Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 347 (2006) 44–50
BBRC
www.elsevier.com/locate/ybbrc

# Bmcystatin, a cysteine proteinase inhibitor characterized from the tick *Boophilus microplus*

Cassia A. Lima, Sergio D. Sasaki, Aparecida S. Tanaka *

*Department of Biochemistry, Universidade Federal de São Paulo, Escola Paulista de Medicina, 04044-020 São Paulo, SP, Brazil*

Received 30 May 2006
Available online 12 June 2006

**Abstract**

The bovine tick *Rhipicephalus* (*Boophilus*) *microplus* is a blood–sucking animal, which is responsible for Babesia spp and *Anaplasma marginale* transmission for cattle. From a *B. microplus* fat body cDNA library, 465 selected clones were sequenced randomly and resulted in 60 Contigs. An open reading frame (ORF) contains 98 amino acids named Bmcystatin, due to 70% amino acid identity to a classical type 1 cystatin from *Ixodes scapularis* tick (GenBank Accession No. DQ066227). The Bmcystatin amino acid sequence analysis showed two cysteine residues, theoretical p*I* of 5.92 and $M_r$ of 11 kDa. Bmcystatin gene was cloned in pET 26b vector and the protein expressed using bacteria *Escherichia coli* BL21 SI. Recombinant Bmcystatin (rBmcystatin) purified by affinity chromatography on Ni–NTA–agarose column and ionic exchange chromatography on HiTrap Q column presented molecular mass of 11 kDa, by SDS–PAGE and the N-terminal amino acid sequenced revealed unprocessed N-terminal containing part of *pelB* signal sequence. Purified rBmcystatin showed to be a C1 cysteine peptidase inhibitor with $K_i$ value of 0.1 and 0.6 nM for human cathepsin L and VTDCE (vitellin degrading cysteine endopeptidase), respectively. The rBmcystatin expression analyzed by semi-quantitative RT-PCR confirmed the amplification of a specific DNA sequence (294 bp) in the fat body and ovary cDNA preparation. On the other hand, a protein band was detected in the fat body, ovary, and the salivary gland extracts using anti-Bmcystatin antibody by Western blot. The present results suggest a possible role of Bmcystatin in the ovary, even though the gene was cloned from the fat body, which could be another site of this protein synthesis.


*Keywords:* Acari; *Boophilus microplus*; Cysteine proteinase inhibitor; Cystatin; Stefin B

Cystatins are biochemically well-characterized as strong inhibitors of cysteine proteinases of the papain family, especially cathepsins and also of some lysosomal caspases, such as legumain [1,2]. Cystatins are divided into three evolutionary related families: stefin (type 1), cystatin (type 2), and kininogen (type 3), among them only stefins are intracellular [3,4].

An important contribution of cystatins in the regulation of the cysteine proteinases is probably in the control of intracellular protein degradations [5,6]. The question of physiological function is particularly interesting for the proteinase inhibitors which have been isolated from parasites, since these molecules could potentially not only fulfill any of the internal protective or regulatory functions suggested for inhibitors from non-parasitic organisms, but also be involved in the parasite and host interaction [7].

The tick *Boophilus microplus* (ixodid) is one of the most important bovine ectoparasites identified in the tropical countries. This tick species affects animal's health not only interfering in blood feeding process but also by transmitting pathogens. Once attached to bovine, *B. microplus* tick is able to ingest a large amount of blood. The parasitic phase initiated by larvae attached and finished when the fully engorged females fall to the ground and during the non-parasitic period when the eggs were laid. In oviparous, nutrients required for embryonic development are produced outside of the ovary and internalized during the development of oocytes. In insects, most egg protein constituents, called yolk protein precursors, are produced by the fat body [8].

* Corresponding author. Fax: +55 11 5572 3006.
*E-mail address:* Tanaka.bioq@epm.br (A.S. Tanaka).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.018

Cysteine proteinases involved in yolk processing during the hard tick *B. microplus* embryogenesis had been reported [9,10], as the vitellin (VT) degrading cysteine endopeptidase (VTDCE) [11] and two aspartic proteases, *Boophilus* Yolk pro-Cathepsin (BYC) and tick heme-binding aspartic proteinase (THAP), from eggs [12,13]. In egg crude extract of the soft tick *Ornithodoros moubata*, proteolytic activity has been essentially attributed to a cathepsin L-like enzyme by substrate and inhibitor specificities [9].

In ticks, genes encoding cysteine proteinase inhibitors (type 2) have already been found in the *Ixodes scapularis* sialoma [14] and in *Amblyomma americanum* facilitate the hematophagy behavior suppressing the antigens, processing and immune recognition of molecules of the tick saliva [15].

Proteinase inhibitors of the cystatin superfamily form tight, but reversible, complexes with most of the cysteine proteinases (C1 family) structurally related to papain [16–19]. Several cystatin-like proteins have been described in mammals, bird, fish, insect, plant, and some protozoan, and their corresponding cDNAs were cloned [17,20–24]. The role of the cystatin-like proteins in tick is still unknown.

Identification of *B. microplus* antigens as candidates for vaccines and the study of host–parasite interactions have been the major research focus since this ectoparasite could acquire resistance after chemical treatments [25]. Tick chemical control also raises problems associated with food chain and environmental contaminations [26]. In this paper, we constructed a fat body cDNA library, and a cystatin gene was found by EST sequence analysis. And for the first time, a tick cysteine proteinase inhibitor, cystatin-like is expressed and biochemically characterized.

## Experimental procedures

*Materials.* PVDF transfer membrane (Amersham Little Chalfont, Buckinghamshire , England). *Bacteria and vector: Escherichia coli* DH5α (F, endA1, hsdR17, sup E44, thi1, λ, recA1, gyrA96, Ø 80 d lacZD15) was used as host for recombinant DNA manipulation. *E. coli* BL 21 (SI) (F –, *ompT, hsdS* (*r* – *m* –), *gal, dcm)* was used as a host for protein production, and both strains were purchased from Invitrogen (Carlsbad, CA). The pET26b expression vector was from Novagen (Madison, WI). *Modification enzymes*: Restriction enzymes *Hin*dIII (Promega, Madison, WI) and *Nco*I (MBI Fermentas, Hanover, MD); *Taq* DNA polymerase was purchased from Promega (Madison, WI). *Chromatography columns*: Ni–NTA–agarose–resin was obtained from Qiagen (Hilden, Germany). HiTrap Q and Sephasil $C_8$ were from GE Healthcare Life (Uppsala, Sweden). *Enzymes and substrates:* Recombinant human cathepsin L (EC 3.4.22.15) was expressed in *Pichia pastoris* as described previously [27], using a plasmid construction kindly provided by Dr. Nägler. Papain (EC 3.4.22.2) and human liver cathepsin B (EC 3.4.22.1) were purchased from Calbiochem (San Diego, CA). VTDCE was gently supplied by Dr. Carlos Termignoni; S2302 (HD-Pro-Phe-Arg-pNA) was obtained from Chromogenix (Mölndal, Sweden).

*Ticks. Boophilus microplus* engorged females were provided by Dr. Itabajara da Silva Vaz Junior of Universidade Federal do Rio Grande do Sul, Brazil.

*cDNA library construction and screening.* Total RNA was extracted from fat body of *B. microplus* using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA library construction using SMART cDNA system was performed according to manufacturer's instructions (Clontech, Palo Alto, CA). The mRNA of *B. microplus* fat body (240 ng) purified by RNeasy Lipid Tissue Mini Handbook Kit (Qiagen, Hilden, Germany) from 20 adult females at 4th day after being detached was reverse transcribed to cDNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI). And *Sfi*I site possessing adaptors were attached to both primer ends of cDNAs. The cDNAs were amplified by PCR and digested with the restriction enzyme *Sfi*I. cDNA fragments longer than 400 bp separated by fractionation column were collected and ligated into pTripIEX plasmid and subsequently submitted to phage packaging reaction using kit from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Approximately 465 cDNA clones from the amplified library were randomly sequenced. The DNA sequence analysis was performed using the BLAST algorithm tools [28]. Alignment of protein sequences was performed with the ClustalW program, version 1.83.

*Cloning of cDNA fragment of Bmcystatin.* The cDNA sequence encoding the tick cystatin was amplified by PCR using an upstream primer (with *Nco*I recognition site) *Nco*I—5′-GCAATCG**CCATGG**AGATG CCTCTC-3′ and a downstream primer *Hin*dIII—5′-CGTAT **AAGCTT**CTGAAAATG-3′. Polymerase chain reaction (PCR) was performed in 50 μl reaction volume containing 5 pmol of gene-specific primers, 100 μM DNTPs, 2 mM $MgCl_2$, and 5 U *Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil). PCR condition was: [94 °C for 10 min, 94 °C for 1 min, and 55 °C for 45 s] × 35 cycles. DNA final extension was carried out at 72 °C during 10 min. The PCR product was purified from agarose gel using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany), and digested with *Hin*dIII and *Nco*I restriction enzymes, followed by ligation into pET26b vector which was previously digested with the same enzymes above. The corrected plasmidial construction was confirmed by DNA sequencing using DYEnamic™ ET Dye Sequencing kit (Amersham Little Chalfont, Buckinghamshire , England) and ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

*Expression of recombinant Bmcystatin.* Bmcystatin cDNA fragment cloned into expression vector pET26b was expressed in *E. coli* BL21 (SI) strain as a protein fused to a leader sequence *pel*B and 6 His-tagged. Previously, the rBmcystatin production was in 2 l TB broth medium containing 30 μg/ml kanamycin and one isolated expression *E. coli* colony. rBmcystatin expression was induced with 0.3 M NaCl (at OD = 0.7) during 15 h at 37 °C. Afterwards, the cells were harvested by centrifugation (4000*g*, 20 min, 4 °C) and suspended in 50 ml of 50 mM Tris–HCl buffer, pH 8.0. The cells were lysed using a French press (1000 psi). The culture supernatant was centrifuged twice (13,000*g*, 45 min, 4 °C).

*Purification of recombinant Bmcystatin.* The resulting supernatant containing rBmcystatin was applied on a Ni–NTA–agarose column previous pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl. After washing step with the same buffer, the protein elution was done with increasing imidazole concentration in the same Tris–HCl buffer (pH 7.2). Solution containing eluted proteins was dialyzed and concentrated using 5 kDa NMWL Ultracel membrane in Amicon system and 40 mM Tris–HCl buffer, pH 8.0. The rBmcystatin was further purified on a HiTrap Q anion-exchange column connected to an ÄKTA system. The protein was eluted by NaCl linear gradient (0–0.7 M) in 50 mM Tris–HCl buffer, pH 8.0, at a flow rate of 1 ml/min. The elution profile was monitored by measuring the absorbance at 215 nm, and the fractions were tested on human cathepsin L inhibition assay. The purified Bmcystatin was analyzed by SDS–PAGE 15% [29].

*HPLC analysis.* Purified rBmcystatin was applied on a Sephasil Peptide $C_8$ 12 μ ST 4.6/250 column connected to an ÄKTA™ purifier system and pre-equilibrated with 0.1% TFA in Milli Q water. The proteins were eluted by an acetonitrile linear gradient (0–80%) in 0.1% TFA, at a flow rate of 1 ml/min (60 min) and monitored by measuring the absorbance at 215 nm.

*Antibody production.* The purified rBmcystatin (10 μg) was subcutaneously injected in Balb C mice (6 animals), 2 months old. Briefly, 10 μg of rBmcystatin was mixed with equal volume of Freund's complete adjuvant (100 μl). The animals received two other injections in a two-week interval each, of rBmcystatin mixed with equal volume of Freund's incomplete adjuvant. The mice were bled 2 weeks after the last injection and antiserum was collected and used in Western blot analysis.

*Western blot analysis.* Extracts of 15 fat body and salivary glands were separately homogenate in saline phosphate buffer, and the supernatants obtained after centrifugation at 20,000*g* for 2 min and eggs (5%) and larvae (3%) extracts of *B. microplus* were analyzed by Western blot. Fat body (25 μl), salivary gland (25 μl) crude extract proteins, and the rBm-cystatin (10 μg) were separated on a 15% SDS–PAGE and blotted onto PVDF membrane using electrophoretic transfer system Mini Trans-Blot Cell (BioRad, Hercules, CA). The PVDF membrane was blocked with blocking buffer (PBS buffer containing 5% skimmed milk) for 1 h at room temperature. The blocked membrane was incubated with anti-serum anti-Bmcystatin (dilution 1:500), overnight at 4 °C. After washing step with blocking buffer, the membrane was incubated with anti-mouse IgG conjugated with peroxidase (dilution 1:20,000) for 1 h at room temperature. The membrane was revealed using SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL) and X-ray film (8 × 10 inch) (Kodak Biomax MS, NY).

*Amino acid sequence analysis.* rBmcystatin purified by HPLC was used for $NH_2$-terminal amino acid determination. The amino acid sequence was performed by Edman degradation using a Sequenator model PPSQ-23 (Shimadzu, Tokyo, Japan).

*Inhibition assay and determination of dissociation constant.* Cysteine proteinase inhibition assays were performed using chromogenic substrate by measuring the residual hydrolytic activity after preincubation with different concentrations of Bmcystatin. The enzymes, papain, cathepsin L, cathepsin B, and VTDCE, were pre activated with 200 mM DTT in 0.1 mM sodium acetate buffer, pH 5.5, containing 60 mM NaCl for 10 min at 37 °C. Afterwards, the activated enzyme was preincubated with different concentrations of rBmcystatin for 10 min at 37 °C. And the synthetic substrate HD-Phe-Arg-pNa (0.2 mM) was added and the absorbance at 405 nm was measured. Apparent $K_i$ values were calculated by fitting the steady-state velocities to the equation $(V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t)$ for tight-binding inhibitors using a non-linear regression analysis [30].

## Results

### *Cloning of tick cystatin gene*

Nucleotide sequence analysis of the cloned fragment by cDNA library revealed a complete amino acid sequence of cysteine proteinase inhibitor present also in other eukaryotes. The nucleotide sequence is deposited in the GenBank database under Accession No. DQ646915 and is referred to as Bmcystatin. BLAST analysis showed that *B. microplus* cystatin possesses 70% identity with cytoplasmatic cystatin of tick *I. scapularis*. Alignments of the homologues of this protein family show that all are small (<100 aa) proteins with key conserved features. The amino acid analysis using the Signal P program did not reveal the presence of a signal peptide, which is typical of cystatin B, indicating intracellular localization common across the phylogenetic scale. The complete nucleotide sequence and its deduced amino acid sequence are shown in Fig 1.

### *Analysis of nucleotide and protein sequences of Bmcystatin*

*Bmcystatin* cDNA fragment encodes a 98 amino acid residue protein. There is an initiation codon (ATG) in nucleotide position +1 and a stop codon (TGA) at 292 bp (Fig. 1).

### *Heterologous expression and purification of Bmcystatin*

For the production of rBmcystatin, the vector containing the *pelB* signal peptide which sends the protein into the bacterial periplasmic space was used. Attempts to purify the Bmcystatin by osmotic shock were unsuccessful. From 16 mg of starting recombinant protein only 0.8 mg of Bmcystatin was obtained. So, the following procedure was adopted, the culture was lysed using French press in 50 mM Tris–HCl buffer, pH 8.0, and NaCl 0.15 M. The rBmcystatin was purified by classical purification methodologies: affinity and ion exchange chromatographies. The Bmcystatin purified by a Ni–NTA–agarose column presents major protein band of 11 kDa, which was further purified by an ion-exchange chromatography on HiTrap Q ion exchange column (Fig. 2A). The purified Bmcystatin migrated as a single band in reduction and two bands in non-reduction conditions on SDS–PAGE, respectively (Fig. 2B). From 2 l of culture, 3.5 mg of purified rBmcystatin was obtained.

### *N-Terminal sequencing and amino acid analysis*

Reverse-phase liquid chromatograph was performed to confirm the purity of rBmcystatin and also to obtain the material for amino acid sequencing. Bmcystatin N-terminal amino acid sequence was obtained by automated Edman degradation (Fig. 2C). Its analysis revealed the presence of the last six amino acid residues of the leader sequence (*pelB*). These results indicate failure in the aminopeptidase cleavage activity. The complete translated amino acid sequence of Bmcystatin showed high similarity to cystatin B-like proteins, which are intracellular proteins (Fig. 3).

```
GCGATGGCCATGGAGATGCCTCTCTGTGGTGGGCTCTCTGAGCAAGTCAAGGATGCCGATGACACAGTC
 A  M  A  M  E  M  P  L  C [G] G  L  S  E  Q  V  K  D  A  D  D  T  V

AAAGAGATCTGCGAAAAGGTTCGCGCCGAAGTGGAAGCGAAGCTGGAGAAGTCCTTTCCAGAGTTTACAC
 K  E  I  C  E  K  V  R  A  E  V  E  A  K  L  E  K  S  F  P  E  F  T

CTCTGAAGTATCGCACGCAGCTGGTGAACGGCATCAACTATTTATTAAGGTTCACGTAGGTAACGACCA
P  L  K  Y  R  T [Q  L  V  N  G] I  N  Y  F  I  K  V  H  V  G  N  D  Q

ACACATCCACGTCCGTGCGCACAAGGCTTTCCAGGGCGAAATATCGTTCTCTGCTGTGCAGGGGGACAAA
  H  I  H  V  R  A  H  K  A  F  Q  G  E  I  S  F  S  A  V  Q  G  D  K

AAGTTGGAAGATCCACTCGAGCATTTTCAGAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCAC
 K  L  E  D  P  L  E  H  F  Q  K  L  A  A  A  L  E  H  H  H  H  H  H
```

Fig. 1. Nucleotide and deduced amino acid sequences of Bmcystatin. Amino acid residues of cystatin involved in enzyme binding are boxed.

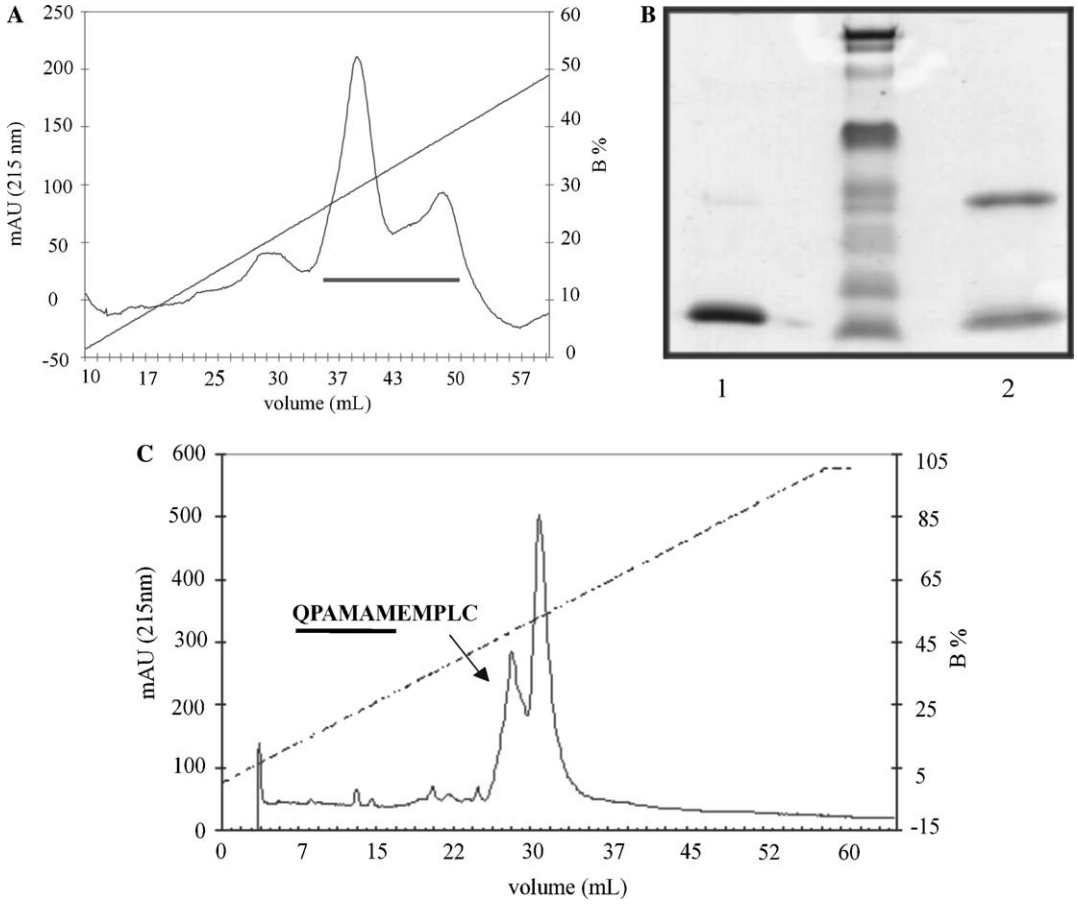


Fig. 2. rBmcystatin purification and characterization. (A) The sample (~8.5 mg) containing Bmcystatin after Ni–NTA–agarose was applied on a HiTrap Q column. The proteins were eluted with linear gradient of NaCl (0–0.7 M). The collected fraction inhibitory activities were pooled in accordance with the protein elution profile. (B) rBmcystatin analysis by SDS–PAGE (15%). (1) rBmcystatin (5 μg) reduced conditions; (2) rBmcystatin (5 μg) non-reduced conditions. (C) Purification of rBmcystatin by reverse-phase chromatography. The rBmcystatin (0.15 mg) was applied on a Sephasil Peptide $C_8$ column previously equilibrated with 0.1% TFA solution in water. Protein elution was with linear gradient of acetonitrile (25–85%) constant flow rate of 1 ml/min. N-Terminal amino acid sequence determined for the first protein peak showed an unprocessed leader sequence which is underlined.

### *Semi-quantitative PCR*

To investigate the tissue-specific Bmcystatin expression, semiquantitative PCR analysis, using gene-specific primers, was performed using cDNA preparation of various tissues isolated from adult ticks. DNA fragments with the predicted size for the tick inhibitors (294 bp) were amplified in fat body and ovary (Fig. 4), while absent in salivary gland. Control semi-quantitative PCR using 18S ribosomal-specific primers confirmed sample integrity. The amplification of Bmcystatin fragment confirmed that this gene is active in *B. microplus* fat body and ovary.

### *Western blot*

Proteins of crude extract of eggs, larvae, ovary previtellogenic period, salivary gland, and fat body were separated by SDS–PAGE, transferred to a polyvinylidene difluoride membrane, and the band corresponding to the 11 kDa protein was only recognized by anti-Bmcystatin antibody in ovary, salivary gland, and fat body (Fig. 5).

### *rBmcystatin inhibitory specificity*

Bmcystatin similar proteins were not previously described in ticks with respect to their natural expression at intracellular level. To analyze Bmcystatin specificity, the recombinant protein was used in inhibition assays of lysosomal cysteine proteinases. As shown in Table 1, Bmcystatin did not inhibit the activity of the papain, in contrast to cystatins isolated from other sources. However, both human cathepsin L and VTDCE of *B. microplus* tick were inhibited by a reversible and competitive mechanism.

## Discussion

The current study describes the expression, purification, and characterization of recombinant tick cystatin, type 1, from hard tick *B. microplus*. This is the first time that an inhibitor of cystatin family has been characterized from tick *B. microplus*. By the EST analysis of fat body cDNA library was found a protein-coding gene which seemed to have biological functions of cystatin-like. The complete

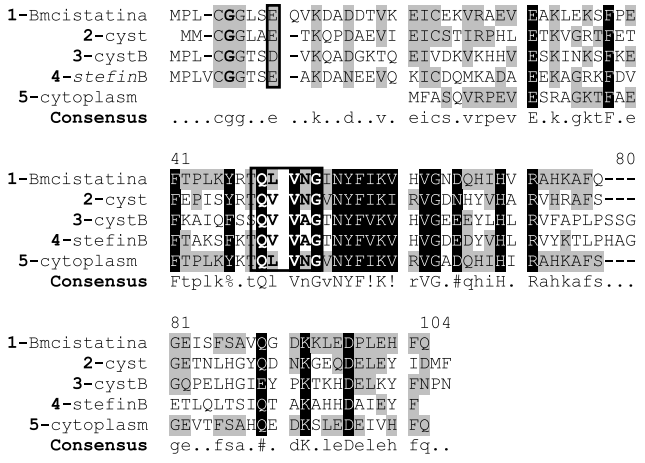


Fig. 3. Alignment of the amino acid sequence of rBmcystatin with cysteine proteinase inhibitors belong to cystatin type 1 family. 1, rBmcystatin (*Boophilus microplus*); 2, cyst, cystatin from *Lepidoglyphus destructor* (Accession No. LDE428051); 3, cystB, stefin B from *Theromyzon tessulatum* (Accession No. AF542131.1); 4, stefinB, similar to cysteine protease inhibitor B from *Danio rerio* (Accession No. XP_698401); 5, cystoplasm, cytoplasmatic cystatin from *Ixodes scapularis* (Accession No. DQ066227). Conserved amino acid residues are shown as grey boxes and residues involved in inhibitory activity are boxed.

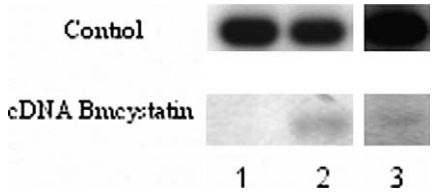


Fig. 4. Semi-quantitative PCR analysis of expression of Bmcystatin cDNA in different tissues of *B. microplus*. Electrophoresis on agarose gel (1%) of PCR products using cDNA preparation of: 1, salivary gland; 2, fat body; and 3, ovary.

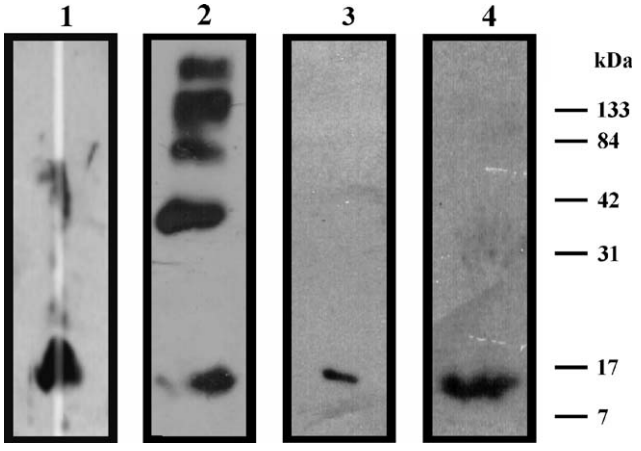


Fig. 5. Western blotting analysis of the Bmcystatin expression in different tissues of *B. microplus*. 1, Ovary pre-vitellogenic period extract; 2, rBmcystatin; 3, salivary gland extract; and 4, fat body extract. PVDF membranes were incubated with anti-rBmcystatin polyclonal anti-serum and revealed with SuperSignal West Pico Chemiluminescent Substrate and X-ray film was used.

Table 1
Dissociation constants ($K_i$) of Bmcystatin for different cysteine proteinases

| Enzyme | $K_i$ (nM) rBmcystatin |
|---|---|
| Human cathepsin L | 0.11 |
| VTDCE[a] | 0.61 |
| Human cathepsin B | 80.00 |
| Papain | n.i. |

n.i., not inhibited using 0.45 μM rBmcystatin.
[a] VTDCE, vitellin degrading cysteine endopeptidase of *Boophilus microplus* tick.

nucleotide sequence was determined and compared with other known sequences of inhibitors from the cystatin family. We cloned the *B. microplus* fat body cysteine proteinase inhibitor gene which encodes a protein of 11 kDa and theoretical p*I* 5.7. The stefins B are intracellular proteins [31] that present p*I* between 5.2 and 7.2. These proteins do not present disulfide bridges, differently Bmcystatin presents two cysteine residues; however they do not differ from the stefins in the molecular mass that is approximately 11 kDa. The central QXVXG region is conserved in the Bmcystatin, as in other stefins [2]. But in contrast, the *B. microplus* cystatin (Bmcystatin) did not inhibit papain B.

Interestingly, we found Bmcystatin expressed in both tick fat body and ovary (Fig. 4), suggesting the possibility for more than one functional role. For example, the ovary cystatin may play a role in regulating endogenous proteolysis during embryogenesis.

In salivary gland, Bmcystatin-like protein was identified by Western blot and it could act as defense proteins or regulating host immune responses. In *Amblyomma americanum*, it is likely that the salivary gland cystatin plays a role in immunomodulating the host response; while midgut cystatin may play a protection role for harmful ingested factors [15].

Kinetic assays revealed that Bmcystatin binds rapidly and tightly to human cathepsin L, with a $K_i$ value ~0.11 nM, and VTDCE, with a $K_i$ value ~0.6 nM. Amongst the attributed functions to ticks cathepsin L-like already described, the activity of degradation egg yolk has been attributed [9,32].

In most non-mammalian oviparous organisms, one of the early events during embryo development is the initiation of proteolytic processing of vitellin (VT). The regulation of this process is still unclear [9,33,34]. Vitellin (VT) is the major yolk protein in the egg of oviparous animals. Its physiological role is described as the source of amino acids to support the embryo development [8,35]. Considering our data that Bmcystatin can inhibit a vitellin degrading cysteine endopeptidase (VTDCE) [11] the following question was raised, whether this molecule could be involved in the regulation of the tick embryogenesis. In fact, several post-fertilization enzyme activities have been demonstrated during arthropod egg development [36,37]. Yamaguchi et al. showed experimentally that a cathepsin L-like participates in the degradation of an egg-derived

tyrosine phosphatase (EDTP) in the *Sargophaga peregina* embryos. EDTP seems to be synthesized and to accumulate in eggs during oogenesis and it disappears rapidly as the embryos develop [37].

Supporting our hypothesis, Western blot experiment probed with a polyclonal anti-Bmcystatin antibody showed the presence of a Bmcystatin-like (11 kDa) in fat body, salivary gland, and ovary extracts. However, it was not detected in eggs extracts (5%) and larvae (3%). In the Western blot, both the monomeric and the dimeric Bmcystatin forms were identified (recombinant form), and these data also confirmed the tendency of cystatin B-like to spontaneously dimerize; the cystatin A does not present this property [38]. Even though, in the semi-quantitative PCR technique, a fragment of the Bmcystatin was amplified only in fat body and ovary cDNAs. The Bmcystatin expression in the ovary might be important to prevent a premature degradation of VT. In cells, stefin B is located mainly in the lysosomes and the cytoplasm, but also in the nucleus [39]. Then regulatory function of a proteinase inhibitor on embryonic development is not known and will be addressed in future studies in the intracellular distribution of Bmcystatin in tick tissues and also by RNA interference techniques.

However, studies on other classes of proteinases have provided indirect evidence that inhibition of proteolytic enzyme activity either by host vaccination or use of specific inhibitors can interfere with the biological capacity of hematophagous parasites [40]. For example, vaccination of cattle by *B. microplus* serine protease inhibitor, showed 72.8% efficacy to interfere in tick development with 69.7% and 71.3% reduction of both tick number and egg weight, respectively [41]. Furthermore, analysis of these molecules combined with Bmcystatin could provide some information for new anti-tick drug candidates. Our future studies may also help in the understanding of the host–parasite relationship.

## Acknowledgments

We thank Dr. Izaura Y. Hirata (UNIFESP, Sao Paulo, Brazil) for performing the amino acid sequencing. Drs. Kelly C. Malavazi-Piza and Simone S. Azzolini for helping the cDNA library construction. Dr. Glória Braz (UFRJ, Rio de Janeiro, Brazil) for performing the ESTs analysis. Dr. Itabajara Vaz da Silva Junior and Dr. Aoi Masuda (UFRGS, Rio Grande do Sul, Brazil) for providing the *B. microplus*. This work was supported by grants from FAPESP (02/13960-8) and CNPq (476869/01-3 and 470070/04-8).

## References

[1] L. Paraoan, I. Grierson, Focus on molecules: cystatin C, Exp. Eye Res. (2006) (in press).
[2] M. Abrahamson, M. Alvarez-Fernandez, C.M. Nathanson, Cystatins, Biochem. Soc. Symp. (2003) 179–199.
[3] V. Turk, W. Bode, The cystatins: protein inhibitors of cysteine proteinases, FEBS Lett. 285 (1991) 213–219.
[4] V. Turk, B. Turk, D. Turk, Lysosomal cysteine proteases: facts and opportunities, EMBO J. 20 (2001) 4629–4633.
[5] B. Lenarcic, A. Ritonja, I. Dolenc, V. Stoka, S. Berbic, J. Pungercar, B. Strukelj, V. Turk, Pig leukocyte cysteine proteinase inhibitor (PLCPI), a new member of the stefin family, FEBS Lett. 336 (1993) 289–292.
[6] F.W. Tsui, H.W. Tsui, S. Mok, I. Mlinaric, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, K.A. Siminovitch, Molecular characterization and mapping of murine genes encoding three members of the stefin family of cysteine proteinase inhibitors, Genomics 15 (1993) 507–514.
[7] P. Willadsen, G.A. Riding, On the biological role of a proteolytic-enzyme inhibitor from the ectoparasitic tick *Boophilus microplus*, Biochem. J. 189 (1980) 295–303.
[8] K.H. Cho, A.S. Raikhel, Organization and developmental expression of the mosquito vitellogenin receptor gene, Insect Mol. Biol. 10 (2001) 465–474.
[9] F. Fagotto, Yolk degradation in tick eggs: I. Occurrence of a cathepsin L-like acid proteinase in yolk spheres, Arch. Insect Biochem. Physiol. 14 (1990) 217–235.
[10] L.A. Abreu, D. Valle, P.P. Manso, et al., Proteolytic activity of *Boophilus microplus* Yolk pro-Cathepsin D (BYC) is coincident with cortical acidification during embryogenesis, Insect Biochem. Mol. Biol. 34 (2004) 443–449.
[11] A. Seixas, P.C. Dos Santos, F.F. Velloso, I. DaSilvaVaz Jr., A. Masuda, F. Horn, C. Termignoni, A *Boophilus microplus* vitellin-degrading cysteine endopeptidase, Parasitology 126 (2003) 155–163.
[12] C. Logullo, S. Vaz Ida, M.H. Sorgine, et al., Isolation of an aspartic proteinase precursor from the egg of a hard tick, *Boophilus microplus*, Parasitology 116 (Pt 6) (1998) 525–532.
[13] M.H. Sorgine, C. Logullo, R.B. Zingali, G.O. Paiva-Silva, L. Juliano, P.L. Oliveira, A heme-binding aspartic proteinase from the eggs of the hard tick *Boophilus microplus*, J. Biol. Chem. 275 (2000) 28659–28665.
[14] J.G. Valenzuela, I.M. Francischetti, V.M. Pham, M.K. Garfield, T.N. Mather, J.M. Ribeiro, Exploring the sialome of the tick *Ixodes scapularis*, J. Exp. Biol. 205 (2002) 2843–2864.
[15] S. Karim, N.J. Miller, J. Valenzuela, J.R. Sauer, T.N. Mather, RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding, Biochem. Biophys. Res. Commun. 334 (2005) 1336–1342.
[16] A.J. Barrett, H. Fritz, A. Grubb, et al., Nomenclature and classification of the proteins homologous with the cysteine–proteinase inhibitor chicken cystatin, Biochem. J. 236 (1986) 312.
[17] D.J. Shyu, W.M. Chou, T.J. Yiu, C.P. Lin, J.T. Tzen, Cloning, functional expression, and characterization of cystatin in sesame seed, J. Agric. Food Chem. 52 (2004) 1350–1356.
[18] M. Abrahamson, A. Ritonja, M.A. Brown, A. Grubb, W. Machleidt, A.J. Barrett, Identification of the probable inhibitory reactive sites of the cysteine proteinase inhibitors human cystatin C and chicken cystatin, J. Biol. Chem. 262 (1987) 9688–9694.
[19] I. Bjork, K. Ylinenjarvi, Interaction between chicken cystatin and the cysteine proteinases actinidin, chymopapain A, and ficin, Biochemistry 29 (1990) 1770–1776.
[20] T. Kartasova, B.J. Cornelissen, P. Belt, P. vandePutte, Effects of UV, 4-NQO and TPA on gene expression in cultured human epidermal keratinocytes, Nucleic Acids Res. 15 (1987) 5945–5962.
[21] R. Jerala, M. Trstenjak, B. Lenarcic, V. Turk, Cloning a synthetic gene for human stefin B and its expression in *E. coli*, FEBS Lett. 239 (1988) 41–44.
[22] H. Inanaga, D. Kobayasi, Y. Kouzuma, C. Aoki-Yasunaga, K. Iiyama, M. Kimura, Protein engineering of novel proteinase inhibitors and their effects on the growth of *Spodoptera exigua* larvae, Biosci. Biotechnol. Biochem. 65 (2001) 2259–2264.
[23] S. Arai, I. Matsumoto, Y. Emori, K. Abe, Plant seed cystatins and their target enzymes of endogenous and exogenous origin, J. Agric. Food Chem. 50 (2002) 6612–6617.

[24] R. Margis, E.M. Reis, V. Villeret, Structural and phylogenetic relationships among plant and animal cystatins, Arch. Biochem. Biophys. 359 (1998) 24–30.
[25] B.J. Mans, A.W. Neitz, Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective, Insect Biochem. Mol. Biol. 34 (2004) 1–17.
[26] C. Nakajima, I. daSilvaVaz Jr., S. Imamura, S. Konnai, K. Ohashi, M. Onuma, Random sequencing of cDNA library derived from partially fed adult female *Haemaphysalis longicornis* salivary gland, J. Vet. Med. Sci. 67 (2005) 1127–1131.
[27] C.J. Linnevers, M.E. McGrath, R. Armstrong, F.R. Mistry, M.G. Barnes, J.L. Klaus, J.T. Palmer, B.A. Katz, D. Bromme, Expression of human cathepsin K in *Pichia pastoris* and preliminary crystallographic studies of an inhibitor complex, Protein Sci. 6 (1997) 919–921.
[28] S.F. Altschul, R. Bundschuh, R. Olsen, T. Hwa, The estimation of statistical parameters for local alignment score distributions, Nucleic Acids Res. 29 (2001) 351–361.
[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[30] J.F. Morrison, Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors, Biochim. Biophys. Acta 185 (1969) 269–286.
[31] A. Grubb, H. Lofberg, Human gamma-trace. Structure, function and clinical use of concentration measurements, Scand. J. Clin. Lab. Invest. Suppl. 177 (1985) 7–13.
[32] F. Fagotto, Yolk degradation in tick eggs: II. Evidence that cathepsin L-like proteinase is stored as a latent, acid-activable proenzyme, Arch. Insect Biochem. Physiol. 14 (1990) 237–252.
[33] X. Liu, J.H. Nordin, Localization of the proenzyme form of the vitellin-processing protease in *Blattella germanica* by affinity-purified antibodies, Arch. Insect Biochem. Physiol. 38 (1998) 109–118.
[34] Y. Yamamoto, S.Y. Takahashi, Cysteine proteinase from *Bombyx* eggs: role in programmed degradation of yolk proteins during embryogenesis, Comp. Biochem. Physiol. B. 106 (1993) 35–45.
[35] X. Liu, R.C. McCarron, J.H. Nordin, A cysteine protease that processes insect vitellin. Purification and partial characterization of the enzyme and the proenzyme, J. Biol. Chem. 271 (1996) 33344–33351.
[36] F. Fagotto, F.R. Maxfield, Yolk platelets in *Xenopus* oocytes maintain an acidic internal pH which may be essential for sodium accumulation, J. Cell Biol. 125 (1994) 1047–1056.
[37] S. Yamaguchi, K. Homma, S. Natori, A novel egg-derived tyrosine phosphatase, EDTP, that participates in the embryogenesis of *Sarcophaga peregrina* (flesh fly), Eur. J. Biochem. 259 (1999) 946–953.
[38] T. Laitala-Leinonen, R. Rinne, P. Saukko, H.K. Vaananen, A. Rinne, Cystatin B as an intracellular modulator of bone resorption, Matrix Biol. 25 (2006) 149–157.
[39] M. Riccio, R. Di Giaimo, S. Pianetti, P.P. Palmieri, M. Melli, S. Santi, Nuclear localization of cystatin B, the cathepsin inhibitor implicated in myoclonus epilepsy (EPM1), Exp. Cell Res. 262 (2001) 84–94.
[40] A. Mulenga, C. Sugimoto, Y. Sako, K. Ohashi, A. Musoke, M. Shubash, M. Onuma, Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated 29-kilodalton protein and its effect as a vaccine against tick infestation in rabbits, Infect. Immun. 67 (1999) 1652–1658.
[41] R. Andreotti, K.C. Malavazi-Piza, S.D. Sasaki, R.J. Torquato, A. Gomes, A.S. Tanaka, Serine proteinase inhibitors from eggs and larvae of tick *Boophilus microplus*: purification and biochemical characterization, J. Protein Chem. 20 (2001) 337–343.