effects and protecting the host from injury or possessing an antimicrobial effect (Mi *et al.*, 2010).

During the preparation of this Chapter, Ogawa and colleagues (2012) identified through the interrogation of the Ensembl database and aligning the sequences of the mammalian *TAC1*, *Tac2* and *TAC3* genes, the FHGLMG motif expressed in four fish species, the teleosts, *Oreochromis niloticus* (tilapia), *Gadus morhua* (Atlantic cod), *Gasterosteus aculeatus* (three-spined stickleback), *Danio rerio* (zebrafish). In their study multiple sequence alignment of the above sequences with mammalian, bird and reptile *TAC1* sequences and phylogenetic analysis showed the newly identified sequences to group into the same clade as the *TAC1* gene and not the *TAC3/Tac2* genes. However, in the study by Ogawa and colleagues (2012), *TAC4* gene sequences were not included in the multiple sequence alignment or the phylogenetic analysis conducted. Our study has shown the expression of the FHGLMG motif also in the fish species *Lates calcarifer* (Asian seabass); moreover, multiple sequence alignment and phylogenetic analysis including sequences of a wide range of species from the three tachykinin genes, grouped the above sequences possessing the FHGLMG motif into a sub-clade (comprising multiple fish species) of the *TAC4* gene clade rather than the *TAC1* gene clade. The biological function of the peptides reported in Figure 5.1 cannot be predicted. Our suggestion is that the elucidation of the anatomical distribution of the above peptides in the fish species expressed is key for the understanding of their specific functions.

Our research also revealed the orthologs of mammalian NKB expressed in the above fish species. We conducted a multiple sequence alignment which suggested that the fish *tac3* gene expresses two tachykinins, the NKB orthologs and a second NKB-like peptide that was made redundant in vertebrate species (Table 5.2). This finding is consistent with previous reports that speculate the redundancy of one tachykinin peptide on the *TAC3* gene (Page *et al.*, 2009). Zhou and colleagues (2012) investigated the evolution of NKB and the NK3 receptor expressed in fish to elucidate the biological role of the piscine NKB/NK3 system. The concomitant expression of the *tac3* and *tac3r* genes (proven by RT-PCR) in neuronal and reproductive related tissues (*i.e.* the ovaries) suggested a neuro-endocrinological and reproductive role of the system in line with the biological role of the homologs in humans. Moreover, they mined the genome database of zebrafish and the EST database from NCBI using the mammalian tachykinin peptides as query sequences; multiple sequence analysis of the *TAC* and *TACR* sequences was conducted and a phylogenetic tree was constructed. Fewer sequences were included in the study of Zhou and colleagues (2012), compared to our study, where they suggest that the fish *tac3* tachykinin genes group as a sub-clade of the mammalian *TAC3* genes. This suggestion was also proposed by Biran and colleagues (2012) performing a similar investigation of mining the protein NCBI databases using the Tac2 peptide sequence as a query; also the GDMHDFFVGLMGKR sequence was used as input to translated blast of fish DNA and EST sequences. The number of sequences included in the phylogenetic analysis is not reported by the authors; however, phylogenetic analysis showed the *tac3* fish genes grouped as a sub-clade of the mammalian *TAC3* genes. The role of fish NKB/NK3 system in the control of zebrafish reproduction was also suggested by Biran and colleagues (2012); revealing by means of *in situ* hybridisation and RT-PCR expression of the NKB/NK3 system in the hypothalamus nuclei and other brain areas expressing the fish kisspeptins, known for their role in the regulation of reproduction and sexual maturity (Biran *et al.*, 2012). The FHGLMG motif was not discovered in mammalian species; potentially this "fish tachykinin" motif evolved into the FFGLMG motif found in mammalian HK-1 or SP (also suggested from the multiple sequence alignment).

According to our research, a common tachykinin ancestor apparently expressed two tachykinin peptides, since the ascidian *Ciona intestinalis* encodes two tachykinins. The

common ancestor gave rise to the clade of the mammalian *TAC3* genes where one tachykinin peptide was made redundant and the two clades of the mammalian *TAC1* and *TAC4* genes, expressing two tachykinins or one tachykinin and the tachykinin-gene related peptides. The non-mammalian species such as fish grouped as subclades derived from a common ancestor giving rise to the mammalian *TAC1* and *TAC4* gene clades, rather than the annotated *tac3* genes being a sub-clade of the mammalian *TAC3* genes that maintained the expression of two tachykinins. Hence, we suggest that the loss of the second tachykinin peptide on the mammalian *TAC3* gene occurred earlier in the course of the evolutionary process. However, the consensus of the above studies and also ours is that through two rounds of gene duplications four *TAC* genes were created, where the one was made redundant through the accumulation of mutations.

The present research has expanded the list of species tachykinin peptides are found expressed in and has shown higher degree of divergence of tachykinins between species. A large number of putative tachykinin-gene related peptides were identified possessing the characteristic tachykinin-gene related motif G/ALLG. The biological role of these peptides cannot be predicted; further investigation of their anatomical distribution would indicate their biological function. Furthermore, the isolation (using purification methods such as size exclusion chromatography and HPLC) and confirmation of molecular weight by means of mass spectrometry and sequencing would confirm the expression of these peptides. To our knowledge the newly identified tachykinin-gene related peptides are orphan and functionality assays on the known mammalian NK1, NK2 and NK3 receptors would have shown a very weak (if any) response. The amidated methionine part of the C-terminus of the tachykinin is key for the activation of the three mammalian tachykinin receptors (Satake & Kawada, 2006). The human EKC/D tachykinin-gene related peptides show a very weak response on the NK3 mammalian tachykinin receptor (Page *et al.*, 2003).

The HK-1 peptides expressed by the mammalian *TAC4* gene are flanked by two dibasic K/R cleavage sites. However, the dibasic cleavage site on the N-terminus of HK-1 is lost in human and also the primates *Gorilla gorilla*, *Macaca mulatta* (macaque), *Pan paniscus* (pygmy chimpanzee), *Pongo abelii* (sumatran orangutan) and *Pan troglodytes*

(chimpanzee) as well as the mammal *Dasypus novemcinctus* (armadillo). The above species are also recommended animal models for placental development, pre-eclampsia and IUGR compared to other mammalian species where pre-eclampsia does not occur (Carter, 2007). For example, the mouse is also characterised by poorly developed newly-born offspring but pre-eclampsia does not develop (Carter, 2007). We suggest a linkage between the loss of the N-terminus HK-1 dibasic cleavage site and certain of the symptoms of pre-eclampsia. The placenta is known as an organ that poorly processes the peptides it produces (Ahmed *et al.*, 2000). Page and colleagues (2006) has suggested that while in normal placentae the TAC4 preprotachykinin precursor is fully processed at its C-terminus at its N-terminus it remains unprocessed. Hence, an extended form of HK-1, namely the EKB is found released from the proteolytic cleavage of the precursor by signal peptidases (at the signal peptide) and convertases at the dibasic cleavage site of the C-terminus. The same author has suggested by means of size-exclusion chromatography and immunoassays that in the pathological condition of pre-eclampsia a shortened tachykinin form, potentially HK-1 released from the fully processed prepro-tachykinin precursor at both the dibasic C-terminus and monobasic N-terminus, is found. A potential suggestion is that processing at not commonly cleaved monobasic K cleavage site occurs as an "emergency" procedure in the pathologic condition of pre-eclampsia or possibly in IUGR cases as well. The processed HK-1 undecapeptide acts on peripheral tachykinin receptors (NK1, NK2, NK3) constricting the portal vein shunting the blood flow towards the placenta; potentially accounting for some of the pre-eclamptic symptoms such as the hypertension.

Chapter 6. Discussion.

Tachykinins are a vast family of signalling peptides, the *classical* members of the tachykinin family in mammals being SP, NKA and NKB which show complete homology among the mammalian species (Severini *et al.*, 2002; Nelson & Bost, 2004). The members of the tachykinin family comprise the common highly conserved signature C-terminal motif –FXGLM-NH_2, where X was thought to be a bulky, hydrophobic residue valine (V), isoleucine (I) or aromatic phenylalanine (F), tyrosine (Y) (Severini *et al.*, 2002; Nelson & Bost, 2004; Page, 2005). Tachykinins as signalling peptides are initially expressed as a part of a preprotachykinin precursor that undergoes cleavage by prohormone convertases at dibasic or monobasic K/R cleavage sites, releasing the elongated or normal (or potentially truncated; Kurtz *et al.*, 2002) forms of these peptides, while the action of carboxypeptidases and peptidylglycine α-amidating monooxygenase produces a biologically active tachykinin peptide (Nelson & Bost, 2004). The field of hemokinins/endokinins was introduced with the cloning of the mouse *Tac4* gene, isolated from B-lymphocytes (Zhang *et al.*, 2000), later found to have peripheral expression and potentially endocrinological role (Kurtz *et al.*, 2002; Patak *et al.*, 2003; Patak *et al.*, 2005). The group of hemokinins/endokinins do not show complete homology between mammalian species (Page *et al.*, 2003; Page, 2004; Page, 2006). The sequence of the mHK-1 peptide was inferred from the cDNA sequence of the mouse/rat *Tac4* gene (Zhang *et al.*, 2000; Kurtz *et al.*, 2002). The translated peptide has not been purified from mouse/rat tissues to elucidate its actual sequence or its' potential post-translational modifications. Moreover, evidence for its expression in the brain has been inconclusive (Zhang *et al.*, 2000; Kurtz *et al.*, 2002, Jin *et al.*, 2009). A purpose of this research was to capture mHK-1 from spleen, a tissue of its expression (Kurtz *et al.*, 2002; Page 2005) and brain in order to investigate the molecular weight and amino acid sequence of the fully processed form along with any potential post-translational modifications. Specific antibodies developed against tachykinins were shown to cross-react with mHK-1, SP, NKB, hHK-1 using antibody titre curves; these antibodies were purified from antiserum and immobilised on Sepharose for the immunoaffinity purification of the tachykinins. Peptide extraction methods were tested for efficiency and applied for the extraction of

peptides from tissues of interest. A reversed phase HPLC method was developed to separate the captured peptides that were detected using MALDI-TOF. mHK-1 was detected in the rat brain and spleen. The potential acetylation detected on mHK-1 in brain infers a role as a neurotransmitter (Barnea & Cho, 1983; Wilkinson, 2006; Zhang *et al.*, 2012). A parallel *in silico* analysis of the NCBI and Ensembl databases was used to identify novel tachykinin peptides. The phylogenetic relationship between the identified species was also investigated. An interesting observation was made in humans and primates (where pre-eclampsia occurs), the evolutionary pressure to maintain the N-terminal dibasic cleavage site of EKA/B has been lost. We have suggested of a linkage between pre-eclampsia and the loss of dibasic cleavage site in those species.

6.1) Summary of the experimental work.

The tachykinins are short linear peptides, that form an α-helix in their secondary structure (Almeida *et al.*, 2004; Nelson & Bost, 2004; Mantha *et al.*, 2004), which possess an immuno-reactive backbone on the FXGLM-NH_2 motif. In agreement with this observation, we successfully showed the cross-reactivity of the polyclonal antibodies raised against the tachykinin EKA/B. In Chapter 2, antibody titre curves have shown, that antibodies developed in sheep against the synthetic EKA/B peptide cross-reacted with the tachykinins mHK-1, SP, NKB and hHK-1. This observation led us to hypothesise about the existence of a tachykinin backbone antibodies recognise, rather than just the five-residue common C-terminal motif. Moreover, we have shown that due to strong extraction procedures SP is oxidised (Chapter 4 results). However, the oxidised SP (oxidised methionine) was still recognised by the immobilised antibodies on the Sepharose-antibody conjugate (as shown in experiment Section 4.3). Therefore we suggest that the oxidation of methionine did not affect the recognition of the endogenous peptide by the SP-specific antibodies.

In Chapter 3, the two peptide extraction buffers, acidified methanol buffer (Sturm *et al.*, 2010, Dowell *et al.*, 2006) and Bennett's solution (Bennett *et al.*, 1981; Nassel *et al.*, 2000) were compared; the rat brain content of peptides was extracted and separated using

RP-HPLC, while fractions were checked for specific anti-tachykinin immunoreactivity with an ELISA assay with immunopurified anti-tachykinin antibodies. Immunoaffinity purification was used as a means of purification and enrichment of tachykinin peptides. A RP-HPLC method was used to fractionate and separate the repertoire of peptides present tissue extracts (either crude or after immunoaffinity purification). MALDI-TOF was used for the identification of the molecular mass of peptides, synthetic to investigate their elution fraction, or endogenous for further separation before detection. The combination of off-line sample separation on RP-HPLC and detection on MALDI-TOF offered the advantages of double confirmation of peptide identity, firstly from the retention time (or elution fraction number) and secondly with confirmation of the molecular mass with MALDI-TOF (Aristoteli *et al.*, 2006). Moreover, for the MALDI-TOF investigation the usage of small quantities of the sample (immunoaffinity eluent) and fractions, offered the non-destructive sample interrogation and the option for repeat analysis (Hatcher *et al.*, 2008). Furthermore, the intrinsic ability of MALDI-TOF to generate predominantly singly-charged ions simplified data analysis (Pan *et al.*, 2009). Hence, prior to ionisation, the sample analyte is concentrated, purified, and fractionated, for the isolation of peptides of interest, the removal of extraneous material, as well as an increase of the likelihood that the peptides reach a detectable threshold for analysis (Mitchell *et al.*, 2011). We investigated the detection of ion peaks of the immunoaffinity enriched eluent (from rat brain) before separation into fractions with RP-HPLC. However, ion suppression led to only SP being detected in the ion spectrum due to its very high abundance in the rat brain (experiment described in Chapter 4). Here, we report mHK-1 as a low abundance peptide in the brain requiring extensive enrichment by means of immunoaffinity purification and further separation by RP-HPLC for efficient detection (see Chapter 4).

6.2) Contributions to knowledge.

A more definite characterisation of the sequence of mHK-1 was required for the identification of its MW as its sequence has only been deduced from the cDNA of the *Tac4* gene and no potential post translational modifications have been suggested, apart from the well established C-terminal amidation. This study clarifies the molecular weight of the mature mHK-1 peptide, and also suggests the presence of an acetylated form of the

peptide in the brain. The mature endogenous mHK-1 was captured from rat spleen and brain and was proven to be an amidated decapeptide with the sequence SRTRQFYGLM-NH_2 identified by means of immunoaffinity purification, RP-HPLC and MALDI-TOF. The spectra obtained from brain tissue also showed the presence of a post-translational modification *i.e.* N-terminal acetylation of Ser^1, a classic neuropeptide post-translational modification (Wilkinson, 2006; Dowell *et al.*, 2006; Lee *et al.*, 2010). Acetylation inhibits ubiqitylation and therefore prolongs the half life of the peptide, or protects the peptide from degrading enzymes *e.g.* neprilysin (Van Dijk *et al.*, 2011).

The peptide mHK-1 was discovered as a hematopoietic-specific tachykinin that regulates B lymphopoiesis (Zhang et *al.*, 2000). In recent reports mHK-1 is a novel humoral-biased molecular adjuvant for DNA vaccines against hepatitis B (Chen *et al.*, 2012). The study suggested mHK-1 induced a higher level of IgG production, a higher percentage of differentiated antibody-secreting plasma cells, and a higher level of T-cell proliferation, hence the adjuvant promoted immunological memory and result in stronger humoral and memory responses (Chen *et al.*, 2012). Apart from the initial autocrine/paracrine function, the role of mHK-1 in pain and nociception was investigated in mice intrathecally administering mHK-1 and comparing the effects with that of SP on NK1 (Endo *et al.*, 2006; Fu *et al.*, 2006, 2007a; Watanabe *et al.*, 2010). Recently, a study investigated the biological effect of mHK-1 in analgesia and its distribution in the brain upon administration and its' interaction with the NK1 receptor (Xia *et al.*, 2013). Studies of ligand-receptor interactions and the subsequent receptor mediated biological effect prerequisites the use of the correct amino acid sequence of the ligand. Therefore we stress the need to determine the precise sequence of mHK-1 and post-translational modifications for the investigation of its neuronal role.

6.3) Future applications of methodology for the elucidation of the role of tachykinins in pre-eclampsia and IUGR.

Pre-eclampsia is a pathologic condition that affects a high percentage (3-10%) of pregnancies and a major cause of maternal morbidity and mortality. The initial stage is asymptomatic and characterised by the abnormal development of the placenta resulting in

placental ischemia and hypoxia during the first trimester of the pregnancy. The poorly perfused placenta releases excessive amounts of placental material into the maternal circulation. Placental peptides can be used as markers of disease onset and the identification of these markers is of great value for the early prevention of pre-eclampsia (Page, 2010). Page and colleagues (2000) identified an up-regulation of the expression of the *TAC3* gene in pre-eclamptic placentas compared to normal and an excessive placental secretion of the NKB peptide into maternal circulation. This peptide binds on the NK3 (and possibly also NK1 and NK2) receptors expressed on the endothelium of the mesenteric and portal veins and possibly acts in order to increase the blood flow towards to the anoxic fetoplacental unit. By causing enhanced venoconstriction, the blood flow is shunted towards the poorly vascularised placenta to the detriment of the liver and the kidneys. In the normotensive placenta NKB-like immunoreactivity of a larger, in terms of MW, peptide was measured potentially corresponding to an N-terminally extended form of NKB, inferring partial processing in spite of the dibasic KR cleavage site (Page *et al.*, 2009). The fully processed variant of NKB *i.e.* the amidated decapeptide was detected in the pre-eclamptic placenta (Page *et al.*, 2001). In parallel, observations reported that the TAC4 precursor is expressed in the placenta as well (Page *et al.*, 2003). Similarly to the NKB processing, significant EKB-like immunoreactivity corresponding to the fully N-terminally processed amidated hHK-1 undecapeptide was found in the pre-eclamptic placenta. In contrast to the normotensive placenta (indicating processing at monobasic site) where EKB-like immunoreactivity was measured as a larger peptide potentially an N-terminally extended, or a partially processed form of EKB (Page, 2006). In the above studies, the processing variants of the TAC3 and TAC4 precursors were determined by means of separation by size-exclusion chromatography and immunoassays, while no reports exist on the definitive identification of the MW or sequencing of the peptides by mass spectrometry. The methodology described in this Thesis is capable of capturing the peptides hHK-1 and EKB from normal and pre-eclamptic placentas or from cases of IUGR, in order to investigate the presence of fully processed forms, or the partial processed forms of these peptides and potential post-translational modifications.

6.4.) General conclusions.

This thesis summarised an experimental strategy for the identification of the molecular weight of mHK-1; presented the observation of a putative post-translational modification and the confirmation of its expression in the brain. The experimental strategy combined four methodologies, peptide extraction, and enrichment by immunoaffinity purification, RP-HPLC separation and MALDI-TOF detection. It has successfully allowed us the identification of the correct molecular weight of mHK-1 and hence the correct sequence of the peptide. The detection of a different molecular weight of mHK-1 in brain tissue compared to the peripheral spleen tissue led to the proposal of an alternative modification of the peptide at a post-translational level. The identification of the acetylation of mHK-1 gives more light on the actual role of the peptide in the brain physiology where it potentially acts as a neuropeptide comprising a signature neuropeptide post-translational modification compared to the spleen tissue where it acts as an endocrine agent.

In parallel, in this study we have conducted *in silico* analyses of the publicly accessible nucleotide and protein databases searching for unannotated transcripts that encode putative tachykinin precursors. Our data include five novel tachykinin precursors which allowed us to successfully identify a novel tachykinin motif the FHGLM-NH_2 expressed in fish. The novel precursors comprised a polar amino acid in the signature motif traditionally considered to possess a hydrophobic or aliphatic amino acid. A comprehensive study of binding assays on the three NK1, NK2 and NK3 receptors would provide information of specificity and preference of the novel peptides for the known receptors or of the need for the identification of a novel tachykinin receptor. The sequencing of the genomes of more species result in the EST, nucleotide and protein searchable databases being updated at a rapid pace giving a rich source for peptide discovery. The novel sequences discovered set the stage for future mass spectral, molecular, anatomical and physiological studies of the identified peptides.

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Appendix 1. Reagents, equipment and their suppliers.

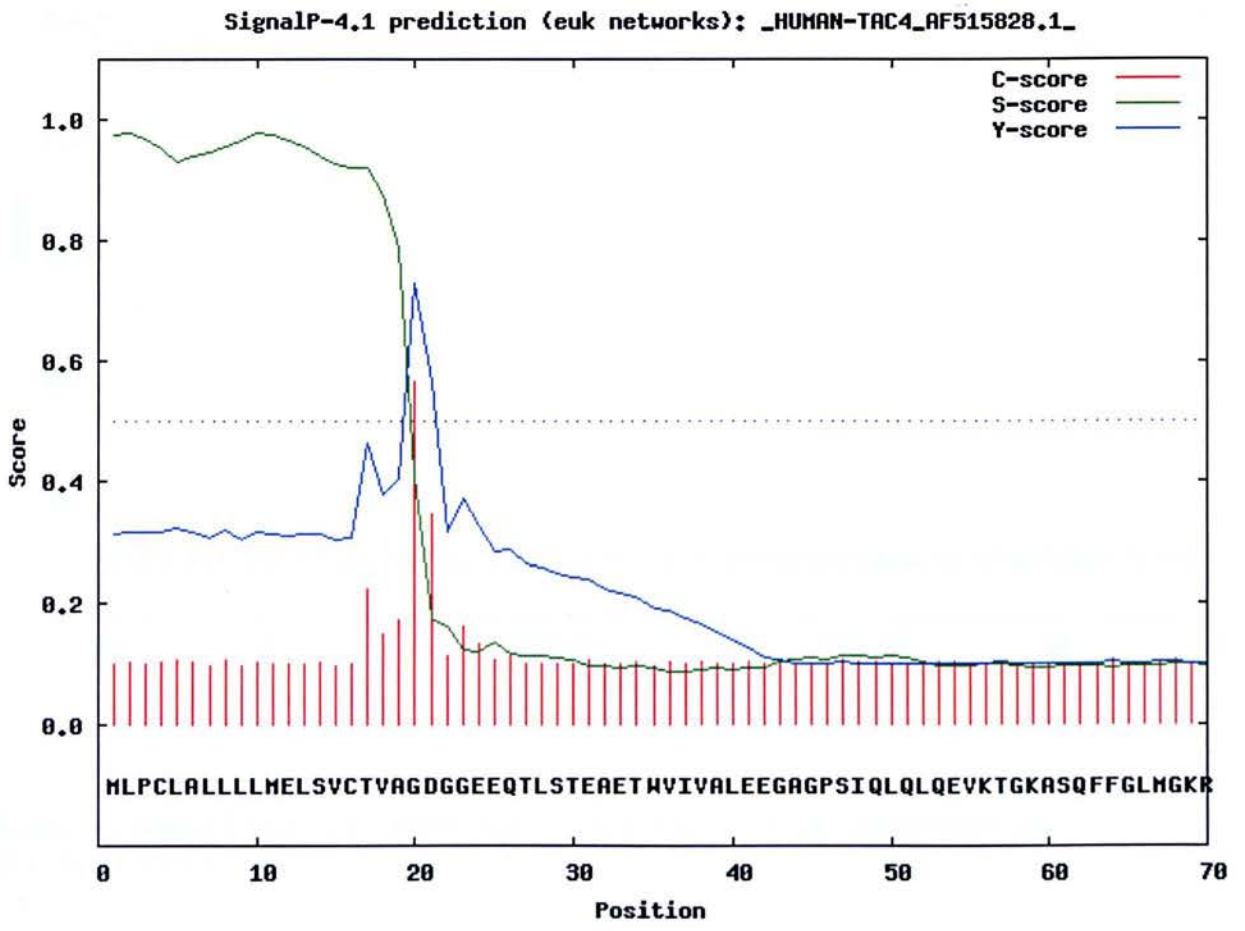
Name of product or reagent	Supplier	Location
Acetic acid, 33209	Sigma-Aldrich	Gillingham, U.K.
Acetonitrile, 34851	Sigma-Aldrich	Gillingham, U.K.
Alpha-Cyano-4-Hydroxycinnamic Acid matrix, G2037A	Agilent Technologies	Berkshire, U.K.
Bovine Serum Albumin, A3294	Sigma-Aldrich	Gillingham, U.K.
Bradford reagent, B6916	Sigma-Aldrich	Gillingham, U.K.
C18 SepPak cartridges, WAT051910	WATERS	Hertfordshire, U.K.
C12 column, Jupiter 4u, Proteo, New column, 00G-4396-BO	Phenomenex	Macclesfield, U.K.
C18 column, Vydac, ULTRASPHERE, 218TP54, 5 mm, 4.6 mm x 250 mm	Beckman Coulter	High Wycombe, U.K.
Centrifuge, Cool Working System, 4236	CAMLAB	Cambridge, U.K.
Centrifuge, spectrafuge 7M	Labnet	NJ, 07095, USA
Centrifuge, Micro centaur, Sanyo	MSE	London, U.K.
Chloroform, 154733	Sigma-Aldrich	Gillingham, U.K.
Citric acid, 251275	Sigma-Aldrich	Gillingham, U.K.
Class II Microbiological Safety Cabinet	LABCAIRE	Stafford, U.K.
Cyanogen bromide-activated Sepharose 4B, C9142	Sigma-Aldrich	Gillingham, U.K.
DMSO, D/4120/PB08	Fischer Scientific	Loughborough, U.K.
Donkey anti-sheep IgG-alkaline phosphatase conjugate, A5187	Sigma-Aldrich	Gillingham, U.K.
Duran bottles (500 ml), BOT5208	Scientific Laboratory Supplies	Nottingham, U.K.
Duran bottles 1L, BOT5210	Scientific Laboratory Supplies	Nottingham, U.K.
Ethanol, E/0650DF/17	Fisher Scientific	Loughborough, U.K.
Flow adaptor/ female leurs, 7318222	BioRad	Hertfordshire, U.K.
Fraction collector, Model 1200	Agilent Technologies	Berkshire, U.K.
Econo-column, 7371012	BioRad	Hertfordshire, U.K.
Ethanolamine, 28306	VWR, BDH	Leicestershire, U.K.
EDTA disodium, E5134	Sigma-Aldrich	Gillingham, U.K.
1.5 ml Eppendorf tubes, CEN7374	SLS	Nottingham, U.K.
50 ml Falcon tubes, 227270	Greiner Bio-One	Gloucestershire, U.K.
15 ml Falcon tubes, 188261	Greiner Bio-One	Gloucestershire, U.K.
Formic acid, 101155F	VWR, BDH	Leicestershire, U.K.
Glycine, G7126	Sigma-Aldrich	Gillingham, U.K.
Mouse hemokinin-1 (custom made sequence SRTRQFYGLM-NH_2)	Designer Biosciences	Cambridge, U.K.
Human hemokinin-1, DBS1120	Designer Biosciences	Cambridge, U.K.
HPLC instrument, Varian ProStar,	Agilent Technologies	Berkshire, U.K.

Model 210		
Hydrochloric acid, 07102	Riedel-de Haen, Sigma-Aldrich	Gillingham, U.K.
IgG antibody from sheep serum, I5131	Sigma-Aldrich	Gillingham, U.K.
KH_2PO_4, P5655	Sigma-Aldrich	Gillingham, U.K.
MALDI-TOF, Bruker Autoflex III, Smartbeam	Bruker, Daltonics	Coventry, U.K.
MALDI-TOF plate, MTP 384, ground steel, S/N 21761	Bruker, Daltonics	Coventry, U.K.
Methanol, 20847307	VWR	Leicestershire, U.K.
NaCl, S-3014	Sigma-Aldrich	Gillingham, U.K.
Na_2CO_3, 102404H	VWR, BDH	Leicestershire, U.K.
$NaHCO_3$, S6297	Sigma-Aldrich	Gillingham, U.K.
Na_2HPO_4, 301584L	VWR, BDH	Leicestershire, U.K.
NaH_2PO_4, 301324Q	VWR, BDH	Leicestershire, U.K.
NaN_3, 0232540	VWR, BDH	Leicestershire, U.K.
NaOH, 301674M	VWR, BDH	Leicestershire, U.K.
Neurokinin B, 55366	Designer Biosciences	Cambridge, U.K.
Nunc Immobilizer™ Amino plates, 743-1277	VWR, LTD	Leicestershire, U.K.
octyl β-D-glucopyranoside, O8001	Sigma-Aldrich	Gillingham, U.K.
pH indicator strips, WHATMAN, PANPEHA, Z134147	Sigma-Aldrich	Gillingham, U.K.
PBS, P4417	Sigma-Aldrich	Gillingham, U.K.
p-nitrophenyl phosphate (*SigmaFast*), N2770	Sigma-Aldrich	Gillingham, U.K.
Rat brain	Charles River	Kent, U.K.
Rat placentae	Charles River	Kent, U.K.
Rat spleen	Charles River	Kent, U.K.
Scalpel, SCA-310-030K	Fisher Scientific	Loughborough, U.K.
Security Guard, MAX-RP, AJO-6073	Phenomenex	Macclesfield, U.K.
Security Guard, Cartridge Kit, KJO-4282	Phenomenex	Macclesfield, U.K.
Serine and cysteine protease inhibitors, #11836170001	Roche	West Sussex, U.K.
Shaking incubator, 211DS	Labnet	NJ, 07095, USA
Silicon tubing, 7318211	BioRad	Hertfordshire, U.K.
Sodium citrate monobasic, 71497	Sigma-Aldrich	Gillingham, U.K.
Substance P, DBS00686-1	Designer Biosciences	Cambridge, U.K.
10 ml syringe, 302188	BD Plastipak, VWR	Leicestershire, U.K.
5 ml syringe, 302187	BD Plastipak, VWR	Leicestershire, U.K.
1 ml syringe, 300013	BD Plastipak, VWR	Leicestershire, U.K.
System controller, pump, Waters 600E, manual injector Rheodyne	Millipore	Watford, U.K.

7725i , Tunable UV absorbance detector, Waters 486		
Trifluoroacetic acid, 302031	Sigma-Aldrich	Gillingham, U.K.
Tris(hydroxymethyl)aminomethane, 252859	Sigma-Aldrich	Gillingham, U.K.
Triton X-100, T9284	Sigma-Aldrich	Gillingham, U.K.
Trypsin sequence grade modified, V511A	Promega	Southampton, U.K.
Tween-20, P1379	Sigma-Aldrich	Gillingham, U.K.
U.V. detector, Varian, ProStar	Agilent Technologies	Berkshire, U.K.
U.V. spectrometer, Cary 100 Scan	Agilent Technologies	Berkshire, U.K.
Varian spectrometer Cary, 50 MPR Microplate Reader	Agilent Technologies	Berkshire, U.K.
Vortex, Miximatic, Julabo	Jencons, VWR	Leicestershire, U.K.
Waterbath	Grant Instruments	Cambridge, U.K.
ZipTips, ZTC18M096	Millipore	Watford, U.K.

Appendix 2. Prediction of the proteolytic processing of TAC4 precursors by signal peptidases using SignalP.

>_HUMAN-TAC4_AF515828.1_



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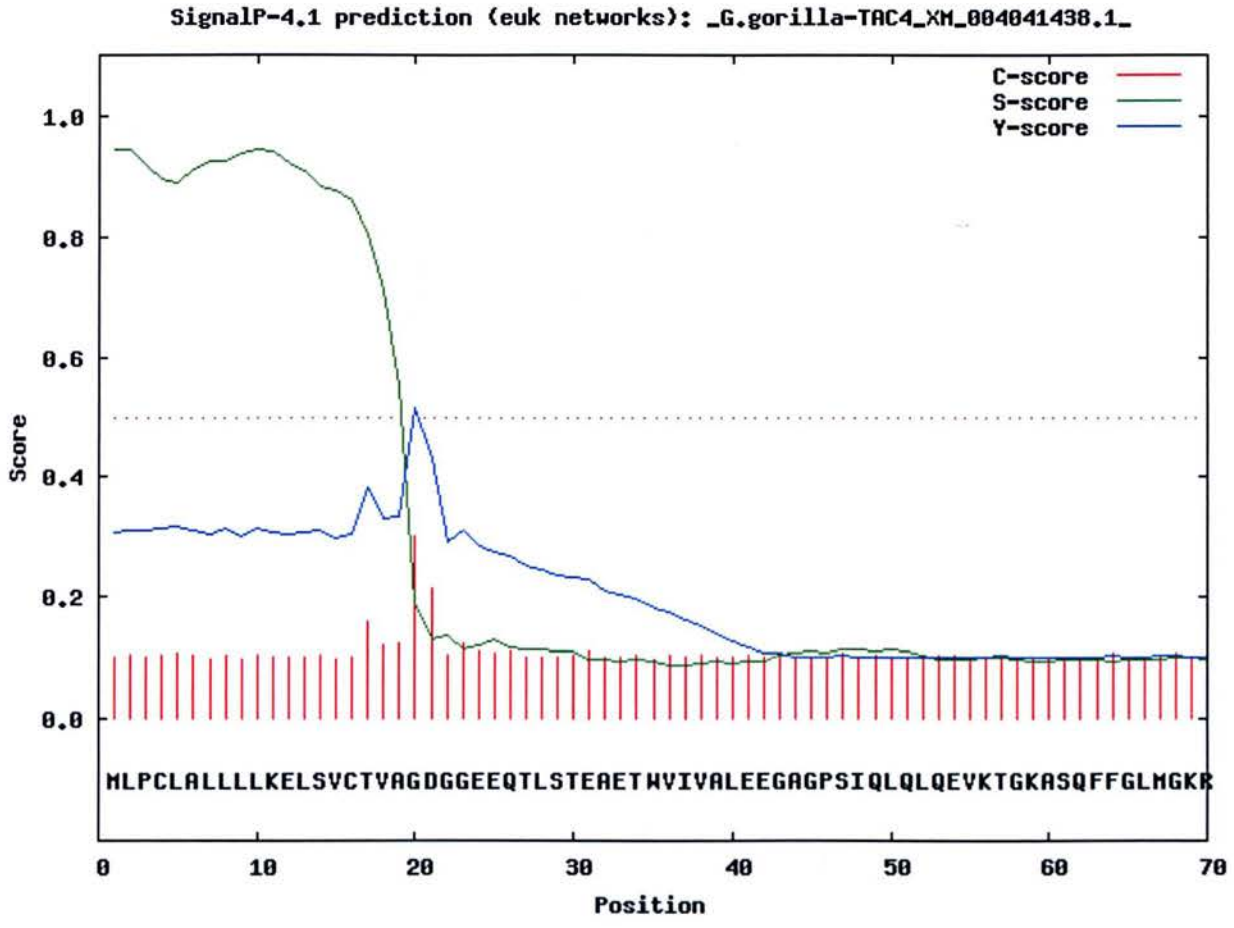

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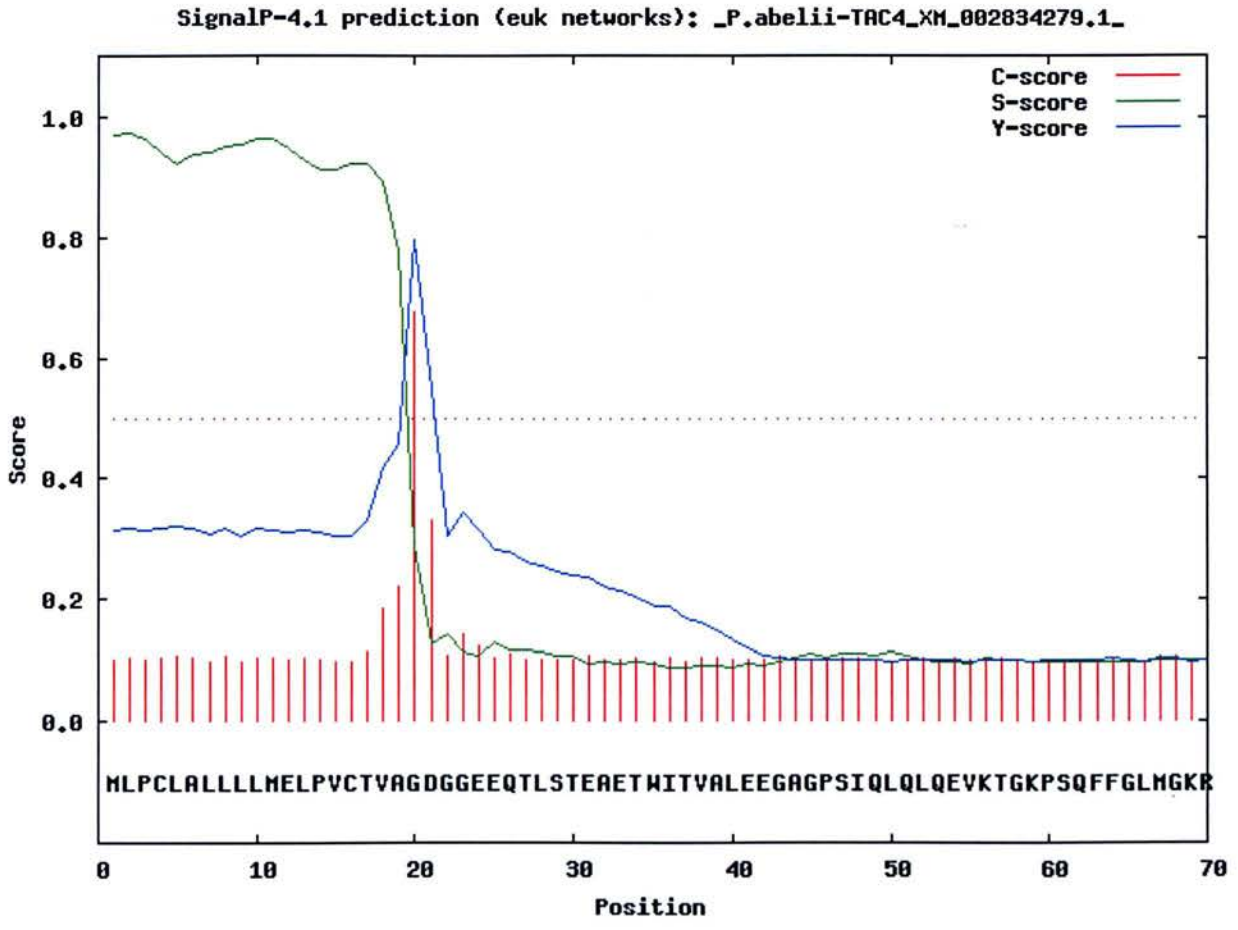

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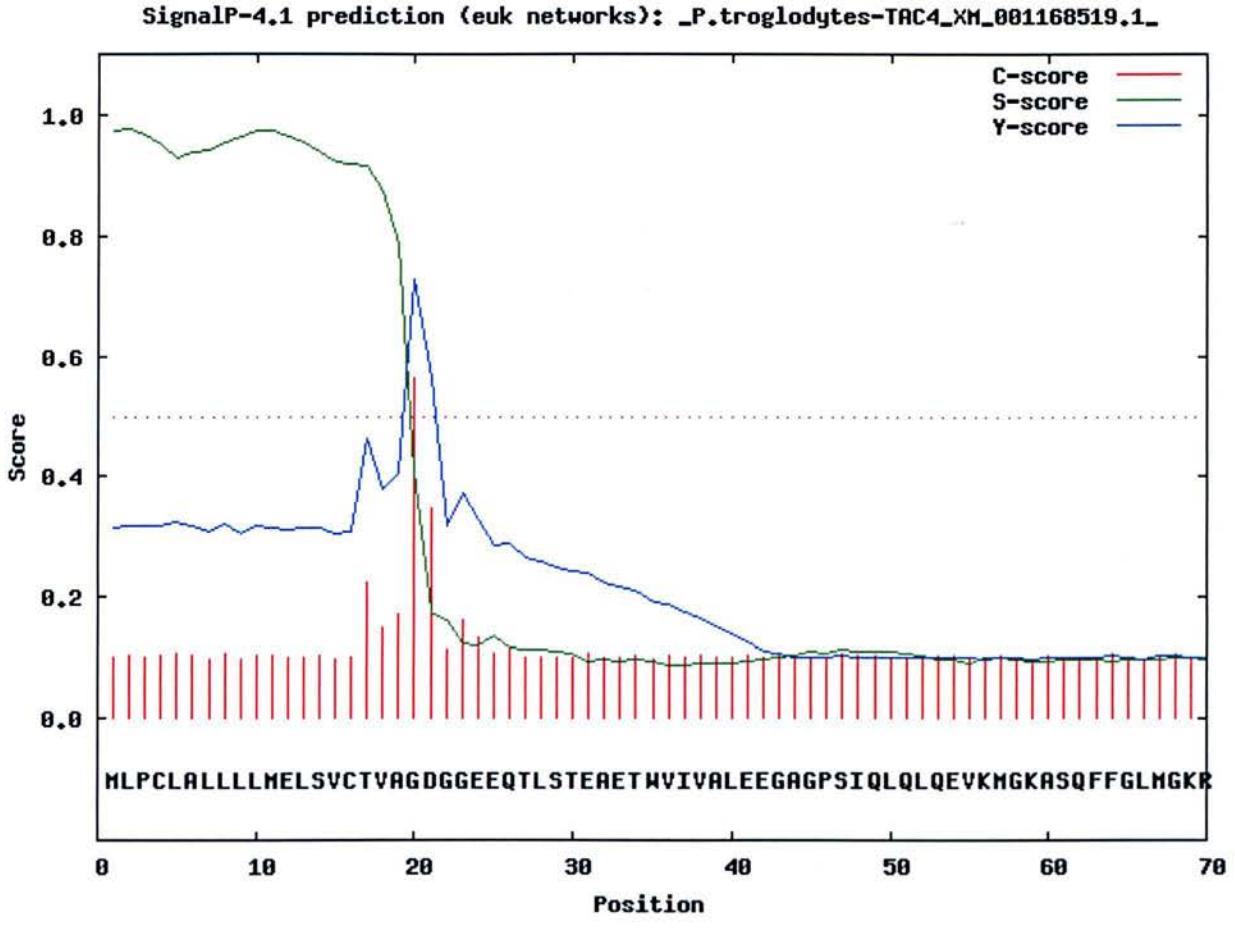

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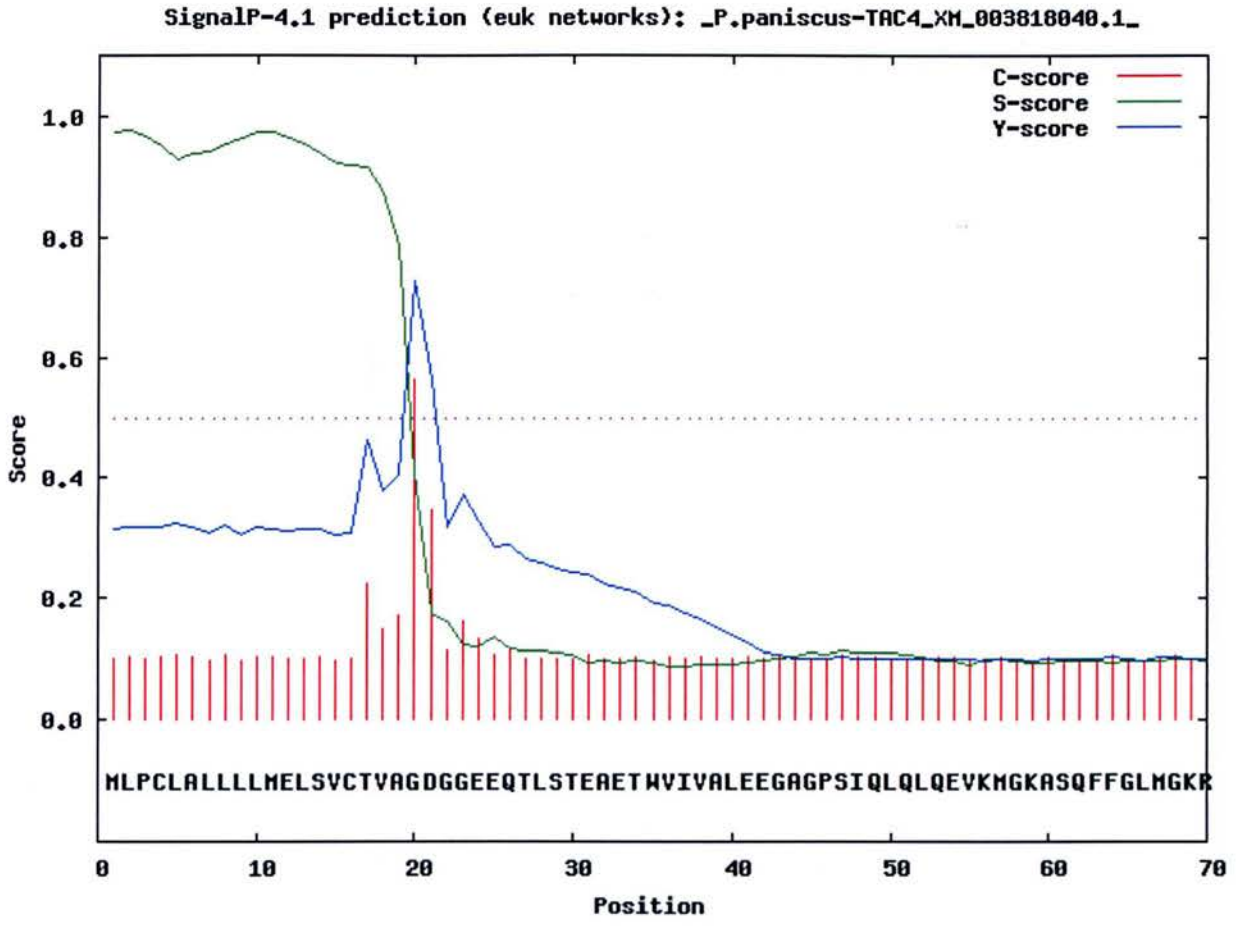

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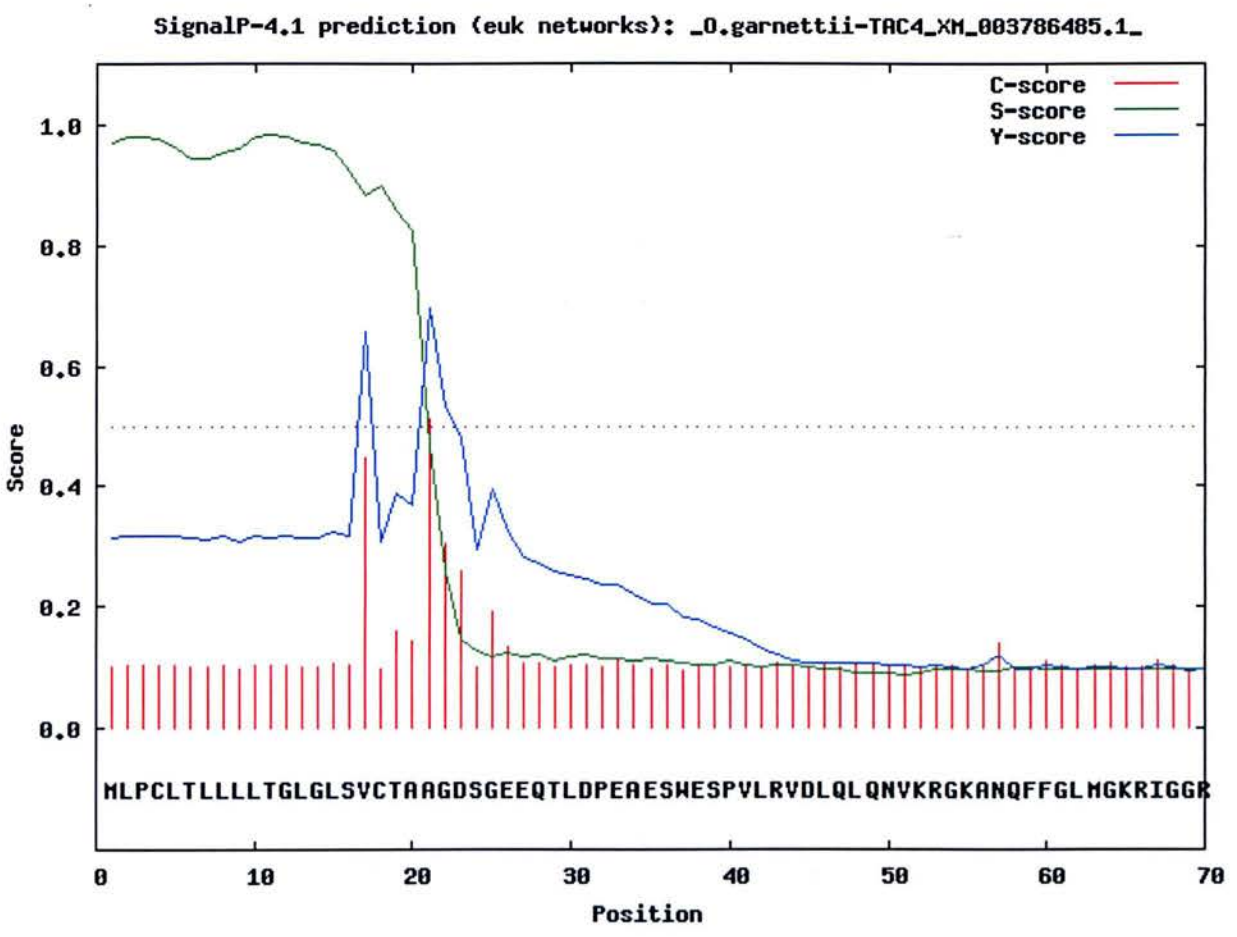

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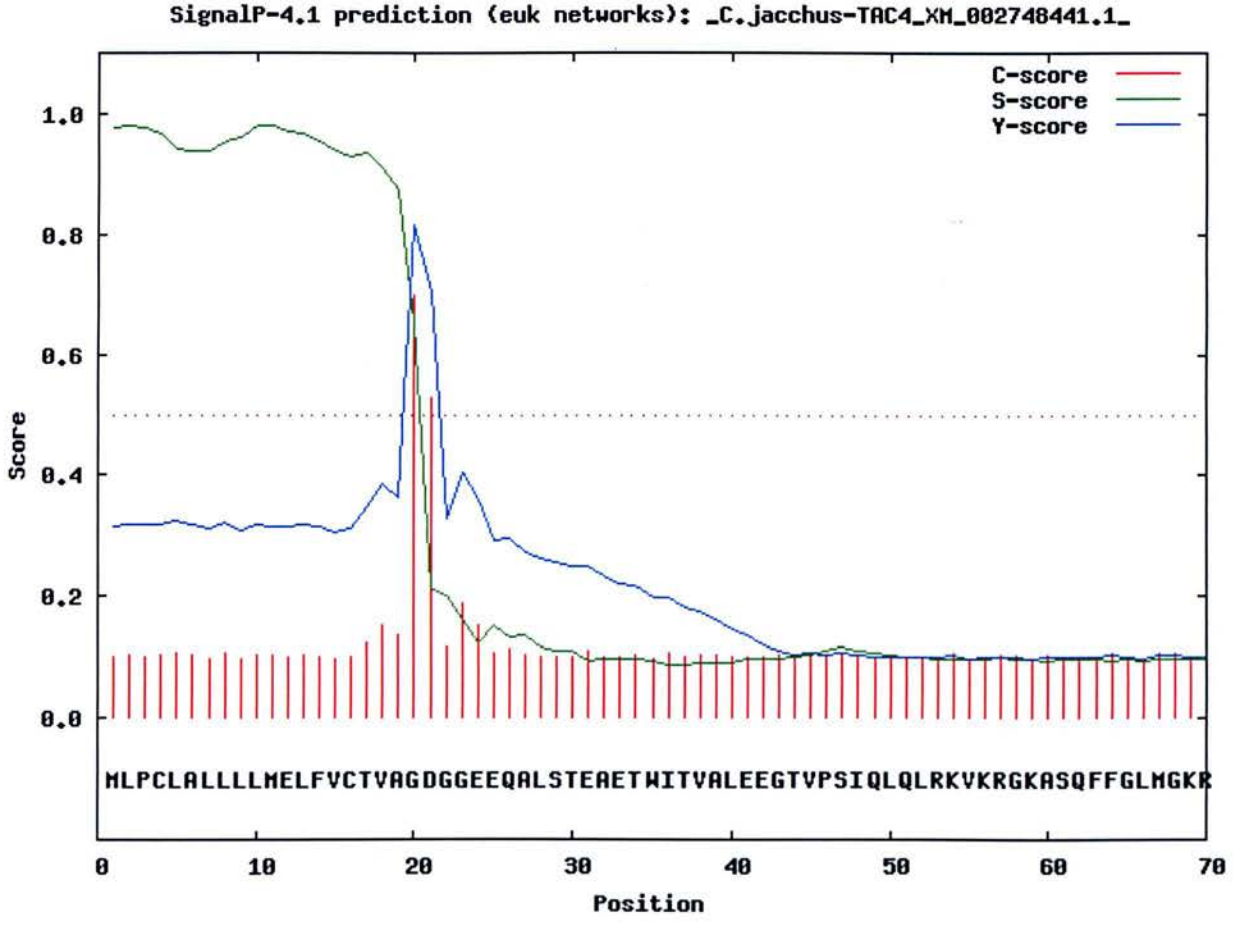

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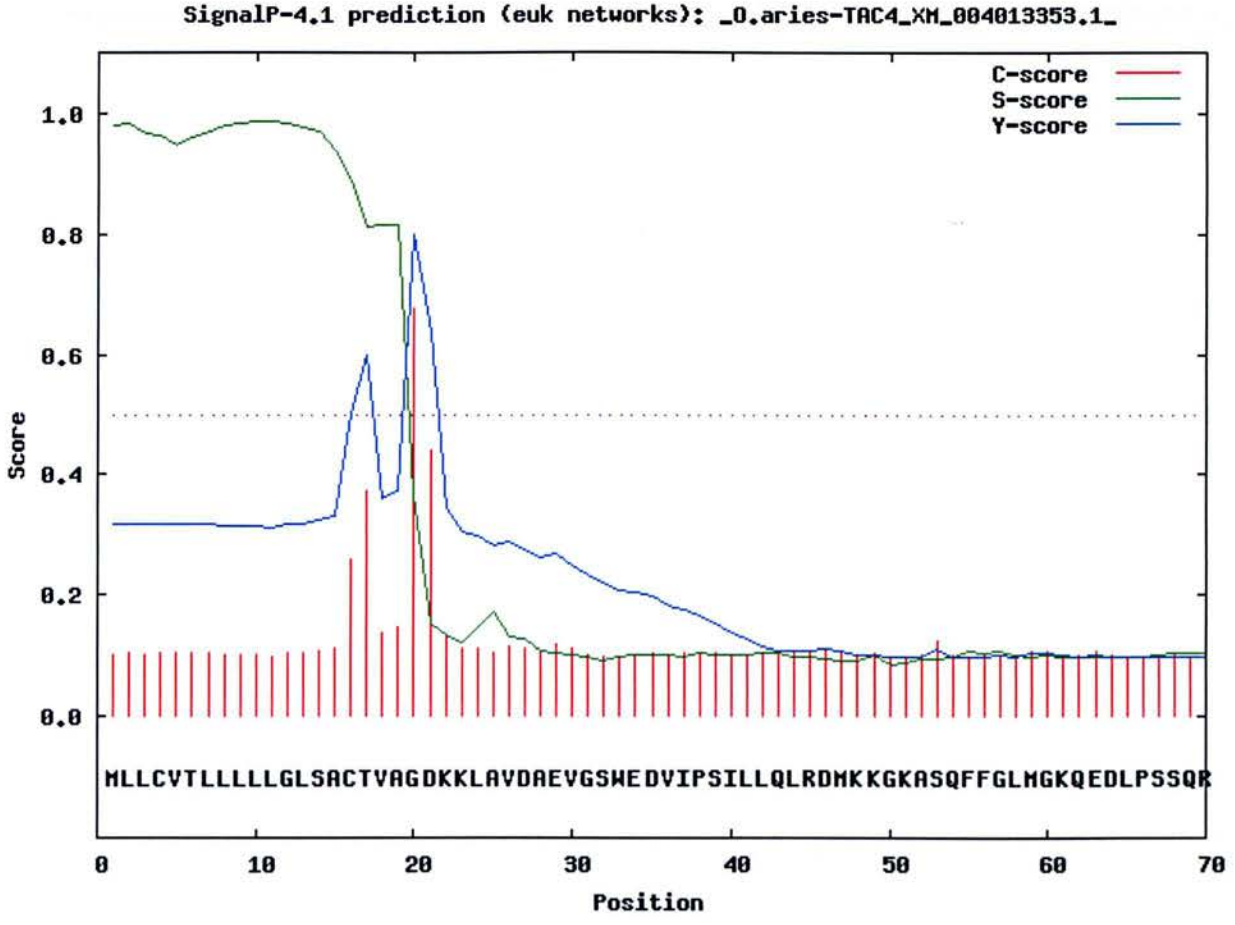

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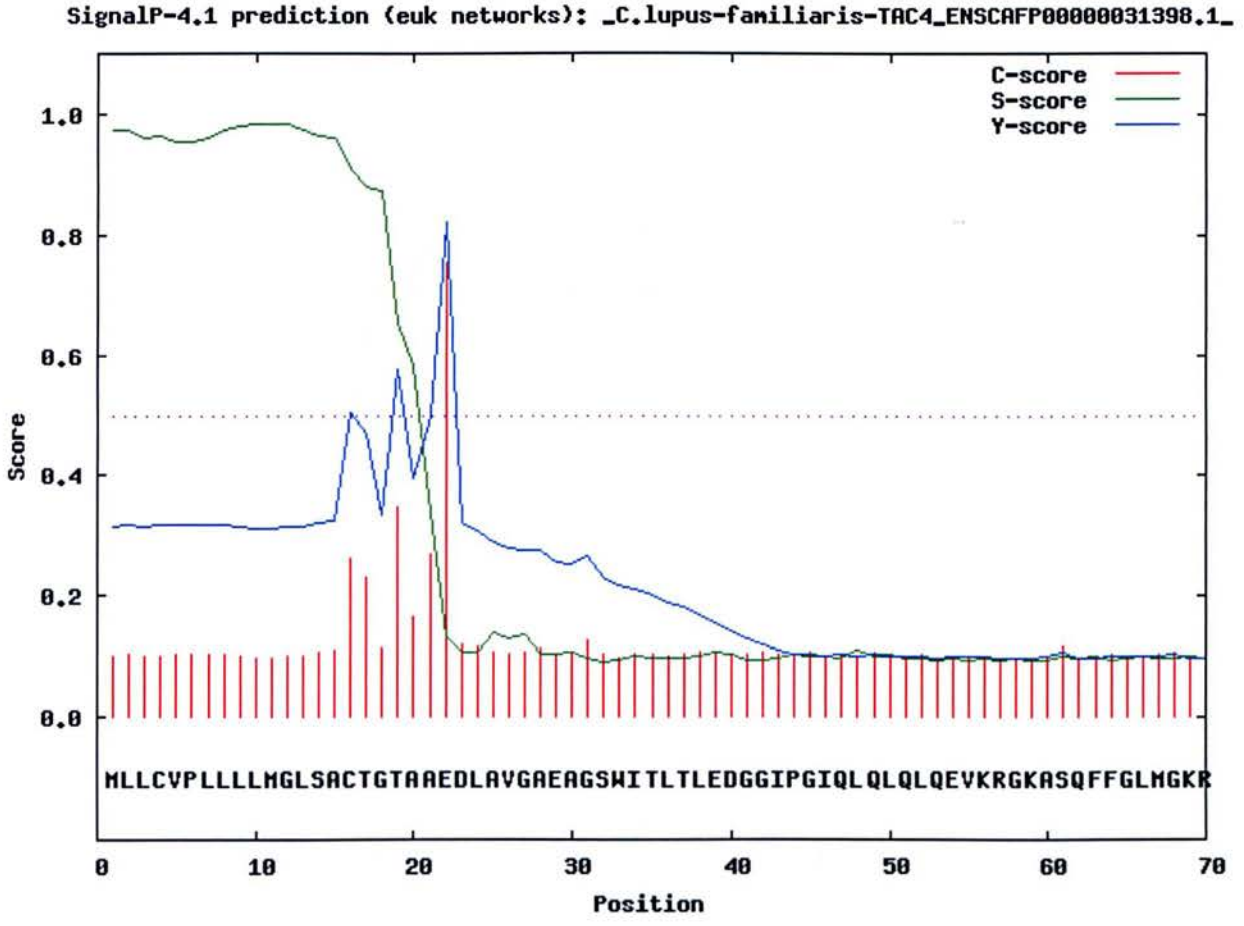

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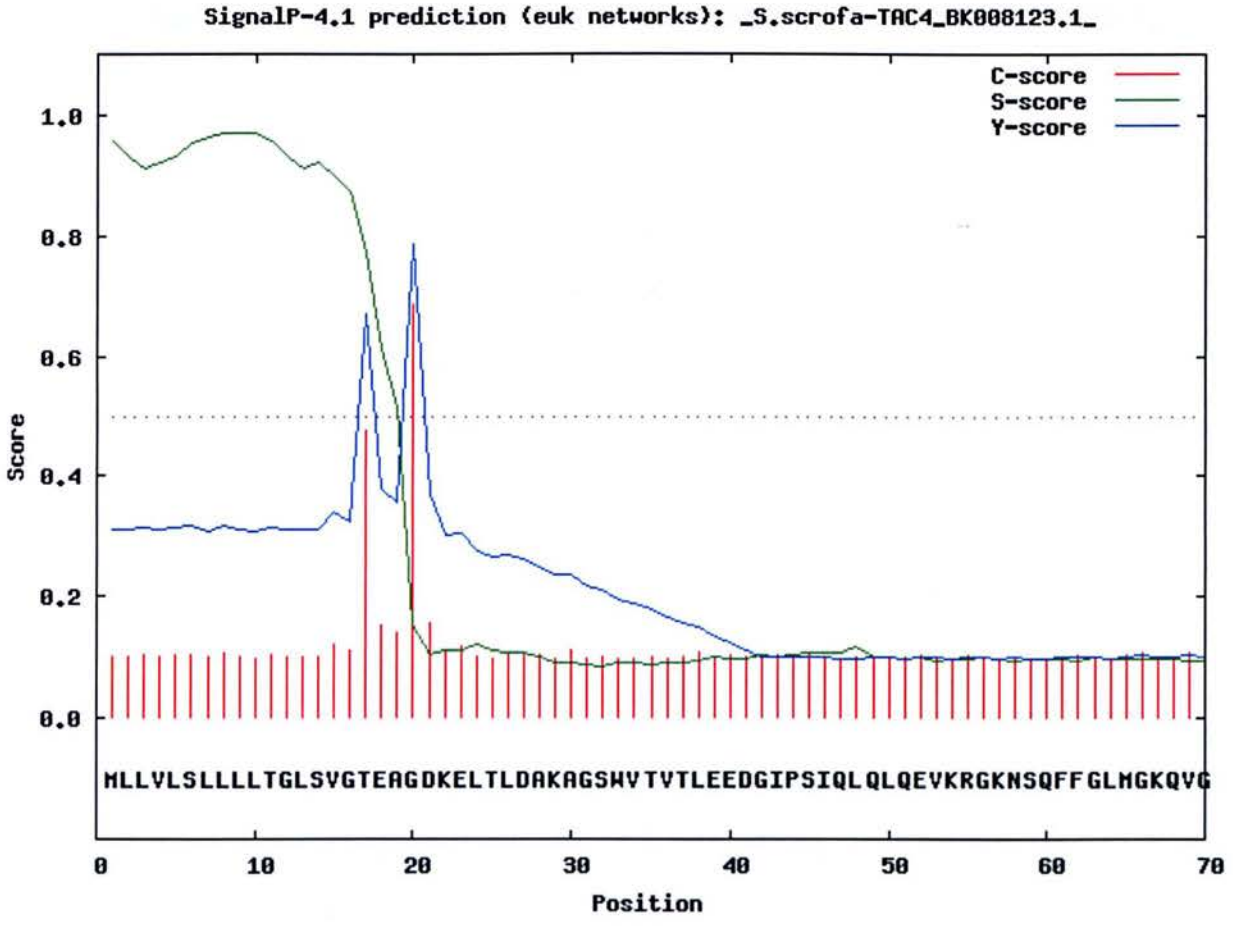
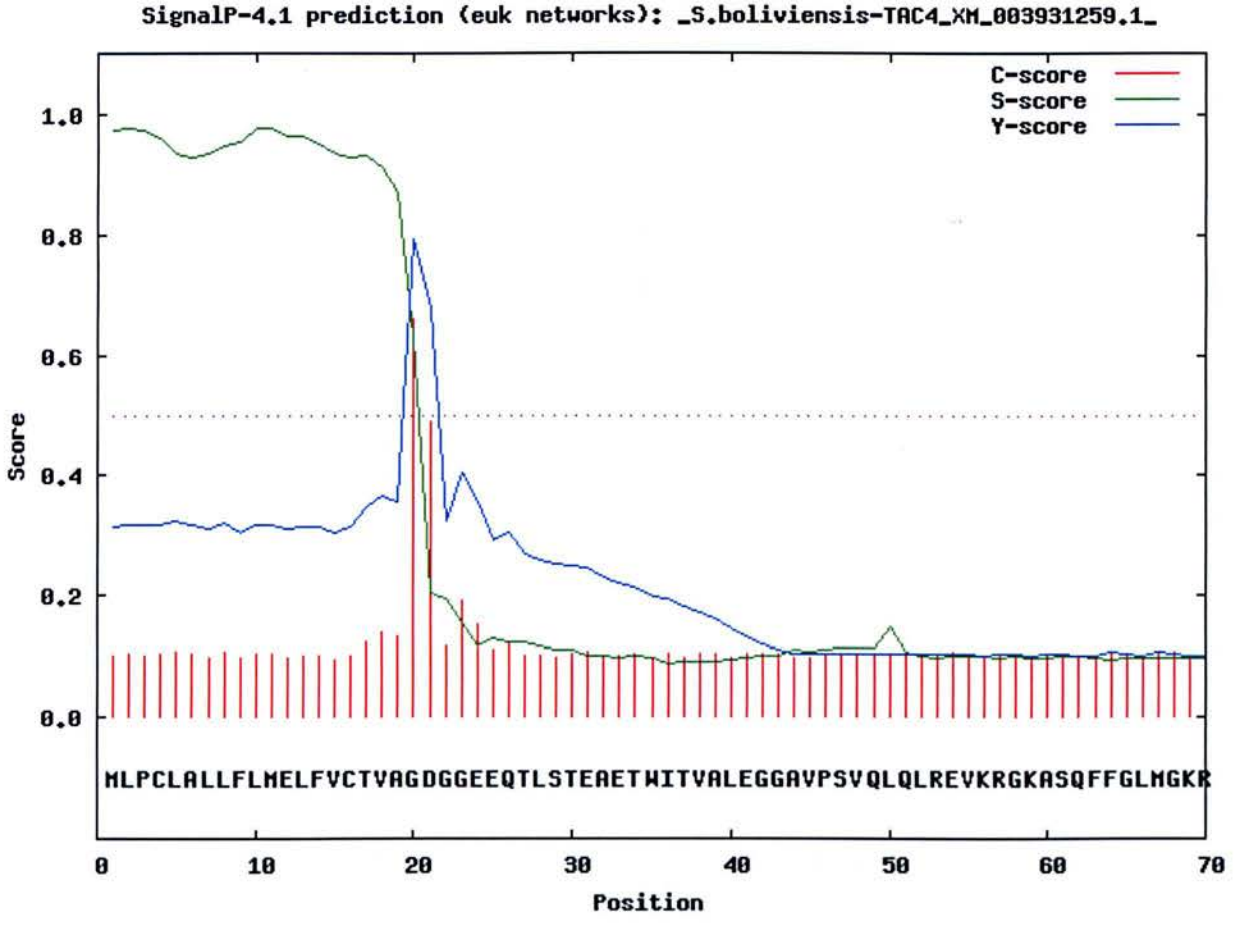


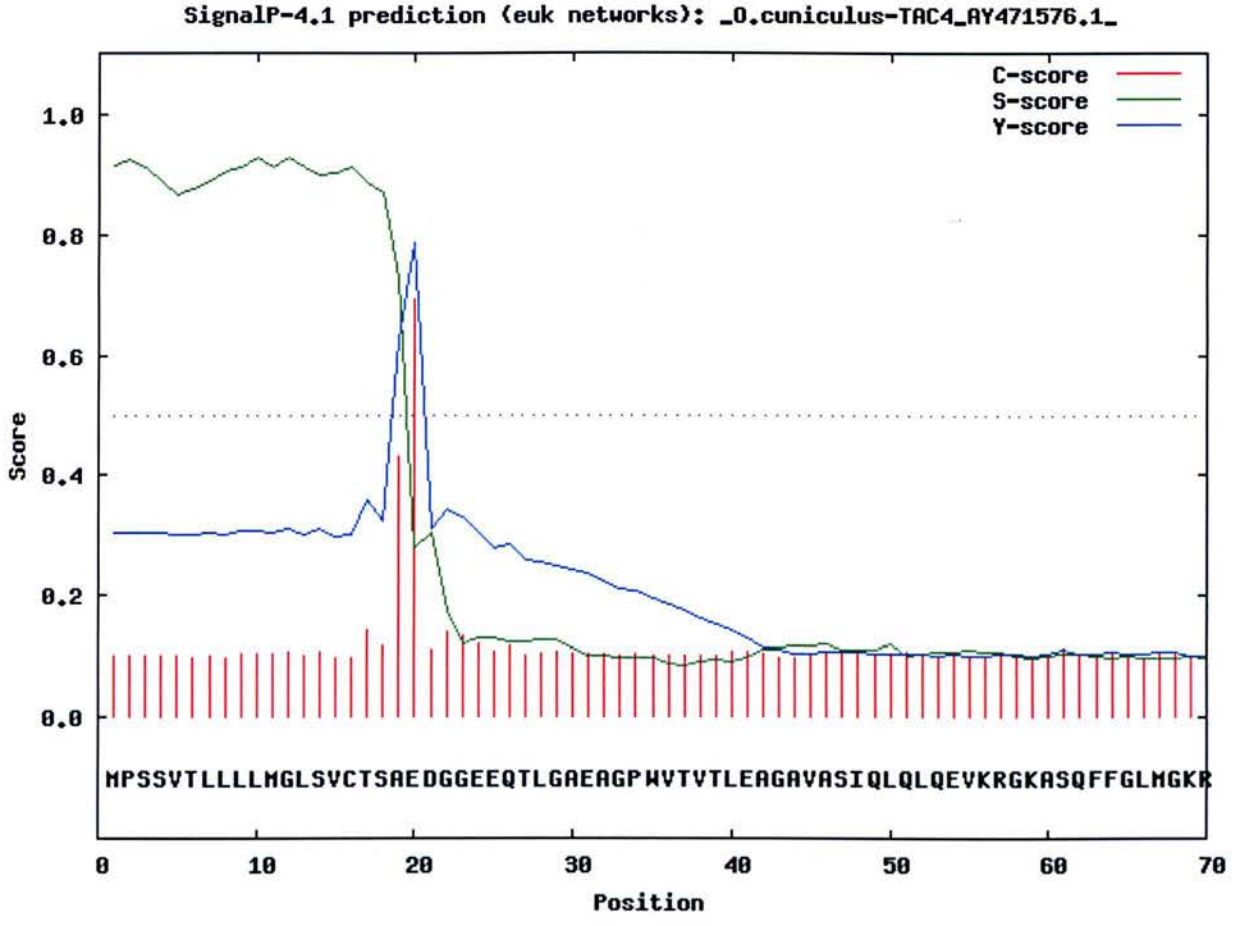
Name=_S.scrofa-TAC4_BK008123.1_ SP='YES' Cleavage site between pos. 19 and 20: TEA-GD

>_S.boliviensis-TAC4_XM_003931259.1_



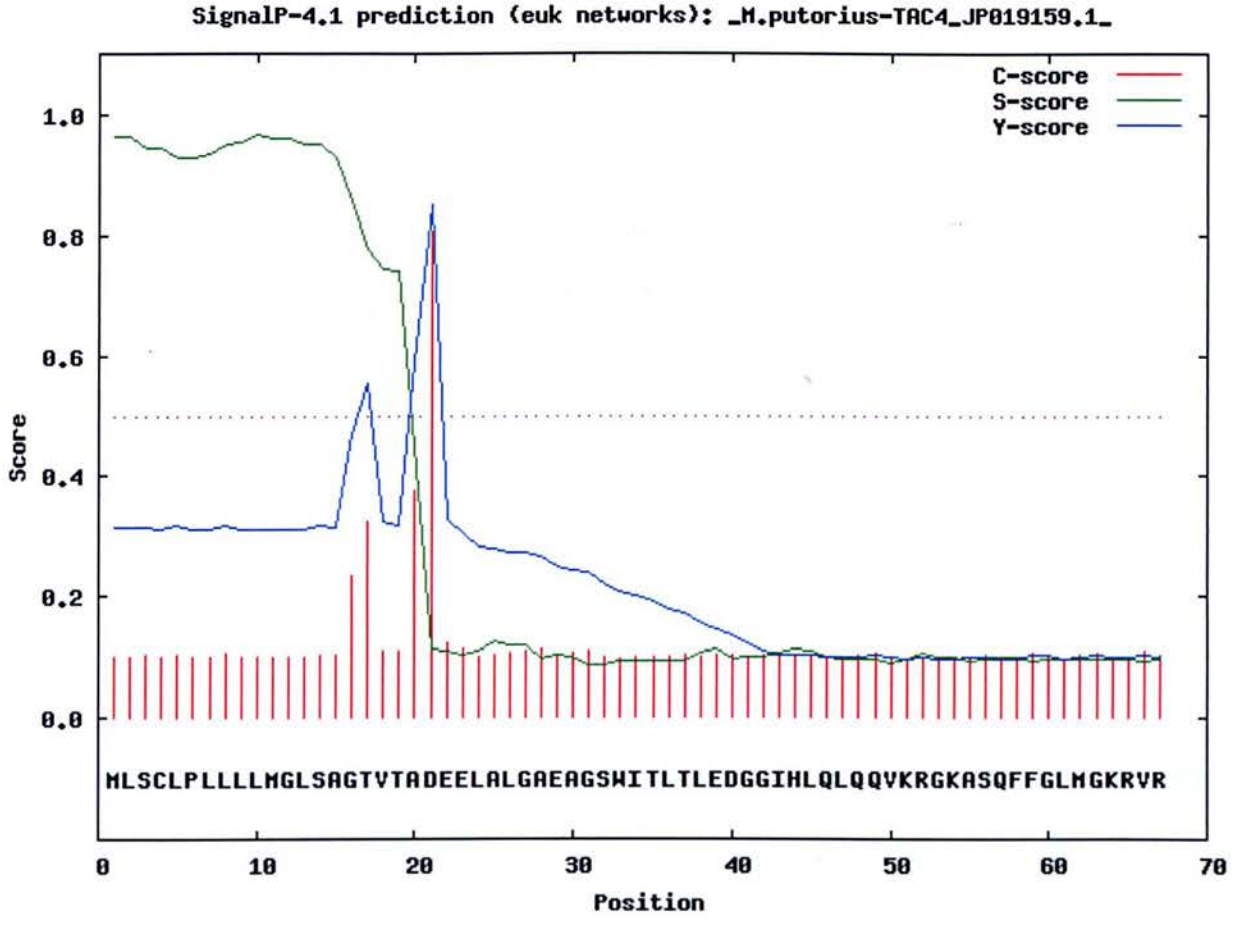
Name=_S.boliviensis-TAC4_XM_003931259.1_ SP='YES' Cleavage site between pos. 19 and 20: TVA-GD D=0.876 D-cutoff=0.450 Networks=SignalP-noTM

>_O.cuniculus-TAC4_AY471576.1_



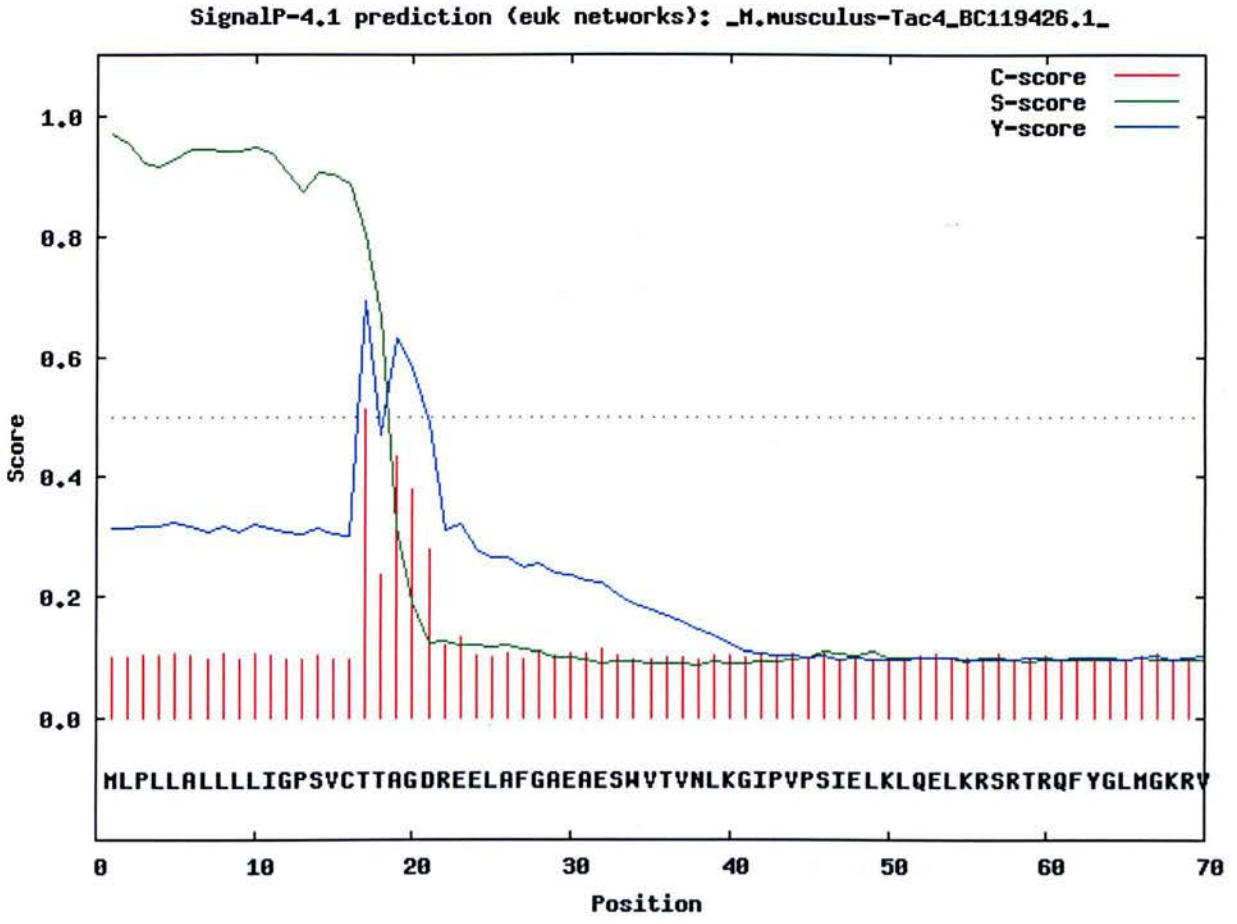
Name=_O.cuniculus-TAC4_AY471576.1_ SP='YES' Cleavage site between pos. 19 and 20: TSA-ED

>_M.putorius-TAC4_JP019159.1_



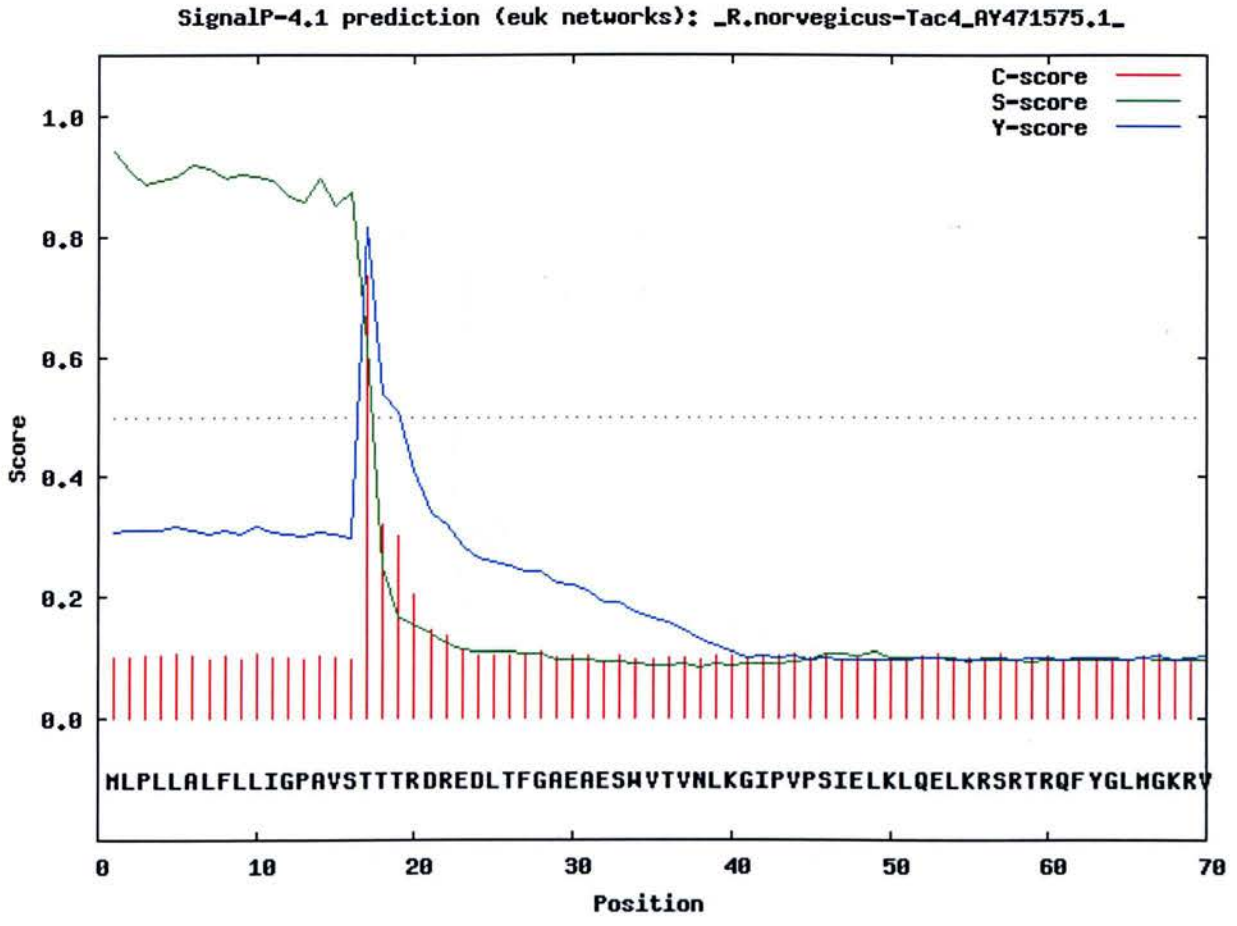
Name=_M.putorius-TAC4_JP019159.1_ SP='YES' Cleavage site between pos. 20 and 21: VTA-DE

>_M.musculus-Tac4_BC119426.1_



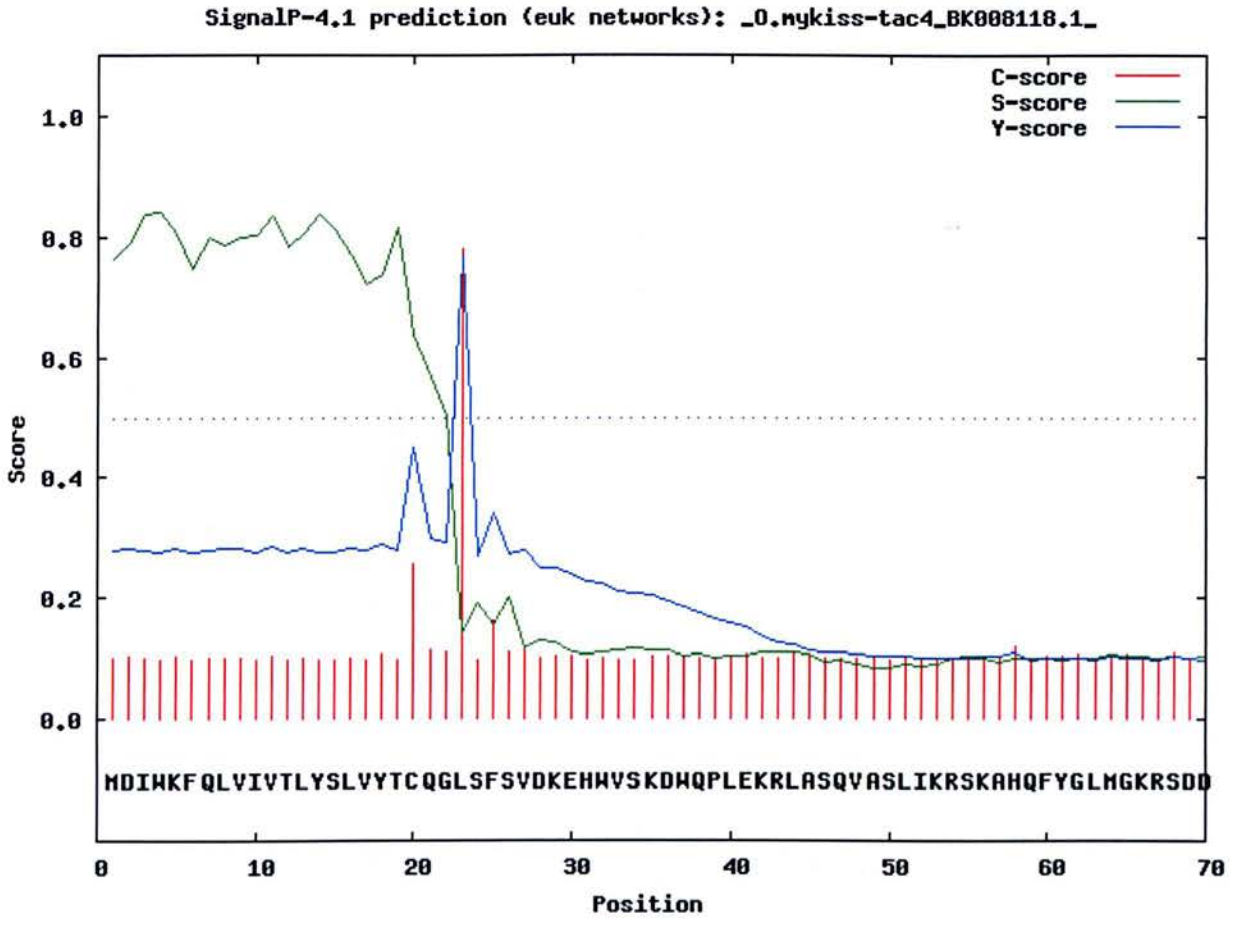
Name=_M.musculus-Tac4_BC119426.1_ SP='YES' Cleavage site between pos. 16 and 17: SVC-TT

>_R.norvegicus-Tac4_AY471575.1_



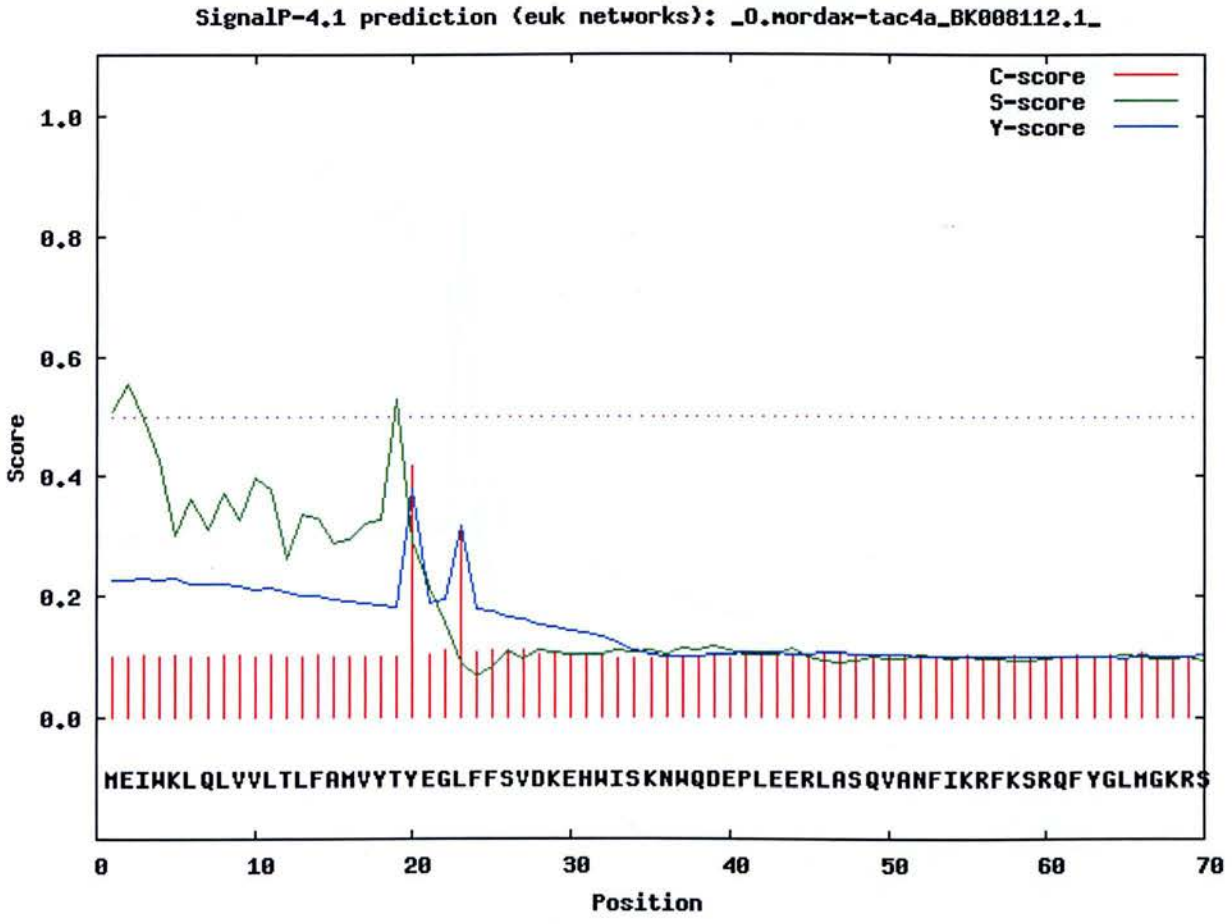
Name=_R.norvegicus-Tac4_AY471575.1_ SP='YES' Cleavage site between pos. 16 and 17: AVS-TT

>_O.mykiss-tac4_BK008118.1_



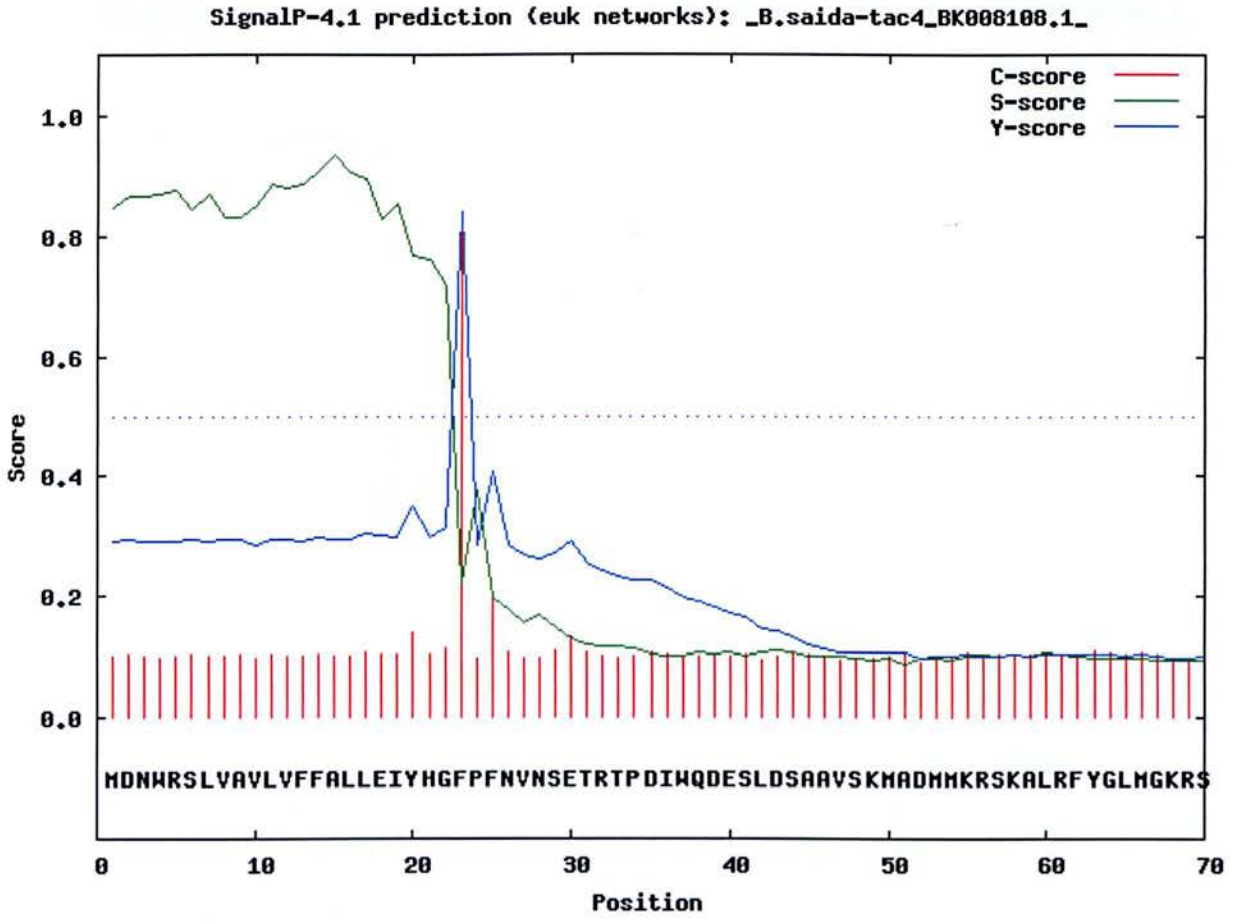
Name=_O.mykiss-tac4_BK008118.1_ SP='YES' Cleavage site between pos. 22 and 23: CQG-LS

>_O.mordax-tac4a_BK008112.1_



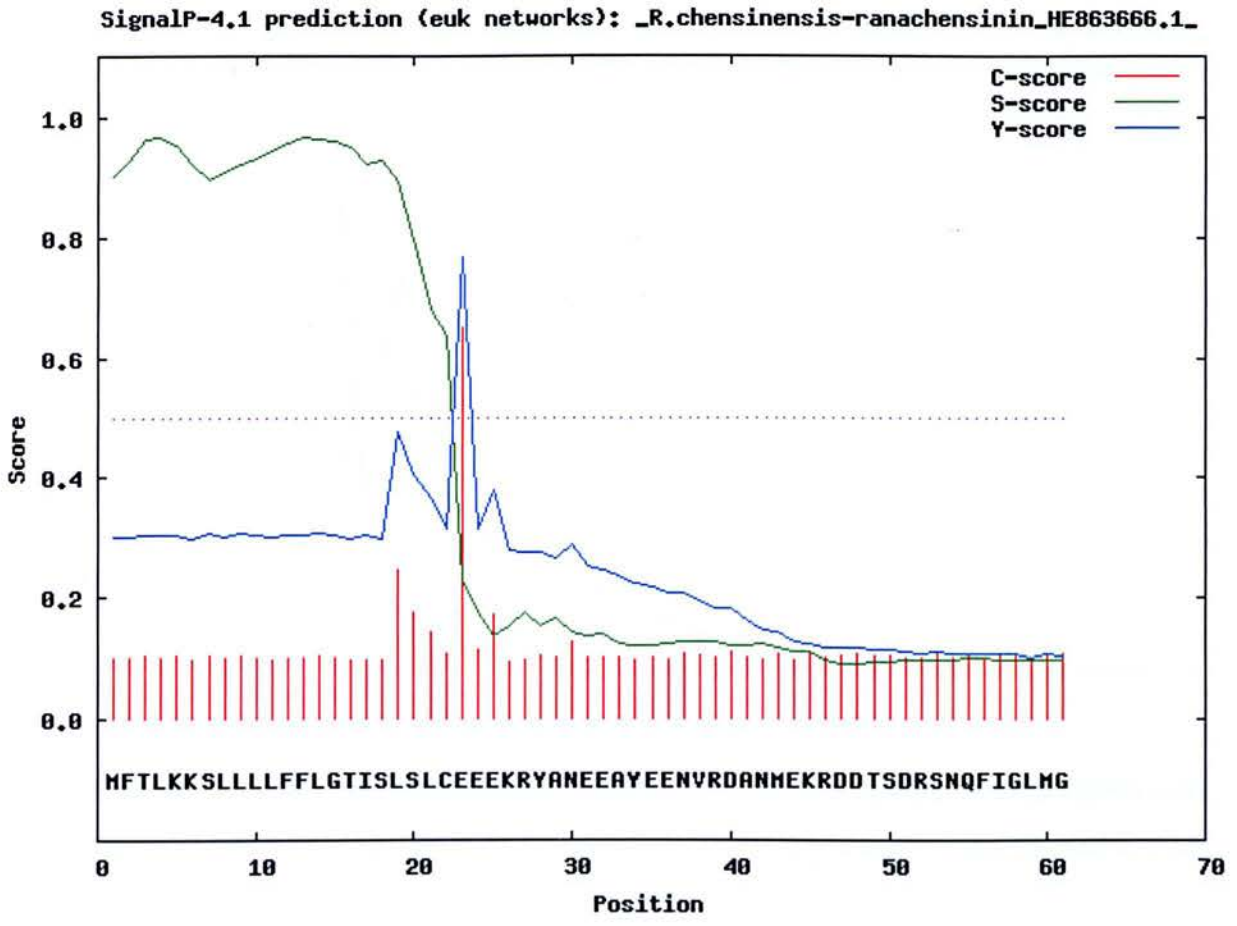
Name=_O.mordax-tac4a_BK008112.1_ SP='NO' D=0.378 D-cutoff=0.500
Networks=SignalP-TM

>_B.saida-tac4_BK008108.1_



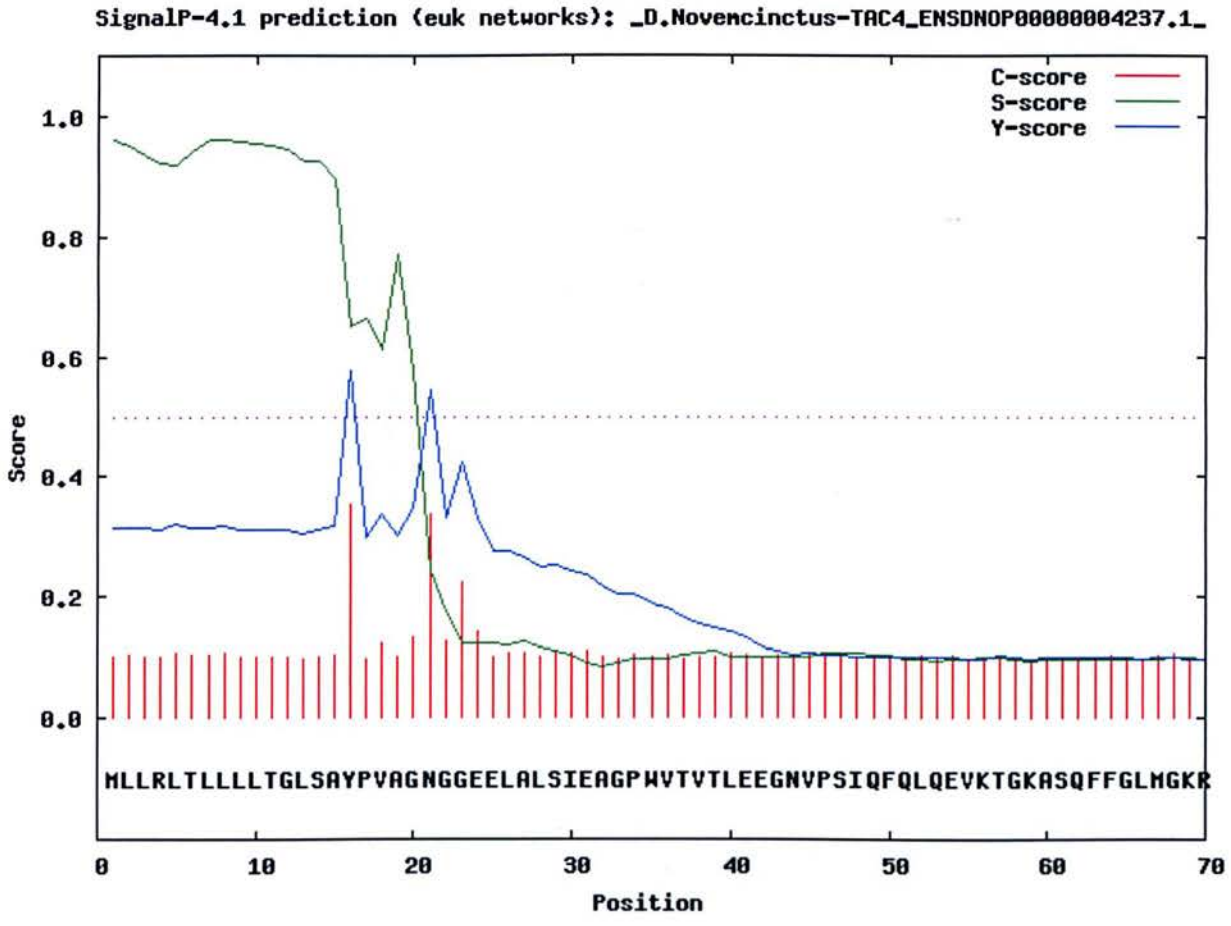
Name=_B.saida-tac4_BK008108.1_ SP='YES' Cleavage site between pos. 22 and 23: YHG-FP

>_R.chensinensis-ranachensinin_HE863666.1_



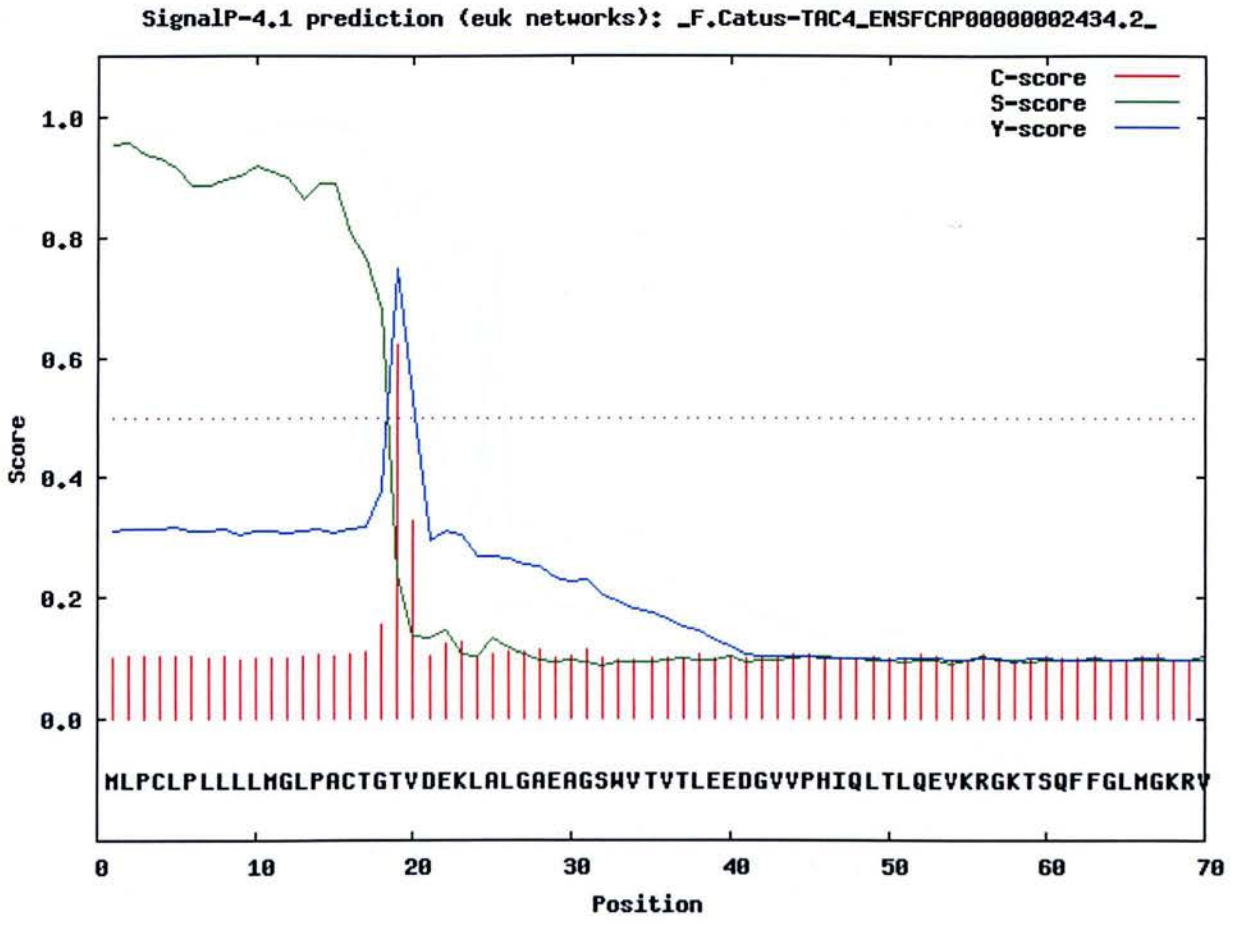
Name=_R.chensinensis-ranachensinin_HE863666.1_ SP='YES' Cleavage site between pos. 22 and 23: SLC-EE

>_D.Novemcinctus-TAC4_ENSDNOP00000004237.1_



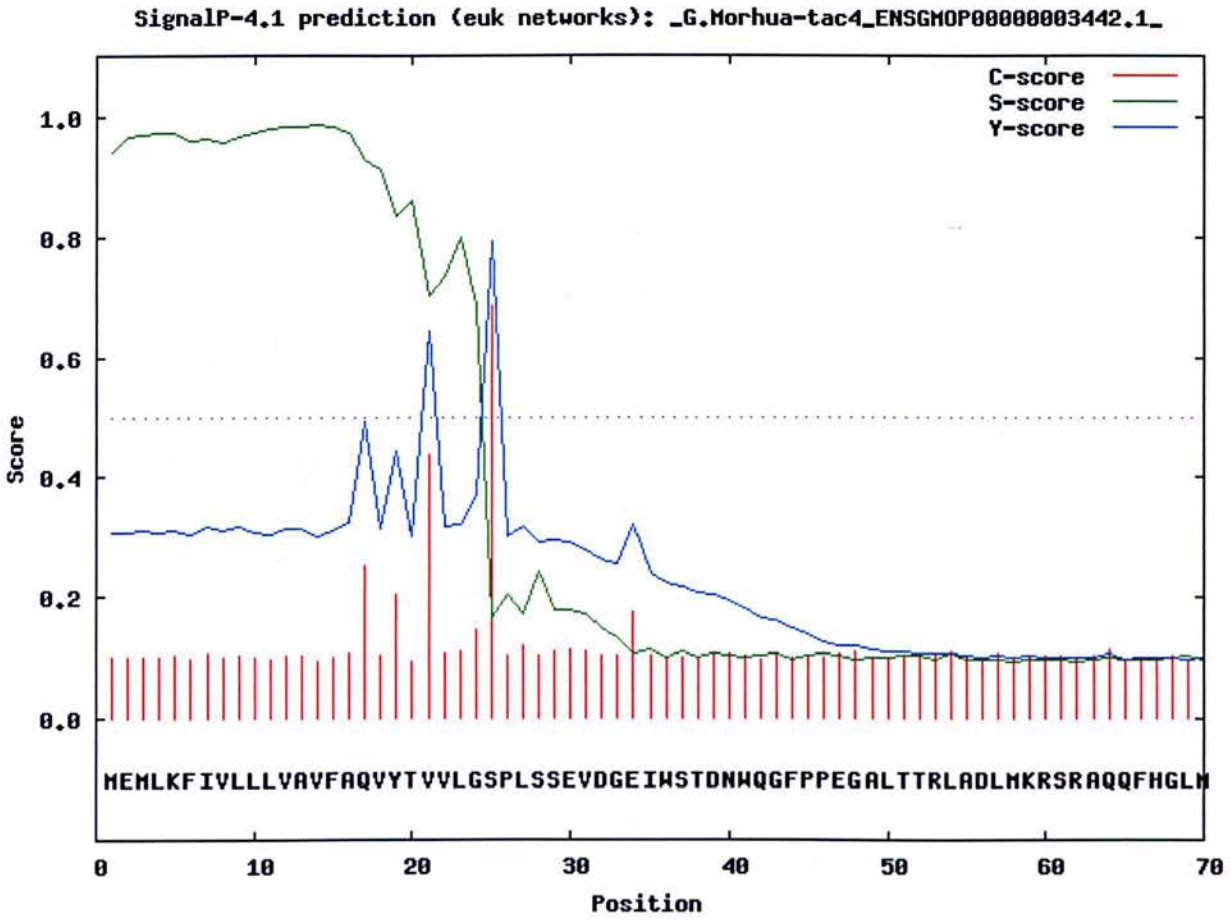
Name=_D.Novemcinctus-TAC4_ENSDNOP00000004237.1_ SP='YES' Cleavage site between pos. 15 and 16: LSA-YP

>_F.Catus-TAC4_ENSFCAP00000002434.2_



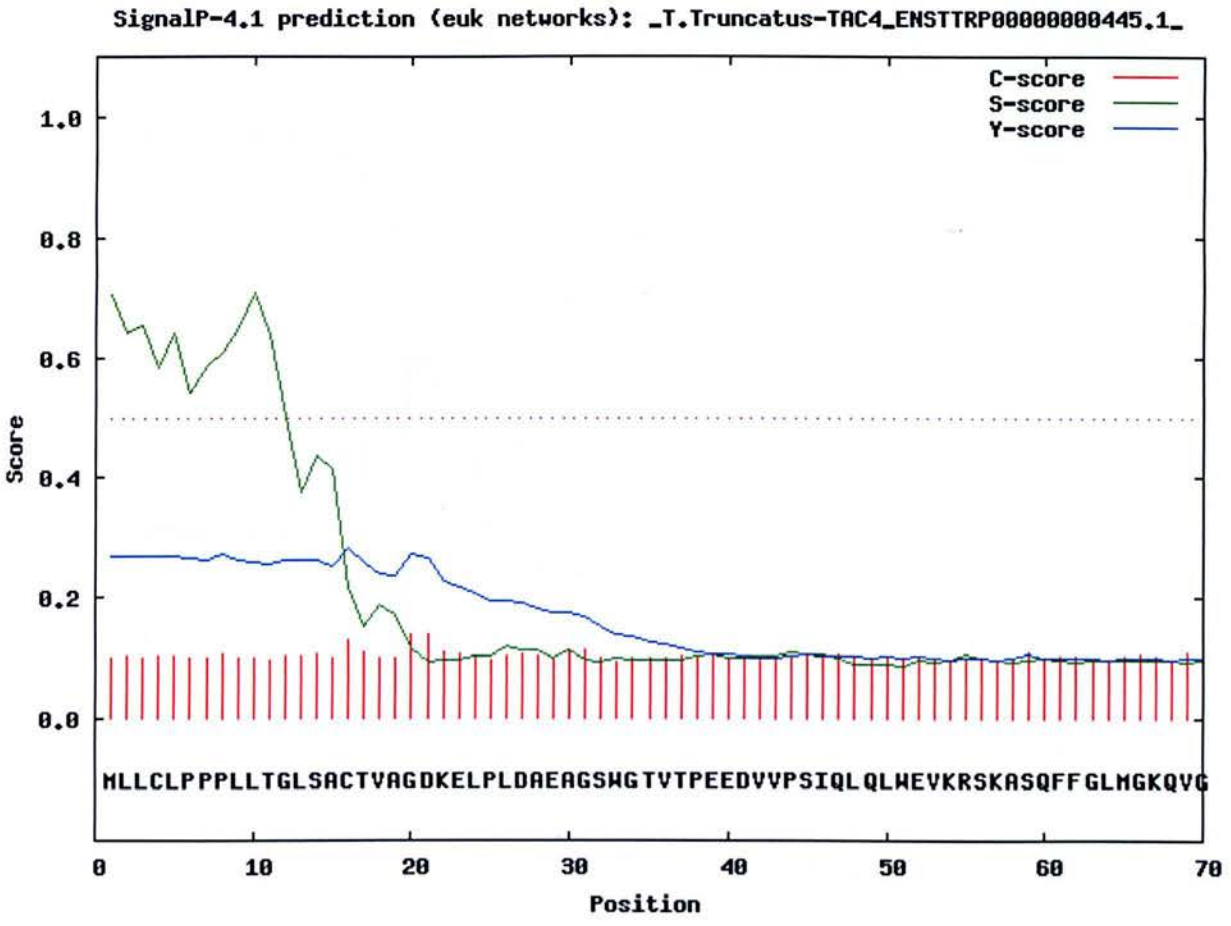
Name=_F.Catus-TAC4_ENSFCAP00000002434.2_ SP='YES' Cleavage site between pos. 18 and 19: CTG-TV

>_G.Morhua-tac4_ENSGMOP00000003442.1_



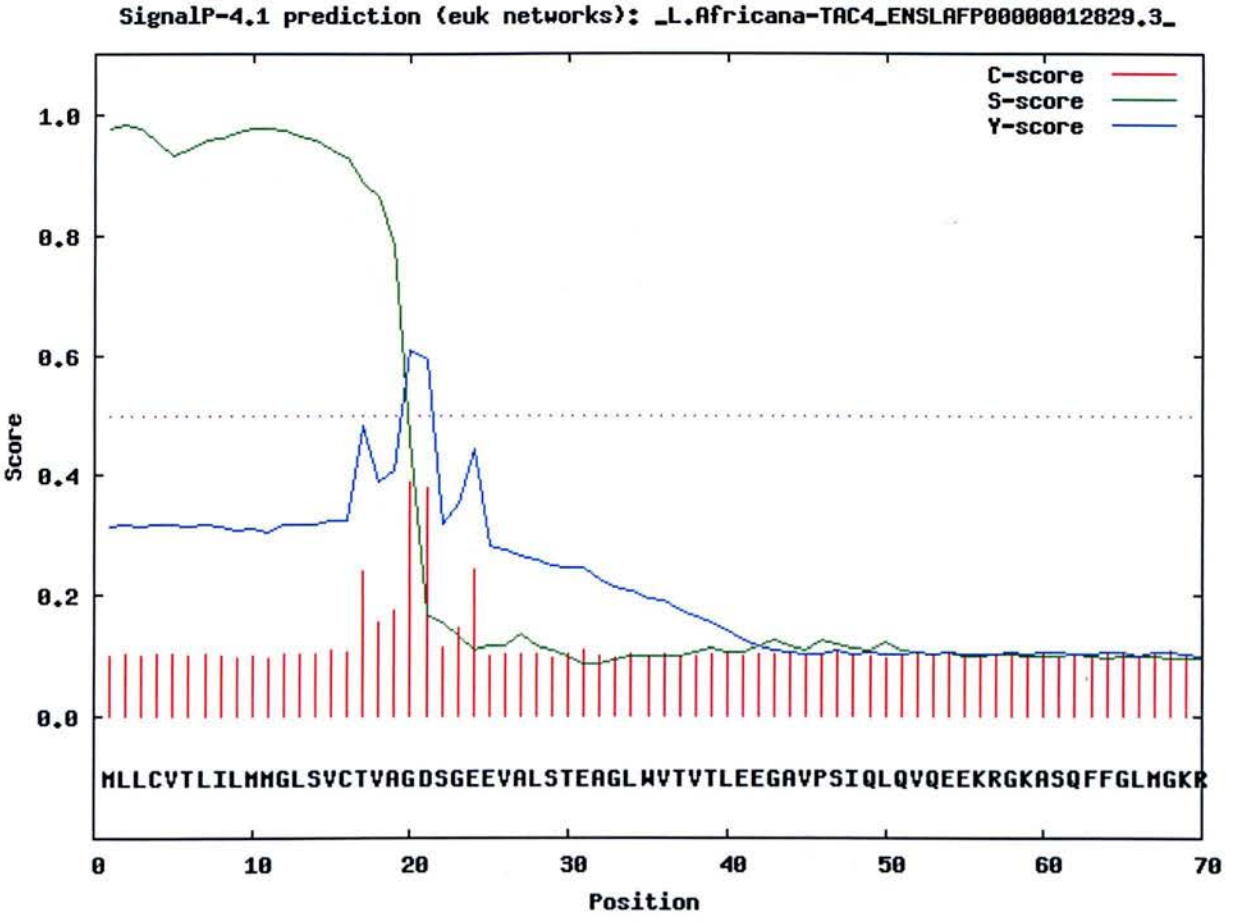
Name=_G.Morhua-tac4_ENSGMOP00000003442.1_ SP='YES' Cleavage site between pos. 24 and 25: VLG-SP

>_T.Truncatus-TAC4_ENSTTRP00000000445.1_



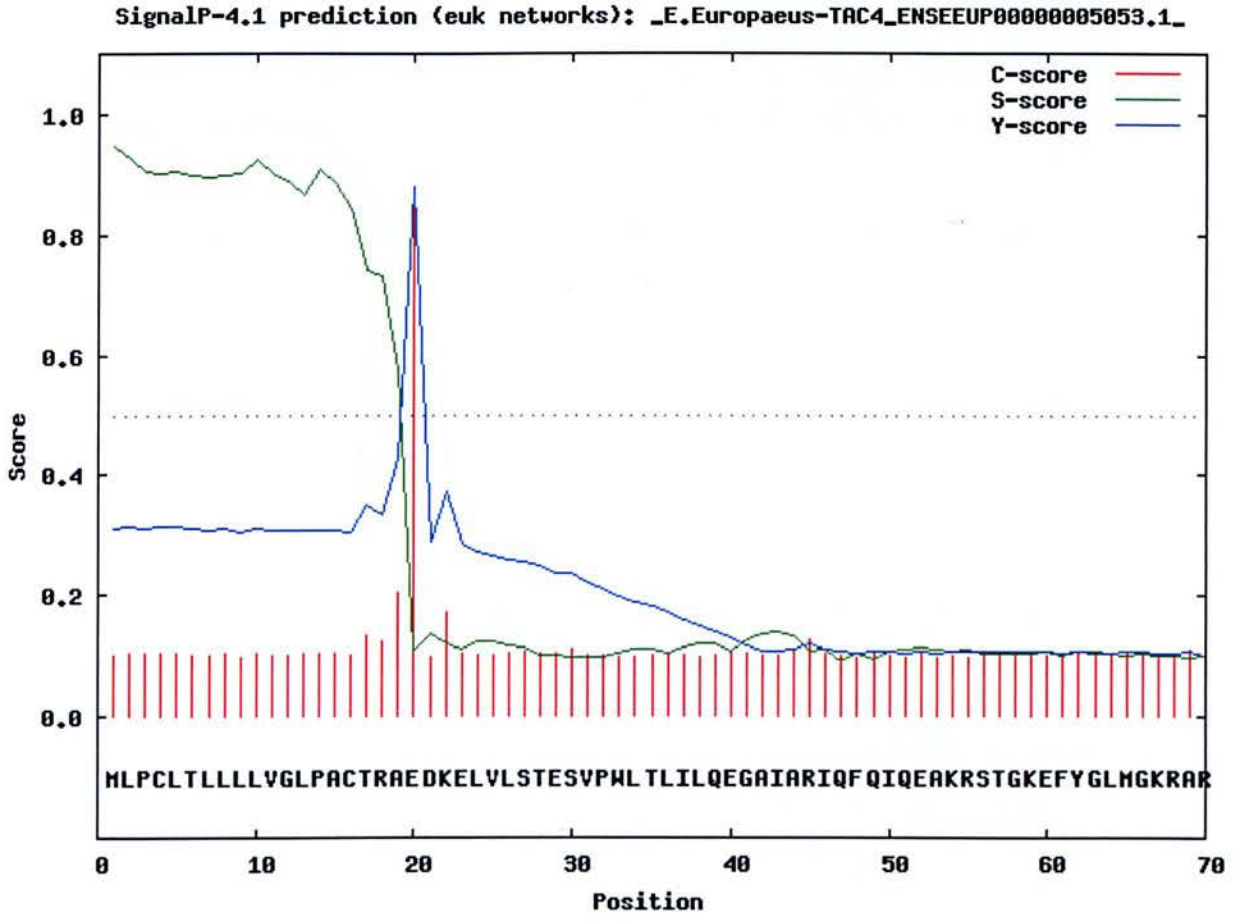
Name=_T.Truncatus-TAC4_ENSTTRP00000000445.1_ SP='NO' D=0.443 D-cutoff=0.450 Networks=SignalP-noTM

>_L.Africana-TAC4_ENSLAFP00000012829.3_



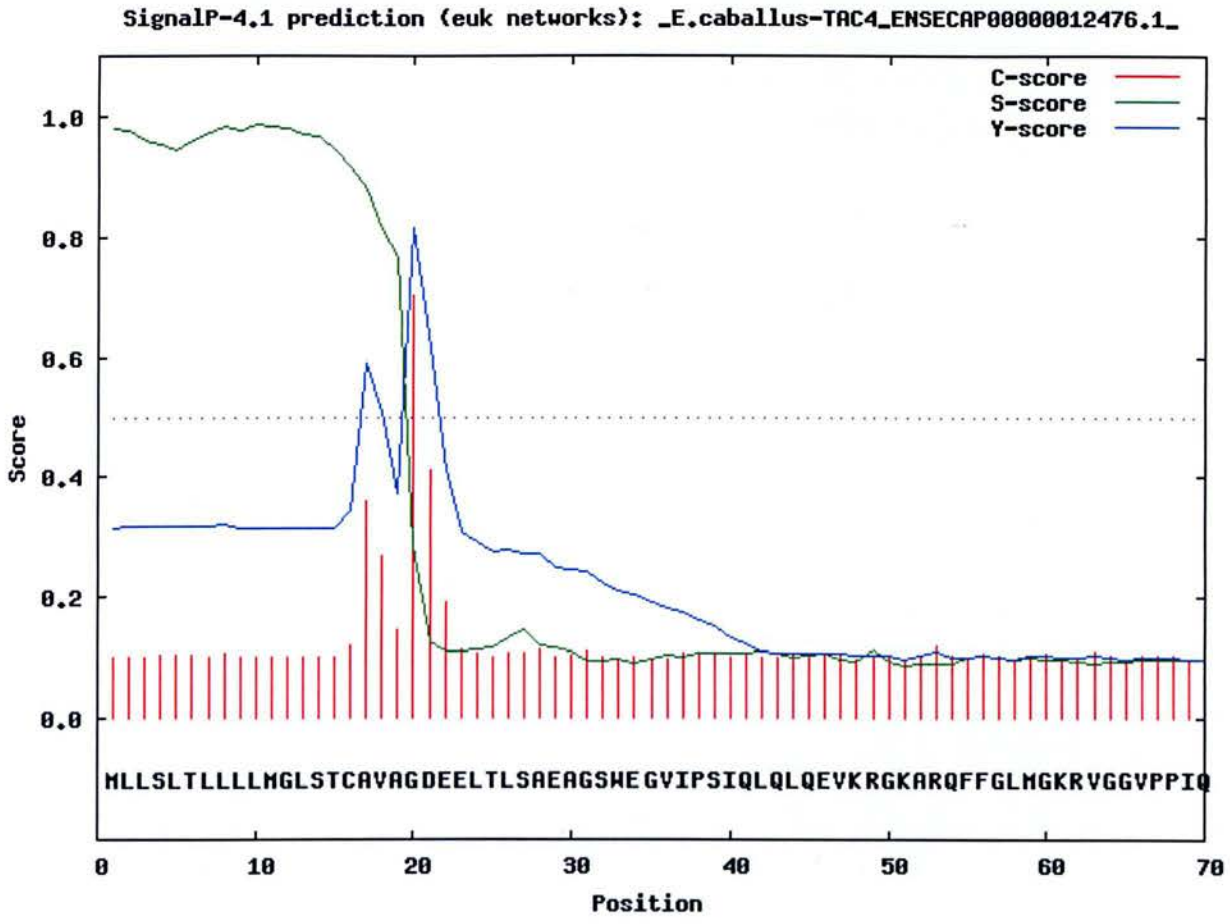
Name=_L.Africana-TAC4_ENSLAFP00000012829.3_ SP='YES' Cleavage site between pos. 19 and 20: TVA-GD

>_E.Europaeus-TAC4_ENSEEUP00000005053.1_



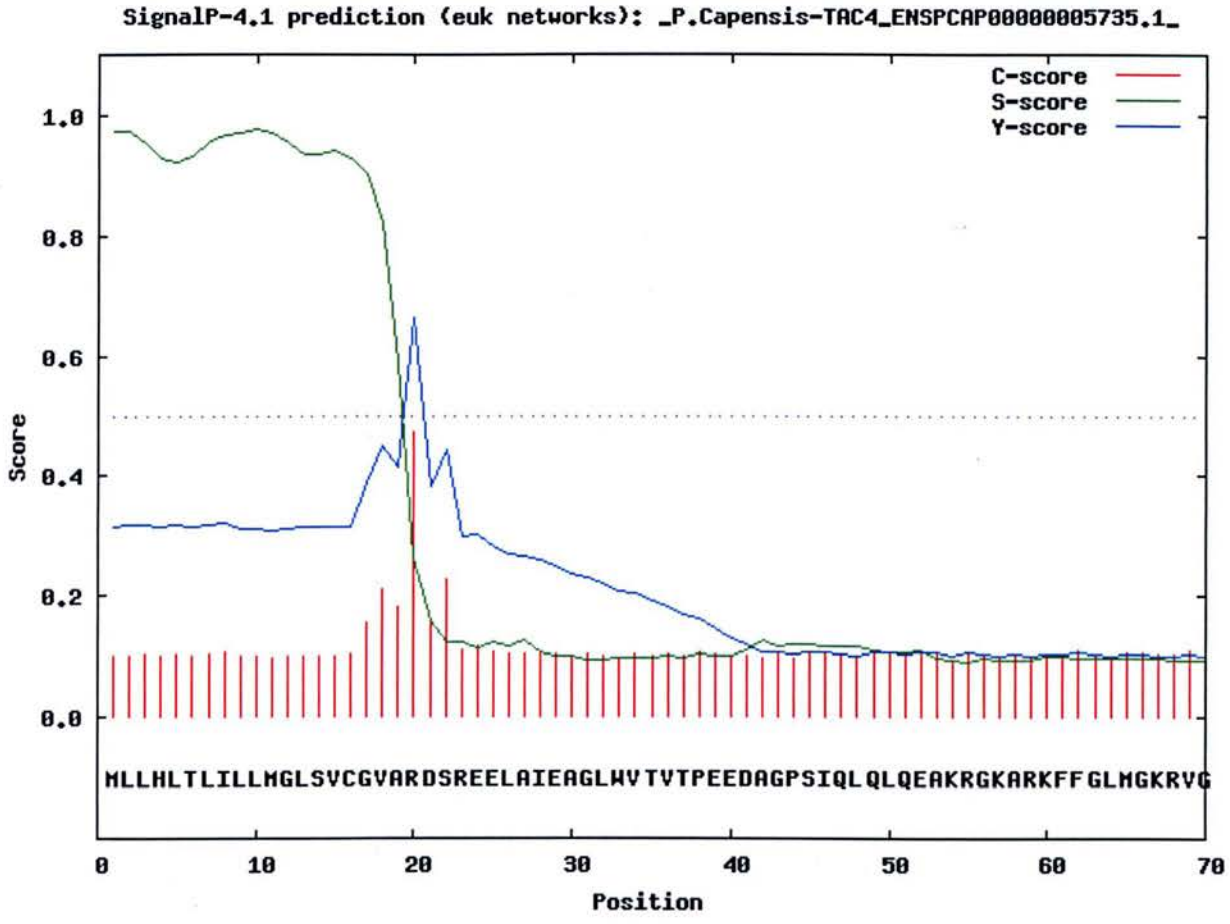
Name=_E.Europaeus-TAC4_ENSEEUP00000005053.1_ SP='YES' Cleavage site between pos. 19 and 20: TRA-ED

>_E.caballus-TAC4_ENSECAP00000012476.1_



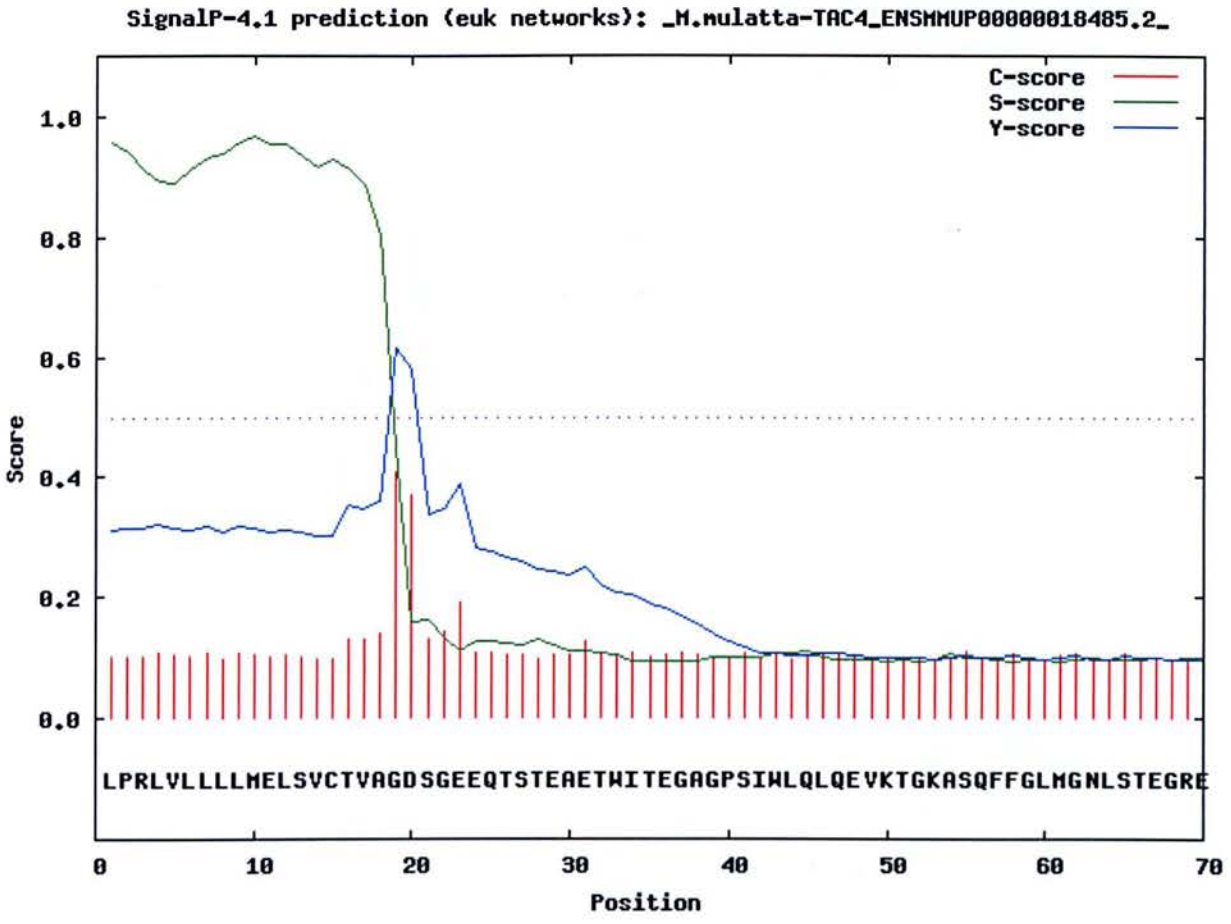
Name=_E.caballus-TAC4_ENSECAP00000012476.1_ SP='YES' Cleavage site between pos. 19 and 20: AVA-GD

>_P.Capensis-TAC4_ENSPCAP00000005735.1_



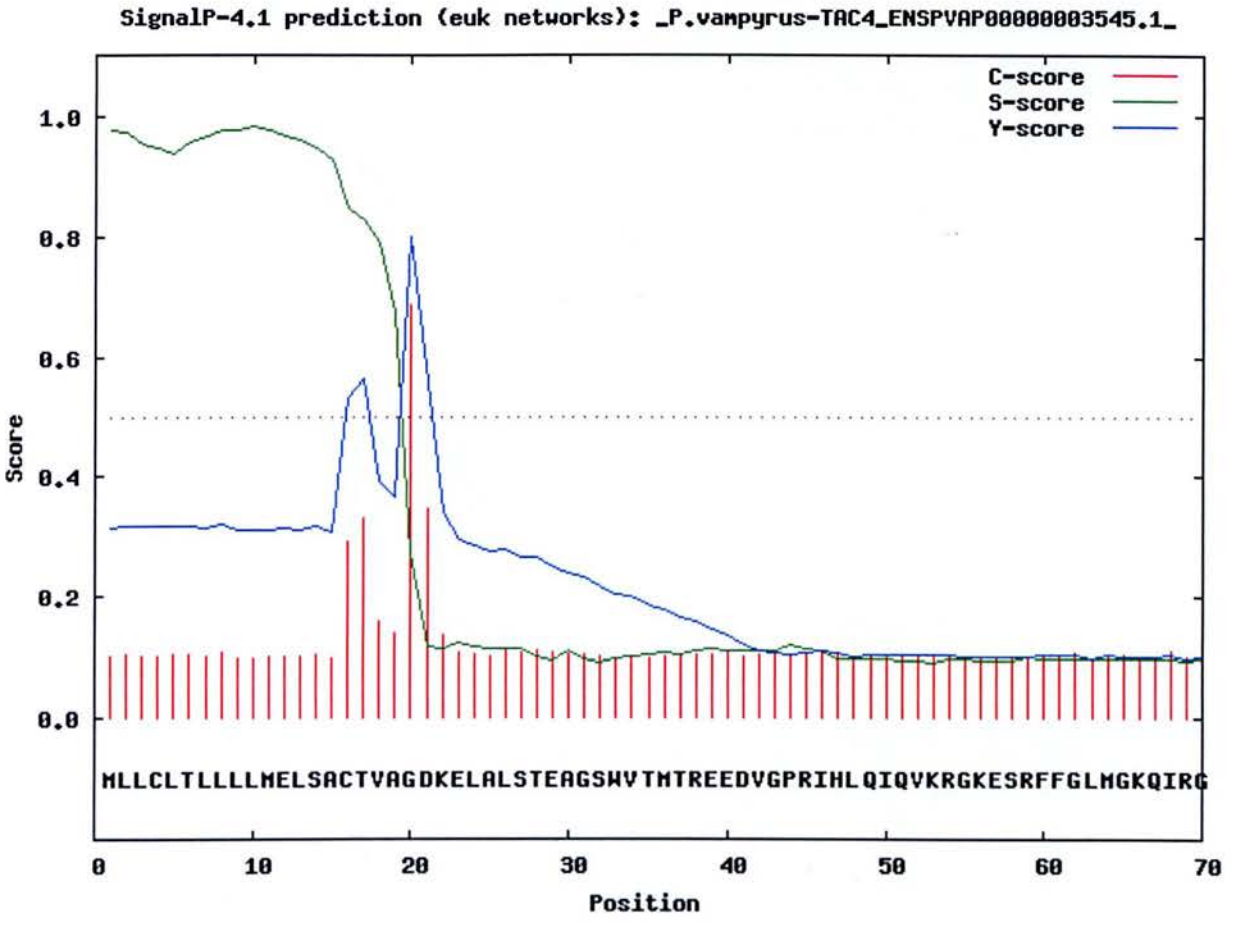
Name=_P.Capensis-TAC4_ENSPCAP00000005735.1_ SP='YES' Cleavage site between pos. 19 and 20: GVA-RD

>_M.mulatta-TAC4_ENSMMUP00000018485.2_



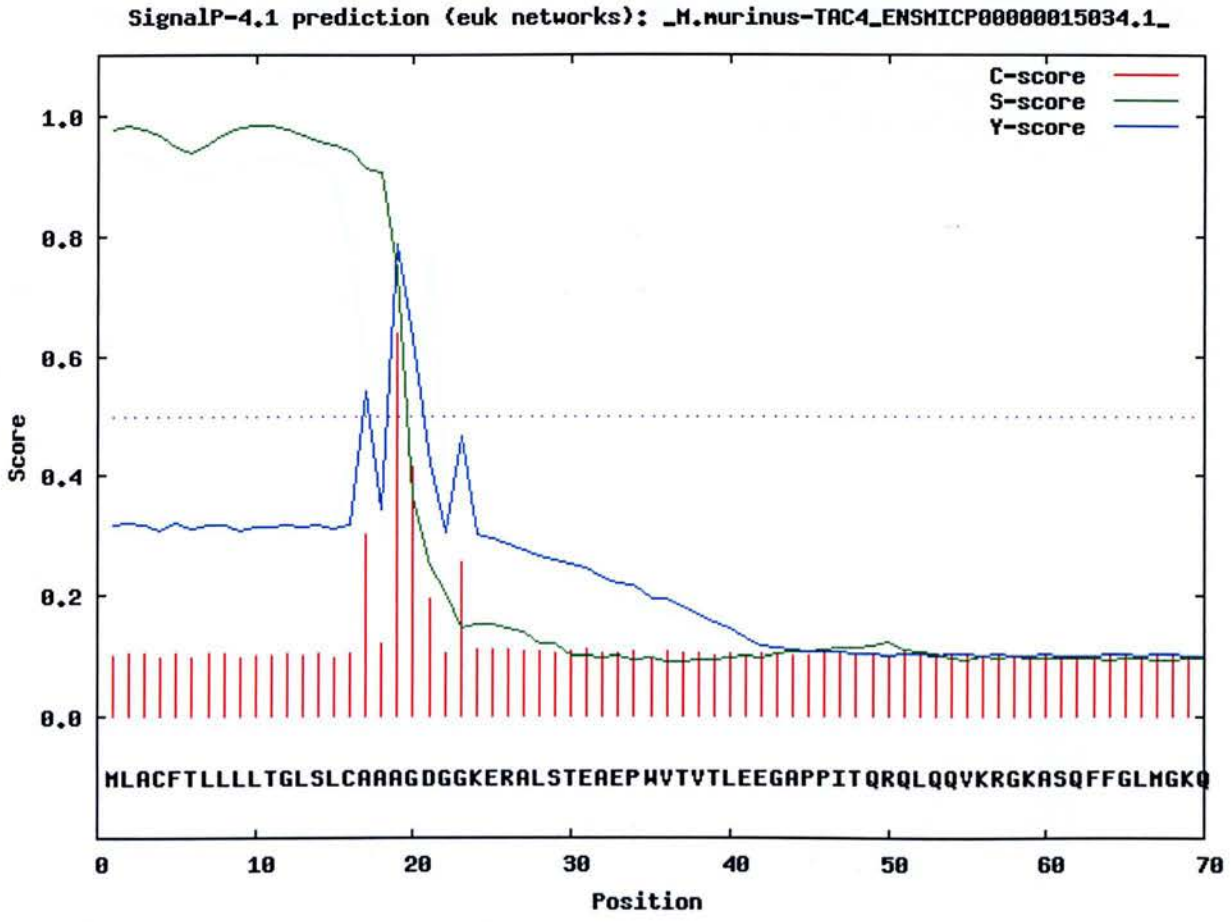
Name=_M.mulatta-TAC4_ENSMMUP00000018485.2_ SP='YES' Cleavage site between pos. 18 and 19: TVA-GD

>_P.vampyrus-TAC4_ENSPVAP00000003545.1_



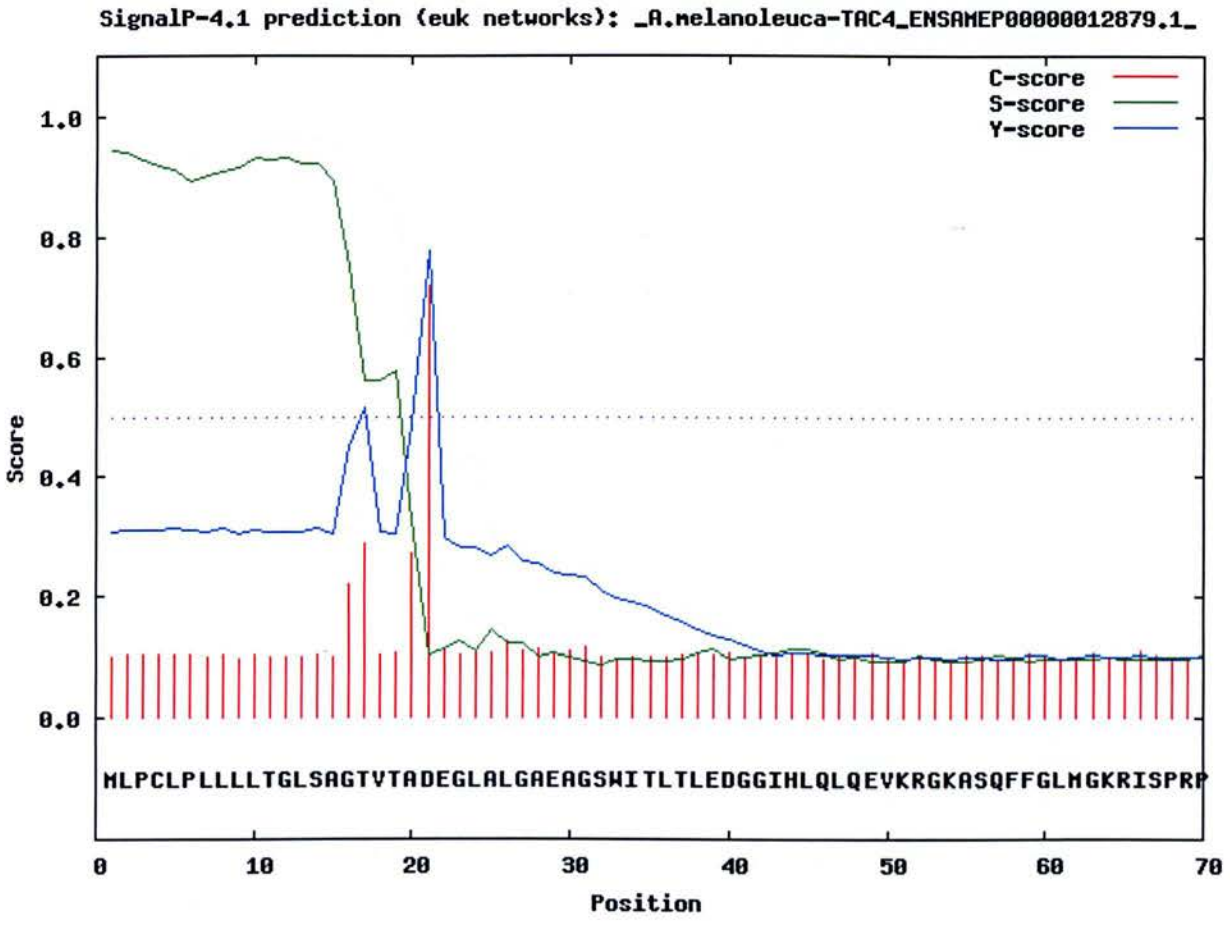
Name=_P.vampyrus-TAC4_ENSPVAP00000003545.1_ SP='YES' Cleavage site between pos. 19 and 20: TVA-GD

>_M.murinus-TAC4_ENSMICP00000015034.1_



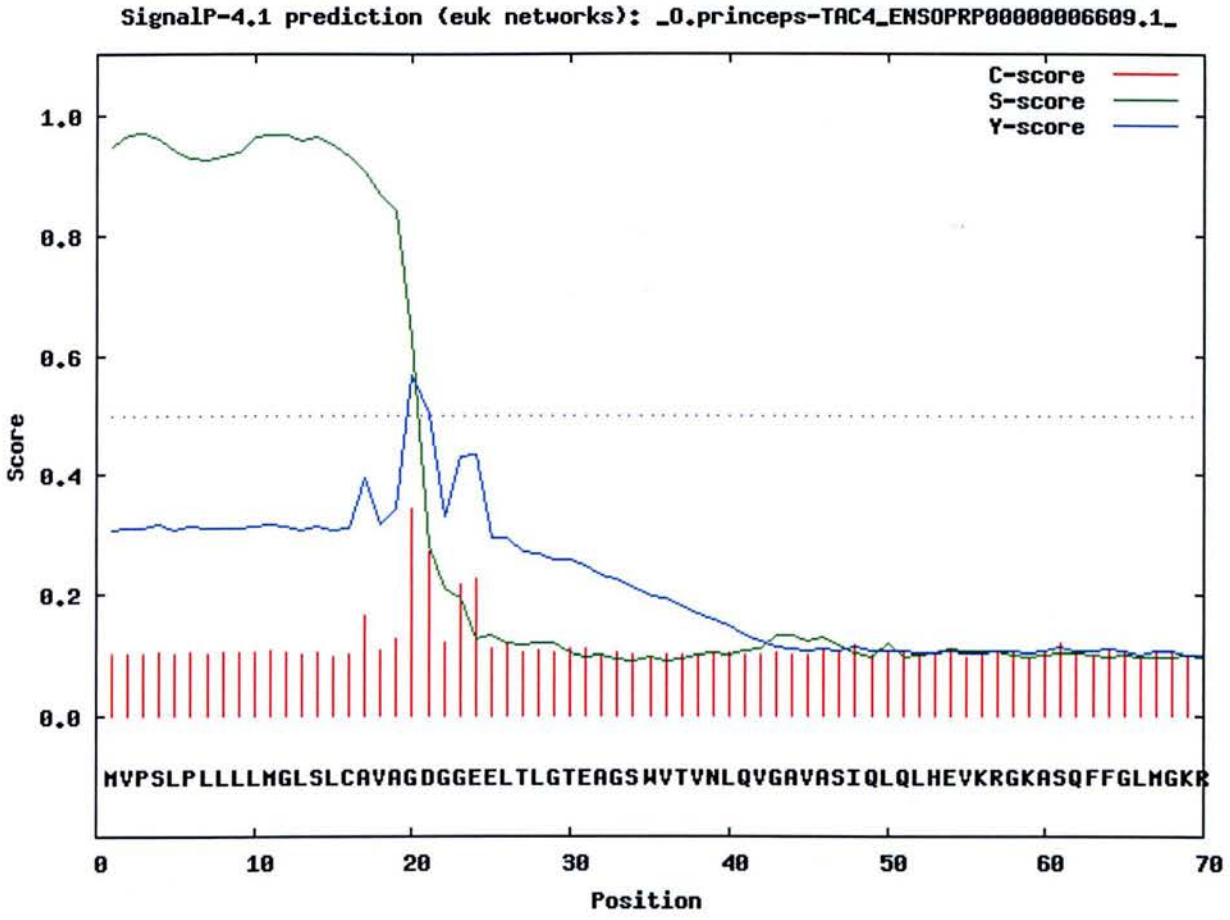
Name=_M.murinus-TAC4_ENSMICP00000015034.1_ SP='YES' Cleavage site between pos. 18 and 19: CAA-AG

>_A.melanoleuca-TAC4_ENSAMEP00000012879.1_



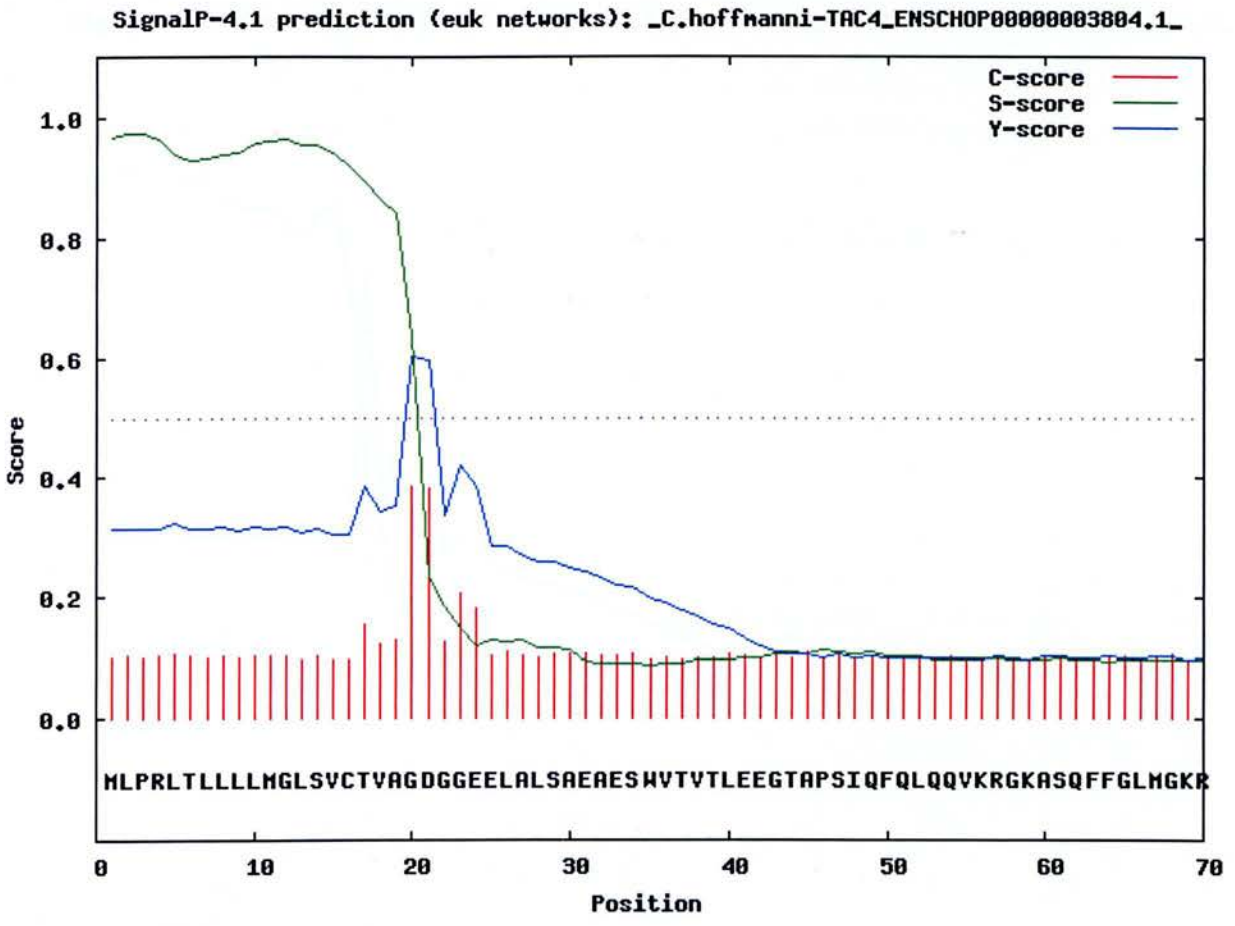
Name=_A.melanoleuca-TAC4_ENSAMEP00000012879.1_ SP='YES' Cleavage site between pos. 20 and 21: VTA-DE

>_O.princeps-TAC4_ENSOPRP00000006609.1_



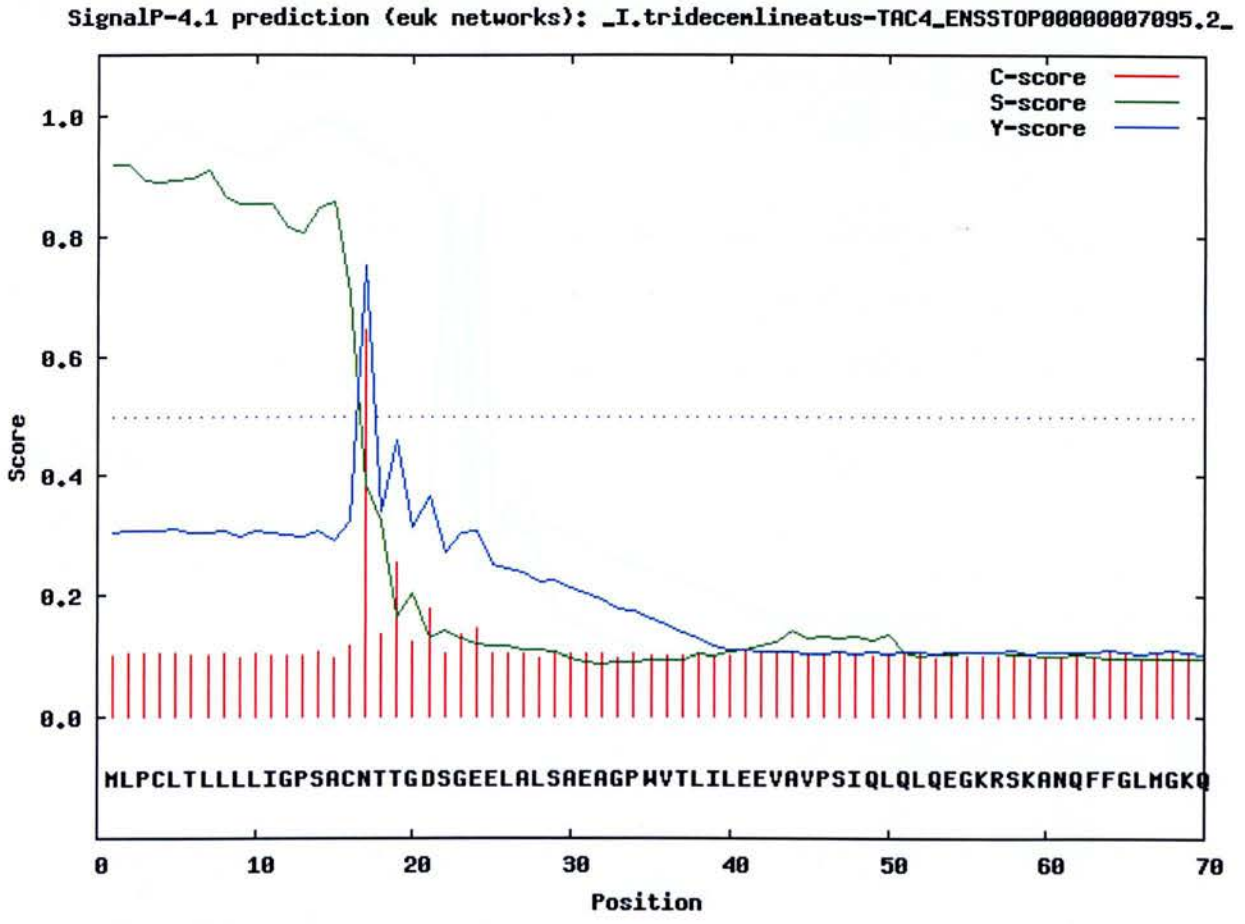
Name=_O.princeps-TAC4_ENSOPRP00000006609.1_ SP='YES' Cleavage site between pos. 19 and 20: AVA-GD

>_C.hoffmanni-TAC4_ENSCHOP00000003804.1_



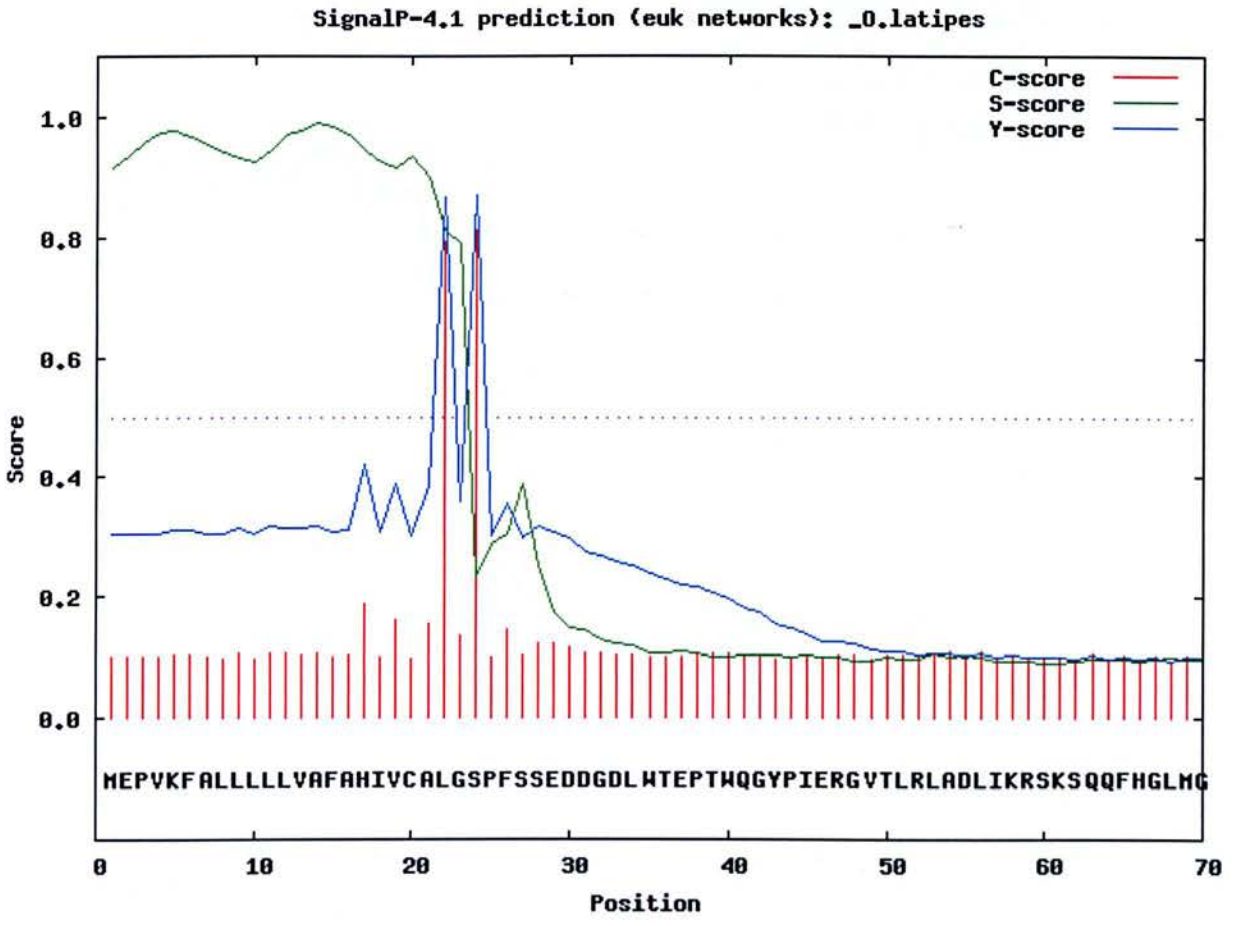
Name=_C.hoffmanni-TAC4_ENSCHOP00000003804.1_ SP='YES' Cleavage site between pos. 19 and 20: TVA-GD

>_I.tridecemlineatus-TAC4_ENSSTOP00000007095.2_



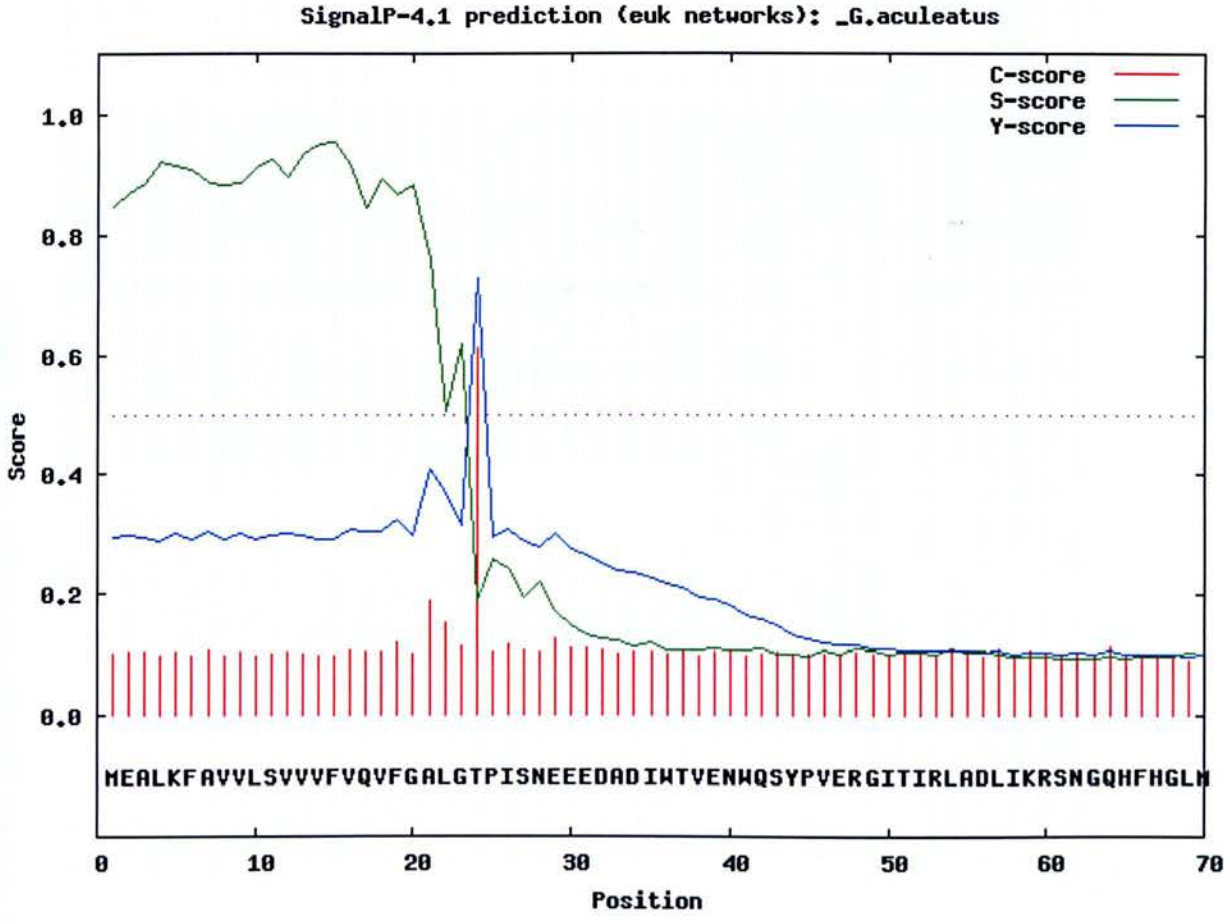
Name=_I.tridecemlineatus-TAC4_ENSSTOP00000007095.2_ SP='YES' Cleavage site between pos. 16 and 17: SAC-NT

>_O.latipes _DK025687_



Name=_O.latipes SP='YES' Cleavage site between pos. 23 and 24: ALG-SP

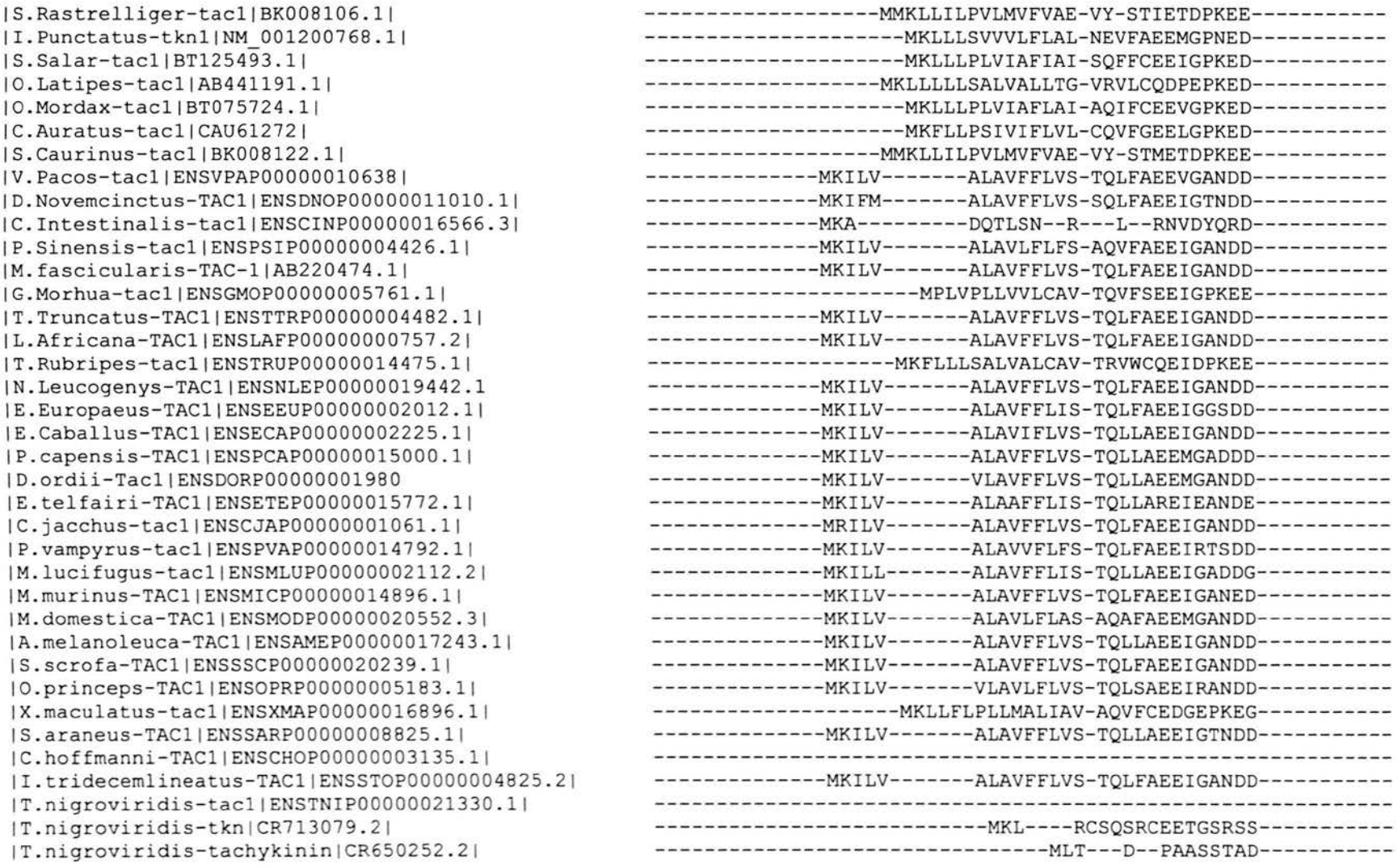
>_G.aculeatus _DV007667_



Name=_G.aculeatus SP='YES' Cleavage site between pos. 23 and 24: ALG-TP

Appendix 3. Multiple sequence alignment of the 176 TAC precursors using Clustal omega.

```
|HUMAN-TAC1|HSU37529|                          ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|G.Gorilla-TAC1|XM_004045800.1|                ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|M.Mulatta-TAC1|ENSMMUP00000035078.1           ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|B.Taurus-TAC1|BC151422.1|                     ----------MKILV-------AVAVIFFIS-TQLSAEEIGANDD----------
|O.Aries-TAC1|NM_001082596.1|                  ----------MKILV-------ALAAIFLVS-TQLSAEEIGANDD----------
|P.Troglodytes-TAC1|XM_001171112.2|           ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|P.Abelii-TAC1|XM_002818228.1|                ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|P.Anubis-TAC1|XM_003896287.1|                ----------MKILV-------ALAVFFLVS-TQLFAEEMGANDD----------
|P.Paniscus-TAC1|XM_003809689.1|              ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|O.Garnettii-TAC1|XM_003782698.1|             ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|C.Porcellus-TAC1|NM_001172899.1|             ----------MKILV-------AVAVFFLVS-TQLSAEEIGANDD----------
|C.Lupus-familiaris-TAC1|XM_532472.3|         ----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
|O.Cuniculus-TAC1|X62994.1|                   ----------MKILV-------ALAVLALVS-TQLSAEDIRANDD----------
|F.Catus-TAC1|XM_003982791.1|                 ----------MKIIV-------ALAVFFLIS-TQLFAEEIGDNDD----------
|S.Harrisii-TAC1|XM_003772510.1|              ----------MKLLV-------ALAVLFLAS-AQAFAEETGANDD----------
|R.Norvegicus-Tac1|NM_012666.2|              ----------MKILV-------AVAVFFLVS-TQLFAEEIGANDD----------
|M.Musculus-Tac1|BC117081.1|                  ----------MKILV-------AVAVFFLVS-TQLFAEEIDANDD----------
|M.Putorius-TAC1|JP019158.1|                  ----------MKILV-------ALAVFFLVS-TQLFAEEIGTSDD----------
|T.belangeri-TAC1|Z50786.1|                   ----------MKILV-------ALAVFFLVS-SQLFAEEIGANDD----------
|S.Boliviensis-TAC1|XM_003921239.1|           ----------MKILV-------ALAVFFLVS-TQLFAEEIGTNDD----------
|T.Guttata-TAC1|XM_002197398.1|               ----------MRLPL-------AFAVLLLAS-SQALGEEMGATDD----------
|G.Gallus-TAC1|BK008126.1|                    ----------MRLPL-------AFTVLLLAS-AQALADEMAAPDD----------
|X.Laevis-tac1|NM_001096537.1|                ---------------------MKILVAFAVILLIS-AQVFAAEIGLNED----------
|X.Tropicalis-tac1|BC155674.1|                ----------MKILV-------AFAVILLVS-AQVFAAEIGFNED----------
|Hyla.simplex-tac1|HM747308.1|                ---------------------MKLILPFAVIMLVS-AQVFAAEIGLDE----------
|O.Vulgaris-OctTK-I|AB085916.1|               ----------MIRVGLILCCIFIA-------G---V--FEASS-AD----------
|O.Vulgaris-OctTK-II|AB085917.1|              ----------MIRVGLILCCIFIV-------G---V--FEASS-AD----------
|A.Aegypti-sialokininI|AF108102.1|            ----------MNMFITVQIVIVLVLAVL-SEAAS---L--PTATETKD----------
|C.parva-TAC1|FJ696706.1|                     ------------------------------------------------DD----------
|G.aculeatus-tac1|ENSGACP00000000114.1|       --------------------MKFVILPLLMFFCAV-AQVFCEENEPKEE----------
|M.Auratus-tkn-I|X80662.1|                    ----------MKILV-------AVAVFFLVS-TQLSAEEIGANDD----------
|D.Rerio-tac1|BK008124.1|                     ---------------------MKFILPTVVIFVVL-CQVFGEELGPKED----------
|O.Mykiss-tac1|BK008119.1|                    ---------------------MKLLLPLVIAFLAI-AQVFCEEIGPKED----------
|S.Fontinalis-tac1|BK008120.1|               ---------------------MKLLLPLVIAFLAI-AQVFCEEIGPKED----------
|A.Fimbria-tac1|JO691854.1|                   -------------------MMKILVLPVLMAFFAV-AQVFCEENDPKEE----------
```


S.Rastrelliger-tac1	BK008106.1
I.Punctatus-tkn1	NM_001200768.1
S.Salar-tac1	BT125493.1
O.Latipes-tac1	AB441191.1
O.Mordax-tac1	BT075724.1
C.Auratus-tac1	CAU61272
S.Caurinus-tac1	BK008122.1
V.Pacos-tac1	ENSVPAP00000010638
D.Novemcinctus-TAC1	ENSDNOP00000011010.1
C.Intestinalis-tac1	ENSCINP00000016566.3
P.Sinensis-tac1	ENSPSIP00000004426.1
M.fascicularis-TAC-1	AB220474.1
G.Morhua-tac1	ENSGMOP00000005761.1
T.Truncatus-TAC1	ENSTTRP00000004482.1
L.Africana-TAC1	ENSLAFP00000000757.2
T.Rubripes-tac1	ENSTRUP00000014475.1
N.Leucogenys-TAC1	ENSNLEP00000019442.1
E.Europaeus-TAC1	ENSEEUP00000002012.1
E.Caballus-TAC1	ENSECAP00000002225.1
P.capensis-TAC1	ENSPCAP00000015000.1
D.ordii-Tac1	ENSDORP00000001980
E.telfairi-TAC1	ENSETEP00000015772.1
C.jacchus-tac1	ENSCJAP00000001061.1
P.vampyrus-tac1	ENSPVAP00000014792.1
M.lucifugus-tac1	ENSMLUP00000002112.2
M.murinus-TAC1	ENSMICP00000014896.1
M.domestica-TAC1	ENSMODP00000020552.3
A.melanoleuca-TAC1	ENSAMEP00000017243.1
S.scrofa-TAC1	ENSSSCP00000020239.1
O.princeps-TAC1	ENSOPRP00000005183.1
X.maculatus-tac1	ENSXMAP00000016896.1
S.araneus-TAC1	ENSSARP00000008825.1
C.hoffmanni-TAC1	ENSCHOP00000003135.1
I.tridecemlineatus-TAC1	ENSSTOP00000004825.2
T.nigroviridis-tac1	ENSTNIP00000021330.1
T.nigroviridis-tkn	CR713079.2
T.nigroviridis-tachykinin	CR650252.2
----------MMKLLILPVLMVFVAE-VY-STIETDPKEE----------
----------MKLLLSVVVLFLAL-NEVFAEEMGPNED----------
----------MKLLLPLVIAFIAI-SQFFCEEIGPKED----------
----------MKLLLLLSALVALLTG-VRVLCQDPEPKED----------
----------MKLLLPLVIAFLAI-AQIFCEEVGPKED----------
----------MKFLLPSIVIFLVL-CQVFGEELGPKED----------
----------MMKLLILPVLMVFVAE-VY-STMETDPKEE----------
----------MKILV-------ALAVFFLVS-TQLFAEEVGANDD----------
----------MKIFM-------ALAVFFLVS-SQLFAEEIGTNDD----------
----------MKA---------DQTLSN--R---L--RNVDYQRD----------
----------MKILV-------ALAVLFLFS-AQVFAEEIGANDD----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MPLVPLLVVLCAV-TQVFSEEIGPKEE----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MKFLLLSALVALCAV-TRVWCQEIDPKEE----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MKILV-------ALAVFFLIS-TQLFAEEIGGSDD----------
----------MKILV-------ALAVIFLVS-TQLLAEEIGANDD----------
----------MKILV-------ALAVFFLVS-TQLLAEEMGADDD----------
----------MKILV-------VLAVFFLVS-TQLLAEEMGANDD----------
----------MKILV-------ALAAFFLIS-TQLLAREIEANDE----------
----------MRILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MKILV-------ALAVVFLFS-TQLFAEEIRTSDD----------
----------MKILL-------ALAVFFLIS-TQLLAEEIGADDG----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANED----------
----------MKILV-------ALAVLFLAS-AQAFAEEMGANDD----------
----------MKILV-------ALAVFFLVS-TQLLAEEIGANDD----------
----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------
----------MKILV-------VLAVLFLVS-TQLSAEEIRANDD----------
----------MKLLFLPLLMALIAV-AQVFCEDGEPKEG----------
----------MKILV-------ALAVFFLVS-TQLLAEEIGTNDD----------

----------MKILV-------ALAVFFLVS-TQLFAEEIGANDD----------

----------MKL----RCSQSRCEETGSRSS----------
----------MLT---D--PAASSTAD----------

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|M.gallopavo-TAC1|ENSMGAP00000010069.2|  ----------MRLPL-------AFTVLLLAS-AQALADEMAAPDD-----------
|M.eugenii-TAC1|ENSMEUP00000003075.1|   ----------MKILV-------ALAVLFLAS-AQAFADEMGANDD-----------
|Human-TAC3|AF537113.1|                  ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|Gorilla-TAC3|XM_004053407.1|           ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|P.troglodytes-TAC3|XM_003313573.1|     ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|M.mulatta-TAC3|XM_001115535.1|         ----------MRIML-LFTAILTF--SLAQS-FGAVCKEPQE--------------
|P.anubis-TAC3|XM_003906628.1|          ----------MRIML-LFTAILTF--SLAQS-FGAVCKEPQE--------------
|P.paniscus-TAC3|XM_003824897.1|       ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|P.abelii-TAC3|XM_003778069.1|          ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|O.garnettii-TAC3|XM_003790546.1|       ----------MRTTL-LFIVILAL--SLAWS-FGATCEESQE--------------
|S.boliviensis-TAC3|XM_003926696.1|     ----------MRITL-LFTAILAF--SLAQS-FGAICNESQK--------------
|N.leucogenys-TAC3|XM_003252783.1|      ----------MRIML-LFTAILAF--SLAQS-FGAVCKEPQE--------------
|E.caballus-TAC3|XM_001488415.3|        ----------MQSTL-LFAAILAL--SLAQS-FGAVCEESQE--------------
|L.africana-TAC3|XM_003405564.1|        ----------MRSTL-LFAAILAV--SLIQS-CRAACEESQE--------------
|A.melanoleuca-TAC3|XM_002916001.1|    ----------MRRSL-LFAAILAI--SLARS-LGAVCEDSQE--------------
|B.taurus-TAC3|NM_181017.1|            ----------MRSTL-LFAVILAL--SSARS-LGAVCEESQE--------------
|O.aries-TAC3|XM_004009563.1|          MSWHKASISERLCGFREVT-KFAKVNSFLLAIGPP-GGSRCSSSKRPEPVQKTQSECQTK
|C.hircus-TAC3|AB499062.1|             ----------------------AVILAL--SLARS-FGAVCEESQE--------------
|S.scrofa-TAC3|AY758208.1|             ----------MRSTL-LFVAILAL--SLAWS-LGAACEESQE--------------
|C.porcellus-TAC3|XM_003475961.1|     ----------MRSTL-LFAAILVL--SLCSS-FGDVCEESQE--------------
|C.lupus-familiaris-TAC3|XM_843938.2|  ----------MRSAL-LFAAILAI--SLASS-FGAVCEDSQE--------------
|F.catus-TAC3|XM_003988924.1|          ----------MRSAL-LFAAILAI--SLVHG-LGAVCEDSRE--------------
|C.jacchus-TAC3|XM_002752636.2|        ----------MRITL-LFTAILAF--SLAQS-FGAICNESQK--------------
|M.musculus-tac2|BC031348.1|           ----------MRSAM-LFAAVLAL--SLAWT-FGAVCEEPQG--------------
|A.mississippiensis-TAC3|BK008115.1|   ----------MRGHL-VLTVLLSL--AAWRL-CQAECRDPT---------------
|X.tropicalis-tac3|BK008110.1|         ----------MKNLAVGLAVLILVAV----RTCRGGCGDSQGEQG-----------
|O.mordax-tac3|BK008111.1|            ----------MRRGLLLVALFLVMKL----RLCQSGCEEPGSRRS-----------
|B.saida-tac3|BK008109.1|              ----------MRISLVLVTLFLVMKL----RSTQSRCEEPGSRRP-----------
|O.latipes-tac3|BK008114.1|            ----------MRSGLLLVTLFIFVKL----RYSQSRCEETASRRS-----------
|G.morhua-tac3|BK008107.1|             ----------MRISLVLVTLFLVMKL----RSTQSRCEEPGSRRP-----------
|P.promelas-tac3|BK008100.1|           ----------------RGLVLLFLVLVLET----RWSESSCQQSESQRS-----------
|I.punctatus-tac3|BK008101.1|          ----------MYRGLLLVFLILLVET----RLSESSCAEQETQRS-----------
|S.salar-tac3A|BK008102.1|             ----------MKNGLLLVTLFLVMKL----RSSQSSCEEPAAGAH-----------
|D.mawsoni-tac3|BK008104.1|            ----------MKGGVLLVTLFLIMKL----RYSHSRCEEPGTRRS-----------
|S.rastrelliger-tac3|BK008105.1|      ----------MRRGLLLVFLFLIMKL----RYSQSRCEEPGARRS-----------
|D.rerio-tac3a|JN392856.1|             ----------MYRGLVLLFLVLVLET----RWSESSCQQSESQRS-----------
|D.rerio-tac3B|JN392857.1|             ----------MSCGWLLALLVHVLLLLACPRLSRSALDYSF---------------
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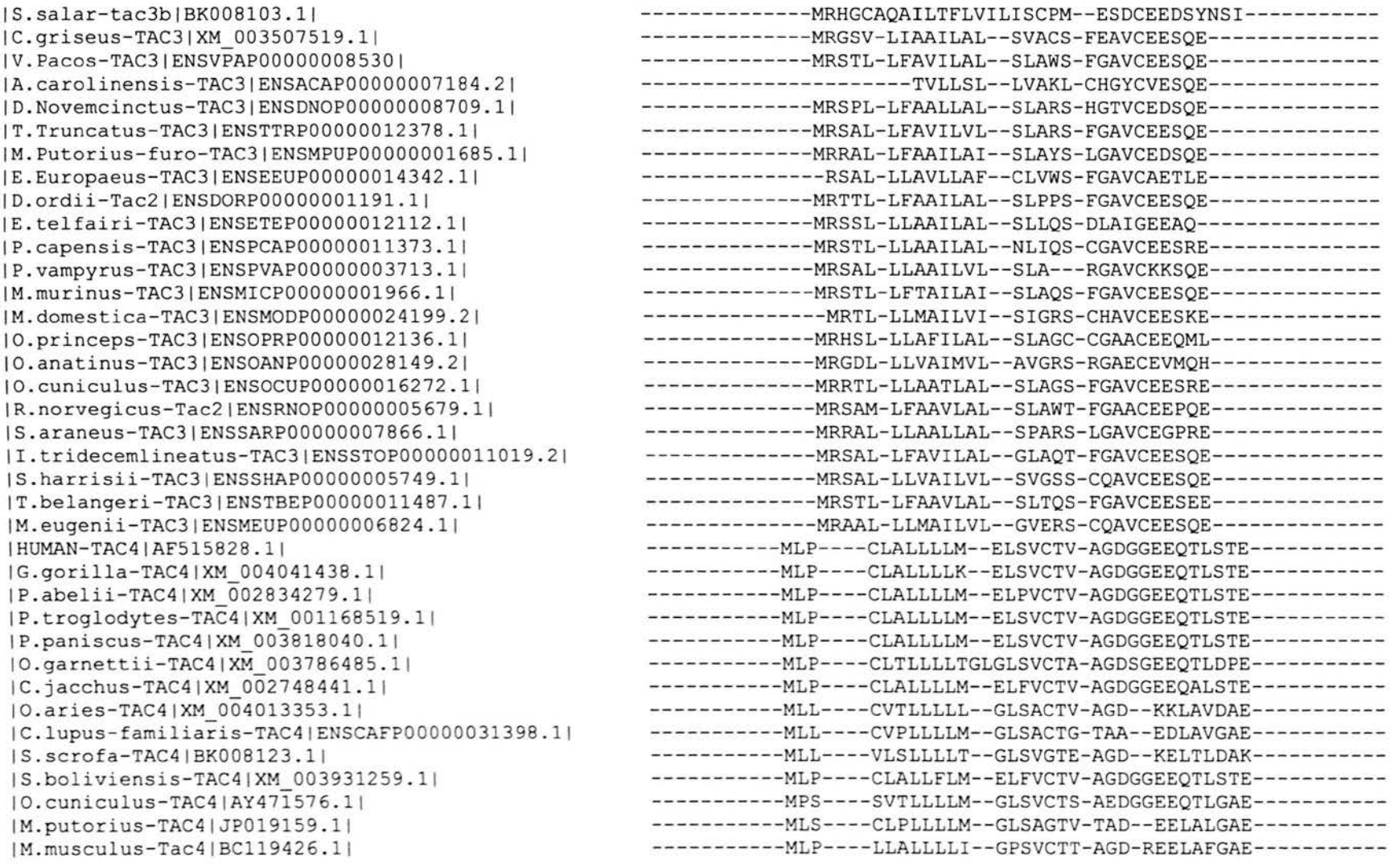
S.salar-tac3b	BK008103.1
C.griseus-TAC3	XM_003507519.1
V.Pacos-TAC3	ENSVPAP00000008530
A.carolinensis-TAC3	ENSACAP00000007184.2
D.Novemcinctus-TAC3	ENSDNOP00000008709.1
T.Truncatus-TAC3	ENSTTRP00000012378.1
M.Putorius-furo-TAC3	ENSMPUP00000001685.1
E.Europaeus-TAC3	ENSEEUP00000014342.1
D.ordii-Tac2	ENSDORP00000001191.1
E.telfairi-TAC3	ENSETEP00000012112.1
P.capensis-TAC3	ENSPCAP00000011373.1
P.vampyrus-TAC3	ENSPVAP00000003713.1
M.murinus-TAC3	ENSMICP00000001966.1
M.domestica-TAC3	ENSMODP00000024199.2
O.princeps-TAC3	ENSOPRP00000012136.1
O.anatinus-TAC3	ENSOANP00000028149.2
O.cuniculus-TAC3	ENSOCUP00000016272.1
R.norvegicus-Tac2	ENSRNOP00000005679.1
S.araneus-TAC3	ENSSARP00000007866.1
I.tridecemlineatus-TAC3	ENSSTOP00000011019.2
S.harrisii-TAC3	ENSSHAP00000005749.1
T.belangeri-TAC3	ENSTBEP00000011487.1
M.eugenii-TAC3	ENSMEUP00000006824.1
HUMAN-TAC4	AF515828.1
G.gorilla-TAC4	XM_004041438.1
P.abelii-TAC4	XM_002834279.1
P.troglodytes-TAC4	XM_001168519.1
P.paniscus-TAC4	XM_003818040.1
O.garnettii-TAC4	XM_003786485.1
C.jacchus-TAC4	XM_002748441.1
O.aries-TAC4	XM_004013353.1
C.lupus-familiaris-TAC4	ENSCAFP00000031398.1
S.scrofa-TAC4	BK008123.1
S.boliviensis-TAC4	XM_003931259.1
O.cuniculus-TAC4	AY471576.1
M.putorius-TAC4	JP019159.1
M.musculus-Tac4	BC119426.1
----------MRHGCAQAILTFLVILISCPM--ESDCEEDSYNSI----------
----------MRGSV-LIAAILAL--SVACS-FEAVCEESQE----------
----------MRSTL-LFAVILAL--SLAWS-FGAVCEESQE----------
----------TVLLSL--LVAKL-CHGYCVESQE----------
----------MRSPL-LFAALLAL--SLARS-HGTVCEDSQE----------
----------MRSAL-LFAVILVL--SLARS-FGAVCEESQE----------
----------MRRAL-LFAAILAI--SLAYS-LGAVCEDSQE----------
----------RSAL-LLAVLLAF--CLVWS-FGAVCAETLE----------
----------MRTTL-LFAAILAL--SLPPS-FGAVCEESQE----------
----------MRSSL-LLAAILAL--SLLQS-DLAIGEEAQ----------
----------MRSTL-LLAAILAL--NLIQS-CGAVCEESRE----------
----------MRSAL-LLAAILVL--SLA---RGAVCKKSQE----------
----------MRSTL-LFTAILAI--SLAQS-FGAVCEESQE----------
----------MRTL-LLMAILVI--SIGRS-CHAVCEESKE----------
----------MRHSL-LLAFILAL--SLAGC-CGAACEEQML----------
----------MRGDL-LLVAIMVL--AVGRS-RGAECEVMQH----------
----------MRRTL-LLAATLAL--SLAGS-FGAVCEESRE----------
----------MRSAM-LFAAVLAL--SLAWT-FGAACEEPQE----------
----------MRRAL-LLAALLAL--SPARS-LGAVCEGPRE----------
----------MRSAL-LFAVILAL--GLAQT-FGAVCEESQE----------
----------MRSAL-LLVAILVL--SVGSS-CQAVCEESQE----------
----------MRSTL-LFAAVLAL--SLTQS-FGAVCEESEE----------
----------MRAAL-LLMAILVL--GVERS-CQAVCEESQE----------
----------MLP----CLALLLLM--ELSVCTV-AGDGGEEQTLSTE----------
----------MLP----CLALLLLK--ELSVCTV-AGDGGEEQTLSTE----------
----------MLP----CLALLLLM--ELPVCTV-AGDGGEEQTLSTE----------
----------MLP----CLALLLLM--ELSVCTV-AGDGGEEQTLSTE----------
----------MLP----CLALLLLM--ELSVCTV-AGDGGEEQTLSTE----------
----------MLP----CLTLLLLTGLGLSVCTA-AGDSGEEQTLDPE----------
----------MLP----CLALLLLM--ELFVCTV-AGDGGEEQALSTE----------
----------MLL----CVTLLLLL--GLSACTV-AGD--KKLAVDAE----------
----------MLL----CVPLLLLM--GLSACTG-TAA--EDLAVGAE----------
----------MLL----VLSLLLLT--GLSVGTE-AGD--KELTLDAK----------
----------MLP----CLALLFLM--ELFVCTV-AGDGGEEQTLSTE----------
----------MPS----SVTLLLLM--GLSVCTS-AEDGGEEQTLGAE----------
----------MLS----CLPLLLLM--GLSAGTV-TAD--EELALGAE----------
----------MLP----LLALLLLI--GPSVCTT-AGD-REELAFGAE----------

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|R.norvegicus-Tac4|AY471575.1|                          ----------MLP----LLALFLLI--GPAVSTT-TRD-REDLTFGAE----------
|O.mykiss-tac4|BK008118.1|                              ----------MDIWKFQLVIVT-LYSLV-YTCQG---L--S---FSVD----------
|O.mordax-tac4a|BK008112.1|                             ----------MEIWKLQLVVLT-LFAMV-YTYEG---L--F---FSVD----------
|B.saida-tac4|BK008108.1|                               ----------MDNWRSLVAVLV-FFALL-EIYHG---F--P---FNVN----------
|R.chensinensis-ranachensinin|HE863666.1|              ----------MFTLKKSLLLLFFLGTISLSLCEE-EKRYANEEA---------------
|V.Pacos-tac4|ENSVPAP00000000575.1|                     ------------------------------------------------------------
|D.Novemcinctus-TAC4|ENSDNOP00000004237.1|             ----------MLL----RLTLLLLT--GLSAYPV-AGNGGEELALSIE----------
|F.Catus-TAC4|ENSFCAP00000002434.2|                     ----------MLP----CLPLLLLM--GLPACTG-TVD--EKLALGAE----------
|G.Morhua-tac4|ENSGMOP00000003442.1|                    ----------MEMLKFIVLLLVAVFAQVYTVVLG---S--PLSSE-VD----------
|T.Truncatus-TAC4|ENSTTRP00000000445.1|                 ----------MLL----CLPPPLLT--GLSACTV-AGD--KELPLDAE----------
|L.Africana-TAC4|ENSLAFP00000012829.3|                  ----------MLL----CVTLILMM--GLSVCTV-AGDSGEEVALSTE----------
|E.Europaeus-TAC4|ENSEEUP00000005053.1|                 ----------MLP----CLTLLLLV--GLPACTR-AED--KELVLSTE----------
|E.caballus-TAC4|ENSECAP00000012476.1|                  ----------MLL----SLTLLLLM--GLSTCAV-AGD--EELTLSAE----------
|P.Capensis-TAC4|ENSPCAP00000005735.1|                  ----------MLL----HLTLILLM--GLSVCGV-ARDSREEL--AIE----------
|D.Ordii-Tac4|ENSDORP00000013872.1|                     ------------------------------------------------------------
|E.telfairi-Tac4|ENSETEP00000010247.1|                  ------------------------------------------------------------
|M.mulatta-TAC4|ENSMMUP00000018485.2|                   ------------LP----RLVLLLLM--ELSVCTV-AGDSGEEQT-STE----------
|P.vampyrus-TAC4|ENSPVAP00000003545.1|                  ----------MLL----CLTLLLLM--ELSACTV-AGD--KELALSTE----------
|M.murinus-TAC4|ENSMICP00000015034.1|                   ----------MLA----CFTLLLLT--GLSLCAA-AGDGGKERALSTE----------
|A.melanoleuca-TAC4|ENSAMEP00000012879.1|               ----------MLP----CLPLLLLT--GLSAGTV-TAD--EGLALGAE----------
|O.princeps-TAC4|ENSOPRP00000006609.1|                  ----------MVP----SLPLLLLM--GLSLCAV-AGDGGEELTLGTE----------
|C.hoffmanni-TAC4|ENSCHOP00000003804.1|                 ----------MLP----RLTLLLLM--GLSVCTV-AGDGGEELALSAE----------
|I.tridecemlineatus-TAC4|ENSSTOP00000007095.2|         ----------MLP----CLTLLLLI--GPSACNT-TGDSGEELALSAE----------
|T.syrichta-TAC4|ENSTSYP00000010450.1|                  ----------MLP----CLMLXXXX--XXXXXXX-XXXXXXXXXXXXX----------
|T.belangeri-TAC4|ENSTBEP00000002423.1|                 ------------------------------------------------------------
|O.latipes|DK025687|                                    ----------MEPVKFALLLLLVAFAHIVCA-LG---S--PFSSE-DD----------
|G.aculeatus|DV007667|                                  ----------MEALKFAVVLSVVVFVQVFGA-LG---T--PISNEEED----------
|D.rerio|XM_001923424|                                  ----------MDIFKLSAL------AFILYLQLH---N--AGASPSEE----------
|O.niloticu|GR699402|                                   ------------------------------------------------------------
|L.Calcarifer|DQ290174|                                 ------------------------------------------------------------
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|HUMAN-TAC1|HSU37529|                          --------LNYWSDWYDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|G.Gorilla-TAC1|XM_004045800.1|                --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|M.Mulatta-TAC1|ENSMMUP00000035078.1           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|B.Taurus-TAC1|BC151422.1|                     --------FNYWSDWSDSDQIKEEM--PEP-FEH-----LLQ-RIARR----------P-
|O.Aries-TAC1|NM_001082596.1|                  --------LNYWSDWSDSDQIKEEM--PEP-FEH-----LLQ-RIARR----------P-
|P.Troglodytes-TAC1|XM_001171112.2|           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|P.Abelii-TAC1|XM_002818228.1|                --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|P.Anubis-TAC1|XM_003896287.1|                --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|P.Paniscus-TAC1|XM_003809689.1|              --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|O.Garnettii-TAC1|XM_003782698.1|             --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|C.Porcellus-TAC1|NM_001172899.1|             --------LNYWSDWSDSDQIKEAL--PEP-FEH-----ILQ-RIARR----------P-
|C.Lupus-familiaris-TAC1|XM_532472.3|         --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|O.Cuniculus-TAC1|X62994.1|                   --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|F.Catus-TAC1|XM_003982791.1|                --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|S.Harrisii-TAC1|XM_003772510.1|              --------LNYWADWSDSDQIKEEL--SEP-FEH-----FLQ-RIARR----------P-
|R.Norvegicus-Tac1|NM_012666.2|               --------LNYWSDWSDSDQIKEAM--PEP-FEH-----LLQ-RIARR----------P-
|M.Musculus-Tac1|BC117081.1|                  --------LNYWSDWSDSDQIKEAM--PEP-FEH-----LLQ-RIARR----------P-
|M.Putorius-TAC1|JP019158.1|                  --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|T.belangeri-TAC1|Z50786.1|                   --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|S.Boliviensis-TAC1|XM_003921239.1|           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|T.Guttata-TAC1|XM_002197398.1|               --------LSYWSDWSDSDQAKEEL--PLP-LEH-----FLQ-RMARR----------P-
|G.Gallus-TAC1|BK008126.1|                    --------LSYWADWADGEQ-KEEL--PLP-LEH-----FLQ-RMARR----------P-
|X.Laevis-tac1|NM_001096537.1|                --------SD-WP---YSDQIQEE--IQGPVIER-----ILQ-RIARK----------P-
|X.Tropicalis-tac1|BC155674.1|                ------------SDWPYSDQIQEDI--QGPVIER-----ILQ-RIARK----------P-
|Hyla.simplex-tac1|HM747308.1|                --------ID-WP---YSDQVQEE--L--PVFEN-----FLQ-RIARK----------P-
|O.Vulgaris-OctTK-I|AB085916.1|               ---------DM----------------------LT------AH-NLIKRSE-----VKPP-
|O.Vulgaris-OctTK-II|AB085917.1|              ---------DI----------------------LT------AH-NLIKRSE-----VKPP-
|A.Aegypti-sialokininI|AF108102.1|            ---------AMDE---GPNQSDEPE--G-SVANTSTEDDDYS-DSLKQDEKYYKVRLLN-
|C.parva-TAC1|FJ696706.1|                     --------LSYWSDWSDSDQIKEEL--PEP-FEH-----VLQ-RMARR----------P-
|G.aculeatus-tac1|ENSGACP00000000114.1|       --------ADYWT---NSNEIQNGW-LANDPFRE-----VLL-RMTRK----------P-
|M.Auratus-tkn-I|X80662.1|                    --------LNYWSDWSDSDQIKEAL--PEP-FEH-----ILQ-RIARR----------P-
|D.Rerio-tac1|BK008124.1|                     --------LDYWT---GNNQIQDEW-IQSDPFRE-----ILR-RMTRK----------P-
|O.Mykiss-tac1|BK008119.1|                    --------LDYWM---ND-QITDEW-LSSDPFGE-----ILR-RMTRK----------P-
|S.Fontinalis-tac1|BK008120.1|                --------LDYWM---ND-AITDEW-LSSDPFGE-----ILR-RMTRK----------P-
|A.Fimbria-tac1|JO691854.1|                   --------ADYWT---SSNDIQDGW-LASDPFRE-----ILR-RMTRK----------P-
|S.Rastrelliger-tac1|BK008106.1|              --------KDYWT---SSNEIQDRW-LSNDPFRE-----ILL-RMTRK----------P-
|I.Punctatus-tkn1|NM_001200768.1|             --------QDYWA---NGSIMQDDWPIQADPFRE-----ILR-RITRK----------P-
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|S.Salar-tac1|BT125493.1|                          --------LDYWT---ND-QITDEW-LSSDPFRE-----ILR-RMTRK----------P-
|O.Latipes-tac1|AB441191.1|                        --------ADYWT---STN-HQDGW-LSSEPLRE-----MLL-RMTRK----------P-
|O.Mordax-tac1|BT075724.1|                         --------PDYWT---NSNQIEDNW-LSTDPFRE-----ILR-RMTRK----------P-
|C.Auratus-tac1|CAU61272|                          --------LDYWT---GSNQVQDEW-LQADPFRE-----IIR-RMTRK----------P-
|S.Caurinus-tac1|BK008122.1|                       --------KDYWT---SSNEIQDRW-LSNDPFRE-----ILL-RMTRK----------P-
|V.Pacos-tac1|ENSVPAP00000010638|                  --------LNYWSDWSDSDQIKEEL--PEP-FEH-----IKQ-RIARR----------P-
|D.Novemcinctus-TAC1|ENSDNOP00000011010.1|        --------VNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|C.Intestinalis-tac1|ENSCINP00000016566.3|        --------DDLRY---LDQLQE----------KR------QR-DLYE--------KNKR-
|P.Sinensis-tac1|ENSPSIP00000004426.1|            --------LNYWSDWSDSDQLKEEL--PLP-FEH-----FLQ-RIARR----------P-
|M.fascicularis-TAC-1|AB220474.1|                 --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|G.Morhua-tac1|ENSGMOP00000005761.1|              --------LDYWT----SNQIQDGW-LAPDPFRE-----ILR-RMTRK----------P-
|T.Truncatus-TAC1|ENSTTRP00000004482.1|          --------LNYWSDWSDRDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|L.Africana-TAC1|ENSLAFP00000000757.2|           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|T.Rubripes-tac1|ENSTRUP00000014475.1|           --------ADYWS----SNRIQDGW-FPNSPLRE-----ILL-RMTRK----------P-
|N.Leucogenys-TAC1|ENSNLEP00000019442.1           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|E.Europaeus-TAC1|ENSEEUP00000002012.1|          --------LNYWSDWSDSDQVKEEL--PQP-FEH-----LLQ-RIARR----------P-
|E.Caballus-TAC1|ENSECAP00000002225.1|           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|P.capensis-TAC1|ENSPCAP00000015000.1|           --------LNYWSYWSDSNQIKEEL--PEP-FEH-----LLP-RITRR----------P-
|D.ordii-Tac1|ENSDORP00000001980                  --------LNYWSDWSDSNQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|E.telfairi-TAC1|ENSETEP00000015772.1|           --------LNYWNDWSDSDQIKEEL--PER-FEH-----LLQ-RIARR----------P-
|C.jacchus-tac1|ENSCJAP00000001061.1|            --------LNYWSDWSDSDQIKXEL--PEP-FEH-----ILQ-RIARR----------P-
|P.vampyrus-tac1|ENSPVAP00000014792.1|           --------LNYWSDWSDNDQIKEEL--SQP-FEH-----LMQ-RIARR----------P-
|M.lucifugus-tac1|ENSMLUP00000002112.2|          --------LNYWSDWSDSDHIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|M.murinus-TAC1|ENSMICP00000014896.1|            --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|M.domestica-TAC1|ENSMODP00000020552.3|          --------LNYWADWSDSDQIKEEL--SEP-FEH-----FLQ-RIARR----------P-
|A.melanoleuca-TAC1|ENSAMEP00000017243.1|        --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|S.scrofa-TAC1|ENSSSCP00000020239.1|             --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|O.princeps-TAC1|ENSOPRP00000005183.1|           --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|X.maculatus-tac1|ENSXMAP00000016896.1|          --------ADYWT---SSNQIQDDW-LTNDPFRE-----ILL-RMTRK----------P-
|S.araneus-TAC1|ENSSARP00000008825.1|            --------LNYWSDWSDGDQIKEEL--PEP-FEH-----VLQ-RMARR----------P-
|C.hoffmanni-TAC1|ENSCHOP00000003135.1|          ----------------------AEL--PEP-FEH-----LLQ-RIARR----------P-
|I.tridecemlineatus-TAC1|ENSSTOP00000004825.2|  --------LNYWSDWSDSDQIKEEL--PEP-FEH-----LLQ-RIARR----------P-
|T.nigroviridis-tac1|ENSTNIP00000021330.1|      ----------------------------SPLRE-----VLL-RMTRK----------P-
|T.nigroviridis-tkn|CR713079.2|                 -----------------SS--ET-----VGLDG-----L-K-NILKR-------YSDL-
|T.nigroviridis-tachykinin|CR650252.2|          --------EERSL---SPAWQDESV--DSSLTNQ------LL-SLMKR----------S-
|M.gallopavo-TAC1|ENSMGAP00000010069.2|         --------LSYWADWADGEQ-KEEL--PLP-LEH-----FLQ-RMARR----------P-
|M.eugenii-TAC1|ENSMEUP00000003075.1|           --------LNYWADWSDSDQIKEEL--SEP-FEH-----FLQ-RIARR----------P-
```

```
|Human-TAC3|AF537113.1|                        -------EVVPGG---GRSK-RDPD------LYQ----------LLQR-------LFKS-
|Gorilla-TAC3|XM_004053407.1|                  -------EVVPGG---GRSK-RDPD------LYQ----------LLQR-------LFKS-
|P.troglodytes-TAC3|XM_003313573.1|            -------EVVPGG---GRSK-RDPD------LYR----------LLQR-------LFKS-
|M.mulatta-TAC3|XM_001115535.1|                -------EMVPGG---GHSK-RDLD------LYQ----------LLQR-------LFKS-
|P.anubis-TAC3|XM_003906628.1|                 -------EMVPGG---GHSK-RDLD------LYQ----------LLQR-------LFKS-
|P.paniscus-TAC3|XM_003824897.1|              -------EVVPGG---GRSK-RDPD------LYQ----------LLQR-------LFKS-
|P.abelii-TAC3|XM_003778069.1|                -------EVVPGR---GRSK-RDLD------LYQ----------LLQR-------LFKS-
|O.garnettii-TAC3|XM_003790546.1|             -------QVVPSG---GHSK-KDSN------LYQ-----LAPSSLLRR-------LYDS-
|S.boliviensis-TAC3|XM_003926696.1|           -------EVASRG---VHNK-KDVD------LYQ----------LVQR-------LYEI-
|N.leucogenys-TAC3|XM_003252783.1|            -------EVVPGG---GRSK-RDPD------LYQ----------LLQR-------LFKS-
|E.caballus-TAC3|XM_001488415.3|              -------QVVPGG---GESK----------LNL-----LPP-SLLRR-------LYDS-
|L.africana-TAC3|XM_003405564.1|              -------QVVPGR---GHSK-KDLD------LYQ-----LPP-SLLRR-------LLDS-
|A.melanoleuca-TAC3|XM_002916001.1|          -------QVAPGG---GHNK-KDAD------LYK-----LPP-SLLRK-------LYDS-
|B.taurus-TAC3|NM_181017.1|                    -------QVVPGG---GHSK-KDSN------LYQ-----LPP-SLLRR-------LYDS-
|O.aries-TAC3|XM_004009563.1|                 AGHCVRSQSSSWR---QNIW-KDSN------LYQ-----LPP-SLLRR-------LYDS-
|C.hircus-TAC3|AB499062.1|                    -------HVGPGG---GHSK-KDSN------LYQ-----LPP-SLLRR-------LYDS-
|S.scrofa-TAC3|AY758208.1|                    -------QLMPGG---GHSK-KDSN------LYQ-----LPS-SLLRR-------LCDS-
|C.porcellus-TAC3|XM_003475961.1|             -------QVVPSG---THN--KDSG------LYQ-----LPP-SLLRR-------LYDS-
|C.lupus-familiaris-TAC3|XM_843938.2|         -------QVVPGG---GHSK-RDSD------LYQ-----LPP-SLLRK-------LYDS-
|F.catus-TAC3|XM_003988924.1|                 -------QVVPGG---GHNK-KDLD------LYQ-----LPP-SLLRK-------LYDS-
|C.jacchus-TAC3|XM_002752636.2|               -------EVASRG---GHNK-KNMN------LYQ----------LVQR-------LYEI-
|M.musculus-tac2|BC031348.1|                  ----------QGG---RLS--KDSD------LYQ-----LPP-SLLRR-------LYDS-
|A.mississippiensis-TAC3|BK008115.1|         ----------AGR---TQIQ-RSSD------LFK-----LPPS-LLRR-------LYQG-
|X.tropicalis-tac3|BK008110.1|               --------S---T---T-QL-KRS-----SDIYK-----LPA-SLLKR-------FY---
|O.mordax-tac3|BK008111.1|                   ------------------SS-GEI-----PGLGE-----MKR-NLLKR-------YSDV-
|B.saida-tac3|BK008109.1|                     ------------------LS-DPS-----LV--------NRR-NIVRR-------FSDL-
|O.latipes-tac3|BK008114.1|                   ------------------TL-GQP-----ISLEE-----FKR-NLLRR-------YTDL-
|G.morhua-tac3|BK008107.1|                    ------------------LS-DPS-----LV--------NRR-NIVRR-------SSDL-
|P.promelas-tac3|BK008100.1|                  ------------------DS-NEI-----PSLRL-----SAH-NLLKR-------YNDI-
|I.punctatus-tac3|BK008101.1|                ------------------AS-DES-----SSFRL-----ATR-NLLKR-------YHDI-
|S.salar-tac3A|BK008102.1|                    --------R---S---TADS-EDT-----PGLEK-----LKR-SILKR-------YNDL-
|D.mawsoni-tac3|BK008104.1|                   ------------------E---KP-----TVLDN-----LKR-NILKR-------YSDL-
|S.rastrelliger-tac3|BK008105.1|             ------------------TS-DQT-----TGLDN-----LKR-NILKR-------YSDL-
|D.rerio-tac3a|JN392856.1|                    ------------------VS-SES-----PSFRM-----STH-NLLKR-------YNDI-
|D.rerio-tac3B|JN392857.1|                    ----------------------T-----DNSDA-----QPE-RYDKR-------YDDI-
|S.salar-tac3b|BK008103.1|                    ------------------L-QDS-----PYYLS-----SGL-TNFKR-------YRDI-
|C.griseus-TAC3|XM_003507519.1|               -------QVAPGG---RHS--KDSD------LYQ-----LPQ-SLLRR-------LYDS-
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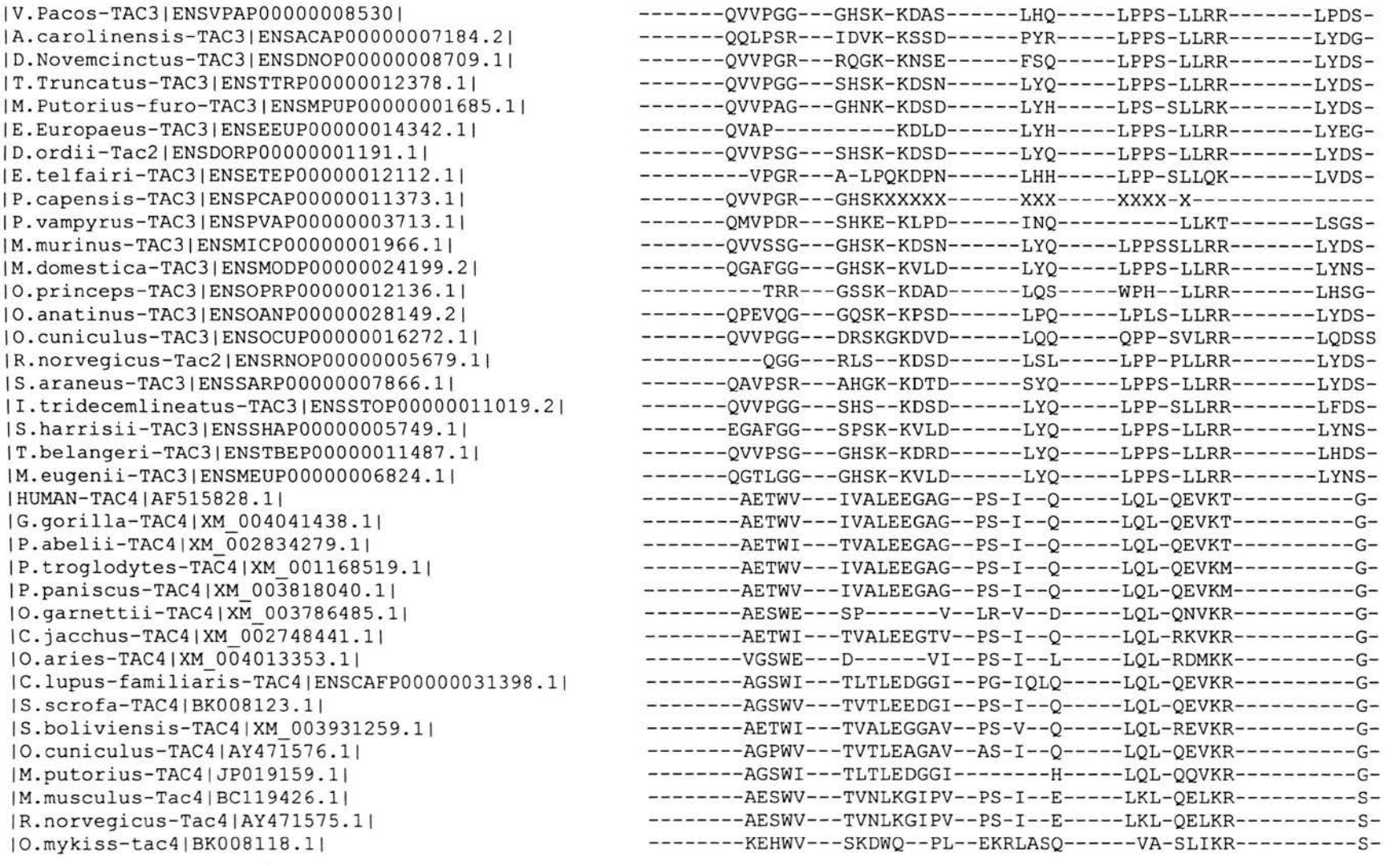
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D.ordii-Tac2	ENSDORP00000001191.1
E.telfairi-TAC3	ENSETEP00000012112.1
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P.vampyrus-TAC3	ENSPVAP00000003713.1
M.murinus-TAC3	ENSMICP00000001966.1
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O.princeps-TAC3	ENSOPRP00000012136.1
O.anatinus-TAC3	ENSOANP00000028149.2
O.cuniculus-TAC3	ENSOCUP00000016272.1
R.norvegicus-Tac2	ENSRNOP00000005679.1
S.araneus-TAC3	ENSSARP00000007866.1
I.tridecemlineatus-TAC3	ENSSTOP00000011019.2
S.harrisii-TAC3	ENSSHAP00000005749.1
T.belangeri-TAC3	ENSTBEP00000011487.1
M.eugenii-TAC3	ENSMEUP00000006824.1
HUMAN-TAC4	AF515828.1
G.gorilla-TAC4	XM_004041438.1
P.abelii-TAC4	XM_002834279.1
P.troglodytes-TAC4	XM_001168519.1
P.paniscus-TAC4	XM_003818040.1
O.garnettii-TAC4	XM_003786485.1
C.jacchus-TAC4	XM_002748441.1
O.aries-TAC4	XM_004013353.1
C.lupus-familiaris-TAC4	ENSCAFP00000031398.1
S.scrofa-TAC4	BK008123.1
S.boliviensis-TAC4	XM_003931259.1
O.cuniculus-TAC4	AY471576.1
M.putorius-TAC4	JP019159.1
M.musculus-Tac4	BC119426.1
R.norvegicus-Tac4	AY471575.1
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-------QVAP----------KDLD------LYH-----LPPS-LLRR-------LYEG-
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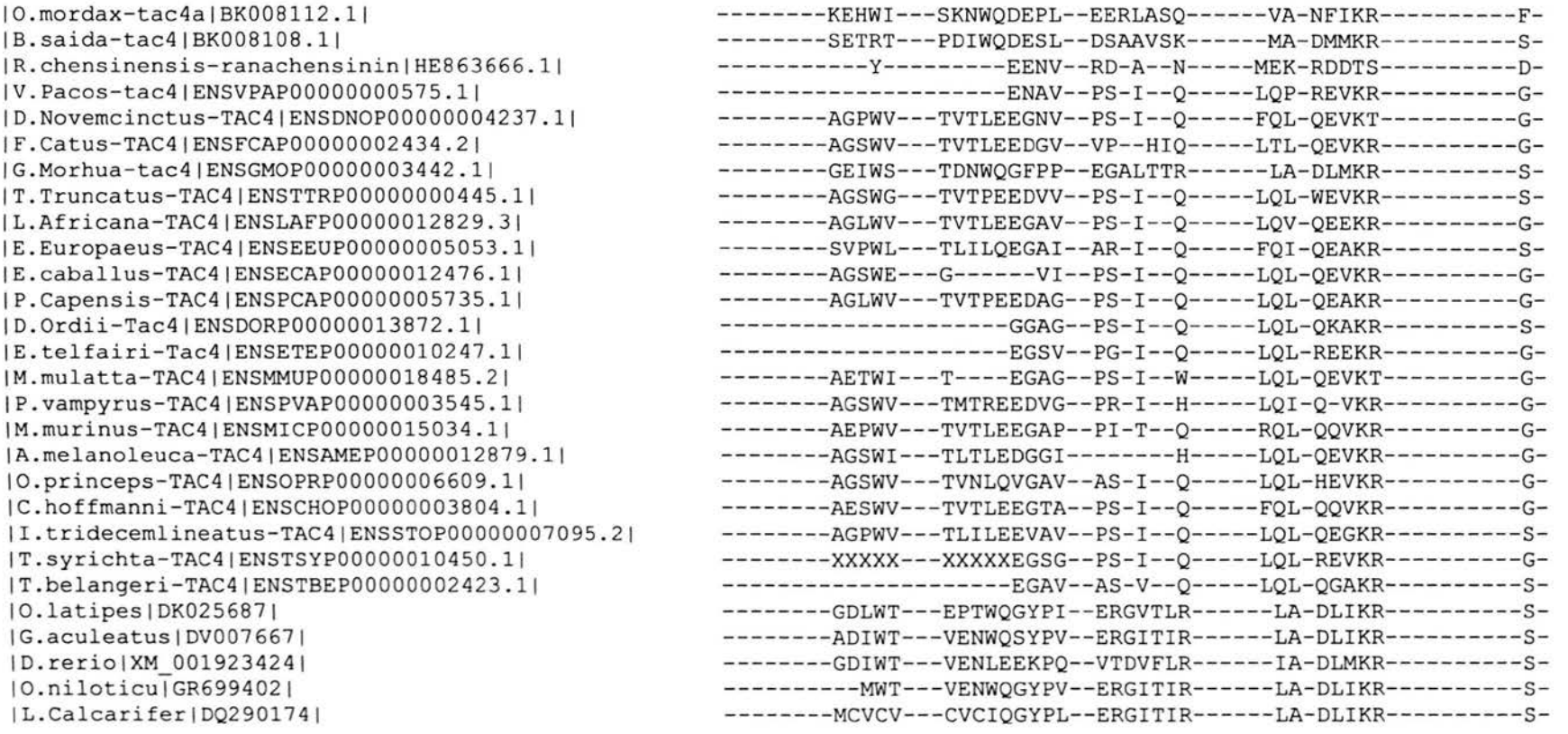
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G.Morhua-tac4	ENSGMOP00000003442.1
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L.Africana-TAC4	ENSLAFP00000012829.3
E.Europaeus-TAC4	ENSEEUP00000005053.1
E.caballus-TAC4	ENSECAP00000012476.1
P.Capensis-TAC4	ENSPCAP00000005735.1
D.Ordii-Tac4	ENSDORP00000013872.1
E.telfairi-Tac4	ENSETEP00000010247.1
M.mulatta-TAC4	ENSMMUP00000018485.2
P.vampyrus-TAC4	ENSPVAP00000003545.1
M.murinus-TAC4	ENSMICP00000015034.1
A.melanoleuca-TAC4	ENSAMEP00000012879.1
O.princeps-TAC4	ENSOPRP00000006609.1
C.hoffmanni-TAC4	ENSCHOP00000003804.1
I.tridecemlineatus-TAC4	ENSSTOP00000007095.2
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T.belangeri-TAC4	ENSTBEP00000002423.1
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G.aculeatus	DV007667
D.rerio	XM_001923424
O.niloticu	GR699402
L.Calcarifer	DQ290174
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--------AGPWV---TLILEEVAV--PS-I--Q-----LQL-QEGKR----------S-
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--------GDIWT---VENLEEKPQ--VTDVFLR------IA-DLMKR----------S-
----------MWT---VENWQGYPV--ERGITIR------LA-DLIKR----------S-
--------MCVCV---CVCIQGYPL--ERGITIR------LA-DLIKR----------S-

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|HUMAN-TAC1|HSU37529|                          -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|G.Gorilla-TAC1|XM_004045800.1|                -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|M.Mulatta-TAC1|ENSMMUP00000035078.1           -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|B.Taurus-TAC1|BC151422.1|                     -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQLSHKRHKTDSFVGLMGKRALNS
|O.Aries-TAC1|NM_001082596.1|                  -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQLSHKRHKTDSFVGLMGKRALNS
|P.Troglodytes-TAC1|XM_001171112.2|           -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|P.Abelii-TAC1|XM_002818228.1|                -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|P.Anubis-TAC1|XM_003896287.1|                -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|P.Paniscus-TAC1|XM_003809689.1|              -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|O.Garnettii-TAC1|XM_003782698.1|             -KPQQFFGL---MGKRDADSSIEKQLALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|C.Porcellus-TAC1|NM_001172899.1|             -KPQQSFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|C.Lupus-familiaris-TAC1|XM_532472.3|        -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|O.Cuniculus-TAC1|X62994.1|                   -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|F.Catus-TAC1|XM_003982791.1|                 -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|S.Harrisii-TAC1|XM_003772510.1|              -KPQQFFGL---MGKRDADTSVEKQVGMLKALY-GHGQMSHKRHKTDSFVGLMGKRSLTS
|R.Norvegicus-Tac1|NM_012666.2|               -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|M.Musculus-Tac1|BC117081.1|                  -KPQQFFGL---MGKRDADSSVEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|M.Putorius-TAC1|JP019158.1|                  -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|T.belangeri-TAC1|Z50786.1|                   -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|S.Boliviensis-TAC1|XM_003921239.1|           -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|T.Guttata-TAC1|XM_002197398.1|               -RPQQFFGL---MGKRD--------------A-GYGQISHKRHKTDSFVGLMGKRSLNS
|G.Gallus-TAC1|BK008126.1|                    -RPQQFFGL---MGKRD--------------A-GYGQISHKRHKTDSFVGLMGKRSLNS
|X.Laevis-tac1|NM_001096537.1|                -RPDQFYGL---MGNGIT---------------DWVKFLANGINQVPFFGLMGKRSL--
|X.Tropicalis-tac1|BC155674.1|                -RPDQFYGL---MGKRNN---------------GFGQISRKRYKSGSFFGLMGKRSLDS
|Hyla.simplex-tac1|HM747308.1|                -RPDQFYGL---MGKRNN---------------GYGQMSR-----------------
|O.Vulgaris-OctTK-I|AB085916.1|               -SSSEFIGL---MGRSEELTRRLIQHP---------------------GSMSETSKRG
|O.Vulgaris-OctTK-II|AB085917.1|              -SSSEFVGL---MGRSEELTRRLIQHP---------------------GSMSETSKRG
|A.Aegypti-sialokininI|AF108102.1|            -TGDKFYGL---MG-------------------------------------------
|C.parva-TAC1|FJ696706.1|                      -KSQQFYGL---MGKRDADSSIEKQMALLKALY-GHGQISHK-----------------
|G.aculeatus-tac1|ENSGACP00000000114.1|       -RPHQFVGL---MGKRSM---------------ANAQITRKRHKVNSFVGLMGKRSQ--
|M.Auratus-tkn-I|X80662.1|                    -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|D.Rerio-tac1|BK008124.1|                     -RPHQFIGL---MGKRSS---------------ANAQITRKRHKINSFVGLMGKRSQ--
|O.Mykiss-tac1|BK008119.1|                    -RPHQFFGL---MGKRSS---------------ANPQITRKRHKINSFVGLMGKRSQ--
|S.Fontinalis-tac1|BK008120.1|                -GPHQFFGL---MGKRSS---------------ANPQITRKRHKINSFVGLMGKRSQ--
|A.Fimbria-tac1|JO691854.1|                   -RPHQFIGL---MGKRSM---------------ANAQITRKRHKVNSFVGLMGKRGQ--
|S.Rastrelliger-tac1|BK008106.1|              -RPHQFIGL---MGKRSM---------------ANAQITRKRHKINSFVGLMGKRSQ--
|I.Punctatus-tkn1|NM_001200768.1|             -RPHQFIGL---MGKRSS---------------ANTQITRKRHKINSFVGLMGKRSQ--
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|O.Latipes-tac1|AB441191.1|                                -RPHQFIGL---MGRRST---------------ANAQITRKRHKVNSFVGLMGKRSQ--
|O.Mordax-tac1|BT075724.1|                                 -RPHQFFGL---MGKRSS---------------ANAQITRKRHKLNSFVGLMGKRSQ--
|C.Auratus-tac1|CAU61272|                                  -RPHQFIGL---MGKRSP---------------ANAQITRKRHKINSFVGLMGKRSQ--
|S.Caurinus-tac1|BK008122.1|                              -RPHQFIGL---MRKRSM---------------ANAQITRKRHKINSFVGLMGKRSQ--
|V.Pacos-tac1|ENSVPAP00000010638|                         -KPQQFFGL---MGKRDADSSVEKQVALLKALY-GLGQISHKRHKTDSFVGLMGKRALNS
|D.Novemcinctus-TAC1|ENSDNOP00000011010.1|                -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|C.Intestinalis-tac1|ENSCINP00000016566.3|                -HVRHFYGL---MGKRSI-GDQPSIF----------------NERASFTGLMGKRGPIP
|P.Sinensis-tac1|ENSPSIP00000004426.1|                   -RPQQFYGL---MGKRDA---------------GYGQMSHKRHKTDSFVGLMGKRSLNS
|M.fascicularis-TAC-1|AB220474.1|                        -KPQQFFGL---MGKRDADSSIEKQVALLKAPY-GHGQISHK----------------
|G.Morhua-tac1|ENSGMOP00000005761.1|                      -RPQQFIGL---MGRRSA---------------ADAQITRKRHKINSFVGLMGKRNT--
|T.Truncatus-TAC1|ENSTTRP00000004482.1|                   -KPQQFFGL---MGKRDADSSVEKQVALLQALY-GHGQLSHKRHKTDSFVGLMGKRALNS
|L.Africana-TAC1|ENSLAFP00000000757.2|                    -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNP
|T.Rubripes-tac1|ENSTRUP00000014475.1|                    -RPHQFIGL---MGKRSM---------------ANAQITHKRHKINSFVGLMGKRSQ--
|N.Leucogenys-TAC1|ENSNLEP00000019442.1                   -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|E.Europaeus-TAC1|ENSEEUP00000002012.1|                   -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQVSHKRHRTDAFVGLMGKRALNS
|E.Caballus-TAC1|ENSECAP00000002225.1|                    -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|P.capensis-TAC1|ENSPCAP00000015000.1|                    -KLQKFYGL---MGKRDADSSVEKQVALLKALY-GHGQTSHKRHKTDSFVGLMGKRALNS
|D.ordii-Tac1|ENSDORP00000001980                          -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|E.telfairi-TAC1|ENSETEP00000015772.1|                    -KPQQFFGL---MGKRDADTALENQVALLKALY-GRGQVSHKRYKTDPFVGLMGKRALNA
|C.jacchus-tac1|ENSCJAP00000001061.1|                     -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|P.vampyrus-tac1|ENSPVAP00000014792.1|                    -KSQQFFGL---MGKRDADSSTEKQVALLKALY-GHGQISHKRYKTDSFVGLMGKRALNS
|M.lucifugus-tac1|ENSMLUP00000002112.2|                   -KPQQFFGL---MGKRDADSSIAKQVALLKALYGHAHFSNKKRHRTDAFIGLMGKRALNS
|M.murinus-TAC1|ENSMICP00000014896.1|                     -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|M.domestica-TAC1|ENSMODP00000020552.3|                   -KPQQFFGL---MGKRDA---------------GHGQMSHKRHKTDSFVGLMGKRSLTS
|A.melanoleuca-TAC1|ENSAMEP00000017243.1|                 -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|S.scrofa-TAC1|ENSSSCP00000020239.1|                      -KPQQFFGL---MGKRDADSSIEKQVALLKALYVGHGQISHKRHKTDSFVGLMGKRALNS
|O.princeps-TAC1|ENSOPRP00000005183.1|                    -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|X.maculatus-tac1|ENSXMAP00000016896.1|                   -RPHQFIGL---MGKRSM---------------ANPQITRKRHKINSFVGLMGKRSQ--
|S.araneus-TAC1|ENSSARP00000008825.1|                     -KSQQFFGL---MGKRDADSSIEKQMALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|C.hoffmanni-TAC1|ENSCHOP00000003135.1|                   -KPQQFFGL---MGKRDADSAIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|I.tridecemlineatus-TAC1|ENSSTOP00000004825.2|            -KPQQFFGL---MGKRDADSSIEKQVALLKALY-GHGQISHKRHKTDSFVGLMGKRALNS
|T.nigroviridis-tac1|ENSTNIP00000021330.1|                -RPQQFIGL---MGRRSM-GESGGQQAASRRPAGANAQITHKRHKINSFVGLMGRRSQ--
|T.nigroviridis-tkn|CR713079.2|                           -DYDSFVGL---MGRRNA-DA-------E------AAQSPQKRELHDIFVGLMGRRNSEP
|T.nigroviridis-tachykinin|CR650252.2|                    -KALRFYGL---MGKRSG-VKKPIQVRRR-------------NKGEAFVGLMGRSISGE
|M.gallopavo-TAC1|ENSMGAP00000010069.2|                   -RPQQFFGL---MGKRDAATLSFFFLSFL-LQP-GYGQISHKRHKTDSFVGLMGKRSLSS
|M.eugenii-TAC1|ENSMEUP00000003075.1|                     -KPQQFFGL---MGKRDADSSVEKQVGMLKALY-GHGQISHKRHKTDSFVGLMGKRSLTS
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|Human-TAC3|AF537113.1|                    -HS-SLEGLLKALSQAST-----------DPKE--STSPEKRDMHDFFVGLMGKRSVQP
|Gorilla-TAC3|XM_004053407.1|              -HS-SLEGLLKALSQAST-----------DPKE--STSPEKRDMHDFFVGLMGKRSVQP
|P.troglodytes-TAC3|XM_003313573.1|        -HS-SLEGLLKALSQAST-----------DPKE--STSPEKRDMHDFFVGLMGKRSVQP
|M.mulatta-TAC3|XM_001115535.1|           -HS-SLEGFLKAMSQART----------DPKE--STSPEKRDMHDFFVGLMGKRSVKP
|P.anubis-TAC3|XM_003906628.1|            -HS-SLEGLLKAMSQAST----------DPKE--STSPEKRDMHDFFVGLMGKRSVKP
|P.paniscus-TAC3|XM_003824897.1|          -HS-SLEGLLKALSQAST----------DPKE--STSPEKRDMHDFFVGLMGKRSVQP
|P.abelii-TAC3|XM_003778069.1|            -HS-SLEGLLKALSQAST----------DPKE--STSPKKRDMHDFFVGLMGKRSIQP
|O.garnettii-TAC3|XM_003790546.1|         -RSVSLEGLLQVLSKASL----------DPKES--SLPQKRDMHDFFVGLMGKRNIQP
|S.boliviensis-TAC3|XM_003926696.1|       -HSFSLEELLIALSQAIL----------DSRGSETPLPRKRNMQDLFVGLMGKRNVQP
|N.leucogenys-TAC3|XM_003252783.1|        -HS-SLEGLLKALSQAST----------DPKE--STSPEKRDMHDFFVGLMGKRSVQP
|E.caballus-TAC3|XM_001488415.3|          -RSVSLDGLLKMLSQASV----------DPKES--SLPQKRDMHDFFVGLMGKRNIQP
|L.africana-TAC3|XM_003405564.1|          -RSVYLDGLLKMLSKASL----------GPKES--SLPQKRDMHDFFVGLMGKRNIQP
|A.melanoleuca-TAC3|XM_002916001.1|      -RSVSLDGLLKMLSKASL----------DPKES--PLPQKRDMHDFFVGLMGKRNIQP
|B.taurus-TAC3|NM_181017.1|              -RVVSLDGLLKMLSKASV----------GPKES--PLPQKRDMHDFFVGLMGKRNLQP
|O.aries-TAC3|XM_004009563.1|            -RVVSLDGLLKMLSKASV----------GPKES--PLPQKRDMHDFFVGLMGKRNLQP
|C.hircus-TAC3|AB499062.1|               -RVISLDGLLKMLSKASV----------GPKES--PLPQKRDMHDFFVGLMGKRNLQP
|S.scrofa-TAC3|AY758208.1|               -RSISLDGLLKMLSKASV----------GAKES--SLPQKRDMHDFFVGLMGKRNIQP
|C.porcellus-TAC3|XM_003475961.1|        -RSVSLEGLLKVLSKASM----------DPKES--SLPQKRDMHDFFVGLMGKRNSQA
|C.lupus-familiaris-TAC3|XM_843938.2|   -GSVSLEGLLKMLSKASV----------DPKES--PLPQKRDMHDFFVGLMGKRNIQP
|F.catus-TAC3|XM_003988924.1|            -RSVSLDGLLKMLSKASV----------DPKEL--PLPQKRDMHDFFVGLMGKRNSQP
|C.jacchus-TAC3|XM_002752636.2|          -HSFSLEELLVALSQAIL----------DSRGSETALPRKRNMQDLFVGLMGKRNVQP
|M.musculus-tac2|BC031348.1|             -RPVSLEGLLKVLSKASV----------GPKET--SLPQKRDMHDFFVGLMGKRNSQP
|A.mississippiensis-TAC3|BK008115.1|    ---VSYEALLQLADKAPV----------GLQAL--APPQKRDMHDFFVGLMGKRTVVP
|X.tropicalis-tac3|BK008110.1|           -DDDSFVGL---MGKRNS-DFK------E------FPSLPLKREMNDFFVGLMGKRNLQT
|O.mordax-tac3|BK008111.1|              -DYDSFVGL---MGRRNA-DI-------N------GVQSQQKREMHDIFVGLMGRRNSEP
|B.saida-tac3|BK008109.1|               -DYDSFVGL---MGRRNT-EAANELPSAN------ELPSANKREMHDIFVGLMGRRNSET
|O.latipes-tac3|BK008114.1|             -DYDSFVGL---MGKRNA-EE-------E------AVQSQPKRDMDDIFVGLMGRRSSEP
|G.morhua-tac3|BK008107.1|              -DYDSFVGL---MGGETQ-RRQM-----N------YRRQIKTGEMHDIFVGLMGRRNSET
|P.promelas-tac3|BK008100.1|            -DYDSFVGL---MGRRNA-DTD-------------DFPPQRKREMHDIFVGLMGRRSADP
|I.punctatus-tac3|BK008101.1|           -DYDSFVGL---MGRRSA-DAAA----AA------EDQSQRKREMHDIFVGLMGRRNSED
|S.salar-tac3A|BK008102.1|              -DYDSFVGL---MGRRGA-DIY------D------LPPSPHKREMDDVFVGLMGRRNLEQ
|D.mawsoni-tac3|BK008104.1|             -DYDSFVGL---MGRRDA-DD-------S------AVPAPQKREMNDIFVGLMGRRNSDP
|S.rastrelliger-tac3|BK008105.1|        -DYDSFVGL---MGRRDA-DA-------N------AVQSPQKREMHDIFVGLMGRRNSEP
|D.rerio-tac3a|JN392856.1|              -DYDSFVGL---MGRRNA-ETD-------------DIPPQRKREMHDIFVGLMGRRSAEP
|D.rerio-tac3B|JN392857.1|              -DYDSFVGL---MGRRST-GIN------R------EAHLPFRPNMNDIFVGLLGRRNTLS
|S.salar-tac3b|BK008103.1|              -HDDTFVGL---MGRRSA-GVND-----L------PSRRSKIRDMDDVFVGLLGRRSSGS
|C.griseus-TAC3|XM_003507519.1|         -RSVSLEGLLKVLSKASM----------GPKET--SLPQKRDMHDFFVGLMGKRNSQP
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|V.Pacos-TAC3|ENSVPAP00000008530|                          -RLVSLNGLLRVLSRAGT-----------GPKES--PLPQKRDMHDFFVGLMGKRNVQP
|A.carolinensis-TAC3|ENSACAP00000007184.2|                 -QGISYEALLRLSGKEEI-----------GPQTL--ASSQKRDMHDFFVGLMGKRTTEL
|D.Novemcinctus-TAC3|ENSDNOP00000008709.1|                 -RSVSLDGLLKMLSKASV-----------DSKEL--SLPQKRDMHDFFVGLMGKRNGQP
|T.Truncatus-TAC3|ENSTTRP00000012378.1|                   -RSVSLDGLLKMLSKASV-----------GPKES--SLPQKRDMHDFFVGLMGKRNIQP
|M.Putorius-furo-TAC3|ENSMPUP00000001685.1|               -RSVSLDGLLKMLSKASV-----------DPKES--SLPQKRDMHDFFVGLMGKRNIQP
|E.Europaeus-TAC3|ENSEEUP00000014342.1|                   -RSASLDGLLKVLSKASM-----------GAKEP--SLPQKRDMHDFFVGLMGKRTLQP
|D.ordii-Tac2|ENSDORP00000001191.1|                       -RSVSLEELLKVLSKASV-----------DSKES--SLPQKRDMHDFFVGLMGKRNSQP
|E.telfairi-TAC3|ENSETEP00000012112.1|                     -QTVSLEGLLKILSSASV-----------GPKKL--SLFQKRDMHDFFVGLMGQRN---
|P.capensis-TAC3|ENSPCAP00000011373.1|                     XXXXXXXXXXXXXXXXX-----------XPKES--SLPQKRDMHDFFVGLMGKRNIQP
|P.vampyrus-TAC3|ENSPVAP00000003713.1|                     -GSVSVDELLKTLGKASK-----------XX-----XXXXSPDMHDFFVGLMGKRNIQP
|M.murinus-TAC3|ENSMICP00000001966.1|                      -RSVSLEGLLKVLSKASV-----------DPKES--SLPQKRDMHDFFVGLMGKRNMQP
|M.domestica-TAC3|ENSMODP00000024199.2|                    -RSISLDGLLRLLSKTSV-----------DSKET--MDYQKRDMHDFFVGLMGKRNIQA
|O.princeps-TAC3|ENSOPRP00000012136.1|                     --RSSLEELLRVLGKASM-----------DPKAV-ASSPQKRDMHDFFVGLMGKRNMQP
|O.anatinus-TAC3|ENSOANP00000028149.2|                     -RAISLDGLLGLLAQTSA-----------DPREL--ASPQKRDMHDFFVGLMGKRRARA
|O.cuniculus-TAC3|ENSOCUP00000016272.1|                   RRPFSLEELLKVLSKASV-----------DPKAA--SLPQKRDMHDFFVGLMGKRNSQP
|R.norvegicus-Tac2|ENSRNOP00000005679.1|                  -RPISLEGLLKVLSKASV-----------GPKET--SLPQKRDMHDFFVGLMGKRNSQP
|S.araneus-TAC3|ENSSARP00000007866.1|                     --SVSLEGLLRVLSRASV-----------GPKAS--SLAQKRDMHDFFVGLMGKRNTQA
|I.tridecemlineatus-TAC3|ENSSTOP00000011019.2|           -RPVSLEGLLKVLSKASV-----------DPKES--SLPQKRDMHDFFVGLMGKRNSQP
|S.harrisii-TAC3|ENSSHAP00000005749.1|                    -RSLSLDGLLKLLSKTSV-----------DSKES--MDFQKRDMHDFFVGLMGKRNIQS
|T.belangeri-TAC3|ENSTBEP00000011487.1|                   -RSVSLEGLLKALSKASV-----------DPKDS--SLPQKRDMHDFFVGLMGKRNIQT
|M.eugenii-TAC3|ENSMEUP00000006824.1|                     -RSLSLDGLLRLLSKTSV-----------DPKET--MDFQKRDMHDFFVGLMGKRNMQS
|HUMAN-TAC4|AF515828.1|                                   -KASQFFGL---MGKRVG-GRPLIQPRRKKA----Y----QLEH---TFQGLLGKRSL--
|G.gorilla-TAC4|XM_004041438.1|                           -KASQFFGL---MGKRVG-GRPLIQPRRKKG----Y----QLEH---TFQGLLGKRSL--
|P.abelii-TAC4|XM_002834279.1|                            -KPSQFFGL---MGKRVG-GRLLIQPRRKKV----Y----QLQH---TFQGLLGKRSL--
|P.troglodytes-TAC4|XM_001168519.1|                      -KASQFFGL---MGKRVG-RRPLIQPRRKKA----Y----QLEH---TFQGLLGKRSL--
|P.paniscus-TAC4|XM_003818040.1|                         -KASQFFGL---MGKRVG-RRPLIQPRRKKA----Y----QLEH---TFQGLLGKRSL--
|O.garnettii-TAC4|XM_003786485.1|                        -KANQFFGL---MGKRIG-GREDEDRGS--------------------------------
|C.jacchus-TAC4|XM_002748441.1|                          -KASQFFGL---MGKRVG-GRPLIRPGRKKC----Y----QLEH---TLQGLLGKRSP--
|O.aries-TAC4|XM_004013353.1|                            -KASQFFGL---MGKQED-L-PSSQRQQGIS------------------ENHWS------
|C.lupus-familiaris-TAC4|ENSCAFP00000031398.1|          -KASQFFGL---MGKRVG-GMPPIQPERRTGPPPGH----QQRP---TEQSLLGRVGP--
|S.scrofa-TAC4|BK008123.1|                               -KNSQFFGL---MGKQVG-GTPPIQPERT-G----Y----QRGP---VIQGLLSPGGP--
|S.boliviensis-TAC4|XM_003931259.1|                       -KASQFFGL---MGKRVG-GRPLIRPGRKKV----Y----QLEH---TLQGLLGKRSP--
|O.cuniculus-TAC4|AY471576.1|                            -KASQFFGL---MGKRVR-GYQMG-----------------------QRGLLGRRAS--
|M.putorius-TAC4|JP019159.1|                             -KASQFFGL---MGKRVR-----------------------------------------
|M.musculus-Tac4|BC119426.1|                             -RTRQFYGL---MGKRVG-----------G----Y----QLGR---IVQDLLGTRGL--
|R.norvegicus-Tac4|AY471575.1|                           -RTRQFYGL---MGKRVE-GVHPIQSAERTG----Y----QLGR---IVQDLLGTRGL--
|O.mykiss-tac4|BK008118.1|                               -KAHQFYGL---MGKRSDDQPQPIGVNRRR-------------DKGEMFVGLMGRRASSG
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|O.mordax-tac4a|BK008112.1|                          -KSRQFYGL---MGKRSD-MKQPIKVYRRR------------NKGDMFVGLMGRRALGK
|B.saida-tac4|BK008108.1|                            -KALRFYGL---MGKRSG-SRQPIQMNRRR------------NKGEMFVGLMGRSITSG
|R.chensinensis-ranachensinin|HE863666.1|            -RSNQFIGL---MG-----------------------------------------------
|V.Pacos-tac4|ENSVPAP00000000575.1|                  -KASQFFGL---MGKHVG-GIPPNQPERA-G----Y----LRGP---VVQGLLDRGRP--
|D.Novemcinctus-TAC4|ENSDNOP00000004237.1|          -KASQFFGL---MGKRAG-GISPIQLVSITG----H----QTGQ---RVQGLLGRKEP--
|F.Catus-TAC4|ENSFCAP00000002434.2|                  -KTSQFFGL---MGKRVE-GIPPIQPERRTA----H----QQAQ---RAWGLLGRAGP--
|G.Morhua-tac4|ENSGMOP00000003442.1|                -RAQQFHGL---MGRSTG-----------------------NKGEMFVGLMGRRGLRG
|T.Truncatus-TAC4|ENSTTRP00000000445.1|              -KASQFFGL---MGKQVG-GIPPIQPERR-A----Y----QRGP---VVQGHLGRGGP--
|L.Africana-TAC4|ENSLAFP00000012829.3|               -KASQFFGL---MGKRVG-GEPLTYPRRKKA----V----E------------------
|E.Europaeus-TAC4|ENSEEUP00000005053.1|             -TGKEFYGL---MGKRAR-----------------------------------------
|E.caballus-TAC4|ENSECAP00000012476.1|               -KARQFFGL---MGKRVG-GVPPIQPGRR--------------------------------
|P.Capensis-TAC4|ENSPCAP00000005735.1|               -KARKFFGL---MGKRVG-GISPIQPLRTIY-----------QQ---VVQGLLGRRGP--
|D.Ordii-Tac4|ENSDORP00000013872.1|                  -KNRQFFGL---MGKRVK-GIPSIQPEGRTV----C------LG---MVQGLLCGTGP--
|E.telfairi-Tac4|ENSETEP00000010247.1|               -KSSQFFGL---MGKRVG-GMSPVQLVRA-----GC----QQGH---VVPGLLDRRGP--
|M.mulatta-TAC4|ENSMMUP00000018485.2|                -KASQFFGL---MGNL------------------------------------------
|P.vampyrus-TAC4|ENSPVAP00000003545.1|               -KESRFFGL---MGKQIR-GIPPIQPERKT-----E----HQKQ---MNQDLLGRGGP--
|M.murinus-TAC4|ENSMICP00000015034.1|                -KASQFFGL---MGKQMG-GATRIQPGRRAG----Y----QSEQ---TVPGFQGLENCVL
|A.melanoleuca-TAC4|ENSAMEP00000012879.1|            -KASQFFGL---MGKRIS-PRPPLQPE--------------------------------
|O.princeps-TAC4|ENSOPRP00000006609.1|               -KASQFFGL---MGKRVR-GIHPIPWRTT-----GY----RTGQ---MVQGSLGRRGL--
|C.hoffmanni-TAC4|ENSCHOP00000003804.1|              -KASQFFGL---MGKRVG-GLSPIQSVSITG----D----QRGR---MNQALLDRKEA--
|I.tridecemlineatus-TAC4|ENSSTOP00000007095.2|      -KANQFFGL---MGKQVE-GIPPIQPERAAG----Y----KLGQ---MVQALLGRRGS--
|T.syrichta-TAC4|ENSTSYP00000010450.1|               -KASQFFGL---MGKRLG-GTPAIQPGRTTG--VGN----QPEH---TVQDLLDR-CL--
|T.belangeri-TAC4|ENSTBEP00000002423.1|              -KPSQFFGL---MGKQIE-GIPPIQPGRT-----GY----QQGQ---PAQSFLGSKGP--
|O.latipes|DK025687|                                 -KSQQFHGL---MGRSSG-ARLPVRLGRKRN-----NN---NNNKGEMFVGLMGRRSSGG
|G.aculeatus|DV007667|                               -NGQHFHGL---MGRSSG-TSQPLRLGKKR------------NKGEMFVGLMGRRSLDG
|D.rerio|XM_001923424|                               -KSQHFHGL---MGSSAG-NTQPLRLGRRR------------NKGEIFVGLMGRSDG--
|O.niloticu|GR699402|                               -KAQQFHGL---MGRS-G-VSHAVRLGRKR------------NKGEMFVGLMGRRSLGG
|L.Calcarifer|DQ290174|                             -KAQQFHGL---MGRSSG-----------------------------------------

|HUMAN-TAC1|HSU37529|                               VAYERSA--M-----------------------------------------------
|G.Gorilla-TAC1|XM_004045800.1|                     VAYERSA--M-----------------------------------------------
|M.Mulatta-TAC1|ENSMMUP00000035078.1                 VAYERSA--M-----------------------------------------------
|B.Taurus-TAC1|BC151422.1|                           VAYERSV--M-----------------------------------------------
|O.Aries-TAC1|NM_001082596.1|                       VAYERSV--M-----------------------------------------------
|P.Troglodytes-TAC1|XM_001171112.2|                 EAYERSA--M-----------------------------------------------
|P.Abelii-TAC1|XM_002818228.1|                       VAYERSA--M-----------------------------------------------
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|P.Anubis-TAC1|XM_003896287.1|                    VAYERSA--M----------
|P.Paniscus-TAC1|XM_003809689.1|                  EAYERSA--M----------
|O.Garnettii-TAC1|XM_003782698.1|                 VAYERSA--M----------
|C.Porcellus-TAC1|NM_001172899.1|                 VGYERSA--M----------
|C.Lupus-familiaris-TAC1|XM_532472.3|            VAYERSA--M----------
|O.Cuniculus-TAC1|X62994.1|                       VAYERSA--M----------
|F.Catus-TAC1|XM_003982791.1|                     VAYERRA--M----------
|S.Harrisii-TAC1|XM_003772510.1|                  ASPYPCC--PVALRGG----------PG
|R.Norvegicus-Tac1|NM_012666.2|                   VAYERSA--M----------
|M.Musculus-Tac1|BC117081.1|                     VAYERSA--M----------
|M.Putorius-TAC1|JP019158.1|                     VAYDRSA--M----------
|T.belangeri-TAC1|Z50786.1|                       VAYERNA--M----------
|S.Boliviensis-TAC1|XM_003921239.1|              VAYERSA--M----------
|T.Guttata-TAC1|XM_002197398.1|                   GSSEGST--A----------
|G.Gallus-TAC1|BK008126.1|                        GSSERSI--A----------
|X.Laevis-tac1|NM_001096537.1|                   -------DSG--------S----------SERN
|X.Tropicalis-tac1|BC155674.1|                   GSSERNS--LLN----------
|Hyla.simplex-tac1|HM747308.1|                   --------KR--------S----------TERN
|O.Vulgaris-OctTK-I|AB085916.1|                  --PPKKV-SRRPYILKK----------
|O.Vulgaris-OctTK-II|AB085917.1|                 --PPKKG-DFNPNELKPESN------IC----------
|A.Aegypti-sialokininI|AF108102.1|               ----------
|C.parva-TAC1|FJ696706.1|                        ----------
|G.aculeatus-tac1|ENSGACP00000000114.1|         -------EEPG-------S----------YEWS
|M.Auratus-tkn-I|X80662.1|                       VAFERSA--M----------
|D.Rerio-tac1|BK008124.1|                        -------EEPE-------S----------YEWG
|O.Mykiss-tac1|BK008119.1|                       -------EKPD-------S----------YEWN
|S.Fontinalis-tac1|BK008120.1|                   -------EKPD-------S----------YEWN
|A.Fimbria-tac1|JO691854.1|                      -------EEPG-------S----------YEWS
|S.Rastrelliger-tac1|BK008106.1|                -------EEPE-------S----------YEWS
|I.Punctatus-tkn1|NM_001200768.1|               -------EEPD-------S----------YDWS
|S.Salar-tac1|BT125493.1|                        -------DEPD-------S----------YEWN
|O.Latipes-tac1|AB441191.1|                      -------EEPE-------S----------YEWS
|O.Mordax-tac1|BT075724.1|                       -------EEPD-------S----------YEWN
|C.Auratus-tac1|CAU61272|                        -------EEPE-------S----------YEWG
|S.Caurinus-tac1|BK008122.1|                     -------EEPE-------S----------YEWS
|V.Pacos-tac1|ENSVPAP00000010638|               VAYERSA--M----------
|D.Novemcinctus-TAC1|ENSDNOP00000011010.1|      VAYERNA--M----------
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|C.Intestinalis-tac1|ENSCINP00000016566.3|        --YGRDSNILNPEPRLPLQDKTYNGDYLFGVPQNDRDAIGPDNVQNDNPVANRMMQAILS
|P.Sinensis-tac1|ENSPSIP00000004426.1|            GSSEKNT-------AQ-----------------------------------------
|M.fascicularis-TAC-1|AB220474.1|                 MAYERSA--M-------------------------------------------------
|G.Morhua-tac1|ENSGMOP00000005761.1|              -------EDPD-------S-------------------------------------YEWS
|T.Truncatus-TAC1|ENSTTRP00000004482.1|          VAYERSA--M-------------------------------------------------
|L.Africana-TAC1|ENSLAFP00000000757.2|          VAYERNA--M-------------------------------------------------
|T.Rubripes-tac1|ENSTRUP00000014475.1|          -------EEPD-------S-------------------------------------YEWS
|N.Leucogenys-TAC1|ENSNLEP00000019442.1          VAYERSA--M-------------------------------------------------
|E.Europaeus-TAC1|ENSEEUP00000002012.1|          VAYERNA--M-------------------------------------------------
|E.Caballus-TAC1|ENSECAP00000002225.1|          VAYERSA--M-------------------------------------------------
|P.capensis-TAC1|ENSPCAP00000015000.1|          -------------------------------------------------------------
|D.ordii-Tac1|ENSDORP00000001980                 VAYERSA--M-------------------------------------------------
|E.telfairi-TAC1|ENSETEP00000015772.1|          AAFERSA--M-------------------------------------------------
|C.jacchus-tac1|ENSCJAP00000001061.1|           VAYERSA--M-------------------------------------------------
|P.vampyrus-tac1|ENSPVAP00000014792.1|          VAYDRSA--M-------------------------------------------------
|M.lucifugus-tac1|ENSMLUP00000002112.2|         VAYERDA--M-------------------------------------------------
|M.murinus-TAC1|ENSMICP00000014896.1|           VAYERS----------------------------------------------------
|M.domestica-TAC1|ENSMODP00000020552.3|         GSSEWSA--PAAALAQ--------------------------------------------
|A.melanoleuca-TAC1|ENSAMEP00000017243.1|       VAYERSA--M-------------------------------------------------
|S.scrofa-TAC1|ENSSSCP00000020239.1|            VAYERSA--M-------------------------------------------------
|O.princeps-TAC1|ENSOPRP00000005183.1|          VAYDSNA--M-------------------------------------------------
|X.maculatus-tac1|ENSXMAP00000016896.1|         -------EEPE-------S-------------------------------------YEWS
|S.araneus-TAC1|ENSSARP00000008825.1|           VAYERSA--M-------------------------------------------------
|C.hoffmanni-TAC1|ENSCHOP00000003135.1|         VAFERSE--T-------------------------------------------------
|I.tridecemlineatus-TAC1|ENSSTOP00000004825.2|  VAYERNA--M-------------------------------------------------
|T.nigroviridis-tac1|ENSTNIP00000021330.1|      -------EDPD-------S-------------------------------------YEWS
|T.nigroviridis-tkn|CR713079.2|                 D-NGPWRRA-Y--PER-----------------------------------------RG
|T.nigroviridis-tachykinin|CR650252.2|          --D-SSN-RIIPASAPAEVL--------E-KPHDQG-----------------FSPEWV
|M.gallopavo-TAC1|ENSMGAP00000010069.2|         GSSERNI--A-------------------------------------------------
|M.eugenii-TAC1|ENSMEUP00000003075.1|           GSSEWST-------AQ-----------------------------------------
|Human-TAC3|AF537113.1|                         EGKTGPFLPSVRVPRPL-------------HPNQLGSTGKSS-----LGT------EEQR
|Gorilla-TAC3|XM_004053407.1|                   EGKTGPFLPSVRVPRPI-------------HPNQLGSKGKPS-----LGT------EELR
|P.troglodytes-TAC3|XM_003313573.1|             EGKTGPFLPSVRVPRPL-------------HSNQLGSTGKSS-----LGT------EEQR
|M.mulatta-TAC3|XM_001115535.1|                 DSPTDVNQENI-----------------------------PS-----SGT------LKYP
|P.anubis-TAC3|XM_003906628.1|                  EGKTGPFLPSVRVPRPL-------------HPNQLGSTGKPS-----LGT------EERR
|P.paniscus-TAC3|XM_003824897.1|               EGKTGPFLPSVRVPRPL-------------HPNQLGSTGKSS-----LGT------EEQR
|P.abelii-TAC3|XM_003778069.1|                  EGKTGPFLPSVRVPRPL-------------HPNQLGSTGKPS-----LGT------EERR
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|O.garnettii-TAC3|XM_003790546.1|          DTSTDVNQENVPSFGTL----------K----------Y-----SPD------AE--
|S.boliviensis-TAC3|XM_003926696.1|        DSPTNGNQENV----------------------------PS-----FGT------FKYP
|N.leucogenys-TAC3|XM_003252783.1|         DSLTDVNQENV----------------------------PS-----FGI------LKYP
|E.caballus-TAC3|XM_001488415.3|           DTPVDVNQENIPSFGTL----------K----------Y-----PPN------AE--
|L.africana-TAC3|XM_003405564.1|           DTPIDVNQENIPSFGNL----------Q----------Y-----PPN------AE--
|A.melanoleuca-TAC3|XM_002916001.1|       DPSTDVNQENIPSFGTL----------K----------Y-----PPN------VE--
|B.taurus-TAC3|NM_181017.1|               DTPVDINQENIPSFGTF----------K----------Y-----PPS------VE--
|O.aries-TAC3|XM_004009563.1|             DTPVDINQENIPSFGTF----------K----------Y-----PPS------VE--
|C.hircus-TAC3|AB499062.1|               DTPVDINQENIPRFGTF----------K----------------------------
|S.scrofa-TAC3|AY758208.1|               GTPVDGNQENAPSLGTF----------K----------Y-----PPS------VE--
|C.porcellus-TAC3|XM_003475961.1|        DTSTDVNQENIPGFGTL----------K----------Y-----ASS------AE--
|C.lupus-familiaris-TAC3|XM_843938.2|    DTPVDVNQEKVPSFGTL----------Q----------Y-----PPS------AE--
|F.catus-TAC3|XM_003988924.1|            DTSIDVNQENVPSFGTL----------K----------Y-----PPS------AE--
|C.jacchus-TAC3|XM_002752636.2|          DSPTDGNQESI----------------------------PS-----FGT------LKYP
|M.musculus-tac2|BC031348.1|             DTPTDVVEENTPSFGIL----------K----------------------------
|A.mississippiensis-TAC3|BK008115.1|    GSPVDESQEPFATFGDP----------WDS----------PS------AE--
|X.tropicalis-tac3|BK008110.1|          GNPTEEENEARP----------------------------------------------DT
|O.mordax-tac3|BK008111.1|              GNAGPWRKA-N--PET-----------------------------------------KG
|B.saida-tac3|BK008109.1|               D-DGPWRKT-D--PER-----------------------------------------RG
|O.latipes-tac3|BK008114.1|             E-NGPWRRD-Y--PDR-----------------------------------------RG
|G.morhua-tac3|BK008107.1|             D-DGPWRKT-D--PER-----------------------------------------RG
|P.promelas-tac3|BK008100.1|           ETGRPLRKD-YPETSG--------------------------------------GG
|I.punctatus-tac3|BK008101.1|          DTERPWRKD-YPET-----------------------------------------RG
|S.salar-tac3A|BK008102.1|             GNMRPLRKEAYPETRR-----------------------------------------GG
|D.mawsoni-tac3|BK008104.1|            D-DGPLRRE-Y--PER-----------------------------------------RG
|S.rastrelliger-tac3|BK008105.1|       D-NGPWRRE-N--TER-----------------------------------------RG
|D.rerio-tac3a|JN392856.1|             ESGRQWRKE-YPEPS-------------------------------------------GG
|D.rerio-tac3B|JN392857.1|             S---------MRKERR-------------------------------------------GN
|S.salar-tac3b|BK008103.1|             AIPQPWREEVYPQPS-------------------------------------------GG
|C.griseus-TAC3|XM_003507519.1|        DTPTDVVEENTPSFGTL----------K----------------------------
|V.Pacos-TAC3|ENSVPAP00000008530|      EGKTGFFLATVKVPWPL----------LPSQLGPTVQLF------LG------AERP
|A.carolinensis-TAC3|ENSACAP00000007184.2| ANPIPPPSRTILFLGRI----------T----------------------------
|D.Novemcinctus-TAC3|ENSDNOP00000008709.1| GK--AEFLAMARLPGPL----------H-SQLGSSDQPS------PR------AERR
|T.Truncatus-TAC3|ENSTTRP00000012378.1|  EGKTGLFLATVRVPLPL----------LTNRLGSTVQPS------PG------AQRR
|M.Putorius-furo-TAC3|ENSMPUP00000001685.1| DTSIDVNQENVPSFGTL----------K----------Y-----PPS------VE--
|E.Europaeus-TAC3|ENSEEUP00000014342.1|  EGNTGLFLDVVRSPQPV----------YSSPPVEAES----------------------
|D.ordii-Tac2|ENSDORP00000001191.1|      VL--------TNASWPL----------LPKQLGFTVESS------WN------SEAT
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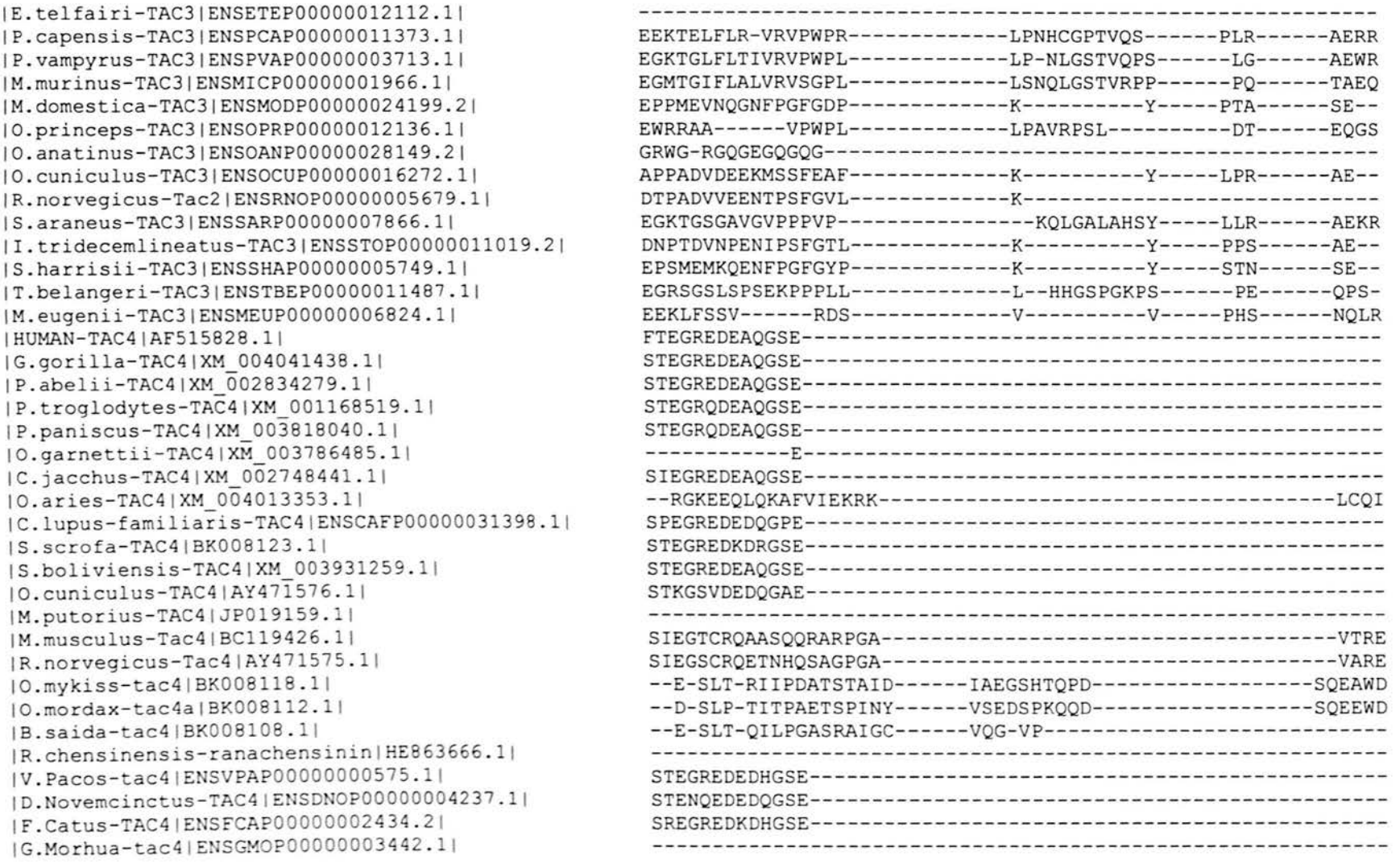
E.telfairi-TAC3	ENSETEP00000012112.1
P.capensis-TAC3	ENSPCAP00000011373.1
P.vampyrus-TAC3	ENSPVAP00000003713.1
M.murinus-TAC3	ENSMICP00000001966.1
M.domestica-TAC3	ENSMODP00000024199.2
O.princeps-TAC3	ENSOPRP00000012136.1
O.anatinus-TAC3	ENSOANP00000028149.2
O.cuniculus-TAC3	ENSOCUP00000016272.1
R.norvegicus-Tac2	ENSRNOP00000005679.1
S.araneus-TAC3	ENSSARP00000007866.1
I.tridecemlineatus-TAC3	ENSSTOP00000011019.2
S.harrisii-TAC3	ENSSHAP00000005749.1
T.belangeri-TAC3	ENSTBEP00000011487.1
M.eugenii-TAC3	ENSMEUP00000006824.1
HUMAN-TAC4	AF515828.1
G.gorilla-TAC4	XM_004041438.1
P.abelii-TAC4	XM_002834279.1
P.troglodytes-TAC4	XM_001168519.1
P.paniscus-TAC4	XM_003818040.1
O.garnettii-TAC4	XM_003786485.1
C.jacchus-TAC4	XM_002748441.1
O.aries-TAC4	XM_004013353.1
C.lupus-familiaris-TAC4	ENSCAFP00000031398.1
S.scrofa-TAC4	BK008123.1
S.boliviensis-TAC4	XM_003931259.1
O.cuniculus-TAC4	AY471576.1
M.putorius-TAC4	JP019159.1
M.musculus-Tac4	BC119426.1
R.norvegicus-Tac4	AY471575.1
O.mykiss-tac4	BK008118.1
O.mordax-tac4a	BK008112.1
B.saida-tac4	BK008108.1
R.chensinensis-ranachensinin	HE863666.1
V.Pacos-tac4	ENSVPAP00000000575.1
D.Novemcinctus-TAC4	ENSDNOP00000004237.1
F.Catus-TAC4	ENSFCAP00000002434.2
G.Morhua-tac4	ENSGMOP00000003442.1
EEKTELFLR-VRVPWPR----------LPNHCGPTVQS------PLR------AERR
EGKTGLFLTIVRVPWPL----------LP-NLGSTVQPS------LG------AEWR
EGMTGIFLALVRVSGPL----------LSNQLGSTVRPP------PQ------TAEQ
EPPMEVNQGNFPGFGDP----------K----------Y-----PTA------SE--
EWRRAA------VPWPL----------LPAVRPSL----------DT------EQGS
GRWG-RGQGEGQGQG----------
APPADVDEEKMSSFEAF----------K----------Y-----LPR------AE--
DTPADVVEENTPSFGVL----------K----------
EGKTGSGAVGVPPPVP----------KQLGALAHSY-----LLR------AEKR
DNPTDVNPENIPSFGTL----------K----------Y-----PPS------AE--
EPSMEMKQENFPGFGYP----------K----------Y-----STN------SE--
EGRSGSLSPSEKPPPLL----------L--HHGSPGKPS------PE------QPS-
EEKLFSSV------RDS----------V----------V-----PHS------NQLR
FTEGREDEAQGSE----------
STEGREDEAQGSE----------
STEGREDEAQGSE----------
STEGRQDEAQGSE----------
STEGRQDEAQGSE----------
------------E----------
SIEGREDEAQGSE----------
--RGKEEQLQKAFVIEKRK----------LCQI
SPEGREDEDQGPE----------
STEGREDKDRGSE----------
STEGREDEAQGSE----------
STKGSVDEDQGAE----------

SIEGTCRQAASQQRARPGA----------VTRE
SIEGSCRQETNHQSAGPGA----------VARE
--E-SLT-RIIPDATSTAID------IAEGSHTQPD----------SQEAWD
--D-SLP-TITPAETSPINY------VSEDSPKQQD----------SQEEWD
--E-SLT-QILPGASRAIGC------VQG-VP----------

STEGREDEDHGSE----------
STENQEDEDQGSE----------
SREGREDKDHGSE----------

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|T.Truncatus-TAC4|ENSTTRP00000000445.1|          STEGPEDEDHWSE----------------------------------------------
|L.Africana-TAC4|ENSLAFP00000012829.3|          ----------------------------------------------------------
|E.Europaeus-TAC4|ENSEEUP00000005053.1|         ----------------------------------------------------------
|E.caballus-TAC4|ENSECAP00000012476.1|          --TGREDEDHGSE----------------------------------------------
|P.Capensis-TAC4|ENSPCAP00000005735.1|          CVGDQE-KDQGPE----------------------------------------------
|D.Ordii-Tac4|ENSDORP00000013872.1|             SIEGAE-DSQGSE----------------------------------------------
|E.telfairi-Tac4|ENSETEP00000010247.1|          ETEDPE-EDQGPE----------------------------------------------
|M.mulatta-TAC4|ENSMMUP00000018485.2|           STEGREDEAQGSE----------------------------------------------
|P.vampyrus-TAC4|ENSPVAP00000003545.1|          HTEGRKDEDHGSE----------------------------------------------
|M.murinus-TAC4|ENSMICP00000015034.1|           FSVGREGEDQGSE----------------------------------------------
|A.melanoleuca-TAC4|ENSAMEP00000012879.1|       ----------------------------------------------------------
|O.princeps-TAC4|ENSOPRP00000006609.1|          SRKGSTDVDQRSE----------------------------------------------
|C.hoffmanni-TAC4|ENSCHOP00000003804.1|         PTG----------------------------------------------------------
|I.tridecemlineatus-TAC4|ENSSTOP00000007095.2|  SMEGTEDEDPGSE----------------------------------------------
|T.syrichta-TAC4|ENSTSYP00000010450.1|          STEGRKDDDQGSE----------------------------------------------
|T.belangeri-TAC4|ENSTBEP00000002423.1|         SIEGREEEDQGAE----------------------------------------------
|O.latipes|DK025687|                            --G-VED-EWNSDSY------------------------------------------------
|G.aculeatus|DV007667|                          --D-VEE-EWNSDS-------------------------------------------------
|D.rerio|XM_001923424|                          ----------------------------------------------------------
|O.niloticu|GR699402|                           --D-MEE-EFKSDSY------------------------------------------------
|L.Calcarifer|DQ290174|                         ----------------------------------------------------------

|HUMAN-TAC1|HSU37529|                           --QNYERRR------------------------------------------
|G.Gorilla-TAC1|XM_004045800.1|                 --QNYERRR------------------------------------------
|M.Mulatta-TAC1|ENSMMUP00000035078.1            --QNYERRR------------------------------------------
|B.Taurus-TAC1|BC151422.1|                      --QDYERRRK-----------------------------------------
|O.Aries-TAC1|NM_001082596.1|                   --QDYERRRK-----------------------------------------
|P.Troglodytes-TAC1|XM_001171112.2|            --QNYERRR------------------------------------------
|P.Abelii-TAC1|XM_002818228.1|                 --QNYERRH------------------------------------------
|P.Anubis-TAC1|XM_003896287.1|                 --QNYERRR------------------------------------------
|P.Paniscus-TAC1|XM_003809689.1|               --QNYERRR------------------------------------------
|O.Garnettii-TAC1|XM_003782698.1|              --QNYERRRK-----------------------------------------
|C.Porcellus-TAC1|NM_001172899.1|              --QNYERRRE-----------------------------------------
|C.Lupus-familiaris-TAC1|XM_532472.3|          --QNYERRRK-----------------------------------------
|O.Cuniculus-TAC1|X62994.1|                    --QNYERRRK-----------------------------------------
|F.Catus-TAC1|XM_003982791.1|                  --QDYERRRK-----------------------------------------
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|S.Harrisii-TAC1|XM_003772510.1|                    VSHGWSPAQTGEAISSSTEKSVLTRVFGMGRST--ELREKA----------
|R.Norvegicus-Tac1|NM_012666.2|                     --QNYERRRK----------------------------------------
|M.Musculus-Tac1|BC117081.1|                       --QNYERRRK----------------------------------------
|M.Putorius-TAC1|JP019158.1|                       --QNYERRR-----------------------------------------
|T.belangeri-TAC1|Z50786.1|                         --QDYERRR-----------------------------------------
|S.Boliviensis-TAC1|XM_003921239.1|                --QNYERRRK----------------------------------------
|T.Guttata-TAC1|XM_002197398.1|                     --QNYERRRK----------------------------------------
|G.Gallus-TAC1|BK008126.1|                         --QNYERRRK----------------------------------------
|X.Laevis-tac1|NM_001096537.1|                     SLLNYYDTRRK---------------------------------------
|X.Tropicalis-tac1|BC155674.1|                     ---YYDTRRK---------------------------------------
|Hyla.simplex-tac1|HM747308.1|                     TIQNYER-RRK---------------------------------------
|O.Vulgaris-OctTK-I|AB085916.1|                    ----------------------------------------------------
|O.Vulgaris-OctTK-II|AB085917.1|                   ----------------------------------------------------
|A.Aegypti-sialokininI|AF108102.1|                 ----------------------------------------------------
|C.parva-TAC1|FJ696706.1|                           ----------------------------------------------------
|G.aculeatus-tac1|ENSGACP00000000114.1|            TLQTYDKRR-----------------------------------------
|M.Auratus-tkn-I|X80662.1|                         --QNYERRRK----------------------------------------
|D.Rerio-tac1|BK008124.1|                          TVQIYDKRR-----------------------------------------
|O.Mykiss-tac1|BK008119.1|                         ALQNYDKRR-----------------------------------------
|S.Fontinalis-tac1|BK008120.1|                     TLQNYDKRR-----------------------------------------
|A.Fimbria-tac1|JO691854.1|                        TIQTYDKRR-----------------------------------------
|S.Rastrelliger-tac1|BK008106.1|                   TIQTYDKRR-----------------------------------------
|I.Punctatus-tkn1|NM_001200768.1|                 LLQNYYERR-----------------------------------------
|S.Salar-tac1|BT125493.1|                          TQQNYNKRR-----------------------------------------
|O.Latipes-tac1|AB441191.1|                        TIRTYDQRR-----------------------------------------
|O.Mordax-tac1|BT075724.1|                         TIQNLDNRR-----------------------------------------
|C.Auratus-tac1|CAU61272|                          TVQIYDKRR-----------------------------------------
|S.Caurinus-tac1|BK008122.1|                       TVQTYDKRR-----------------------------------------
|V.Pacos-tac1|ENSVPAP00000010638|                  --QNYERRRK----------------------------------------
|D.Novemcinctus-TAC1|ENSDNOP00000011010.1|        --HNYERRRK----------------------------------------
|C.Intestinalis-tac1|ENSCINP00000016566.3|        TILNKYCDEN----------------------------------------
|P.Sinensis-tac1|ENSPSIP00000004426.1|            ---NYERRRK----------------------------------------
|M.fascicularis-TAC-1|AB220474.1|                 --QNYERRR-----------------------------------------
|G.Morhua-tac1|ENSGMOP00000005761.1|              SRHDYEQRRR---------------------------------------
|T.Truncatus-TAC1|ENSTTRP00000004482.1|          --QNYERRHK----------------------------------------
|L.Africana-TAC1|ENSLAFP00000000757.2|           --QNYERRRK----------------------------------------
|T.Rubripes-tac1|ENSTRUP00000014475.1|            TIQTYDKRR-----------------------------------------
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|N.Leucogenys-TAC1|ENSNLEP00000019442.1          --QNYERRR----------
|E.Europaeus-TAC1|ENSEEUP00000002012.1|          --QDYERRRK---------
|E.Caballus-TAC1|ENSECAP00000002225.1|           --QNYERRRK---------
|P.capensis-TAC1|ENSPCAP00000015000.1|           ----------
|D.ordii-Tac1|ENSDORP00000001980                 --PTYERRRK---------
|E.telfairi-TAC1|ENSETEP00000015772.1|          --QNYERRRK---------
|C.jacchus-tac1|ENSCJAP00000001061.1|           --QNYERRRK---------
|P.vampyrus-tac1|ENSPVAP00000014792.1|          --QNYERRRK---------
|M.lucifugus-tac1|ENSMLUP00000002112.2|         --QNYERRRK---------
|M.murinus-TAC1|ENSMICP00000014896.1|           ----------
|M.domestica-TAC1|ENSMODP00000020552.3|         ---NYERRRK---------
|A.melanoleuca-TAC1|ENSAMEP00000017243.1|       --QNYERRR----------
|S.scrofa-TAC1|ENSSSCP00000020239.1|            --QNYERRR----------
|O.princeps-TAC1|ENSOPRP00000005183.1|          --QNYERRRK---------
|X.maculatus-tac1|ENSXMAP00000016896.1|         TIQRR------------
|S.araneus-TAC1|ENSSARP00000008825.1|           --QNYERRRK---------
|C.hoffmanni-TAC1|ENSCHOP00000003135.1|         --QNYERRRK---------
|I.tridecemlineatus-TAC1|ENSSTOP00000004825.2|  --QNYERRRK---------
|T.nigroviridis-tac1|ENSTNIP00000021330.1|      TLQRSGRR----------
|T.nigroviridis-tkn|CR713079.2|                 ILLNKCRLRFLQGP-----
|T.nigroviridis-tachykinin|CR650252.2|         QMLN-------------
|M.gallopavo-TAC1|ENSMGAP00000010069.2|        --QNYERRRK---------
|M.eugenii-TAC1|ENSMEUP00000003075.1|          ---NYERRRK---------
|Human-TAC3|AF537113.1|                        P------L----------
|Gorilla-TAC3|XM_004053407.1|                  P------L----------
|P.troglodytes-TAC3|XM_003313573.1|           P------L----------
|M.mulatta-TAC3|XM_001115535.1|               L------RAE---------
|P.anubis-TAC3|XM_003906628.1|                P------L----------
|P.paniscus-TAC3|XM_003824897.1|              P------L----------
|P.abelii-TAC3|XM_003778069.1|                P------L----------
|O.garnettii-TAC3|XM_003790546.1|            ----------
|S.boliviensis-TAC3|XM_003926696.1|          P------SAK---------
|N.leucogenys-TAC3|XM_003252783.1|           P------RAE---------
|E.caballus-TAC3|XM_001488415.3|             ----------
|L.africana-TAC3|XM_003405564.1|             ----------
|A.melanoleuca-TAC3|XM_002916001.1|          ----------
|B.taurus-TAC3|NM_181017.1|                   ----------
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|O.aries-TAC3|XM_004009563.1|                                  ----------
|C.hircus-TAC3|AB499062.1|                                     ----------
|S.scrofa-TAC3|AY758208.1|                                     ----------
|C.porcellus-TAC3|XM_003475961.1|                             ----------
|C.lupus-familiaris-TAC3|XM_843938.2|                         ----------
|F.catus-TAC3|XM_003988924.1|                                 ----------
|C.jacchus-TAC3|XM_002752636.2|                              P------SAK----------
|M.musculus-tac2|BC031348.1|                                  ----------
|A.mississippiensis-TAC3|BK008115.1|                         ----------
|X.tropicalis-tac3|BK008110.1|                               RYSTKCRMKFRM----------
|O.mordax-tac3|BK008111.1|                                   IFYNKCRLRFRRGL----------
|B.saida-tac3|BK008109.1|                                    VFLNKCRLRFRRGF----------
|O.latipes-tac3|BK008114.1|                                  VLFNKSRLRFLQGL----------
|G.morhua-tac3|BK008107.1|                                   VFLNKCRLRFRRGF----------
|P.promelas-tac3|BK008100.1|                                 IFFNKCKLRFRRGL----------
|I.punctatus-tac3|BK008101.1|                               LFFNKCRLRFRRGL----------
|S.salar-tac3A|BK008102.1|                                   LFFNKCRLRFRRGL----------
|D.mawsoni-tac3|BK008104.1|                                  IFLNKCRLRFLQGL----------
|S.rastrelliger-tac3|BK008105.1|                            IFLNKCRLRFLQGL----------
|D.rerio-tac3a|JN392856.1|                                   IFFNKCKLRFRRGL----------
|D.rerio-tac3B|JN392857.1|                                   IFFKDGRLRFCCGV----------
|S.salar-tac3b|BK008103.1|                                   ILIKKGRLRFVPGV----------
|C.griseus-TAC3|XM_003507519.1|                             ----------
|V.Pacos-TAC3|ENSVPAP00000008530|                           -------LY----------
|A.carolinensis-TAC3|ENSACAP00000007184.2|                  ----------
|D.Novemcinctus-TAC3|ENSDNOP00000008709.1|                  P------LG----------
|T.Truncatus-TAC3|ENSTTRP00000012378.1|                     -------PL----------
|M.Putorius-furo-TAC3|ENSMPUP00000001685.1|                 ----------
|E.Europaeus-TAC3|ENSEEUP00000014342.1|                     -------LLE----------
|D.ordii-Tac2|ENSDORP00000001191.1|                         F----------
|E.telfairi-TAC3|ENSETEP00000012112.1|                      ----------
|P.capensis-TAC3|ENSPCAP00000011373.1|                      P------L----------
|P.vampyrus-TAC3|ENSPVAP00000003713.1|                      P------L----------
|M.murinus-TAC3|ENSMICP00000001966.1|                       G------PLL----------
|M.domestica-TAC3|ENSMODP00000024199.2|                     ----------
|O.princeps-TAC3|ENSOPRP00000012136.1|                      H------LG----------
|O.anatinus-TAC3|ENSOANP00000028149.2|                      ----------
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|O.cuniculus-TAC3|ENSOCUP00000016272.1|                  ----------------------------------------------------
|R.norvegicus-Tac2|ENSRNOP00000005679.1|                 ----------------------------------------------------
|S.araneus-TAC3|ENSSARP00000007866.1|                    P---------------------------------------------------
|I.tridecemlineatus-TAC3|ENSSTOP00000011019.2|          ----------------------------------------------------
|S.harrisii-TAC3|ENSSHAP00000005749.1|                   ----------------------------------------------------
|T.belangeri-TAC3|ENSTBEP00000011487.1|                  ----------------------------------------------------
|M.eugenii-TAC3|ENSMEUP00000006824.1|                    P------PILRPGIKK--------TYW---------------------------
|HUMAN-TAC4|AF515828.1|                                  ----------------------------------------------------
|G.gorilla-TAC4|XM_004041438.1|                         ----------------------------------------------------
|P.abelii-TAC4|XM_002834279.1|                          ----------------------------------------------------
|P.troglodytes-TAC4|XM_001168519.1|                    ----------------------------------------------------
|P.paniscus-TAC4|XM_003818040.1|                       ----------------------------------------------------
|O.garnettii-TAC4|XM_003786485.1|                      ----------------------------------------------------
|C.jacchus-TAC4|XM_002748441.1|                        ----------------------------------------------------
|O.aries-TAC4|XM_004013353.1|                          NLEFDCRNYRRRNLN-----------WANGG----------P-------------
|C.lupus-familiaris-TAC4|ENSCAFP00000031398.1|        ----------------------------------------------------
|S.scrofa-TAC4|BK008123.1|                             ----------------------------------------------------
|S.boliviensis-TAC4|XM_003931259.1|                    ----------------------------------------------------
|O.cuniculus-TAC4|AY471576.1|                          ----------------------------------------------------
|M.putorius-TAC4|JP019159.1|                           ----------------------------------------------------
|M.musculus-Tac4|BC119426.1|                           SLQS--REEDEAPLTTSNV-------------------------------------
|R.norvegicus-Tac4|AY471575.1|                         SLQS--QRGRSEPPNHQQH-----VALSLGTEEDDQSSERAPRDASQMMPRPSRP
|O.mykiss-tac4|BK008118.1|                            QLLYDYS-------------------------------------------------
|O.mordax-tac4a|BK008112.1|                           KLQYY--------------------------------------------------
|B.saida-tac4|BK008108.1|                             ----------------------------------------------------
|R.chensinensis-ranachensinin|HE863666.1|             ----------------------------------------------------
|V.Pacos-tac4|ENSVPAP00000000575.1|                   ----------------------------------------------------
|D.Novemcinctus-TAC4|ENSDNOP00000004237.1|           ----------------------------------------------------
|F.Catus-TAC4|ENSFCAP00000002434.2|                   ----------------------------------------------------
|G.Morhua-tac4|ENSGMOP00000003442.1|                  ----------------------------------------------------
|T.Truncatus-TAC4|ENSTTRP00000000445.1|               ----------------------------------------------------
|L.Africana-TAC4|ENSLAFP00000012829.3|                ----------------------------------------------------
|E.Europaeus-TAC4|ENSEEUP00000005053.1|               ----------------------------------------------------
|E.caballus-TAC4|ENSECAP00000012476.1|                ----------------------------------------------------
|P.Capensis-TAC4|ENSPCAP00000005735.1|                ----------------------------------------------------
|D.Ordii-Tac4|ENSDORP00000013872.1|                    ----------------------------------------------------
|E.telfairi-Tac4|ENSETEP00000010247.1|                ----------------------------------------------------
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|M.mulatta-TAC4|ENSMMUP00000018485.2|          ----------
|P.vampyrus-TAC4|ENSPVAP00000003545.1|        ----------
|M.murinus-TAC4|ENSMICP00000015034.1|         ----------
|A.melanoleuca-TAC4|ENSAMEP00000012879.1|     ----------
|O.princeps-TAC4|ENSOPRP00000006609.1|        ----------
|C.hoffmanni-TAC4|ENSCHOP00000003804.1|       ----------
|I.tridecemlineatus-TAC4|ENSSTOP00000007095.2| ----------
|T.syrichta-TAC4|ENSTSYP00000010450.1|        ----------
|T.belangeri-TAC4|ENSTBEP00000002423.1|       ----------
|O.latipes|DK025687|                           ----------
|G.aculeatus|DV007667|                         ----------
|D.rerio|XM_001923424|                         ----------
|O.niloticu|GR699402|                          ----------
|L.Calcarifer|DQ290174|                        ----------
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Appendix 4. Published abstracts. Poster presentation at the Winter Neuropeptides Meeting.

IDENTIFICATION OF NOVEL TACHYKININ PEPTIDES BY *IN SILICO* ANALYSIS AND ELUCIDATION OF THEIR FUNCTIONAL EVOLUTION.

Deliconstantinos, G.[1]; Barton, S. J.[2]; Soloviev, M.[3] & Page, N. M.[1]

[1]School of Life Sciences, Kingston University London, UK; [2]School of Pharmacy & Chemistry, Kingston University London, UK; [3]School of Biological Sciences, Royal Holloway, University of London, UK.

Aims: Tachykinins comprise a large family of signaling peptides based on similarities in their sequence receptor binding and signal transduction. They comprise a signature motif FXGLM-NH_2 where X is known to be substituted by a hydrophobic residue (F, Y, I, V). They are found in both vertebrates and invertebrates. Although, the phylogenetic relationship of tachykinin receptors has been reported, there is little previous evidence of phylogenetic or functional divergence analysis of the tachykinins themselves. In the present study, we aim to identify and expand the known tachykinin sequences and motifs, determine the role of motif divergence and the role of gene duplication and tachykinin redundancy.

Methods: Tachykinins are cleaved from preprotachykinin sequences normally at dibasic cleavage sites (KK/KR/RR) that are preceded by a glycine. Therefore, the sequence FXGLMGK/R (replacing X for all 20 amino acids) was used to interrogate the nucleotide collection (nr/nt) and expressed sequence tags (est) databases. The tblastn algorithm parameters were set as: Expect threshold: 1,000,000; Word size: 2; Matrix: BLOSUM62; Gap Costs: Existence 12 Extension 1. All 100% matches were collected and translated in all 6 frames using purpose built software. Tachykinins were selected from false-positives on scoring parameters and a database of novel and known tachykinins was built with sequences prepared in FASTA format for ClustalW and phylogenetic analysis.

Results: Interrogation of the nr/nt nucleotide collection and est collections revealed 1616 sequences and 2170 sequences respectively that had 100% identity with the searched motif. We report a novel motif FHGLM expressed in fish and frog and also the FGGLM motif expressed in fish, frog, amphibians, birds and a mammal. We also suggest proteolytic cleavage occurring at double serine splicing site (SS). Our present results showed that tachykinins are expressed in 45 species including humans, mammals, birds, fish, cephalopod mollusks (octopus), amphibians, insects and viruses.

Conclusions: Our investigation has expanded the family of tachykinins and their motifs to include a signature motif comprising histidine a hydrophilic positively charged residue. This indicates an evolution from a hydrophilic ligand to a hydrophobic and an accompanying evolution of receptors. The divergence of tachykinin peptide sequences among species will elucidate the evolution of splicing mechanisms. Tachykinin motifs may direct peptide assembly modulate their roles in diverse biological processes.

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Presenter: Ph.D. student