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# Three serine proteinases from midguts of the hard tick *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization

A. MULENGA[1,*], O. MISAO[2] and C. SUGIMOTO[3]

*[1]Dept. of Microbiology and Immunology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, Maryland 21201, USA; [2]Graduate School of Veterinary Medicine, Hokkaido University, Kitaku, Kita 18, Nishi 9, 060-0818 Sapporo, Hokkaido, Japan; [3]The National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 080-8555 Obihiro, Hokkaido, Japan; *Author for correspondence (e-mail: amvle001@umaryland.edu; phone: +1 410 706 3337; fax: +1 410 706 0282)*

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**Abstract.** *Rhipicephalus appendiculatus* is one of the most economically important ticks distributed in south central and eastern Africa where little or no progress has been made on attempts to develop a vaccine. We have used a combination of RT-PCR, the 3′ and 5′rapid amplification of cDNA ends (RACE) to clone and sequence three cDNAs encoding full-length *R. appendiculatus* midgut serine proteinases (RAMSP). RT-PCR degenerate primers were designed from amino acid sequences surrounding active sites, $His^{57}$ and $Ser^{195}$ conserved among most known serine proteinase-like genes (Mulenga et al. 2001). Northern blotting analysis of total RNA extracted from unfed and partially fed adult ticks revealed that mRNAs for RAMSP-1 and -2 were expressed only in partially fed ticks, while RAMSP-3 mRNA was not only expressed in both unfed and partially fed ticks, it was also up-regulated as tick feeding progressed. Expression analysis by RT-PCR revealed that RAMSP-3 was predominantly expressed in midguts when compared to salivary glands. For RAMSP-1 and -2, they were expressed at equivalent levels in both midguts and salivary glands. Based on key amino acid sequence features as well as similarity comparisons from the database, we speculated that polypeptides encoded by RAMPSP-1 to -3 are structurally more closely related to chymotrypsin- than trypsin-like serine proteinases. We have based our comments on the potential of serine proteinases as candidates for tick vaccines.

## Introduction

In eastern, central and southern Africa, *Rhipicephalus appendiculatus* is among the most economically important ticks infesting livestock (Wang and Nuttal 1995; Mukhebi 1992). The importance of this tick, *R. appendiculatus*, is its role as a vector of *Theileria parva parva and T. parva lawreinci*, the causative agents of cattle disease, East coast fever and corridor diseases respectively. The monetary loses due to effects of East coast fever and corridor disease on livestock production as well as the costs of controlling these diseases amounts to hundreds of thousands of dollars (Mukhebi 1992). Suppression of the tick vector population is at present the number one method of choice to reduce the impact of tick-borne diseases of live-

stock. Ticks are routinely controlled by use of chemical acaricides (Willadsen 1987; Willadsen et al. 1996; Tellam et al. 1992). Chemical acaricides have serious limitations such as environmental and food chain contamination and these have stimulated research into alternative methods to control ticks (Willadsen 1987; Sonenshine 1993). Among the several alternative tick control methods that have been considered, immunological protection of hosts against tick infestation has been shown to be practical and sustainable (Sonenshine 1993; Mulenga et al. 2000). Studies by an Australian group (reviewed in Tellam et al. (1992), Willadsen et al. (1995) and Willadsen and Jongejan (1999)) leading to commercialization of the first ever anti-arthropod vaccine provided evidence that ticks can be immunologically controlled. For immunological control of ticks to succeed as an alternative to acaricide use, identification of key vaccine antigens is a necessary pre-requisite (Mulenga et al. 2000; Elvin and Kemp 1994). We are interested in tick proteins that are involved in mediation of blood meal acquisition and digestion by ticks.

Serine proteinases are considered potential target tick vaccines antigens because of their involvement in regulation of several physiological and developmental processes in a wide range of organisms ((Neurath 1984, 1986; Rao et al. 1998; Krem and Di Cera 2001)). It is postulated that if serine proteinase function can be interfered with, an appreciable degree of tick physiology will be compromised ((Lehane 1994; Mulenga et al. 2000, 2001)). One other advantage of targeting serine proteinases as candidate vaccine antigens is the fact that they are generally secreted in the extra cellular environment and hence they are likely to be exposed to host antibodies (Vaughan and Azad 1988; Ben-Yakir 1989; Allingham et al. 1992). In the present study we have described molecular cloning by rapid amplification of cDNA ends (RACE) and preliminary characterization of three serine proteinase genes from midguts of the brown ear tick, *R. appendiculatus*.

## Materials and methods

### *Tick dissection*

A colony of *R. appendiculatus* ticks that was used in this study was kindly provided by the international livestock research institute (ILRI) in Kenya. Tick dissection was carried out as described elsewhere (Ribeiro 1988; Mulenga et al. 1999). Briefly, adult female *R. appendiculatus* ticks partially fed on rabbits for 4 d were submerged in sterile DPEC treated water and held down using a soft tissue forcept under a dissection light microscope. The dorsal cuticle was cut out with a scapel blade and organs were separated using an 18 gauge needle. The separated tick organs, midguts, salivary glands and carcasses (remnants of the tick body after removal of midguts and salivary glands) were transferred directly into the RNA extraction reagent, TRIZOL (GIBCO BRL, USA).

*Extraction of tick total RNA*

Total RNA was extracted from adult female ticks, unfed or partially fed for 48 and 96 – 120 h as well dissected tick organs (salivary glands, midguts and carcasses). Partially fed ticks were detached from rabbit ears by traction and left at room temperature for about 1 h to shed off remnants of rabbit pieces of tissue and hair. Whole ticks were pulverized in liquid nitrogen prior to extraction of total RNA. For rabbit RNA, 20 ml of rabbit blood was collected in acid dextrose and centrifuged at room temperature at 2500 rpm to separate the buffy coat (Mulenga et al. 2001). The buffy coat was mixed with the RNA extraction solution, the TRIZOL reagent (GIBCO-BRL, USA) at a 1:3 ratio. Total RNA was extracted using the TRIZOL reagent (GIBCO-BRL, USA) according to the manufacturer's instructions.

*Reverse Transcription-Polymerase Chain Reaction (RT-PCR) amplification of the serine proteinase gene fragment*

Design of degenerate primers used here was described elsewhere (Mulenga et al. 2001). Total RNA (5 µg) extracted from tick midguts was used in a standard RT-PCR reaction to generate the first strand cDNA. A one µl aliquot of the RT-PCR reaction mix was used in a hot-start touch-down PCR reaction with annealing temperatures decreasing from 55 °C to 40 °C over 30 cycles and the final 20 cycles maintained at 50 °C. The resulting PCR fragment was purified using a gene clean kit according to the manufacturer's instructions (Promega, USA) and ligated into pGEM-T cloning vectors (Promega, USA). The resulting plasmid was transfected and subsequently amplified in *Escherichia coli* strain DH 5α (Promega, USA) according to standard protocols. DNA sequencing was carried out as described below using T7 and sp6 promoter primers (Promega, USA).

*Rapid amplification of cDNA ends (RACE) to clone the full-length tick* R. appendiculatus *midgut serine proteinase genes (RAMSP)*

Details of the cloning protocol were described elsewhere (Mulenga et al. 2001). Briefly, following cloning and DNA sequencing of the RT-PCR product (1st PCR), anti-sense gene specific primers (GSP) for use in 5′ RACE (2nd PCR) were designed. After cloning and determining the nucleotide sequence of the 5′ end gene fragments, sense GSPs were designed and used in a 3′ RACE protocol to clone the full length genes (3rd PCR). PCR fragments were routinely cloned into pGEM-T vectors (Promega, USA) and sequenced using T7 and SP6 promoter primers (Promega) and gene specific primers where necessary. The cloned cDNAs were identified as possible members of the serine proteinase gene family by presence of consensus motifs, highly conserved among most known serine proteinases ((Elvin et al. 1993, 1994; Greer 1990; Sakanari et al. 1989; Mulenga et al. 2001)) and homology matching to other known proteins on the SWISS-PROT data base.

*Restriction enzyme analysis*

To investigate the possibility of different types of serine proteinase genes being present in the amplified PCR product (1st PCR product), 60 cloned serine proteinase fragments were subjected to restriction enzyme digestion using *Sac*II. The digestion products were analyzed on a 2% agarose gels containing 1 µg/µl ethidium bromide. Different serine proteinase fragments were identified based on the different migration profiles. Representative clones for each migration profile was sequenced as described below.

*DNA sequencing and analysis of the cloned PCR products*

Nucleotide sequences were determined using the dye terminator system (Applied Biosystems, USA) and an automated sequencer (Applied Biosystems, 310 Genetic analyzer). The template for sequencing was generated by purification of recombinant plasmid DNA using miniprep spin columns according to instructions by the manufacturer (Qiagen, USA). DNA Sequence analysis was done using the GENETX MAC software packages in combination with the GENBANK and SWISS-PROT databases for comparison with other known protein sequences.

*Northern blotting analysis*

To confirm sizes of transcripts and to determine whether expression of the tick serine proteinases encoded by the cloned cDNAs was influenced by blood meal uptake, total RNA extracted from adult ticks, unfed or partially fed for 96 to 120 h was subjected to northern blotting analysis. Total RNA extracted from rabbit white blood cells was used for negative control. Sample (20–30 µg total RNA per lane) treatment, electrophoresis and transfer to Hybond N+ membranes were done as described in NorthernMax protocol (Ambion, USA). Following transfer, RNA was cross-linked by exposing the membranes for 3 min to a UV (312 nm wave length) trans-illuminator (UVP Inc., USA). Hybridization probes (RAMSP-1 to -3) were labeled with Psolaren-Biotin as described by the manufacturer (Ambion, USA). Hybridization was carried out overnight at 42 °C in Ultrahyb (Ambion, USA) hybridization buffer. The membranes were washed to final stringency of 0.1X SSC plus 0.1 % SDS at 42 °C twice for 15 min each and subsequently analyzed using a multi-image scanner (Biorad, USA) up to 4 h when the optimum signal was achieved.

*Expression analysis by RT-PCR*

To investigate the organ/tissue expression pattern for RAMSP-1 to -3, total RNA extracted from dissected tick organs (midguts and salivary glands) and carcasses was subjected to RT-PCR analysis. Total RNA (5 µg) was used in a standard RT reaction to generate the first strand cDNA. One (l aliquots of the RT products were used in a PCR reaction with gene specific primers designed from the extreme ends

of the ORFs for RAMSP-1 to -3. Aliquots (10 µl) of the PCR products were analyzed on a 1.5% agarose gel containing 1 µg/ml ethidium bromide.

## Results

*cDNA Cloning and DNA sequence analysis*

Degenerate primers were successfully used to amplify a 460 bp *R. appendiculatus* tick serine proteinase gene fragment (results not shown). Restriction enzyme analysis of 60 cloned serine proteinase fragments revealed that at least three different serine proteinase were amplified. Three representative clones for each group were sequenced and herein named as *R. appendiculatus* midgut serine proteinases (RAMSP)-1 to -3. The full-length RAMSP-1 cDNA is about 1.3 kb and encodes a 298 amino acid polypeptide with 32.3 kDa predicted molecular mass (Figure 1a). For RAMSP-2 and -3, both cDNAs are 1.6 kb and encode 474 and 461 amino acid polypeptides with 51.2 and 49.5 kDa predicted molecular mass respectively (Figure 1b and c). Nucleotide sequences for full-length cDNAs have been deposited in GenBank with the following accession numbers AY078093, AY078094 and AY078095 for RAMSP-1 to -3 respectively. The Respective predicted amino acid sequences for RAMSP-1 to -3 (see Figure 1a – c) show conservation of the serine proteinase consensus catalytic triad (See Greer (1990) and Krem and Di Cera (2001) for comparison) including His at positions 89, 294, 252, Asp at positions, 132, 341, 300 and Ser at positions 224, 417, 396. By use of the web based PsortII signal (http//psort.nibb.ac.jp) peptide prediction program only RAMSP -3 deduced polypeptide seemed to have a 15 amino acid residue predicted signal peptide while RAMSP-1 and -2 do not have. On visual analysis, polypeptides for RAMSP-1 to -3 have one potential N-glycosylation site each, while RAMSP-2 has two sites (Figure 1a – c). The chymotrypsin- and trypsin-like serine proteinases N-terminal consensus motif, IleValGlyGly (Zhu and Baker 1999) is partially conserved in all three serine proteinases (RAMSP-1 to -3) reported here at positions 45 – 48 (Figure 1a), 251 – 254 (Figure 1b) and 209 – 212 (Figure 1c). This motif (IleValGlyGly) is by consensus preceded by either Arg or Lys (Zhu and Baker 1999). In all three serine proteinase genes (RAMSP-1 to -3) Arg is conserved (Figure 1a to c). In RAMSP-1, the first and third amino acid residues of the motif, IleValGlyGly are replaced by Val and Asp respectively (Figure 1a) while for RAMSP-2, Ile is replaced by Val (Figure 1b). For RAMSP-3, the third amino acid residue, Gly is replaced by Ala. The three amino acid residues $Gly^{411}$, $Gly^{434}$, and $Asp^{444}$ that determine chymotrypsin specificity (Kraut 1977; Zhu and Baker 2000) are conserved in RAMSP-2 (Figure 1b). For RAMSP-3, $Gly^{390}$ and $Gly^{414}$ are conserved while $Met^{424}$ replaces Asp (Figure 1c). In RAMSP-1, $Thr^{218}$ and $Lys^{245}$ replace the first Gly and Asp respectively (Figure 1a). Multiple sequence alignment of RAMSP-1 to -3 predicted amino acid sequences with the two serine proteinases from *H. longicornis*, HLSG-1 and -2 (Mulenga et al. 2001) revealed that, in addition to classical serine proteinase

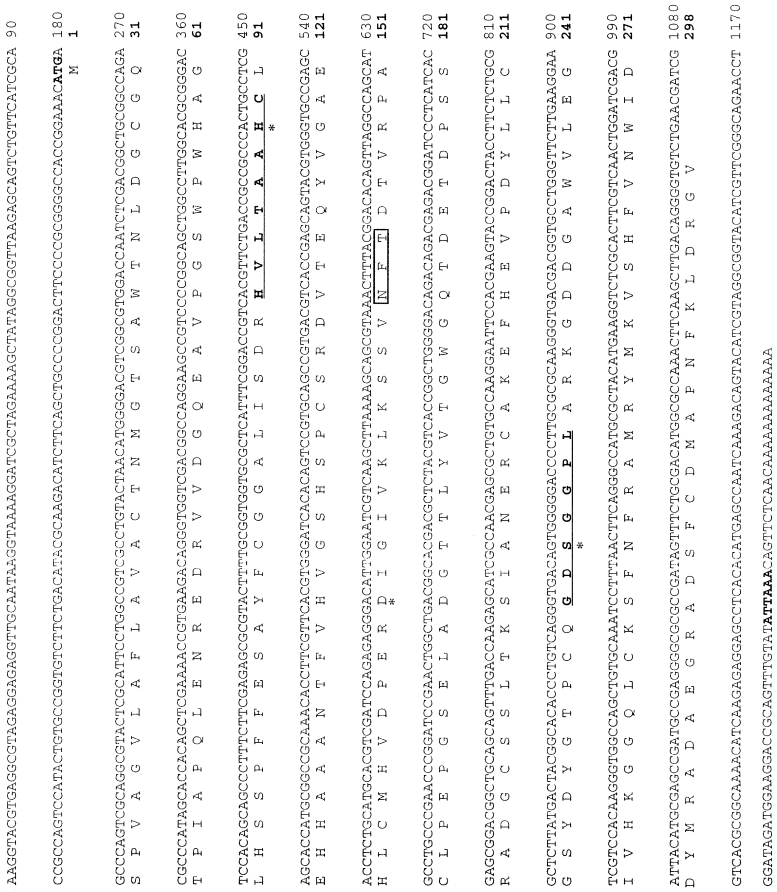

*Figure 1.* The nucleotide and deduced amino acid sequence of RAMSP-1 (a), RAMSP-2 (b) and RAMSP-3 (c) cDNAs. The upper and lower numbers, represent nucleotide and amino acid sequence positions respectively. The predicted signal peptides are underlined, while the potential N-glycosylation sites are boxed. The consensus amino acid sequence motifs that were used to design primers are boldfaced and underlined. The start codon (ATG) and polyadenylation signal AATAAA are boldfaced. The star sign (*) denotes the catalytic triad residues. The nucleotide sequences for RAMSP-1 to -3 have been deposited in GenBank, with accession numbers, AY078093, AY078094 and AY078095 respectively.

motifs, the 6 consensus cysteine (C) residues were conserved in RAMSP-1 to -3 (Figure 2, see Greer (1990) for comparison). The cysteine residues are necessary for formation of disulfide bonds (Wang et al. 1995).

Predicted amino acid sequences for RAMSP-1 to -3 were scanned against known proteins on the database. Overall similarities to the best matches listed in (Table 1) ranged between 33 – 42%, 35 – 36% and 34 – 35% for RAMSP-1 to -3 respectively. Of the top 10 best matches to RAMSP-1, 9 were chymotrypsin A-like including a serine proteinase from the hard tick, *H. longicornis* (Mulenga et al. 2001). For RAMSP-2, it showed similarity predominantly to the homo sapiens membrane

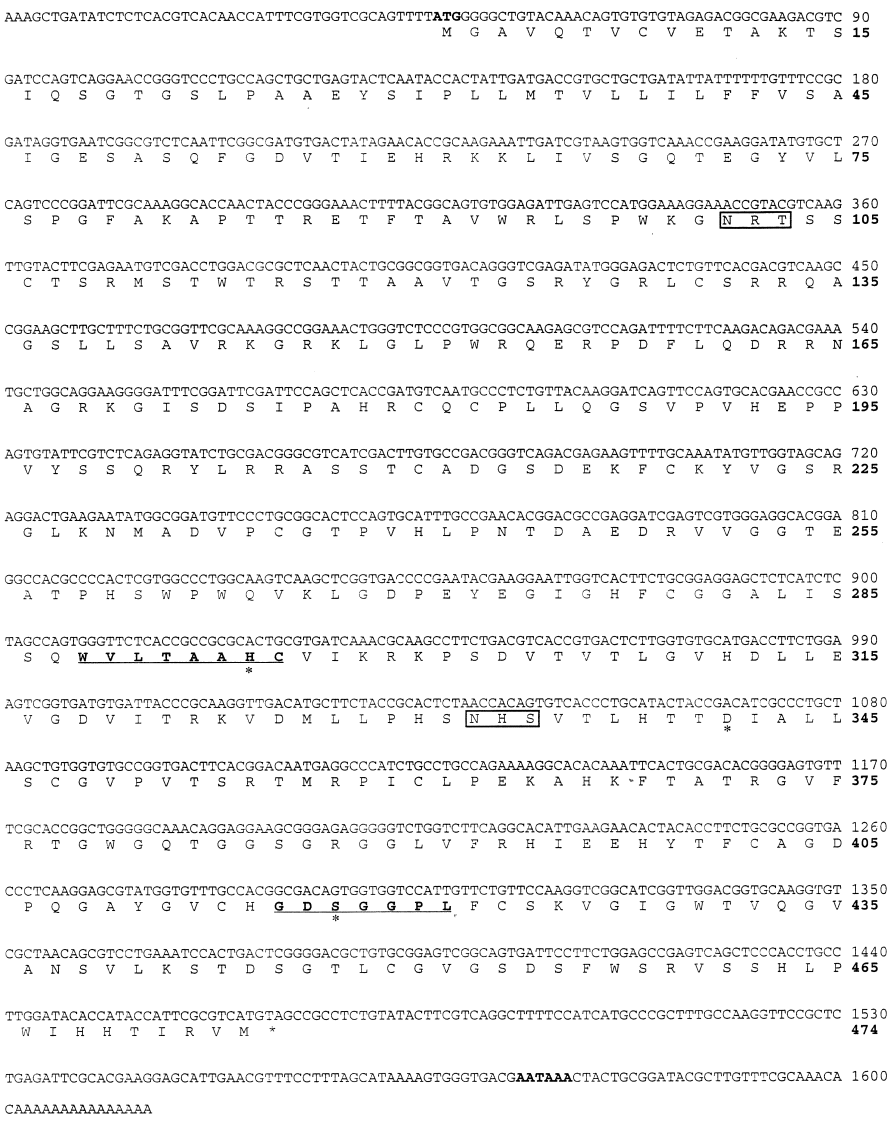


*Figure 1.* Continued

type serine proteinases, while 8 of the 10 best matches to RAMSP-3 were chymotrypsin B-like.

*Northern blotting analysis*

Total RNA extracted from adult female ticks, unfed or partially fed for 96 – 120 h as well as rabbit white blood cells were subjected to Northern blotting analysis as

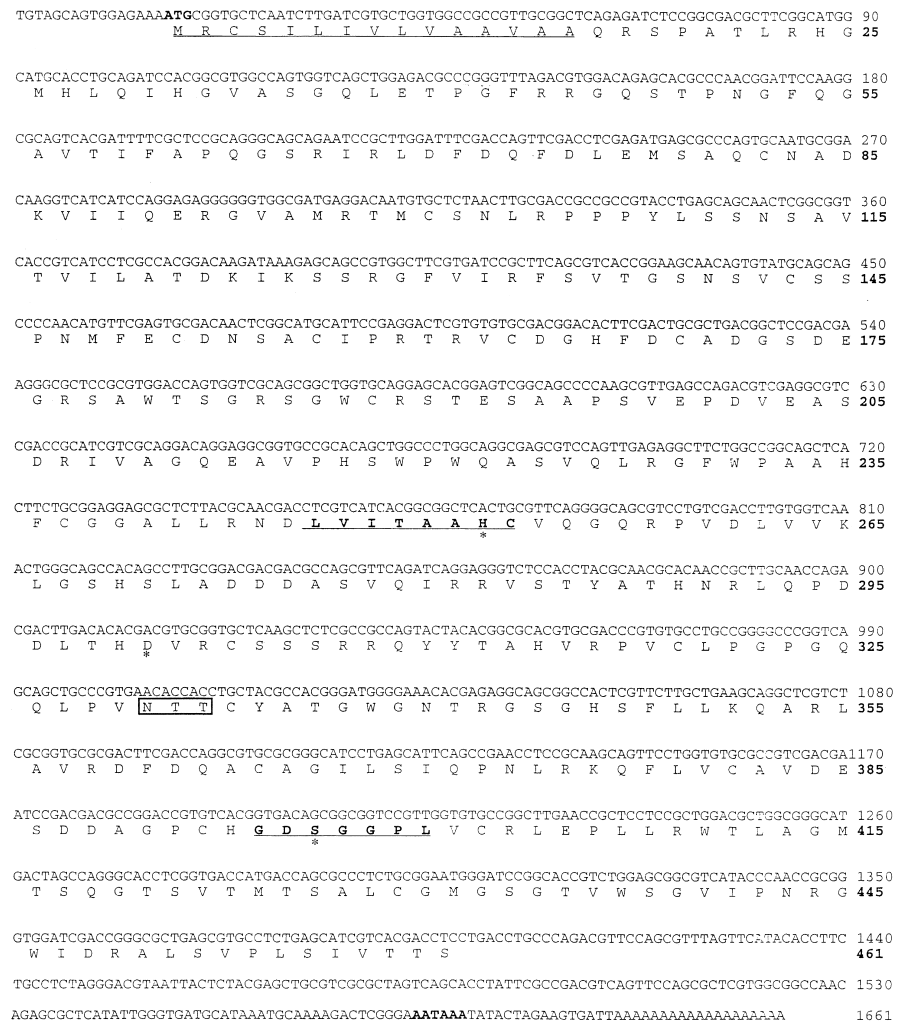


*Figure 1.* Continued

described under materials and methods. As shown in Figure 3a and 3b, Positive messages were only detected on RNA blots from partially fed ticks for RAMSP-1 and -2, while RMASP-3 (Figure 3c) hybridized bands on both unfed and partially fed tick RNA blots. Additionally, RAMSP-3 hybridized a more dense band on partially fed tick RNA blots than unfed tick RNA blots indicating that RAMSP-3 was up-regulated during feeding. All three probes did not hybridize any bands on rabbit blots (Figure 3a – 3c). Consistent with the expected size ranges, RAMSP-1 (Figure 3a) hybridized a band about 1.35 kb, while bands hybridized by RAMSP-2 and -3 were slightly higher than 1.35 kb (Figure 3b and 3c).

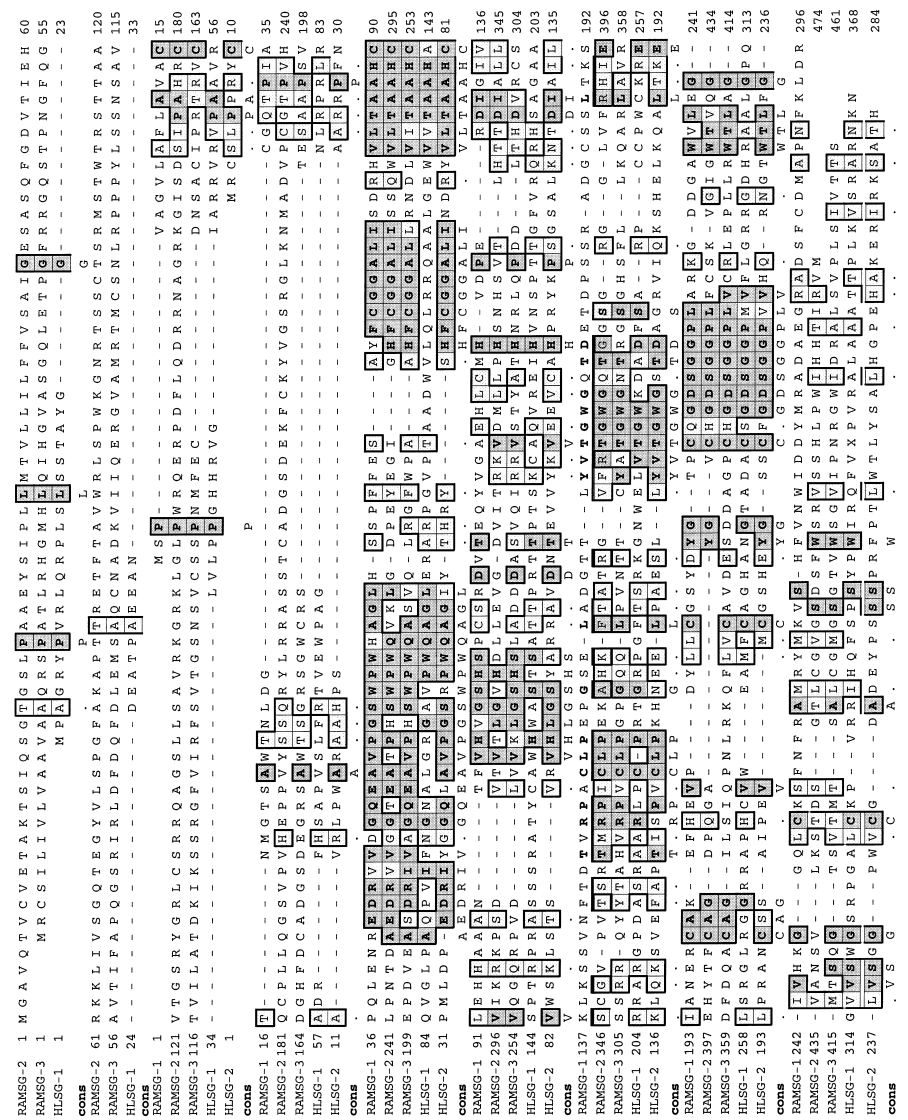

*Figure 2.* Multi-sequence alignment of the deduced RAMSP-1 to -3 amino acid sequences with *H. longicornis* serine proteinase (HLSG)-1 and -2 (Mulenga et al. 2001). Regions of high amino acid similarity are boxed and shaded gray with highly conserved (CONS) amino acid residues indicated at the bottom. Dots indicate partially similar residues while dashed lines indicate gaps to facilitate alignment similar residues. RAMSP-1 to -3 represent *R. appendiculatus* midgut serine proteinase -1 to -3.

## *Tissue specific expression analysis by RT-PCR*

To predict the tissue distribution profiles for cloned tick serine proteinases, total RNA extracted from dissected tick organs was analyzed by RT-PCR as described.

*Table 1.* Similarity comparisons of *R. appendiculatus* midgut serine proteinases (RAMSP) -1 to -3 to known proteinases on the database.

| RAMSP | Database best match | Accession # | % similarity |
|---|---|---|---|
| 1 | HLSG-2 | AB020544 | 42 |
| | HACHY | EC. 3.4.21.1 | 33 |
| | BCHYA | P00766 | 33 |
| 2 | Matripase | 2517353A | 36 |
| | HSMTP | AF133086-1 | 36 |
| | | AF118224-1 | |
| | | AF051145-1 | |
| | HST14 | Q9Y5Y6 | 36 |
| 3 | LDRP/*Mus musculus*/human | AB013874-1 | 35 |
| | | JE0135 | |
| | ATPCE | Q92319 | 35 |
| | *Gadus morhua* CHYB | AJ242521-1 | 34 |

HLSG-2: *Haemaphysalis longicornis* serine proteinase -2; HACHY: human αchymotrypsin; BCHYA: bovine chymotrypsin A; HSMTP: homo sapiens membrane type serine proteinase 1 protein; HST14: human suppressor of tumorigenicity 14; LDRP: low density lipoprotein receptor related protein 4; ATPCE: atrial natriuretic peptide converting enzyme; CHYB: chymotrypsin B.

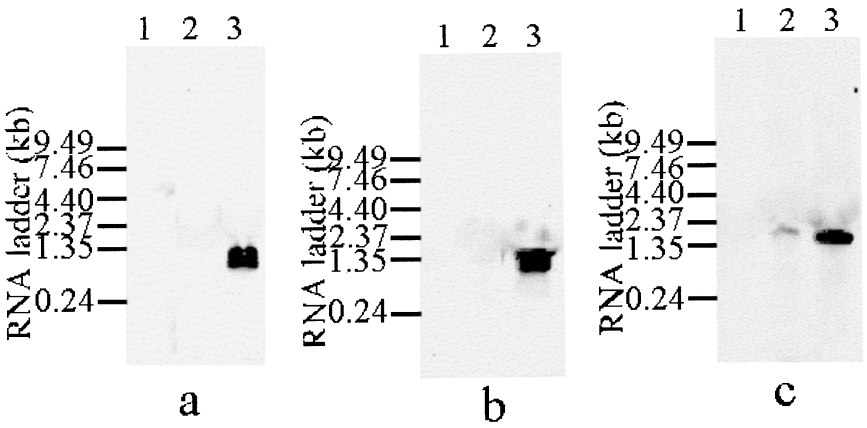


*Figure 3.* Northern blotting analysis of total tick RNA. Total RNAs extracted from rabbit white blood cells (lane 1), unfed ticks and ticks partially fed for 96 – 120 h (lanes 2 & 3 respectively) were subjected to Northern blotting analysis as described and probed with psolaren-biotin-labelled *R. appendiculatus* midgut serine proteinase (RAMSP) -1 (a), -2 (b) and -3 (c).

Results shown in Figure 4 indicate that in addition to midguts, RAMSP-1 to -3 were also expressed in other tick organs. Based on the intensity of the observed bands, it appears RAMSP-1 is expressed at equivalent levels in all tick organs, while RAMSP-2 was weakly expressed in both salivary glands and midguts (Figure 4a and 4b respectively). For RAMSP-3, it appears to be more predominantly expressed in midguts than salivary glands (Figure 4c).

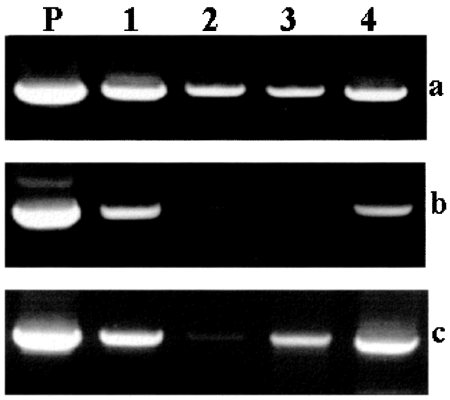


*Figure 4.* Tissue expression analysis by RT-PCR. Total RNA extracted from dissected salivary glands (lane 2), midguts (lane 3) and carcass (tick remnant after removal of salivary glands and midguts) (lane 4) was subjected to RT-PCR and analyzed as described under materials and methods. Cloned RAMSP -1 to -3 cDNAs and RNA extracted from whole ticks were used for positive controls (lanes P and 1 respectively).

## Discussion

Members of the serine proteinase gene family have been cloned from several arthropods ((Elvin et al 1993, 1994)) and a number of functions have been proposed for them in the biology of these arthropods. Here we report the first serine proteinase cDNA sequences from the tick, *R. appendiculatus*. The cloned cDNAs were identified as members of the serine proteinase gene family by presence of consensus motifs, highly conserved among most known serine proteinases ((Elvin et al. 1993, 1994; Greer 1990; Sakanari et al. 1989; Mulenga et al. 2001)) and homology matching to other known proteins on the SWISS-PROT data base. Based on current data, we are unable to determine the biological functions of the polypeptides encoded by the three cDNAs reported here. However based on key features of the RAMSP-1 to -3 predicted amino acid sequences it can be speculated that the native proteins encoded by the three cDNAs are likely to be chymotrypsin-like other than trypsin. Consistent with the observed key amino acid features, 9 and 8 of the 10 best matches to RAMSP-1 and -3 were chymotrypsin-like respectively. Hybridization of bands only to partially fed tick RNA blots by RAMSP-1 and -2 as well up-regulation of RAMSP-3 during tick feeding suggested that expression of the genes reported in this study is linked to events regulating blood meal uptake or digestion by ticks. The blood meal is the only source of nutrients for ticks and as such these tick midgut proteins are considered potential candidate tick vaccine antigens (Lehane 1994). This should be interesting, in that it raises the possibility that *R. appendiculatus* feeding activities can be interfered with through host immunization with recombinant RAMSP-1 to -3. Successful blood meal uptake is central to the tick's capacity both as vector and pest ((Sauer et al. 1995, 1996; Ribeiro 1987)) and hence a vaccine with capacity to stop ticks from a successful blood meal up-

take is mostly desired. Other studies also linked serine proteinases to blood in other hematophagous arthropods such as *H. longicornis* (Mulenga et al. 2001), *Aedes aegypti* (Jiang et al. 1997; Noriega et al. 1996), *Anopheles gambiae* (Muller et al. 1993) and the black fly, *Simulium vittatum* (Xiong and Jacobs-Lorena 1995). Because RAMSP-1 to -3 were amplified from RNA that was extracted from midguts that were dissected from rabbit-fed ticks, there was a possibility that the RNA used here was contaminated with rabbit RNA. The fact that none of the three probes, RAMSP-1 to -3 hybridized on rabbit RNA blots, ruled out the possibility that RAMSP-1 to -3 were amplified from rabbit RNA. Expression analysis by RT-PCR of total RNA extracted from dissected tick organs, the salivary glands, midguts as well as the carcass revealed that the three genes, RAMSP-1 to -3 are expressed in other tick organs in addition to midguts. This data should however be interpreted with caution because of the potential for cross-tissue contamination during tick dissection. Tick tissue is very tender and as such cross-tissue contamination is a possibility and this may in the final analysis affect the outcome of expression analysis by RT-PCR. Immunolocalization data is awaited to validate the current findings.

While the roles of proteinases in the pathogenesis of both helminthological and protozoan parasitic diseases have been extensively studied (Coombs and Mottram 1997; Dubremetz and McKerrow 1995; Rosenthal 1999), studies on ectoparasitic proteinases, particularly ticks have been quite limited. To date there has been no elaborate studies to demonstrate importance of serine proteinases in tick physiology. Despite this lack of baseline information with regard to the role of serine proteinases in the biological success of ticks both as vectors and pests, there is abundant compelling indirect evidence that makes this group of proteins appealing as target antigens for tick vaccine development. For example, addition of serine protease inhibitors such as soybean trypsin to a blood meal inhibited egg production up to 71% in *Stomoxys calcitrans* (Spates 1979). Inclusion of low levels of serine protease inhibitors such as soybean trypsin or ovomucoid in the blood meal can either kill *H. irritans* or inhibit egg development (Elvin et al. 1993). Casu et al. (1994) also observed a reduced growth rate of *Lucilia cuprina* larvae when they fed *in vitro* with added soybean trypsin inhibitor. Vaccination of cattle against *Hypoderma lineatum* with a purified serine protease stimulated a strong cellular and humoral immunity leading to 95% protection (Baron and Cowell 1991). While evidence on other arthropods may not necessarily represent events in ticks, observations from the above studies are certainly encouraging. We intend to evaluate the roles of RAMSP-1 to -3 in the biology of *R. appendiculatus* and subsequently assess their suitability as tick vaccine candidate antigens.

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