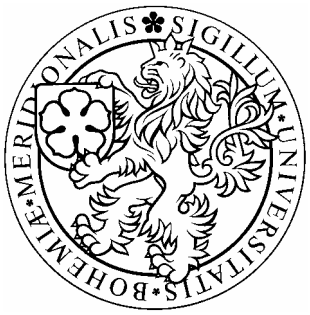
University of South Bohemia
Faculty of Biological Sciences
Department of Molecular Biology and Biochemistry



Ph.D. thesis

Immune-related molecules in the gut of the soft tick *Ornithodoros moubata*

Lenka Grunclová

Supervisor: RNDr. Petr Kopáček, CSc.
Biology Centre of the Academy of Sciences of the Czech Republic, v.v.i.
Institute of Parasitology
Laboratory of Vector Immunology

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Annotation: Several genes coding for proteins which are affected by blood-meal in the midgut of the soft tick *Ornithodoros moubata* are presented. This set comprises tick gut lysozyme (TGL), two genes coding for putative inhibitors of cysteine peptidases of cystatins family (Om-cystatin1 and Om-cystatin 2), putative thyroglobulin domain containing protease inhibitor (Om-thyropin) and serine protease homolog (SPH). Complete cDNA sequences, tissue expression profiles, regulation of expression by blood meal, localization of native proteins in tick tissues and eventual activities of recombinant fusion proteins are illustrated.

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I declare that this thesis was performed by me or in collaboration with co-authors and with the help of cited literature.

České Budějovice, May 9, 2007 Lenka Grunclová

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Contents:

Introduction

Ticks are blood feeding external parasites and very important vectors of agents causing human and animal diseases, transmitting a number of protozoan, bacterial, rickettsial, spirochaetal and viral pathogens.

The midgut in ticks does not contain extracellular digestive enzymes and serves mainly as a major storage organ of a blood meal. Digestion occurs intracellularly by the cells of midgut epithelium (Sonenshine, 1991). The absence of proteolytic enzymes in the midgut lumen would make this organ a highly favorable environment for survival of ingested microbial pathogens in case there would not be any active defense mechanisms (Munderloh et al., 1995). Naturally occurring antimicrobial peptides (AMPs) from a first line of host defense against pathogens are involved in innate immunity (Bulet et al., 2004). Only few AMPs have been isolated from ticks.

In this work, several immune-related molecules from the gut of the soft tick *Ornithodoros moubata* are characterized:

Lysozyme from the midgut of *O. moubata* is likely adapted to the digestive function. However it still shares some features with other antibacterial lysozymes that its defense role in the tick gut targeted especially against Gram-positive bacteria seems to be quite probable.

We have reported two members of the cystatin family in the soft tick *O. moubata*, and presented detailed functional and expression analyses. Our work suggests that the tick cystatin inhibitors most likely play several distinct roles and are regulated in time and space

We characterized and localized first tick cysteine protease inhibitor containing thyroglobulin type-I domain. The *O. moubata* Om-thyropin has all characteristics of other thyropin-family inhibitors, i. e. thyroglobulin type I domain containing three disulfide bonds and inhibition activity against cysteine protease cathepsin L.

The gut specific serine protease homolog was examined. Function of this homolog is unclear, it could be co-factor to serine proteases including to digestion, or interact with inhibitors of these proteases.

I. Ticks

Ticks are blood feeding external parasites of mammals, birds and reptiles occurring throughout the world. Currently 850 species have been described worldwide (Furman et al., 1984). There are two well-established families of tick, the Ixodidae (hard ticks) and Argasidae (soft ticks). Next to mosquitoes, ticks are very important vectors of agents causing human and animal diseases, transmitting a number of protozoan, bacterial, rickettsial, spirochaetal and viral pathogens. Both families of ticks differ in many respects (Tab. 1): adaptation of integument to different feeding strategy, mating and oviposition strategy, and differs in salivary gland morphology, regulation of water balance, blood meal digestion, etc.

Table 1: Survey of differences between hard ticks and soft ticks. Properties associated with blood feeding. Data obtained from Balashov (1972), Sonenshine (1991), Mans et al. (2004)

	Soft ticks (Argasidae)	Hard ticks (Ixodidae)
Integument	Leathery integument allows for fast feeding	Sclerotized scutum protects during long attachment periods but needs a growth phase to allow for rapid engorgement
Feeding	Multiple fast feeding, escapes host immune response	Single slow feeding, locates host only once and imbibes large blood meal
Mating strategy	Mate while unfed or when fed: does not limit feeding event to host location	Mate after initial attachment only. Mating essential for rapid engorgement
Oviposition strategy	Lay several eggs batches, once after every feeding event	Lay one batch of eggs, after which female dies
Salivary gland morphology	One simple granular acinus fully developed before feeding	Multiple granular acini, that develop and changes before rapid engorgement
Regulation of water balance	Secrete water from blood meal via the coxal organs	Secrete water from blood meal via the salivary glands
Blood meal digestion	Feed first, digest later. First phase of digestion includes development of midgut epithelium	Low rate of feeding and digestion during preparatory feeding phase when ticks grows to allow rapid engorgement

II. The soft tick *Ornithodoros moubata*

The soft tick *Ornithodoros moubata* is widely distributed throughout eastern and central Africa and the northern part of South Africa (Van der Merwe, 1968; Walton, 1979). The vast majority of the soft ticks are nest parasites, residing in sheltered environments such as burrows, caves, or nests. The life stages of soft ticks are not readily distinguishable. The first life stage to come out of the egg, a six legged larva, molts directly to the first nymphal stage. Unlike hard ticks, many soft ticks go through multiple nymphal stages, gradually increasing in size until the final molt to the adult stage. Some soft ticks pass through up to seven nymphal molts before they become adults (Sonenshine, 1991).

Soft ticks feed several times during each life stage, and females lay multiple small batches of eggs between blood meals. The time to completion of the entire life cycle is generally much longer than that of hard ticks, lasting over several years. Additionally, many soft ticks have an extremely resistance to starvation, and can survive for many years without a blood meal (Furman et al., 1984). *O. moubata* is a vector of various pathogenic agents, including African swine fever virus (ASFV), *Borrelia duttoni*, causing African tick-borne relapsing fever, *Coxiella burunetti*, *Rickettsia* sp. (Burgdorfer et al., 1968; Derrick, 1939; Hess, 1971; Novy et al., 1906). *O. moubata* has become a useful laboratory model because of its size and relative simplicity of artificial feeding using ParafilmM® membrane (Schwan et al., 1991).

Figure 1: Soft tick *Ornithodoros moubata*. The development, from larvae to adult, of the soft tick; ventral and dorsal view on *O. moubata*, respectively.

III. The midgut of the soft ticks

1. Midgut structure and blood meal digestion

The midgut of the soft ticks consists of central ventriculus (stomach), numerous broad diverticula (caeca) and small, narrow tube, the intestine, which directs the undigested waste residues to the hindgut (Fig. 2). Intestine is absent in *O. moubata* and *Ornithodoros savignyi*. The waste products are accumulated in gut lumen through its life.

The midgut in ticks differs from insect in that it does not contain extracellular digestive enzymes and serves mainly as a major storage organ of a blood meal. Digestion occurs intracellularly by the cells of midgut epithelium (Sonenshine, 1991). The rich nutrient is only gradually absorbed by midgut cells and intact proteins can be found in gut contents of soft ticks even after several months after blood uptake (Balashov, 1972).

Digestive process in the Argasidae can be divided in three phases: (1) hemolysis, taking 2-3 days post feeding, (2) rapid digestion, beginning during or immediately after hemolysis, continuing several weeks to several months after feeding, and (3) slow digestion, lasting months or years in some species.

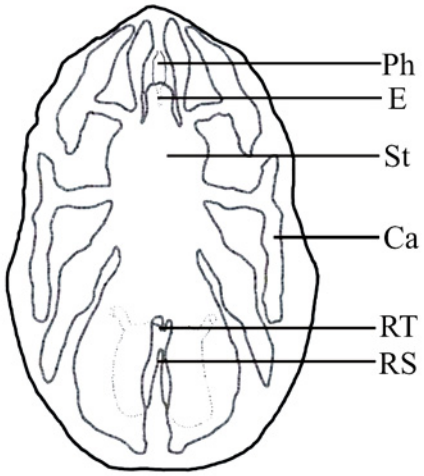

Figure 2: A schematic structure of gut of the soft ticks.
Ph-pharynx, E-esophagus; St-stomach (central ventriculus); Ca-caeca (diverticula); RS-rectal sac; RT-rectal tube (intestine), absent in *Ornithodoros savignyi* and *O. moubata*

During the first phase of blood meal, hematin filled digestive cells and exhausted secretory cells are sloughed off. In *Argas persicus*, most erythrocytes agglutinate and are lysed rapidly. Some erythrocytes, thrombocytes and most leucocytes are ingested directly by the tick digestive cells by phagocytosis. Digestive cells form large vacuoles in which the ingested blood cells may remain evident for several days. Hemolysis is complete within 6

days in *A. persicus* (Tatchell, 1964). During the second phase, rapid digestion, the digestive cells enlarge and ingest protein and cellular element by the endocytosis. Endocytotic vesicles fuse to form larger inclusions, which in turn fuse with lysosomes to form heterolysosomes. Phagocytosis is the main process of blood absorption in *O. moubata* (Grandjean, 1984). A lytic process in the soft tick digestive cells is similar to hard tick digestion. Waste products are produced and excreted from digestive cells. As digestion is completed, during the third phase, the digestive cells detach from the basal lamina and the hematine granules are launched into the gut lumen as so-called residual bodies (Sonenshine, 1991). The structure of *O. moubata* midgut in phase towards the end of the rapid digestion is shown in Figure 3.

In contrast, the digestive cells of the hard tick midgut take up components, especially hemoglobin, by receptor mediated and fluid phase endocytosis and phagocytosis (Sonnenshine, 1991). Huge amount of heme is released during hemoglobin digestion in the hard ticks. Most of this heme is detoxified by accumulation into residual bodies in specialized organelles, the hemosomes, in midgut epithelium cells of *Boophilus microplus* (Lara et al., 2003).

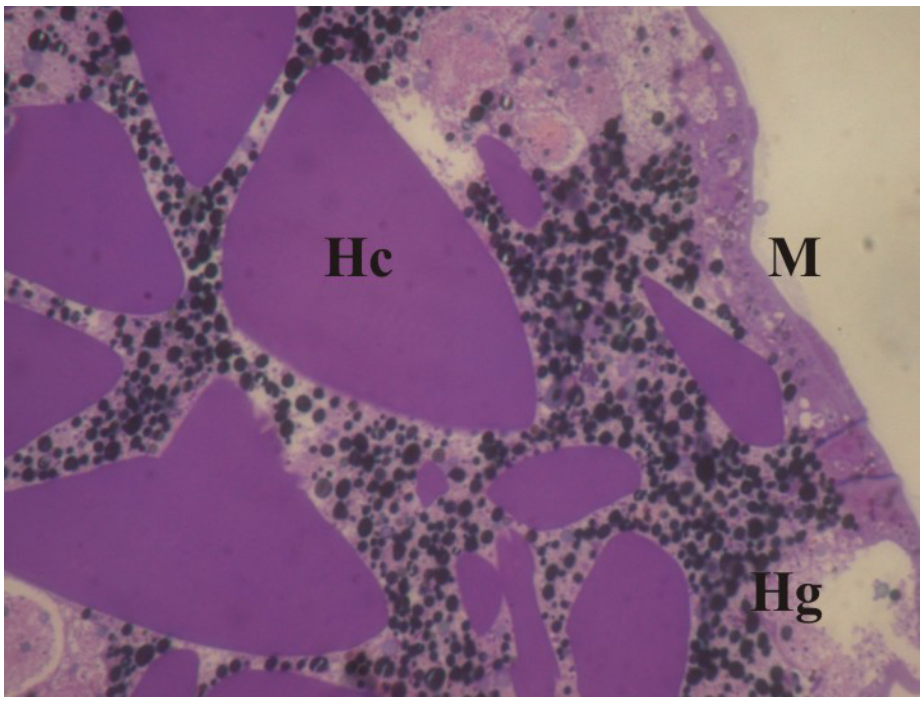

Figure 3: *O. moubata* midgut structure, midgut epithelium and gut contents. Semi-thin section of the gut of *O. moubata* female 6 week after blood meal, stained with toulidine blue. M-midgut epithelium, Hg-hematin granules (residual bodies), Hc-hemoglobin crystals. Hemoglobin crystals appear irregularly shaped triangles and are composed of host hemoglobin (Smit et al., 1977).

Little known is about the metabolic processes involved in the intracellular digestion in ticks. Cysteine and aspartic peptidases are believed to conduce to hemoglobinolysis (Coons et al., 1986; Mendiola et al., 1996). The papain-type cysteine peptidase – cathepsin L from *Boophilus microplus* (Renard et al., 2000; 20002) and aspartic peptidase from *Haemaphysalis longicornis* (Boldbaatar et al., 2006) were sufficiently characterized and proven to be involved in blood digestion. Recently, an asparaginyl endopeptidase, a CD clan cysteine protease, from *Ixodes ricinus* was described in our laboratory as an enzyme which may activate *in-trans* other efficient peptidases of the digestion system (Sojka et al., 2007).

2. Tick immunity

Immune related processes and molecules

Ticks have the ability to control infection when challenged with various bacteria (Podboronov, 1991; Ceraul et al., 2002). Until now, only a limited number of processes and molecules related to innate immunity response in ticks have been described (Tab. 2).

On the other hand, the components and processes of the tick immunity known so far fit well to the overall scheme of the invertebrate immune system (Fig. 4):

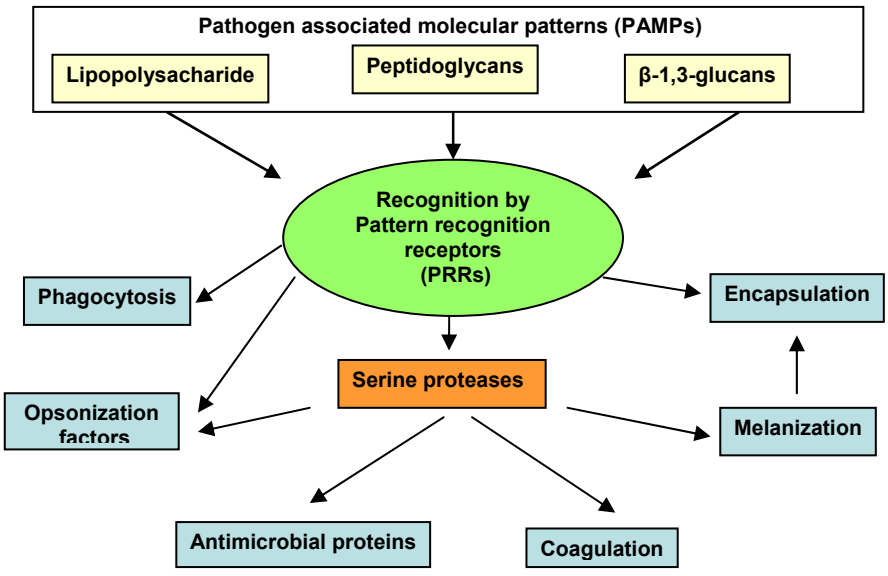

Figure 4: Schematic overview of invertebrate immune system response. Detection of microbial pathogens (microbial elicitors indicated in yellow) by recognitions proteins (green) activates an array of interconnected and synergistic host defense mechanisms (blue)

(1) Several lectins have been described as proteins able to recognize different patterns of glycan epitopes in *O. moubata*: DorinM (Kovář et al., 2000), OMFREP (Rego et al., 2005) and Omgalectin (Huang et al., 2007).

(2) In tick hemolymph were reported cellular immune mechanisms such as nodulation and encapsulation (Ceraul et al., 2002), cell mediated response (Johns et al., 1998; 2000), phagocytosis (Inoue et al., 2001; Burešová et al., 2006) and production of reactive oxygen species by hemocytes (Pereira et al., 2001).

(3) Serine proteases from *Haemaphysalis longicornis* (Mulenga et al., 2001), *Rhipicephalus appendiculatus* (Mulenga et al., 2003) and cubilitin-related serine protease from *H. longicornis* (Myioshi et al., 2004a) were identified.

(4) A broad variety of antimicrobial peptides (AMPs), forming the first line of host defense against pathogens, have been describe in invertebrates (Bulet et al., 2004). Only few AMPs have been isolated from ticks (Tab. 2) and can be divided into several groups:

Hemoglobin fragments

Tick digestion of blood meal hemoglobin may result in peptides that can destroy invading microorganisms. Hemoglobin fragments were reported in the midgut contents of the hard tick *Boophilus microplus* (Fogaça et al., 1999; Sforça et al., 2005). This fragment of bovine hemoglobin was active against Gram-positive bacteria and fungi. Two other AMP derivated from rabbit hemoglobin were isolated from the midgut of the soft tick *O. moubata*. Their activity increased markedly after blood feeding (Nakajima et al., 2003). Antimicrobial hemoglobin fragments were also found in the gut of *D. variabilis* (Sonenshine et al., 2005).

Defensins

Defensins are widespread throughout the animal kingdom, both in vertebrates and invertebrates. On exposure to bacterial challenge, they are induced rapidly and exhibit bactericidal or bacteriostatic activity against numerous bacteria (Beerntsen et al., 2000; Gillespie et al., 1997). Defensins are small peptides (4 kDa), usually containing six cysteine residues. These peptides attack primarily Gram-positive bacteria, leading to cell lysis by the formation of membrane penetrating channels (Gillespie et al., 1997) and intramolecular bridges (Beerntsen et al., 2000).

Table 2. : Summary of immune-related proteins in ticks.

	Process	Molecule	Tick species	Tissue	References
PRRs	recognition by the pattern recognition receptors	DorinM	*O. moubata*	hemolymph	Kovář et al. (2000)
		OMFREP	*O. moubata*	hemolymph	Rego et al. (2005)
		OMGalectin	*O. moubata*	hemocytes, midgut	Huang et al. (2007)
Serine protease	transference system and the intensification of the signal	HLSG1, HLSG2	*H. longicornis*		Mulenga et al. (2001)
		RAMSP1-3	*R. appendiculatus*		Mulenga et al. (2003)
		Cubilitin-related serine protease	*H. longicornis*		Myioshi et al. (2004a)
Protease inhibitors	broad spectrum protease inhibitor	tick-α_2-macroglobulin	*O. moubata*	hemolymph	Kopáček et al. (2000) Saravanan et al. (2003)
Cellular immunity	nodulation, encapsulation phagocytosis		*D. variabilis*	hemolymph	Ceraul et al., (2002)
			O. moubata	hemolymph	Inoue et al. (2001)
			I. ricinus	hemolymph	Burešová et al. (2006)
	production of reactive oxygen species		*B. microplus*	hemolymph	Pereira et al. (2001)
Humoral response	antimicrobial peptides (AMPs)	hemoglobin fragments	*O.moubata*	gut contents	Nakajima et al. (2003)
			B. microplus	gut contents	Fogaça et al. (1999)
			D.variabilis	gut contents	Sonenshine et al. (2005)
		defensins	*O. moubata*	hemolymph, midgut	Nakajima et al. (2001, 2002)
			D. variabilis	hemocytes, midgut	Johns et al. (2001), Sonenshine et al. (2002)
			B. microplus	hemocytes, fat body	Fogaça et al. (2004)
			A. hebraeum	hemolymph	Lai et al. (2004)
		lysozymes	*O. moubata*	midgut	Kopáček et al. (1999) Grunclová et al. (2003)
			D.variabilis	hemolymph	Simser et al. (2004a) Johns et al. (1998)
			D. andersoni	cell line	Mattila et al. (2007) Simser et al.(2004a)
	AMPs-protease inhibitors	cystatin- Hlcyst-2	*H. longicornis*	midgut, hemocytes	Zhou et al. (2006)
		serpin-Ixodidin	*B. microplus*	hemocytes	Fogaça et al. (2006)
	AMPs-unclassified	hebraein	*A. hebraeum*	hemolymph	Lai et al. (2004)
		microplusin	*B. microplus*	hemocytes, ovaria, fatbody	Fogaça et al. (2004)

In ticks, defensins were characterized from the hemolymph and midgut of *O. moubata* (Nakajima et al., 2001, 2002) and *Dermacentor variabilis* (Johns et al., 2001; Sonenshine et al., 2002) and hemolymph of *Boophilus microplus* (Fogaça et al., 2004) and *Amblyomma hebraeum* (Lai et al., 2004).

Lysozymes

The bacteriolytic function of lysozyme is widely employed in the innate immunity, in both animal and plant kingdoms (Hultmark, 1996). Lysozyme occurs widely in the cells and secretory fluids of vertebrates, where it may function as a bactericidal agent or help to dispose bacteria after they have been killed by other means. The known lysozymes are grouped into several types. Within the animal phyla, three main lysozyme types are recognized: chicken type (c), goose-type (g), and invertebrate type (i). Lysozyme hydrolyses the 1,4-β-glycosidic bound between N-acetylmuramic acid and N-acetylglucosamine in cell wall peptidoglycans of Gram-positive bacteria (Hultmark, 1996). In ticks, the lysozyme was first purified from the midgut of the fed *O. moubata* (Kopáček et al., 1999) and its expression increased after the uptake of a blood meal (Grunclová et al., 2003). The immune-responsive lysozyme was identified from the hemocytes of the hard tick *D. variabilis* and embryonic cell line of *Dermacentor andersoni* (Simser et al., 2004a) and cell line DAE15 of *D. andersoni* (Mattila et al., 2007).

Protease inhibitors

Cystatins and serpins are inhibitors of cysteine proteases and serine proteases, respectively. They can regulate the endogenous and/or exogenous (pathogenic) incidence of proteases. Blood feeding and LPS inoculation significantly increased expression of cystatin, a cysteine protease inhibitor from the hard tick *Haemaphysalis longicornis* (Zhou et al., 2006). Growth inhibition of *E. coli* and *M. luteus* was embodied by ixodidin, a serine protease inhibitor from the cattle tick *Boophilus microplus* (Fogaça et al., 2006). A broad spectrum protease inhibitor, an α_2-macroglobulin, was described in the soft tick *O. moubata* (Kopáček et al., 2000; Saravanan et al., 2003)

Unclassified AMPs

The cysteine-rich AMP, called microplusin, was isolated from the hemolymph of *Boophilus microplus*. It has no sequence similarity to any known AMPs, is expressed in hemocytes, ovaries and fat body and prevents the growth of *Micrococcus luteus* (Fogaça et al., 2004). Other antimicrobial protein named as hebraein was purified from the hemolymph of fed *Amblyomma hebraeum* female ticks. This AMP had antimicrobial activity against wide

spectrum pathogens included Gram-positive bacterium *Staphylococcus aureus* and Gram-negative *E. coli* (Lai et al., 2004).

Tick-pathogen interaction

It is known that most tick-borne pathogenic agents are able to pass through the midgut epithelium and enter the hemocoel (Fujisaki et al., 1984; Han et al., 2000; Rudzinska et al., 1982). *E. coli* as an environmental microbe can not pass through the midgut epithelium of *O. moubata* and enter the hemocoel (Matsuo et al., 2004). On the other hand, the Gram-negative bacterium *Chryseobacterium indologenes* was demonstrated to be pathogen of the soft tick *O. moubata*; the concentration of bacteria grew exponentially in the midgut post infective feeding. In addition, examination of orally infected ticks revealed that the amount of bacteria increase abundantly in the hemolymph of *O. moubata*. The pathogenicity of *C. indologenes* to *O. moubata* contrasted to the very effective and fast clearance of this bacterium from the gut of the hard tick *Ixodes ricinus* (Burešová et al., 2006).

Tick-borrelia interaction

The tick gut epithelium barrier passing by *Borrelia burgdorferi* has been intensively studied. *B. burgdorferi* adapts to the transmission between the arthropod vector and mammalian host in part by preferential gene expression (Fig. 5).

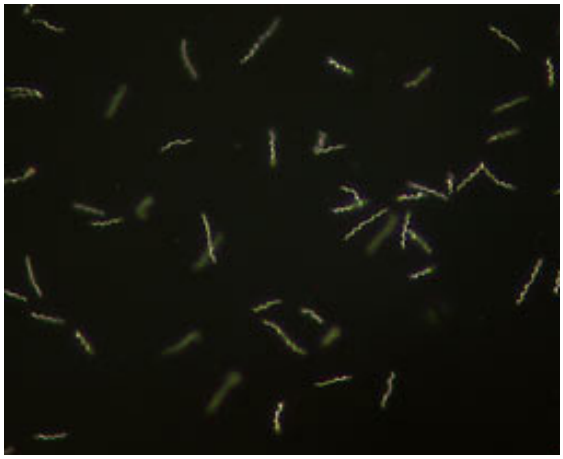

Fig. 5: *Borrelia burgdorferi* belongs into the group of spirochetes causing Lyme disease. *B. burgdorferi* has evolved remarkable abilities to survive in a wide range of organisms such as arthropods, and vertebrates like birds and mammals. www3.niaid.nih.gov/. ../borrelia_burgdorferi.jpg

The gene coding for an outer surface protein (Osp) A is expressed by *B. burgdorferi* within the tick *I. scapularis* gut (de Silva et al., 1996) and down regulated during transmission to the mammalian host (Ohnishi, et al., 2001; de Silva et al., 1996). OspA is essential for *B. burgdorferi* to persist within the tick. OspA-deficient spirochetes failed to survive within *I.*

scapularis and these spirochetes could not bind to the tick gut (Yang et al., 2004). The tick receptor for OspA (TROSPA) that binds OspA is required for spirochetal colonization of *I. scapularis* (Pal et al., 2004). The time and the location of TROSPA expression accord with the natural cycle of spirochetes. Inside ticks, *B. burgdorferi* express OspA (Schwan et al., 2000; de Silva et al., 1996) and, at the same time, *I. scapularis* expresses TROSPA. In ticks with silenced TROSPA (antiserum or dsRNA), small amount of spirochetes was still able to colonize and persist in ticks and be transmitted to a naive host. This indicates that TROSPA may not be the only ligand utilized by spirochetes in ticks. After start of tick engorgement, TROSPA and OspA expression decreased (Pal et al., 2004). Simultaneously with the disappearance of OspA, expression of outer surface protein C (OspC) begins. These processes correlate with the exit of spirochetes from midgut, dissemination through the hemolymph and passage via the salivary glands of feeding tick to the mammalian host (Schwan et al., 2002).

Relative few studies have examined the distribution of relapsing fever spirochetes in tissues of argasid tick. The study of Burgdorfer (1951) has shown that the process of *B. duttoni* transmission varies with the stage of the soft tick *O. moubata*. Nymphs transmit *B. duttonii* in the saliva; adults transmit *B. duttonii* via coxal fluid.

The soft tick *Ornithodoros hermsii* was first identified as a vector of relapsing fever spirochetes in 1935 by Wheeler. *O. hermsii* is capable of transmitting this spirochete *Borrelia hermsii*, in contrast to other species *O. tunicata* and *O. parkeri*, which transmit other species of spirochetes (Davis, 1942). The mechanisms of this species specifity of spirochetes transmission are unknown. The distribution of *B. hermsii* in *O. hermsii* organs was observed and spirochetes were found in midgut, salivary gland and synganglion (Schwan et al., 1998). Vsp33 protein, the *B. hermsii* homolog of OspC, was shown to be expressed by spirochetes colonized salivary glands and was hypothesized as a protein involved in the spirochetes dissemination from the midgut (Marconi et al., 1993). In the fast feeding soft tick, there is no time for spirochetes to disseminate out of the midgut, penetrate the salivary gland and synthesize a new outer surface protein during the feeding. That is why *B. hermsii* upregulates the synthesis of Vsp33 directly after infecting *O. hermsii* and all spirochetes express the protein during persistent infection of the salivary glands. In contrast to OspC of *B. burgdorferi*, Vsp33 does not seem to be needed for dissemination from the midgut to the salivary glands (Schwan et al., 2002).

3. Tick gut immunity

The absence of proteolytic enzymes in the midgut lumen would make this organ a highly favorable environment for survival of ingested microbial pathogens in case there would not be any active defense mechanisms (Munderloh et al., 1995). Any successfully transmitted pathogens have to overcome several barriers within the tick vector, including: (1) physical barriers; (2) epithelial barriers; (3) production of antimicrobial peptides.

In hematophageous insect, peritrophic matrix is the first barrier, which can affect the survival and virulence of ingested pathogens (Elvin et al., 1996). Unlike the hematophageous insect, little known is about the structure or molecular composition of this matrix in ticks. During the initial phases of the blood feeding, a peritrophic matrix was observed in the midgut of *O. moubata* (Grandjean. 1984), *Ixodes dammini* (Rudzinska et al., 1982) and *I. ricinus* (Zhu et al., 1991). Recently, the peritrophic matrix structure of tick female of genus *Ixodes* was reported (Grigor'eva et al., 2004).

In order to avoid bacterial growth and decomposition of the ingested blood by multiplying bacteria, ticks have to posses an efficient protective mechanism within their gut. In the midgut of both, soft and hard ticks, the antimicrobial fragments of host hemoglobin are contributed to the rapid elimination of most invading microorganisms. Antimicrobial bovine hemoglobin fragments were first time reported in the gut lumen of the hard tick *Boophilus microplus* (Fogaça et al., 1999). The functional and structural features of hemoglobin fragments were explained recently. The details of the mode of action are unclear, but the synthetic proteolytic products of bovine hemoglobin (peptide Hb33-61) were shown to be able to disrupt the bacterial membrane of *Micrococcus luteus* (Sforça et al., 2005). Fragments of α- and β-chain rabbit hemoglobin were observed also in the midgut of *Dermacentor variabilis* (Sonenshine et al., 2005) and the soft tick *O. moubata* (Nakajima et al., 2003).

Next to hemoglobin fragments, defensins and lysozyme are the integral part of active immune defense in the midgut of the soft ticks. Expression of the genes encoding four isoforms of *O. moubata* defensins was strongly upregulated by blood meal (Nakajima et al., 2001, 2002). The induction of A and B defensins was observed in the midgut after experimental infection with bacteria (Nakajima et al., 2001). Defensin A was isolated from the gut contents; it indicated that this isoform is secreted by the gut epithelium cells into the gut lumen (Nakajima et al., 2002). Another AMP, lysozyme, was purified from the midgut of the fed *O. moubata* (Kopáček et al., 1999). Tick gut lysozyme seems to be an interesting case

in which the features of lysozymes with antimicrobial and digestive function are combined. Blood meal strongly induced the expression of lysozyme in the midgut (Grunclová et al., 2003).

In the hard ticks, the main tissue of defensins as well as lysozymes expression is the hemolymph. The challenge of midgut with different bacteria did not increase defensin expression in the gut of the hard tick *D. variabilis*, in contrast to hemolymph (Sonenshine et al., 2005). The gene expression profile of defensin from the hard tick *Boophilus microplus* showed that the major sites of expression are hemocytes and the fat body (Fogaça et al., 2004). The immune responsive lysozymes were identified in hemocytes of *D. variabilis* (Johns et al., 1998; Simser et al., 2004a), but the lysozyme transcript was also found in the midgut of *D. variabilis* (Sonenshine et al., 2005). The *D. variabilis* defensin was detected only in the hemolymph of ticks inoculated with *Borrelia burgdorferi* and *Bacillus subtilis*. Although the *D. variabilis* defensin alone was not immediately effective against *B. burgdorferi*, tick defensin together with lysozyme killed much better the cultured *B. burgdorferi* (Johns et al., 2001). The cystatin (cysteine protease inhibitor) identified in the midgut and hemolymph of the hard tick *H. longicornis* was reported to have growth inhibitory effect on cultured *Babesia bovis* (Zhou et al., 2006).

Results and discussions

- **Tick gut lysozyme from the soft tick *Ornithodoros moubata* (Paper I)**
- **Identification of genes affected by blood feeding from *O. moubata* gut-specific cDNA library**
- **Cystatins from the soft tick *Ornithodoros moubata* (Paper II)**

Unpublished results:

- **Thyropin from the soft tick *Ornithodoros moubata***
- **Serine protease homolog from the soft tick *Ornithodoros moubata***

I. Tick gut lysozyme from the soft tick *Ornithodoros moubata*

The bacteriolytic function of lysozyme is widely employed in both animal and plant kingdoms. Lysozyme inducible by bacterial challenge is probably the most comprehensively studied defense molecule in the immune system of invertebrates (Jollès, 1996). In some animals, the lysozyme was adapted to digestive purposes like in the stomach of ruminants, where the enzyme digest ingested bacteria providing nitrogen and phosphorus necessary for milk production (Dobson et al., 1984; Irwin, 1996). A similar adaptation of lysozyme to the digestive function has been reported for langur ruminant monkeys (Stewart et al., 1987), leaf-eating bird-hoatzin (Kornegay et al., 1994) and also for lysozymes from the midgut of flies *Drosophila melanogaster* (Hultmark, 1996; Regel et al., 1998) and *Musca domestica* (Lemos et al., 1993; Ito et al., 1995).

Lysozymes in ticks

In eighties, a systematic analysis on tick lysozymes was performed by Russian authors. They tested the lysozymes activity in different tick species comprising *Alveonasus lahorensis*, *Ornithodoros papillipes*, *O. moubata*, *Hyalomma asiaticum* and *Ixodes persulcatus*. They also observed an increasing lytic activity in the whole-body homogenates of *O. moubata* challenged by blood meal infected with variety of bacterial strains (Podboronov et al. 1991).

The first sufficiently characterized tick lysozyme was purified from the gut contents of the adult soft tick *O. moubata* in our laboratory (Kopáček et al., 1999). The enzyme designated as tick gut lysozyme (TGL) was responsible for high lytic activity of gut contents against the bacteria *Micrococcus luteus*. The homogeneous TGL had a mass of 14006 +/- 20 Da as determined by mass spectroscopy. Similarly to the common basic c-type lysozymes, TGL possesses a high isoelectric point (pI≥9.67). In contrast to it, digestive lysozyme isolated from the midgut of *M. domestica* (Lemos et al., 1993) and *D. melanogaster* (Regel et al., 1998) were reported to have neutral or even acidic isoelectric point. On the other hand, TGL displayed N-terminal amino-acid motif DRCSLA occurring specifically in the digestive lysozymes of several dipteran insects. These rather controversial preliminary data did not make it possible to answer unequivocally a question whether TGL is adapted for digestive function or plays a defense role against ingested bacteria in the tick gut. Thus characterization of the whole primary structure of TGL followed by comprehensive phylogenetic analysis and

study of TGL post blood meal expression was necessary to understand the main role of TGL in the tick digestive tract.

Sequence analysis of tick gut lysozyme

TGL sequence was determined using degenerated primers derived from known N-terminus and highly conserved motifs from C-terminal part of vertebrate and invertebrate lysozymes RACE PCR techniques were used to determine the 5' and 3' untranslated sequences The complete cDNA sequence of TGL, deposited in the GenBank under Accession No. AF425264, was the first lysozyme representing the subphylum Chelicerata (Paper I). Multiple alignment of TGL with selected mature lysozymes revealed that TGL is clearly of the c-type (Paper I; Bachali et al., 2002; Ting et al., 2002).

TGL possesses all of the 8 conserved cysteine residues and two residues (Glu^{33} and Asp^{51}) responsible for the catalytic activity of c-type lysozymes. The most striking difference in the TGL sequence is the presence of histidine (His^{52}), which replaces the highly conserved tyrosine found in the vast majority of c-type lysozymes at this position (Paper I; Fig. 2). The same substitution was found in the gene products agCP6542 from *Anopheles gambiae* strain PEST (GenBank EAA11630) and CG8492-PA from *D. melanogaster* (GenBank AAF50522) containing multiple lysozyme-like sequences. Among tick lysozyme-like sequences available in the meantime from the GenBank, this replacement was found in one EST (GenBank CV457331, clone BEAH196) from *Boophilus microplus* here designated as Bm2 clone. Moreover, Bm2 clone apparently shares other amino-acid residues and motifs similar to TGL indicating that these lysozymes may be related (Fig. 6).

The phylogenetic analysis revealed that the TGL is more related to the family of the *Drosophila* digestive lysozyme, than to other c-type lysozymes which do not have the digestive function (Paper I, Fig. 3). Surprisingly, TGL differs significantly from lysozymes from other (hard) tick species described later as immune-responsive c-type lysozyme identified from the hard *D. variabilis* hemocytes (Dv Lys) and a *D. andersoni* embryonic derived cell line DAE100. These enzymes are more closely related to a number of c-type lysozymes from *A. gambiae* and D. *melanogaster* than to TGL from *O. moubata* (Simser et al., 2004a).

Kinetic of TGL expression

The tick gut lysozyme is markedly up-regulated at the transcriptional level by a blood meal. First, using semi-quantitative RT-PCR analysis, we observed a maximum mRNA level of

TGL one day after blood meal and then the mRNA level slowly declined to the basic level within the next 14 days. The detailed post blood-meal kinetics of TGL mRNA expression was also analyzed by Northern blotting. This experiment showed that TGL mRNA level in the guts started to increase quickly 12 h PBM and reached its maximum already at 16 h PBM (Paper I, Fig. 4 and 5). In contrast to this, the lytic activity of lysozyme in the gut contents was rather constant and stable at least for one month PBM (Kopáček et al., 1999).

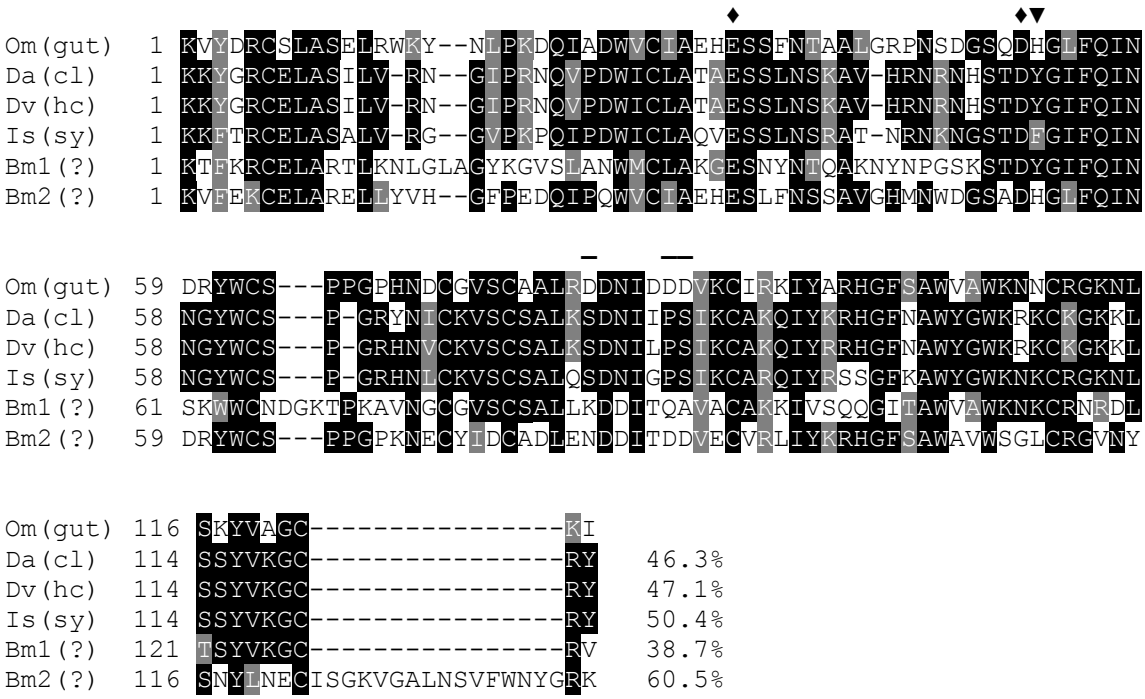

Figure 6: Multiple alignment of the TGL from *O. moubata* and available lysozyme sequences from ticks and no annotated ESTs of tick origin; percent identity: (▼) indicates position of histidine H52, (♦) mark residues (Glu33 and Asp51) responsible for the catalytic activity of c-type lysozymes, (–) point Asp residues involved in Ca^{2+} binding in α-lactalbumins. Om - Gut lysozyme of *O. moubata* (AAL17868); Dv - hemocyte lysozyme of *D. variabilis* (AAO49740); Da - lysozyme from the cell line DAE100 of *D. andersoni* (AAO23571); Is - clone IS_SYNG_15F10_TEx2Seq1of *Ixodes scapularis* (EL515757); Bm1, Bm2 - two cDNA clones BEAAA08, BEAH196 of *B. microplus* (CK174158 and CV457331, respectively).

The expression of immune-responsible lysozyme from hemocytes of the hard tick *D. variabilis* increases 72h post-inoculation with *E. coli* and dropped back 96h after infection (Simser et al., 2004a). *D. variabilis* lysozyme message was not upregulated by the blood meal, mechanical injury or PBS control. Recently, the influence of bacteria on lysozyme expression of the *I. scapularis* cell line IDE12 and the *D. andersoni* cell line DAE15 was reported (Mattila et al., 2007). The expression of lysozymes was upregulated after stimulation with heat killed *E. coli* and *M. luteus*. In contrast to it, tick endosymbiont *Rickettsia peacockii*

did not modify lysozyme expression. These results suggest that tick endosymbionts may avoid recognition by the tick immune system, and infection may not affect humoral immune responses to bacteria (Mattila et al., 2007).

In conclusion, our data indicate that lysozyme from the midgut of *O. moubata* is likely adapted to the digestive function. However, TGL still shares some features with other antibacterial lysozymes that its defense role in the tick gut targeted especially against Gram-positive bacteria seems to be quite probable. The fundamental differences in the sequence characteristics, phylogenetic placement and compartmental and temporal regulations between the hard tick and soft tick lysozymes point to different adaptation c-type enzymes in tick. So far, no hard tick have been reported to display lysozyme activity in its gut indicating that different feeding strategy and physiology reflect different need for efficient immune reactions.

Paper I

Grunclová, L., Fouquier, H., Hypša, V., Kopáček, P. (2003).

Lysozyme from the gut of the soft tick *Ornithodoros moubata*: the sequence, phylogeny and post-feeding regulation.

Dev. Comp. Immunol. 27: 651–660.

Lysozyme from the gut of the soft tick *Ornithodoros moubata*: the sequence, phylogeny and post-feeding regulation

Lenka Grunclová, Hélène Fouquier, Václav Hypša, Petr Kopáček
Dev. Comp. Immunol. 2003. 27: 651–660.

Sequence of a tick gut lysozyme (TGL) from the soft tick *Ornithodoros moubata* was determined by cloning and sequencing of overlapping polymerase chain reaction (PCR) and RACE PCR products. It is the first lysozyme sequence representing the subphylum Chelicerata. The resulting open reading frame codes for a putative signal peptide of 22 amino-acid residues and a mature protein composed of 124 amino-acids. Calculated mass of the protein is 14037.75 Da and a theoretical isoelectric point is 8.16. The phylogenetic analysis revealed that the TGL belongs to the c-type lysozymes. It forms a distinct monophyletic group together with multiple lysozyme-like sequences found in the gene products agCP6542 from *Anopheles gambiae* strain PEST and CG8492-PA from *Drosophila melanogaster*. This group is referred to as an H-branch due to a unique histidine residue at position 52 which replaces the highly conserved tyrosine present in the vast majority of c-type lysozymes. TGL seems to be an interesting case in which the features of lysozymes with anti-bacterial and digestive function are combined. Semiquantitative RT-PCR and Northern blotting analysis demonstrated that TGL is strongly up-regulated at the transcriptional level after a blood meal. The maximum lysozyme mRNA level was detected 16 h post blood meal and the message remained stable for 5 days and then it slowly dropped down to the level of non-fed ticks within 2 weeks.

II. Identification of genes affected by blood feeding from *O. moubata* gut-specific cDNA library

The post blood meal up-regulation of defensins (Nakajima et al., 2001; 2002) and lysozyme (Grunclová et al., 2003) in the gut of the soft tick *O. moubata* (see above) led us to search for other genes which may be affected by blood-meal in this species. This idea basically followed a work of Sanders et al., (2003), who observed global changes in the midgut gene expression in the malaria vector *Aedes aegypti*.

As a first step, the gut specific cDNA library was constructed using SMART^{TM} cDNA Library Construction Kit (Clontech, BD Biosciences) according to the manual provided by the supplier. Briefly, guts of 10 adult *O. moubata* ticks were dissected 24 hours after membrane feeding and 1μg of isolated total RNA was taken for the first-strand cDNA synthesis using PowerScript^{TM} reverse transcriptase. Two microlitres of resulting reaction was used for cDNA amplification using Advantage 2 PCR kit (Clontech, BD Biosciences). Double stranded cDNA was fractionated on the provided gel filtration columns and only fractions ≥500 bp were collected and ligated into the λ TripleEx2 vector. Packaging reaction was performed with Gigapack® III Gold Packaging Extract (Stratagene). Clones of un-amplified phage cDNA library were multiple converted to a plasmid library in *E. coli* BM25.8 host cells. Individual colonies were picked from LB/ampicilin plates and transferred to 9 x 384-well plates using GeneTAC G3 robot (Genomic Solutions). Bacteria were grown in 100 μl of LB/carbenicilin medium supplemented with 7.5% glycerol overnight at 31° C and stored at -80°. Colonies plates were robotically gridded onto Hybond N^{+} membranes at the density of 3,456 per 12 x 8 cm membrane.

Membrane chips were hybridized with [α-^{32}P] dATP labeled ss cDNAs prepared by reverse transcription (SuperScript^{TM}, Invitrogen) of 1 μg of total RNA isolated from engorged ticks (24 pbm), then stripped and hybridized again with [α-^{32}P] dATP labeled ss cDNAs prepared from un-fed ticks (at least two month after last blood meal). The images of both membrane chips were carefully examined for spots clearly differing in the signal intensity. This approach also allowed us to avoid the most abundant house-keeping genes (Nelson et al., 1999). A number of 64 clones selected for further sequencing are listed in the Table 3. Thirty one isolated clones contained full coding sequence out of which 12 genes possess a clear signal peptide predicted by the SignalP V1.1 server according to Nielsen et al. (1997).

The search for the genes differentially expressed in the midgut of the soft tick *O. moubata* after blood meal led to the identification of several genes coding for proteins of potentially interesting function (Table 3). This set contained an already described gene coding for defensin isoform A (Nakajima et al., 2002), namely the PK-9. We have further focused on two genes coding for putative inhibitors of cysteine peptidases apparently belonging to the family of cystatins (Om-cystatin1 and Om-cystatin 2) and a putative thyroglobulin domain containing protease inhibitor (thyropin), because of their possible function in regulation of hemoglobin digestion (endogenous proteases control) and/or tick-gut immunity (inhibition of exogenous microbial proteases). The other gene selected for further study was the chymotrypsin-like protein characterized later as a serine protease homolog (SPH). The aim of this work was to contribute by any data to the challenging discovery of the real function of such a molecule in the tick gut. The same motivation was in the case of a gene coding for a protein similar to the mosquito "mucin-like perithrophin" which was concern of the Master thesis of Franta (2005).

Gene Code	Identical to	Gene length	Full Clone	Signal sequence	Blast search/similarity to (Highest score)	Appropriate for GenBank
LG-1			Yes	**Yes**	**serine protease homolog**	**AY376147**
PK-1	PK-2, PK-10	>600 bp	?		??? 3-isopropylmalate dehydratase (31)	
PK-2	PK-1, PK-10	~646bp	Yes	Yes!	??? 3-isopropylmalate dehydratase (31)	**AY526113**
PK-3	PK-12	611bp	Yes	**Yes!**	**!!! cystatin (**putat. cystatin Ixodes ricinus, 92)	**AY521024**
PK-4		512bp	Yes	Yes!	??? uknown protein Arab.thaliana (35)	**AY524181**
PK-5	PK-26	? > 900 bp	Yes?	Yes?	??? Adhesion protein MAA1	?
PK-6		? > 500 bp	?	?	no similarity	
PK-7		562bp	Yes?	No!	Ring box protein, ubuiquitin ligase (**180!**)	
PK-8		790 bp	Yes	No?	CDP antigen, tetraspanin, RIKEN (93)	**AY527385**
PK-9		378bp	Yes	Yes	O. moubata DefensinA (**109!)**	no
PK-10	PK-1, PK-2	>600 bp	No		3-isopropylmalate dehydratase (31) ???	
PK-11		871bp	Yes	Yes	similar to Niemann Pick protein -N-term. (108)	**AY524182**
PK-12	PK-3				**!!! cystatin (**putat. cystatin Ixodes ricinus, 92)	yes
PK-13		735bp	yes	Yes?	N-terminal similar to **mucin-like perithrophin** (40)	**AY521026**
PK-14		636 bp	?	No!	similar to 3-hydroxyanthranilate 3,4-dioxygenase, C-terminal half (99)	yes?
PK-15		409 bp	?	no	O. moubata mitochondrial DNA (440!)	no
PK-16		372 bp	yes	yes CMS-IP	NADH ubiquinone oxidoreductase, **6.3 kDa salivary gland peptide in Anopheles**	**AY524183**
PK-17		146 bp	**no**	no	3' end of something	no
PK-18		>600 bp	yes	No!	glyceraldehyde-3-phosphate dehydrogenase (285!)	yes
PK-19		>600 bp	yes	No!	acetylcoenzym-A acetyl;transferase (199!)	yes
PK-20		372 bp	no	no	O. moubata mitochodnrial DNA (196)	no
PK-21		671 bp	Yes	Yes! VSA-AS	**similar to eqcuistatin (protease inhibitor), thrombin domain**	**AY521025**
PK-22		863 bp	Yes	No!	**Proteasome alpha chain, macropain (300!)**	**AY521027**
PK-23		465 bp	no	no	O. moubata mitochondral DNA	no
PK-24		1139 bp	yes	No?	conserved hypothetical protein (140), domains - oligoketide cyclase, aromatic rich protein family, lipid metabolism?	yes
PK-25		>600 bp	no	no	O/ moubata mitochondrial DNA cytochrome b oxidase	no
PK-26	PK-5 (or similar)	? > 900 bp	Yes?	Yes?	??? Adhesion protein MAA1 (34)	?
PK-27		> 1000 bp	No-partiall	No	**Fasciciclin-like** domain, C-terminal similar to hypothetical protein Plasmodium falciparum	?
PK-28		> 600 bp	Yes	No	similar to **eukaryotic translation initiation factor** 3	yes?
PK-29	PK-13	735bp	yes	Yes?	N-terminal similar to **mucin-like perithrophin** (40)	yes
PK-30		200 bp	no	no	similar to ribosomal protein fragment	no
PK-31		1077 bp	yes	No!	**SNF7 - homolog (170!)**	**AY526884**
PK-32		> 600 bp	?	?	no similarity	no
DS-1	PK-5,PK-26, DS-27	>900 bp	Yes?	Yes?	???Adhesin MAA1, juvenile hormone binding	?
DS-2		~1200 bp	Yes	No!	(beta) Actin (**377!**)	**AY547732**
DS-3		587 bp	Yes	No!	60S ribosomal protein L 21(**200!**)	**AY547733**
DS-4		354 bp	?		O. moubata mitochodrial DNA	no
DS-5		>700 bp	No ~1/2 C-t	No!	selenium binding protein cytoplasmic (**200!**)	?
DS-7		231 bp		no	3' end of something	no
DS-8				no?	similarity to hepatitis B interacting virus (50)	no
DS-10		>700 bp	no 1/2	no	elongation factor 2, C-terminal part	no
DS-11		> 600 bp	?	no	no similarity	no
DS-12		> 600 bp	?	?		no
DS-13		>600 bp	Yes?	No!	clathrin adaptor (**150!**)	yes
DS-14		~700 bp	Yes	no	40S ribosomal protein S5 (**361!**)	**AY547734**
DS-15		>700 bp	Yes	Yes SQG-TS	**cystatin-like BIG! different from PK3,12**	**AY547735**
DS-16		>700 bp	no	no	similar to zinc finger protein and others	no
DS-17		> 600 bp	yes?	No	similar to micorfibrillar associated protein	no
DS-18		~650 bp		Yes?? NSR-QE??	no similarity	yes?
DS-19		~600 bp	no	?	O. moubata mitochodrial DNA	no
DS-20		~650 bp	yes	no	40S ribosomal proteinS14 (158!)	yes
DS-21		~220 bp	no	no	3'-end of O. moubata NADH dehydrogenase 4	no
DS-22		~700 bp	yes	yes? VWG -KP	heat shock protein, alpha crystalin B-chain (60)	yes
DS-23		>700 bp	no	no	O. moubata mitochodrial DNA	no
DS-24		>700bp	?	?	similar to heatshock, rhodanese -SH transferase	?
DS-25		>700 bp	yes		ubiquitin conjugating enzyme (**220!**)	yes
DS-26		?	?		no similarity	no
DS-27	PK-5,PK-26, DS-1	> 900 bp	yes	yes?	???Adhesin MAA1, juvenile hormone binding	yes
DS-28		>700 bp	yes?	?	similar to GP from Drosophila + Anopheles	?
DS-29		>700 bp	?	?	low similarity to amino-acid transporter	?
DS-30		?	?	?	unknown	
DS-31		~5000 bp!	~180 AA fro	no	adenylate cyclase (109!]	
DS-32		> 700 bp	?	?	uknown	?

Table 3: Table of identified gut-specific genes from the soft tick *Ornithodoros moubata*

Legend:

In red and yellow - the most interesting genes selected for further work

LG-1 – Serine protease homolog

PK-3 (PK-12) – Om-cystatin1

DS-15 – Om-cystatin2

PK-21 – Om-thyropin

PK-13 (PK-29) – mucin-like perithrophin

In green: The clones worth to complete sequence

In blue: Already known gene – defensin A

In white: Genes of low interest

In gray: housekeeping or ballast

III. Cystatins from the soft tick *Ornithodoros moubata*

The superfamily of cystatins comprises three major structural families: Type 1 cystatins (also known as stefins) are cytoplasmatic, single domain proteins of about 100 amino-acid residues lacking the disulfide bridges and signal sequence. Cystatins of type 2 are secreted inhibitors of about 120 residues having two disulfide bridges and typical signal peptide. Cystatins of type 3 (called kininogens) are multidomain proteins presenting three repeated domains of cystatins type 2 (Barret et al., 1986; Turk et al., 1991).

Secreted cystatins have been found in various invertebrate organisms. Nematode cystatins were shown to play a crucial role in evasion of the host defense system and in modulation of the immune response via their capability to inhibit the activity of various cysteine proteases (Hartmann et al., 2003). The *Onchocerca volvulus* cystatin was shown to inhibit cysteine proteases cathepsin L and S that are involved in the proteolytic processing of polypeptides (Schonemeyer et al., 2001).

Cysteine peptidase and cysteine peptidase inhibitors of protozoan parasites play crucial role in host invasion, modulation of host immunity, nutrition etc. Malaria parasite *Plasmodium falciparum* degraded hemoglobin to amino acids for parasite protein synthesis. Degradation of hemoglobin by papain-family cysteine protease, falcipain, can be inhibited by incubation of erythrocytic parasites in the presence of cysteine protease inhibitor (Olson et al., 1999). Experimental inhibition of cysteine protease cruzain of *Trypanozoma cruzi* caused life cycle interruption in the stage of intracellular amastigots (Engel et al., 1998). Cysteine protease from tick-born pathogen *Theileria sergenti* was isolated. It seems to be involved in the process of erythrocytes and tissue invasion (Sako et al., 1999). Similar role plays cysteine protease of other tick-born pathogen *Babesia equi*. Propagation of intraerythrocytic stage of *B. equi* was inhibited in the presence of cysteine peptidase inhibitor in-vitro (Holman et al., 2002). Recombinant cystatin from the tick *Haemaphysalis longicornis* showed a significant grow-inhibitory effect on *B. bovis* cultured in-vitro (Zhou et al., 2006).

Last but not least, cystatins were shown to function as antimicrobial proteins in the hemolymph of the horseshoe crab *Tachypleus tridentatus*. L-cystatin, expressed in hemocytes, served as defense molecule against invading Gram-negative microbes, together with other proteins secreted into extracellular fluid (Agarwala et al., 1996b).

These facts imply that cystatins may be the potential regulators of protein turnover and defense against pathogens, the balance of the host-parasite immune relationship as well as regulation of endogenous cysteine peptidases involved in blood digestion in tick.

Although the knowledge of molecules involved in blood digestion in ticks is still rather limited, the main role is apparently played by acidic cysteine and aspartic peptidases (Mendiola et al., 1996, Lara et al., 2005; Boldbaatar et al., 2006). Cloning and sequencing of two cathepsin L genes and the sequence of midgut cathepsin B (GenBank Acc. No. AY208820) from the midgut of hard tick *H. longicornis* have been reported (Mulenga et al., 1999). Cysteine peptidase cathepsin L from the hard tick *Boophilus microplus* was capable of effective hemoglobin hydrolysis and was find to be expressed in exocytosis vesicles of gut secretion cells (Renard et al., 2000, 2002).

Recently, several group published results on cystatins from different tick species in parallel to our work on two cystatins from *Ornithodoros moubata* (Paper II). Cystatin from the *Amblyomma americanum* expressed in the salivary gland and the midgut (Karim et al., 2005); secreted cystatin from *H. longicornis* (Zhou et al., 2006); sialostatin L from *Ixodes scapularis* (Kotsyfakis et al., 2006) and cytosolic BMcystatin from *B. microplus* (Lima et al., 2006). In addition to it, several tick putative cystatin cDNA sequences has become available in the GenBank database (Fig. 7).

Sequence analysis of Om-cystatins

In our laboratory, cystatins from the midgut of *O. moubata* were characterized. Om-cystatin 1 and 2 belong to type 2 cystatins; they contain clearly predictable signal peptide, display a conserved pattern of four cysteine residues known to form two internal disulfide bridges, possess three typical segments (P-I, P-II and P-III) that create a wedge-shaped binding site involved in reversible binding in the active site of cysteine peptidases of the papain family (Bode et al., 1988).

Interestingly, the terminal (P-III) hairpin loop motif containing proline and tryptophane is shared only with cystatin from the midgut of the hard tick *Ixodes ricinus*, but not with cystatins expressed in the salivary glands of hard ticks *I. scapularis* (Ribeiro et al., 2006) and *A. americanum* (Karim et al., 2005) (Fig. 7). All tick cystatins known to date, lack the domain responsible for inhibition of asparaginyl endopeptidases (legumain-like peptidases) from the C13 family (see AEP in Fig. 7) (Alvarez-Fernandez et al., 1999).

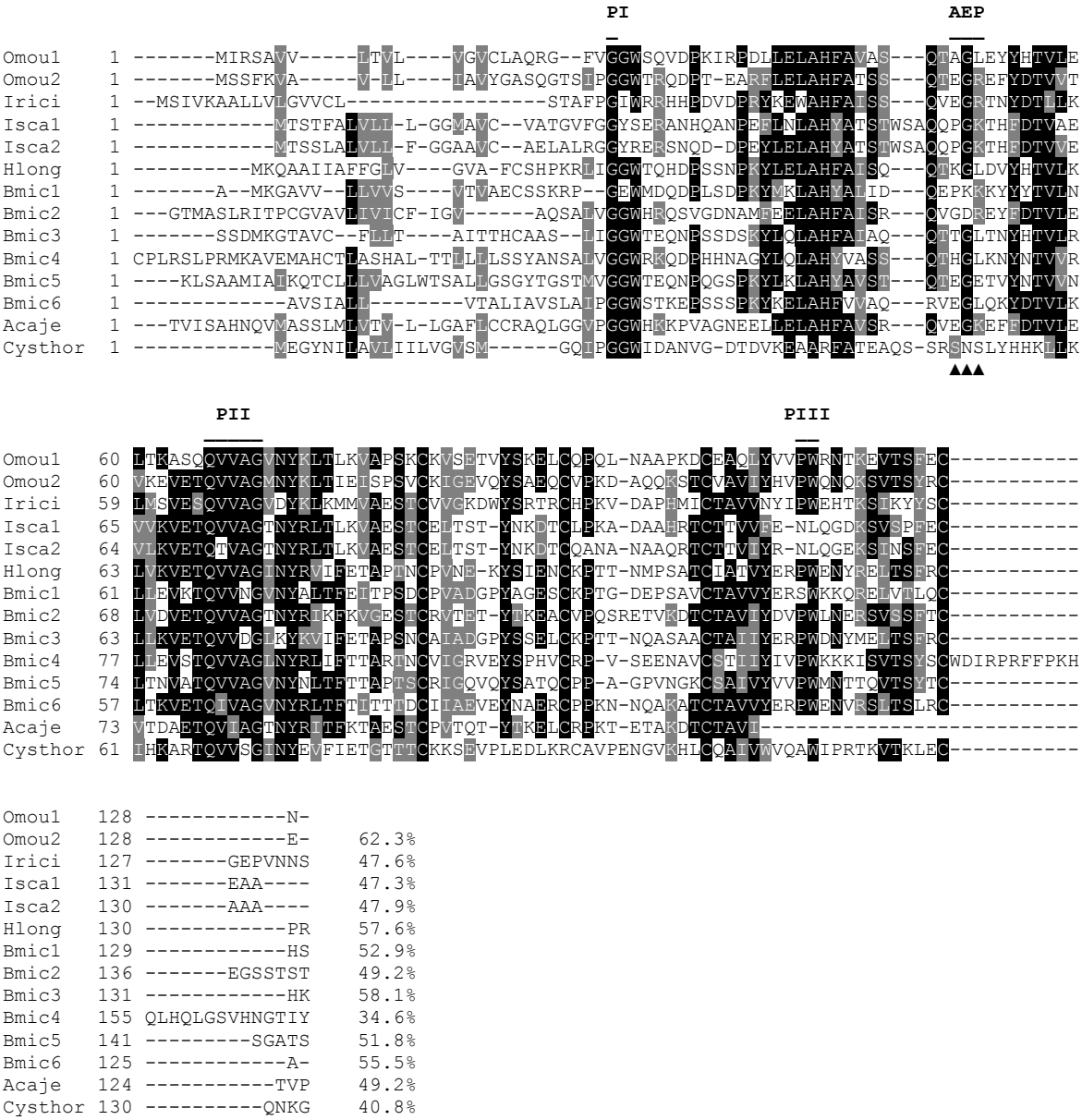

Figure 7: Multiple alignment of Om-cystatin 1 and 2 from *O. moubata* and available cystatin sequences from tick and no annotated ESTs of tick origin; percent identity to Om-cystatin 1: PI–PIII, papain-binding domains; AEP, asparaginyl endopeptidase (legumain)-binding domain position (▲▲▲ – AEP in horseshoe crab cystatin). Omou1 and Omou2 – Om-cystatins from the gut of *Ornithodoros moubata* (AAS01021; AAS55948); Irici – gut cystatin from *Ixodes ricinus* (CAD68002); Isca1 and Isca2 – secreted cystatins from *Ixodes scapularis* (AAM93646; AAY66685); Hlong – secreted cystatin from *Haemaphysalis longicornis* (ABC94582); Bmic1-6 - cDNA clones BEAAN91, BEAA778, BEAF056, BEAFZ42, BEABG74, BEAB489 from *Boophilus microplus* (GenBank CK176418, CK173752, CV444905, CV450827, CK181298, CK179250 respectively); Acaje – cDNA clone ACAH15A10 from *Amblyomma cajennense* tick library; cysthor – L-cystatin from the horseshoe crab *Tachypleus tridentatus* (Agarwala et al., 1996)

Tissue expression profile of Om-cystatins

The presence of mRNA Om-cystatin 1 is specific for the tick gut, expression of Om-cystatin 2 was found also in other tissues tested: salivary glands, ovaries and Malphigian tubules (Paper II, Fig. 2). A strong band corresponding to authentic Om-cystatin 2 was detected in the gut contents of unfed ticks using Western blot analysis (Paper II, Fig. 3). These localizations were confirmed using indirect immunolocalization on semi-thin sections. Om-cystatin 2 was secreted into the gut lumen of partially digested ticks and was significantly associated with the residual bodies. The presence of authentic Om-cystatins in hemosomes and residual bodies which are accumulated in the gut lumen was confirmed using electron microscopy on ultra-thin sections labeled by anti-Om-cystatin imunogold particles (Paper II, Fig. 5). Residual bodies are raised from none crystalline heme aggregate and contain yet unidentified proteins (Lara et al., 2003). The presence of Om-cystatins in the hemosomes and further in the residual bodies which accumulate in the gut lumen implies also their role in heme detoxification (Paper II).

Om-cystatin 2 was also clearly detected in salivary gland extracts of unfed ticks, whereas this amount decreased considerably 1 day after blood meal (abm) (Grunclová et al., 2006, Fig. 3). Based on localization by indirect immunofluorescence, Om-cystatin 2 was found to be expressed in salivary glands and synthesized specifically by the type 2 secretory cells (Balashov, 1979) of unfed ticks. Its expression was significantly suppressed in salivary glands of ticks 1 day after blood meal (Paper II, Fig. 4).

Inhibitory activity of cystatins

Tick cystatins are not capable to inhibit legumain-like activity therefore they are discriminated from their interference with MHC II antigen presentation dependent on asparaginyl-endopeptidase processing (Manoury et al., 1998). Cystatin 2 inhibited model mammalian exopeptidase cathepsin C (a dipeptidyl peptidase I) (Paper II), which is present in the greatest amount in cytotoxic T-lymphocytes, macrophages and mast cells. Cathepsin C is required for full activation of neutrophil-derived serine peptidases (cathepsin G, neutrophil elastase, proteinase 3) which are involved in inflammation processes (Brown et al., 1993; Wolters et al., 1998). Regulation of neutrophil elastase is important for the primary host defense against bacterial infection. These facts support the idea, that Om-cystatin 2 can modulate the host immune response. Our preliminary results suggest the ability of Om-cystatin 2 to modulate cytokine response of murine leucocytes (Salát, personal communication). The saliva from the hard tick *I. scapularis* display anti-cathepsin L activity

due to the presence of cystatin called sialostatin L. The authors further demonstrated that the recombinant sialostatin L inhibited papain-like proteases, targeting mainly cathepsin L and C. Sialostatin L had an anti-inflammatory activity and inhibited proliferation of cytotoxic lymphocytes (Kotsyfakis et al., 2006).

Silencing of cystatin expression in the salivary glands of *A. americanum* by ds RNAi significantly reduced the ability of ticks to feed successfully on rabbits (Karim et al. 2005). Moreover, rabbits previously exposed to cystatin silenced ticks exhibited resistance to repeated tick feeding. Salivary glands cystatin thus seem to help ticks to evade host immune and inflammatory response during blood feeding.

Differences in cystatin expression in hard and soft ticks

The hard ticks and the soft tick differ in timing of cystatin protein expression which is likely related to different feeding strategy. A secreted cystatin from the tick *H. longicornis* was mostly expressed in the tick midgut and hemocyte throughout the developmental stages and its distribution showed significant tissue specifity (Zhou et al., 2006). The relative amount of cystatin mRNA of *H. longicornis* increases during the feeding and culminates 4th day of feeding (Zhou et al., 2006). In contrast to the hard tick, *O. moubata* feeds for a 20 - 30 minutes period. Hence, if there is Om-cystatin 2 somehow involved to mediate host immune response it should be stored and instantly ready for the feeding. This is in accord to our data showing that mRNA level of Om-cystatin 2 is lowered after blood meal and the amount of Om-cystatin 2 markedly decreased in salivary gland secretory cells (Grunclová et al., 2006, Fig. 4). *B. microplus* cystatin did not have a signal peptide, which indicates its intracellular localization. Protein was only recognized in ovary, salivary glands and fat body (Lima et al., 2006).

Antimicrobial activities

In contrast to Zhou et al. (2006) demonstrating an immune function of cystatin from *H. longicornis* and the proven antibacterial activity of cystatin from the horseshoe crab (Argawala et al., 1996b), we were not able to confirm any antimicrobial activity of Om-cystatins in grow-inhibition assays.

In conclusion, we have reported two members of the cystatin family in the soft tick *O. moubata*, and presented detailed functional and expression analyses. Our work suggests that the tick cystatin inhibitors most likely play several distinct roles and are regulated in time and space. The potential inhibition targets comprise both endogenous and exogenous proteases. Rather than regulation of intracellular digestion, the cystatins in the gut contents are most likely targeted to eliminate the undesired action of cysteine peptidases released into the lumen from detached digestive cells. The presence of cystatin in salivary glands suggests the immunomodulatory role in tick-host interaction.

Paper II

Grunclová, L., Horn, M., Vancová, M., Sojka, D., Franta, Z., Mareš, M., Kopáček, P. (2006).

Two secreted cystatins of the soft tick *Ornithodoros moubata*: differential expression pattern and inhibitory specificity.

Biol. Chem. 387: 1635–1644.

Two secreted cystatins of the soft tick *Ornithodoros moubata*: differential expression pattern and inhibitory specificity

Lenka Grunclová, Martin Horn, Marie Vancová, Daniel Sojka, Zdeněk Franta, Michael Mareš, Petr Kopáček

Biol. Chem. 2006. 387: 1635–1644.

Two genes coding for cysteine peptidase inhibitors of the cystatin family (Om-cystatin 1 and 2) were isolated from a gut-specific cDNA library of the soft tick *Ornithodoros moubata*. Both cystatins were clearly down-regulated after a blood meal. Om-cystatin 1 is mainly expressed in the tick gut, while Om-cystatin 2 mRNA was also found in other tick tissues. Authentic Om-cystatin 2 was significantly more abundant than Om-cystatin 1 in the gut contents of fasting ticks and was associated with hemosome-derived residual bodies accumulated in the gut lumen. Om-cystatin 2 was also expressed by type 2 secretory cells in the salivary glands of unfed ticks. The inhibitory specificity of recombinant Om-cystatins 1 and 2 was tested with mammalian cysteine peptidases, as well as endogenous cysteine peptidases present in the tick gut. Both cystatins efficiently inhibited papain-like peptidases, including cathepsin B and H, but differed significantly in their affinity towards cathepsin C and failed to block asparaginyl endopeptidase. Our results suggest that the secreted cystatin isoinhibitors are involved in the regulation of multiple proteolytic targets in the tick digestive system and tick-host interaction.

IV. Thyropin from the soft tick *Ornithodoros moubata*

Abstract

The gene coding for putative cysteine peptidase inhibitors containing thyroglobulin type-I domain (Om-thyropin) was isolated from a gut-specific cDNA library of the soft tick *Ornithodoros moubata*. RT-PCR showed Om-thyropin mRNA message in several tick tissues like gut, salivary glands, ovaria and Malphigian tubes. Om-thyropin was down regulated after blood meal. Immunolocalization using antibodies against recombinant protein revealed that Om-thyropin is evenly distributed in the gut contents of fasting ticks. The preliminary data suggest that recombinant Om-thyropin protein is able to inhibit papain-like peptidases as cathepsin L, the typical target protease of inhibitors of thyropin type.

Introduction

Thyroglobulin type-I domain inhibitors belong to the clan IX, family I31 according to the MEROPS database classification. The cysteine protease inhibitory function of thyroglobulin domains was established when a fragment of the class II invariant chain was isolated from human kidney bound to the cathepsin L, a papain family cysteine protease (Ogrinč et al., 1993). This fragment is normally part of the major histocompatibility complex (MHC). The p41 form has a region with the significant homology with the thyropin domain of thyroglobulin. Crystallography of this p41 domain in complex with cathepsin L revealed that the domain assumes a wedge-shape conformation containing three loops stabilized by three disulfide bridges and it is lodged in the active site of cathepsin L. Thus the inhibition mechanism resembles that of other cysteine proteinase inhibitors, namely the cystatin/stefin family. They are both wedge-shaped structures, which fill the active site cleft with three short binding segments; three hairpin loops of the p41 fragment versus the N-terminal and two hairpin loops of stefins (Gunčar et al., 1999). Additionally the p41 fragment can discriminate between two closely related enzymes, cathepsin L and cathepsin S, inhibiting only the former (Bevec et al., 1996).

In the last decade, several proteins containing thyroglobulin domain-type I (Tg-I) were identified in variety of species. Saxiphilin, a bullfrog serum protein that binds neurotoxin (Lenarčič et al., 2000), equistatin from a sea anemone (Lenarčič et al., 1997), human testican (Bocock et al., 2003), oocytes protease inhibitors (OPI-1 and -2) from rainbow trout (Wood et al., 2004) also have one or more thyropin domains. Except OPIs, these proteins inhibited

cathepsin L proteolytic activity. Only one of the two Tg-I domains present in saxiphilin inhibits cathepsins L or B (Lenarčič et al., 2000). Similarly, the first of three equistatin domains exhibited broad specificity against papain-like cysteine peptidases, while the subsequent two are non-inhibitory (Lenarčič et al., 1999; Galeša et al., 2003). Furthermore, the second Tg-1 domain of equistatin is unique in inhibiting also the aspartic peptidase cathepsin D (Galeša et al., 2003).

Cathepsin L is a ubiquitously expressed protease efficiently secreted into the lysosomes, where low pH allows for optimal activity to mediate proteolysis (Kornfeld et al., 1989). In addition to it, it participated in developmental processes and antigen processing (Turk et al., 2000). In ticks three cathepsins L have been described. The recombinant enzyme from *Boophilus microplus* was capable of hydrolyzing gelatin, tick vitellin and bovine hemoglobin. This cathepsin L was immunolocalized in probable secretory cells of the gut (Renard et al., 2000, 2002) and was likely involved in hemoglobin digestion process. Two forms of cathepsin L-like cysteine proteinase genes from the hard tick *H. longicornis* were characterized. Both proteases displayed homology with invertebrate cathepsin L-like cysteine proteinase genes and conserved motifs of cysteine proteases were identified in both (Mulenga et al., 1999).

To our knowledge Om-thyropin is the first protein containing thyroglobulin type-I domain in ticks. It has been cloned, sequenced and partially characterized.

V. Serine protease homolog from the soft tick *Ornithodoros moubata*

Abstract

The gene coding for a serine protease homolog (SPH) was isolated from a gut-specific cDNA library of the soft tick *Ornithodoros moubata*. SPH falls into the S1A non-peptidase family (chymotrypsin subfamily) comprising secreted endopeptidase homologs with substituted amino acids in the catalytic triad. Moreover, SPH contains an (HD)-rich domain of unknown function. Using RT-PCR, mRNA message of SPH was present strictly in the tick gut. Authentic SPH protein was detected by Western blot analysis in the gut tissue homogenate and secreted into the gut contents. Although function of SPH remains unknown, we speculate that it may play a role as an antimicrobial peptide or be involved in processing of blood-meal by interference with clotting serine protease of the host origin.

Introduction

Extracellular serine proteinases mediate rapid local reactions to physiological or pathological impulses in vertebrates and invertebrates. In mammals, these enzymes are involved in cascade reactions like complement activation or blood clotting. In arthropods similar proteinase systems function in response to injury and infection (for review see Iwanaga et al., 2006)

Based on the structure and sequence similarity, serine proteinases are divided into several clans counting from A to G (Barrett and Rawlings, 1995). Representatives of serine proteinase gene families have been cloned and characterized from variety of invertebrate species. Generally, beside their crucial role in immunity response (Dimopoulos et al., 2001), serine proteases perform a rapid blood meal digestion in some haematophageous arthropods like mosquitoes or tsetse flies.

Several serine proteases have been described also in ticks. The first tick serine proteases cDNA named as HLSG-1 and -2 were cloned and sequenced from the ixodid tick *Haemaphysalis longicornis* (Mulenga et al., 2001). Both enzymes displayed a conservation of the consensus serine protease catalytic triad, namely His, Asp and Ser. HLSG-1 is highly similar to the coagulation factor C precursor from the coagulation cascade of the horseshoe crab (Agarwala et al., 1996a). HLSG-2 is predominantly related to chymotrypsinogen-like precursor enzymes. Both serine proteases are expressed in gut and salivary glands and their expression increases in partially fed ticks (Mulenga et al., 2001). The same authors described

three serine proteases from the hard tick *Rhipicephalus appendiculatus* (RAMSP-1 to -3). The mRNAs for RAMSP-1 and -2 were expressed in both midgut and salivary glands of partially fed ticks, while RAMSP-3 mRNA was gradually upregulated as tick feeding progressed in midgut (Mulenga et al., 2003). In the hard tick *H. longicornis*, a cubilitin-related serine protease (HlSP) was described in all developmental stages and its expression was upregulated during a blood feeding process (Miyoshi et al. 2004a). The RNAi silencing of HlSP inhibited the growth of treated ticks compared to control ticks (Miyoshi et al. 2004b). Recently, it was shown that HlSP plays a crucial role in the hemolysis of the erythrocytes from the ingested blood. Thus HlSP seems to be the enzyme initiating the whole complex process of blood digestion in the tick gut (Miyoshi et al., 2007).

In serine protease homologs, one, two or three amino-acid residues of the conserved catalytic triad are replaced. Although it is almost nothing known about their function, a serine protease homolog expressed in hemocytes, ovaries and salivary glands of the tick *Dermacentor variabilis* was reported to play a role in a response to bacterial infection (Simser et al. 2004b).

In this work, we report a characterization a gut-specific serine protease homolog from the soft tick *O. moubata* and show some preliminary data for assessment its function.

3. Summary of results

- Tick gut lysozyme from the soft tick *Ornithodoros moubata* was the first lysozyme representing the subphylum Chelicerata. The phylogenetic analysis revealed that the TGL belongs to the c-type lysozymes having a rare histidine residue at position 52 which replaces the highly conserved tyrosine (H-branch group). Semiquantitative RT-PCR and Northern blotting analysis demonstrated that TGL is strongly up-regulated at the transcriptional level after a blood meal. TGL seems to be an interesting case in which the features of lysozymes with anti-bacterial and digestive function are combined.

- The search for the genes differentially expressed in the midgut of the soft tick *Ornithodoros moubata* after blood meal led to the identification of several genes coding for proteins which may be affected by blood-meal in this species. This set contained i. a. two genes coding for putative inhibitors of cysteine peptidases of cystatins family (Om-cystatin1 and Om-cystatin 2), putative thyroglobulin domain containing protease inhibitor (thyropin) and chymotrypsin-like protein characterized later as a serine protease homolog (SPH).

- Both cystatins (Om-cystatin 1 and 2) were clearly down-regulated after a blood meal. Om-cystatin 1 is mainly expressed in the tick gut, while Om-cystatin 2 mRNA was also found in other tick tissues. Authentic Om-cystatin 2 was associated with hemosome-derived residual bodies accumulated in the gut lumen. Om-cystatin 2 was also expressed by type 2 secretory cells in the salivary glands of unfed ticks. Both cystatins efficiently inhibited papain-like peptidases, including cathepsin B and H, but differed significantly in their affinity towards cathepsin C and failed to block asparaginyl endopeptidase.

- The gene coding for a putative cysteine peptidase inhibitor containing thyroglobulin I domain (Om-thyropin) was characterized. RT-PCR showed Om-thyropin mRNA message in several tick tissues like gut, salivary glands, ovaria and Malphigian tubes. Om-thyropin was down-regulated after a blood meal. Immunolocalization revealed that Om-thyropin is evenly distributed in the gut contents of fasting ticks. The preliminary data suggest that recombinant Om-thyropin protein is able to inhibit papain-like peptidases as cathepsin L, the typical target protease of inhibitors containing thyroglobulin domain.

- Serine protease homolog (SPH) from *O. moubata* falls into S1A non-peptidase family (chymotrypsin subfamily) comprising secreted endopeptidase homologs with substituted amino acids in catalytic triad. Moreover SPH contains (HD)-rich domain of unknown function. The mRNA message of SPH was observed to be present strictly in tick gut. Authentic SPH protein was detected by Western blot analysis in gut tissue homogenate and secreted into gut contents. Although its function remains unknown, we speculate the role of SPH in immunity or/and blood meal processing in gut lumen of the soft tick *O. moubata*.

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