Cloning and functional expression of a *Boophilus microplus* cathepsin L-like enzyme

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Abstract

A cysteine proteinase gene homologous to cathepsins L genes was isolated from a *B. microplus* cDNA library. The precursor protein deduced from the nucleotide sequence contains 332 amino acid residues consisting of a signal sequence (pre-region), a pro-region and a mature proteinase. The DNA fragment coding for the proenzyme was cloned and expressed using the *E. coli* expression vector pMAL-p. The recombinant protein (MBP + PROCP) once activated is able to hydrolyze synthetic substrates as well as protein substrates like hemoglobin, vitellin and gelatin. Its optimal enzymatic activity on both fluorogenic and protein substrates was found to occur at an acidic pH. Expression of the proteinase gene was tested by RT-PCR with tick larvae RNA. Detection of amplified sequences indicates that the gene is expressed at this stage of the tick life cycle and the molecule is therefore potentially a target for chemotherapy or an immunogen in a vaccine. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Boophilus microplus*; Cysteine proteinase; Cathepsin L; Gene expression; cDNA

1. Introduction

The bovine tick *Boophilus microplus* is a blood sucking ectoparasite that causes severe production losses by its feeding activity and through transmission of other pathogens (Jongejan and Uilenberg, 1994). Current control methods depend heavily on the use of acaricides. However, the accompanying problems of resistance to the acaricides (Solomon, 1983; Nolan et al., 1989; Gonzales, 1995) and the presence of chemical residues in meat and milk show the need for alternative control methods (Kunz and Kemp, 1994). Since proteinases are involved in key functions in many organisms they could be potential targets to which novel antiparasite chemotherapy and/or immunoprophylaxis could be directed. In fact, an antigen derived from *B. microplus* (Bm91) shown to be protective to cattle infestation was characterized as a carboxydipeptidase similar to mammalian angiotensin-converting enzymes (Riding et al., 1994; Jarmey et al., 1995). Other midgut derived enzymes with cathepsin L cysteine and aspartic proteinases activities were described in *B. microplus* (Mendiola et al., 1996). Midgut cysteine and serine proteinase activities have also been detected in *Haemaphysalis longicornis* (Mulenga et al., 1999). Yolk protein degradation during...
embryogenesis has been attributed to a cathepsin L-like proteinase (Fagotto, 1990). More recently, an aspartic proteinase precursor also involved in yolk degradation was isolated from B. microplus eggs (Logullo et al., 1998) and it was shown that cattle vaccinated with this protein were partially protected against tick infestation (Da Silva Vaz et al., 1998).

In the present study we report cloning, expression and characterization of a functional B. microplus cysteine proteinase. The papain superfamily of cysteine proteinases represents the largest group of proteinases described so far. They have been isolated from a wide range of sources including plant and animal tissues. Different physiological roles associated with parasitic life have been assigned to these proteinases. Potential functions include degradation of extracellular matrix for parasite penetration (Scholze and Tannich, 1994; Rhoads and Fetterer, 1996), involvement in molting processes (Richer et al., 1993) and the C3 component of complement (Reed et al., 1989) as an escape mechanism from the host immune response. The availability of enough quantities of the active enzyme will enable studies on the protein structure and of their role in tick physiology. This cysteine proteinase can also be tested as a putative immunogen for the development of a vaccine.

2. Materials and methods

Restriction endonucleases were supplied by Cbiot (Porto Alegre, Brazil), Gibco-BRL Life Technologies (Gaithersburg, USA) and Pharmacia Biotech Inc. (São Paulo, Brazil), T4 DNA Ligase by Gibco-BRL Life Technologies and T4 DNA Polymerase by Cbiot. Amylase resin was from New England Biolabs. Inc. (Beverly, USA). Other molecular biology grade chemical compounds were purchased from Merck (Rio de Janeiro, Brazil), Sigma (St. Louis, USA) and Pharmacia Biotech Inc. Chromogenic substrate H-D-Val-Phe-Lys-pNA was supplied by Chromogenix AB (Molndal, Sweden). Fluorogenic substrates N-Cbz-Phe-Arg-MCA, N-Cbz-Arg-Arg-MCA, N-t-Boc-Gly-Arg-Arg-MCA, N-t-Boc-Gln-Ala-Arg-MCA, N-t-Boc-Val-Pro-Arg-MCA, N-t-Boc-Glu-Ala-Arg-MCA, N-t-Boc-Ile-Glu-Gly-Arg-MCA, N-t-Boc-Glu-Lys-Lys-MCA and N-t-Boc-Gln-Gly-Arg-MCA were purchased from Sigma.

2.1. Ticks

B. microplus ovipositing females, eggs and larvae (Porto Alegre strain) were maintained in the laboratory at 28°C and 85% relative humidity and their parasitic life cycle was completed in calves, housed in individual pens on slatted floors. Twenty day old larvae were collected after egg hatching and kept at −70°C until used.

2.2. PCR amplification and cloning of a DNA fragment representing a conserved region from a B. microplus cysteine proteinase gene

A DNA fragment was amplified by PCR using tick larvae cDNA and three oligonucleotides based on the sequences flanking the conserved residues of cysteine and asparagine present in the active site of cysteine proteinases (Eakin et al., 1990; Sakanari et al., 1989). RNA was obtained using a single-step purification procedure (Chomczynski and Sacchi, 1987). Poly (A)+ RNA was purified by oligo(dT)-cellulose chromatography as described in Sambrook et al. (1989). cDNA was synthesized from Poly (A)+ RNA using the cDNA Synthesis System Plus kit (Amersham). The oligonucleotides synthesized were a 33-mer corresponding to the region flanking the cysteine residue, 5’-ACAGAAATTCCARGGCAARGGTCGAGGTCGG-3’ (primer 411) and two 33-mer corresponding to the region flanking the asparagine residue: 5’-TTAAAGCTTCCACCCGATCTTGACRATCCG-3’ (primer 413) and 5’-TTAAAGCTTCCAGGARTTYTTGACRATCCG-3’ (primer 414). The primer 411 had a 5’ recognizing site for the region flanking the cysteine residue, 5’-ACAGAAATTCCARGGCAARGGTCGAGGTCGG-3’ (primer 413) and 5’-TTAAAGCTTCCAGGARTTYTTGACRATCCG-3’ (primer 414). The primer 411 had a 5’ recognizing site for the region flanking the cysteine residue, 5’-ACAGAAATTCCARGGCAARGGTCGAGGTCGG-3’ (primer 413) and 5’-TTAAAGCTTCCAGGARTTYTTGACRATCCG-3’ (primer 414). The primer 411 had a 5’ recognizing site for the HindIII (underlined sequence) and the primers 413 and 414 a 3’ recognizing site for HindIII (underlined sequence). PCR conditions were as follows: the first step at 94°C for 5 min, followed by 45 cycles of 30 s at 94°C, 2 min at 55°C and 2 min at 72°C, followed by a final step at 72°C for 7 min. An approximately 500 bp long PCR product (referred to as CP1) was obtained and cloned into the pBluescript vector DNA digested with EcoRI and HindIII restriction enzymes.

2.3. cDNA library screening

A cDNA library was made in λZAPII vector (Stratagene, La Jolla, USA) using poly A+ RNA from the tick B. microplus at different stages of its life cycle (larvae, nymps, young females, adult males and semi-engorged adult females). Five thousand individual cDNA clones were screened on nitrocellulose membranes (Schleicher and Schuell, USA) using the 500 bp long DNA fragment of B. microplus cysteine proteinase (CP1) labeled with [α-32P]dATP by random priming (Sambrook et al., 1989). Hybridization was carried out overnight at 65°C in hybridization buffer (6× SSC (Sambrook et al., 1989) containing 5% (w/v) cow non-fat dry milk, 200 μg/ml denatured salmon sperm DNA and the CP1 probe at 107 c.p.m.). Filters were then washed for 30 min each in 6× SSC at room temperature followed by 2× SSC, 1× SSC, 0.5× SSC and 0.2× SSC at 65°C.
2.4. Nucleotide sequencing and sequence analysis

A clone hybridizing to the CP1 probe was selected and sequenced using M13 universal forward and reverse primers and subsequently gene specific primers. Sequencing was performed using Sequencing Kit (Pharmacia). Analyses of nucleotide and deduced amino acid sequences were carried out using Genepro software program (Riverside Scientific Enterprises). Database searches were carried out using the FASTA e-mail server to search nonredundant set of databases (Pearson and Lipman, 1988). The putative signal peptide and the corresponding cleavage point (Von Heijne, 1986) were identified using the SIGSEQ program (Rockefeller University, 1989). The cloned gene was named Bmcl1.

2.5. Subcloning of Bmcl1 DNA fragment corresponding to the proproteinase and genomic DNA amplifications

The following oligonucleotides were used: primer 1 (5'-GGGAATTCAGCTCTCAAGAAATCCTACGCA-3') corresponding to nucleotides 52-73 and primer 2 (5'-GGGAATTCTGCAAAAGTTGTCGAC-3') corresponding to nucleotides 345-363 of Bmcl1 nucleotide sequence, and primer 3 (5'-GGGAGCTCTGTGTAGCAGGAGGGGTA-3') corresponding to the inverse complement of nucleotides 986-1003 in Bmcl1. Primers 1 and 2 have an additional 5' recognizing site for the restriction enzyme EcoRI (underlined) and primer 3 has an additional 3' recognizing site for XhoI (underlined). PCR were performed using two primers (1+3 or 2+3) and Bmcl1 DNA as template. After denaturation at 94°C for 5 min amplification was performed with 30 cycles of 94°C for 30 s, 60°C for 2 min and 72°C for 2 min followed by a final extension at 72°C for 5 min. A 950 bp and a 660 bp DNA fragment were obtained. The first one was cloned in the pMOSBlue vector (Amersham). After digestion with the restriction enzyme EcoRI, the fragment was subcloned into the pMAL-p expression vector (New England Biolabs) to produce a pro Bmcl1 fused with MBP (pMAL.PROCP). The reading frame was confirmed by sequencing.

The two pair of primers were also used when tick genomic DNA was used as template. Genomic DNA was extracted from 2 g of 12 days old larvae ground in a mortar and pestle under liquid nitrogen. The grounded material was suspended in 20 ml of extraction buffer (0.15 M NaCl, 0.1 M EDTA pH 8); 1 ml of RNase (0.5 mg/ml), 100 µl of proteinase K (100 mg/ml) and 1 ml of sarcosyl 10% were added and the mixture incubated at 37°C for 2 h. Protein was extracted with phenol/chloroform and the lysate was precipitated with ethanol.

2.6. RNA isolation and RT-PCR

Total RNA was isolated from 20 days old B. microplus larvae using the TRIzol™ reagent as described by the manufacturer (Gibco-BRL Life Technologies). Five µg of total RNA were submitted to reverse transcription (RT) before PCR. The RT reaction was done at 37°C in the presence of oligo(dT) (Pharmacia Biotech Inc.) and M-MLV Reverse Transcriptase (Gibco-BRL Life Technologies) according to the manufacturer’s instructions. The PCR was performed using 1 µl of the RT reaction, 10 pmol of each primer (1+3 or 2+3) and 2.5 U Taq polymerase in a final volume of 50 µl. Samples were denatured for 5 min at 94°C and amplification was achieved through 30 cycles of 30 s at 94°C, 30 s at 54°C and 30 s at 72°C. Following the last cycle a final extension (72°C, 10 min) was carried out. A parallel cDNA sample with actin-specific primers was also amplified (340 pb) as a positive control.

2.7. Expression of proBmCL1 in E. coli cells and its purification

After induction with 0.3 mM IPTG for 2 h at 37°C, the cells containing pMAL.PROCP were harvested and lysed by freezing and thawing and homogenized in a French Press. Purification of the fusion protein by amylose affinity chromatography was performed according to the manufacturer’s instructions (New England Biolabs). The buffer utilized was 20 mM Tris-HCl, 200 mM NaCl, 1mM EGTA, 1 mM EDTA, 10 mM β-mercaptoethanol and 1 mM PMSF, pH 7. A pool of the eluted fractions from the affinity chromatography containing the recombinant BmCL1 proenzyme was applied onto a DEAE-Sepharose CL-6B column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tris-HCl pH 7. The column was eluted with a linear gradient of 100–1000 mM NaCl in 20 mM Tris-HCl pH 7. Fractions were tested for activity using the fluorogenic substrate N-Cbz-Phe-Arg-MCA.

2.8. Polyacrylamide gels containing copolymerized substrates

PAGE was adapted from the method described by Heussen and Dowdle (1980) with the exception that the gel did not contain SDS. After the electrophoretic separation (using 0.15–0.2 pmol protein per slot) in a 10% non-denaturing polyacrylamide gel containing copolymerized substrates (0.05% gelatin, 0.05% hemoglobin, 0.05% tick vitellin, 0.05% bovine serum albumin or 0.05% ovalbumin), the gels were incubated in 0.1 M sodium acetate buffer (pH 3.5 and pH 5.5) containing 5 mM DTT for 16 h at 37°C. Subsequently, the gels were washed with water and stained in 5% methanol/10%
acetic acid/water containing 0.1% Coomassie Brilliant Blue R-250.

2.9. Enzyme assays

The mature cysteine proteinase was generated by incubating the purified BmCL1 fusion proenzyme at 37°C for 1 h in 25 mM sodium acetate pH 3.5 and 5 mM DTT. Assays with the activated enzyme were performed with chromogenic (0.5 mM) and fluorogenic (20–30 μM) substrates in a final volume of 100 μl containing 20 mM citric acid/54 mM sodium phosphate pH 5.5 and 5 mM DTT. Fluorogenic substrates assays were done with 51.6 μg/ml (6.79 pmols) of enzyme except for N-Cbz-Phe-Arg-MCA where 25.8 μg/ml was used. For the chromogenic substrate, 40 μg/ml (5.27 pmol) of enzyme was used. Fluorogenic assays were monitored by continuous fluorimetry in a fmax Microplate Reader (Molecular Devices Corporation). The wavelength pair for emission and excitation was 320–430 nm. Enzyme-catalyzed release of p-nitroanilide with chromogenic substrates was monitored at 405 nm in a Spectra Max 3. Results and discussion

3.1. Cloning of a DNA fragment corresponding to the conserved region of a cysteine proteinase gene and isolation of a full cDNA clone

Tick larvae cDNA and degenerate oligonucleotides (see Materials and methods) based upon sequences flanking the active site cysteine and asparagine amino acids (Sakanari et al., 1989; Eakin et al., 1990) were used to amplify by PCR a conserved sequence of a cysteine proteinase gene of the bovine tick B. microplus. A DNA fragment of approximately 500 bp was obtained and cloned into the pBluescript KS vector. Nucleotide sequence analysis of the cloned fragment revealed a conserved sequence present in other eukaryotic cysteine proteinases (results not shown). The cloned fragment, referred to as CP1, was used to probe a B. microplus cDNA library in λZAPII. Screening of 5×10³ plaques resulted in the detection of a cDNA clone with an insert of 1123 bp. The sequence is deposited in Genbank database under accession number AF227957 and is referred to as Bmcl1.

3.2. Sequence analysis of Bmcl1

The total nucleotide sequence of Bmcl1 and its deduced amino acid sequence are shown in Fig. 1. A presumed initial ATG codon (Kozak, 1991) is found 32 nucleotides downstream of the 5′ end. Three stop codons (nucleotides 997, 1039, 1063), a 94 bp of non-coding region including a presumed polyadenylation signal (Proudfoot and Brownlee, 1976), AATAAA (nucleotides 1065–1070) are found at the 3′ end. Within the cDNA sequence a single long open reading frame is found which encodes a predicted prepropeptide of 332 amino acids (BmCL1). It presents a putative signal peptide (pre-region) formed by 18 amino acid residues, a pro-region containing 97 amino acid residues and a predicted mature enzyme with 217 amino acid residues. The calculated molecular masses for the preprotein and mature enzyme are 34.51 kDa and 23.46 kDa, respectively. Similar molecular sizes are found for other cysteine proteinases; examples are: Dictyostelium discoideum (Pears et al., 1985), Fasciola sp. (Yamasaki and Aoki, 1993; Roche et al. et al., 1997), Nephrops norvegicus (Le Boulay et al., 1995) and Toxocara canis (Loukas et al., 1998). Comparing the deduced amino acid sequence with sequences in the GenBank™ we found that BmCL1 is most similar to the papain-family of proteinases. The pre-region is composed predominantly of hydrophobic residues characteristic of a signal peptide (Von Heijne, 1983). The putative cleavage point (Von Heijne, 1986) for the release of the signal peptide is located at Ser^-98-Ser^-99 (Fig. 1). Either secreted or lysosomal enzymes of the papain family are synthesized with signal peptides

2.10. Inhibitor studies

The following known proteinase inhibitors were tested: E-64 at 50 μM, leupeptin at 0.1 mM, PMSF at 1 mM, EDTA at 10 mM, pepstatin A at 1 μM and antipain at 0.1 μM. The inhibitory activity was determined by measuring the residual enzyme activity upon 0.5 mM H-D-Val-Phe-Lys-pNA after preincubating the activated enzyme for 15 min with the inhibitor.
Fig. 1. Nucleotide and the corresponding deduced amino acid sequence of *B. microplus* cysteine proteinase gene (*Bmcl1*). The regions used for the synthesis of primers 1, 2 and 3 are underlined. The arrows indicate the presumed cleavage sites for the pre and proenzyme, respectively. The asterisk indicates a potential glycosylation site. The conserved residues involved in catalysis are double underlined. In bold is the polyadenilation signal.

...and have a propeptide at the N-terminus. To protect cells from the potentially disastrous consequences of uncontrolled degradative activity, essentially all known cellular and bacterial proteolytic enzymes are synthesized as inactive precursors (or zymogens) (Carmona et al., 1996; Khan and James, 1998). Acidic limited proteolysis is necessary for activation of these zymogens (Mason et al., 1987; Rawlings and Barret, 1994). The potential cleavage site of the pro-region of the mature enzyme was estimated to be between residues Ser$^{-1}$ and Leu$^{1}$ (Fig. 1). Many members of the papain family contain a proline residue at position 2 in the mature enzyme. This is also observed in BmCL1. The proline may serve to prevent unwanted N-terminal proteolysis (Rawlings and Barret, 1994). Glycosylation with mannose 6-phosphate has been shown to be an important sorting signal for routing proteins into lysosomes. Glycosylation occurs in lysosomal proteinases, including mammalian cathepsins B, L, ...
S and H, and cathepsins L-like proteinases from protozoan parasites (Yamasaki and Aoki, 1993; Kirschke et al., 1993). N-glycosylation sites have been detected also in the pro-region sequence of papain (Vernet et al., 1990), cathepsin S (Kirschke, et al., 1993), cathepsin F (Wang et al., 1998) and in a cysteine proteinase of Blattella germanica (Liu et al., 1996). In some cases it is located in the C-terminal region of the propeptide (Padilla-Zúñiga and Rojo-Domínguez, 1998). A single potential N-linked glycosylation motif was observed in the propeptide region of BmCL1 (Fig. 1). This implies that targeting via mannose-6-phosphate receptor would only be possible for the precursor and not for the mature enzyme.

Like in papain, the recombinant BmCL1 contains the conserved Cys25, His159 and Asn175 (papain numbering) residues involved in the catalysis and also the six cysteine residues involved in disulfide bond formation (Cys22, Cys56, Cys65, Cys108, Cys157 and Cys206). The sequences around the Cys22, His164 and Asn184 residues are also well conserved in cysteine proteinases (Rawlings and Barret, 1994). Cysteine proteinases similar to the mammalian cathepsins L and H contain in their propeptide the ERFNIN motif (Karrer et al., 1993) and a set of conserved amino acids (Ishidoh et al., 1987) not present in cathepsin B-like proteinases. Both characteristics are present in the propeptide region of BmCL1 and are represented by Glu73, Arg69, Phe65, Asn62, Ile58 and Asn54, and by Asn39, Phe37, Gly36, Asp35, Leu34, Leu33, His31, Glu30 and Phe29, respectively. It was suggested that the ERFNIN motif serves to inhibit proteinase activity and that the removal of the propeptide converts the protein into its enzymatically active form (Karrer et al., 1993). Another motif conserved among cysteine proteinases, including the cathepsin B-like proteinases, is the GCNGG motif. It is invariant with the exception of the central asparagine residue. This motif is also present in BmCL1 (Gly64 to Gly68) and has a glutamic acid residue in place of asparagine. It was suggested that it has an important structural role (Karrer et al., 1993).

The proteinase sequences found in the GenBank and Swiss-Prot databases highly similar to the proform of BmCL1 were cathepsin L proteinases from several organisms. BmCL1 showed 61.68% identity with Sarcoptagia peregrina cathepsin L (Q26636), 63.66% with Penaeus vannamei cathepsin L (Q27759), 58.17% with Homarus americanus cysteine protease (P25782), 57.65% with Nephrops norvegicus cathepsin L (Q27708), 55.06% with Rattus norvegicus cathepsin L (P07154), 53.01% with Sus scrofa peptidase (Q28944) and 60.85% with Bombyx mori (Q26425). At the nucleotide level BmCL1 showed 73.61% identity with Haemaphysalis longicornis cathepsin L-like cysteine proteinase A mRNA (Ab202492), 67.41% with H. longicornis cathepsin L-like cysteine proteinase B mRNA (Ab202491) and 64.25% with P. vannamei cathepsin L mRNA (X99730).

3.3. RT-PCR and genomic DNA amplification

The forward primers 1 or 2 and the reverse primer 3 (see Materials and methods) were used in RT-PCR to analyze the expression of Bmcl1 in 20 day old larvae using total RNA as template. DNA fragments with the predicted size for the proenzyme (950 bp) and for the mature region (660 bp) transcripts were amplified (Fig. 2). Control RT-PCR amplifications using actin-specific primers confirmed sample integrity. The amplification of Bmcl1 fragments and the CP1 clone from larvae cDNA (used as probe in the library screening) indicate that this gene is active in this B. microplus developmental stage. Fragments with approximately 950 pb and 660 pb were also obtained when PCR was carried out using tick genomic DNA as template and primers 1+3 and 2+3 (Fig. 2). Since the PCR amplified fragments have the same size when cDNA or genomic DNA were used as template, it can be inferred that large introns are not present within the amplified portion of the Bmcl1 gene.

3.4. Expression of BmCL1 in E. coli

A DNA fragment of approximately 950 bp corresponding to the proprotease was amplified by PCR from Bmcl1 and expressed in E. coli as MBP-fusion protein with the pMAL-p vector. The expressed fusion protein, BmCL1, has an apparent molecular mass of 76 kDa.

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Fig. 2. PCR fragments generated from Bmcl1, genomic DNA and larvae cDNA. Lanes 2 and 5 show the fragments amplified from Bmcl1, lanes 3 and 6 the fragments amplified from genomic DNA, and lanes 4, 7 and 8 the fragments amplified from larvae cDNA. The primers used (see material and methods) were 1+3 for the pro-region (lanes 2, 3 and 4), 2+3 for the mature region (lanes 5, 6 and 7) and specific B. microplus actin primers for larva cDNA control (lane 8). In lane 1 are the molecular weight markers (100 bp ladder, Pharmacia). Samples were submitted to electrophoresis on a 2% agarose gel stained with ethidium bromide.
After a first purification step using amylose affinity chromatography, the sample containing the fusion protein still showed some impurities when analyzed on SDS-PAGE. For further purification the sample was applied onto a DEAE Sepharose column. Using a linear gradient from 100–1000 mM NaCl the fusion protein was eluted in a pure form at 300 mM NaCl.

3.5. Enzyme activity assays

The fraction eluted at 300 mM NaCl from the DEAE Sepharose column containing the purified fusion protein, BmCL1, was assayed for its activity upon chromogenic, fluorogenic and protein substrates. On polyacrylamide gels containing copolymerized protein substrates the enzyme was able to hydrolyze gelatin, bovine hemoglobin and tick vittelin at pH 3.5 (Fig. 3). It also hydrolyzes gelatin at pH 5.5 but not bovine serum albumin, ovalbumin, hemoglobin and vittelin (data not shown). Similar findings were described by Brady et al. (1999) for Schistosoma mansoni cathepsin L when hemoglobin was used as substrate. Controls done with total bacteria (XL1Blue) extracts with and without pMAL-p vector induced under the same conditions did not show any proteolytic activity when tested in polyacrylamide gels containing gelatin (data not shown). On the other hand, with the synthetic substrate H-D-Val-Phe-Lys-pNA BmCL1 exhibited activity over a wide pH range (pH 2.5 to 6.5) with optimum activity at pH 5.5 (Fig. 4). No activity was observed at pH 7. Similar pH activity profiles were also shown with cysteine proteinases purified from Xenopus laevis embryos (Yoshizaki et al., 1998), Blattella germanica yolk (Liu et al., 1996), rat liver lysosomes (Kirschke et al., 1989) and human liver (Mason et al., 1986). This distinguishes them from cathepsin S which is active at neutral pH (Kirschke et al., 1993).

Like most members of the papain family (Rawlings and Barrett, 1994; Kirschke et al., 1993; Wang et al., 1998) BmCL1 hydrolyzes substrates containing a bulky hydrophobic residue in P2 and arginine or lysine in P1 (Table 1). Almost no hydrolysis is observed when a proline is in the P2 position (Table 1). This type of result was also shown for other cathepsins L (Mason et al., 1984; Brömme et al., 1989) which also discriminate

Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Activity (µmol/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>H-D-Val-Phe-Lys-pNA</td>
<td>0.39</td>
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<tr>
<td>N-Cbz-Phe-Arg-MCA</td>
<td>31.2</td>
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<tr>
<td>N-Cbz-Arg-Arg-MCA</td>
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<tr>
<td>N-tBoc-Gly-Arg-Arg-MCA</td>
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<td>0.002</td>
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<td>N-tBoc-Glu-Ala-Arg-MCA</td>
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<td>NH</td>
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<td>N-tBoc-Glu-Lys-Lys-MCA</td>
<td>NH</td>
</tr>
<tr>
<td>N-tBoc-Gln-Gly-Arg-MCA</td>
<td>NH</td>
</tr>
</tbody>
</table>

a NH, not hydrolyzed.
Table 2
Inhibition BmCL1 by various protease inhibitors. The purified B. microplus recombinant cysteine protease was preincubated with the indicated inhibitors and assayed for residual activity using H-D-Val-Phe-Lys-pNA as substrate. Assays were done in duplicate.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Specificity</th>
<th>Concentration (mM)</th>
<th>Inhibition %</th>
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<tbody>
<tr>
<td>E-64</td>
<td>all cysteine proteinases</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>most cysteine and trypsin-like serine proteinases</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>serine proteinases</td>
<td>1</td>
<td>0</td>
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<tr>
<td>EDTA</td>
<td>Metalloproteinases</td>
<td>10</td>
<td>0.1</td>
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<tr>
<td>Pepstatin</td>
<td>Asparticproteinases</td>
<td>0.001</td>
<td>2.37</td>
</tr>
<tr>
<td>Antipain</td>
<td>trypsin-like serine and some cysteine proteinases</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>Without DTT</td>
<td></td>
<td>0</td>
<td>83.3</td>
</tr>
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</table>

Table 3
Reaction kinetics of BmCL1 on chromogenic and fluorogenic peptide substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$K_{cat}$ (µM s$^{-1}$)</th>
<th>$K_m/K_m$ (µM$^{-1}$ s$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>H-D-Val-Phe-Lys-pNA</td>
<td>282</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>N-Cbz-Phe-Arg-MCA</td>
<td>18.8</td>
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<tr>
<td>N-Cbz-Arg-Arg-MCA</td>
<td>23.6</td>
<td>15.5</td>
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</table>

* ND, not determined.

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