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Boophilus microplus cathepsin L-like (BmCL1) cysteine protease: Specificity study using a peptide phage display library

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ABSTRACT

The tick *Rhipicephalus (Boophilus) microplus* is one of the most important bovine ectoparasites, a disease vector responsible for losses in meat and milk productions. A cysteine protease similar to cathepsin L, named BmCL1, was previously identified in *R. microplus* gut, suggesting a role of the enzyme in meal digestion. In this work, BmCL1 was successfully expressed in *Pichia pastoris* system, yielding 54.8 mg/L of culture and its activity was analyzed by synthetic substrates and against a *R. microplus* cysteine protease inhibitor, Bmcystatin. After rBmCl1 biochemical characterization it was used in a selection of a peptide phage library to determine rBmCL1 substrate preference. Obtained sequenced clones showed that rBmCL1 has preference for Leu or Arg at P_1 position. The preference for Leu at position P_1 and the activation of BmCL1 after a Leu amino acid residue suggest possible self activation.

1. Introduction

The tick *Rhipicephalus (Boophilus) microplus* is one of the most important bovine ectoparasites, a disease vector held responsible for the transmission of diseases such as babesiosis and anaplasmosis, which cause massive losses in livestock production (Sauer et al., 1995). Once attached to a bovine, *R. microplus* is able to ingest a large volume of blood, in a process that increases its body weight by more than 50 times, when compared with its original weight, most intensely during the last 24 h of engorgement. Blood digestion in ticks is an intracellular process via phagocytosis by desquamated epithelial cells in the midgut (Koh et al., 1991; Sonenshine, 1991). Recently, Lara et al. (2005) showed that the midgut digestive cells endocytose blood components and release large amounts of heme during hemoglobin digestion. Proteolytic enzymes secreted in the tick midgut may be required for various functions that are essential to tick survival via blood feeding behavior (Ribeiro, 1987), and play critical roles in pathogen transmission (Tsuji et al., 2008). Thus, proteolytic enzymes may become interesting candidates as drug targets and a component of vaccines for both tick and tick-borne disease controls (Rand et al., 1989; Renard et al., 2002; Willadsen and Kemp, 1988). Among the tick proteolytic enzymes, several cysteine proteases belonging to the papain superfamily have been identified (Estrela et al., 2007; Renard et al., 2000, 2002; Seixas et al., 2003; Tsuji et al., 2008). Cysteine proteases are expressed in organisms from bacteria to humans. In parasites it has been suggested that cysteine proteases are involved in the invasion of host tissues and evasion of

Abbreviations: rBmCL1, recombinant *Boophilus microplus* cathepsin L-Like; K_{cat}, turnover number; K_i, inhibition constant; K_m, Michaelis–Menten constant; V_{max}, maximum velocity.

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the host immune system (reviewed in McKerrow (1989) and Roche et al. (1999)). In hematophagous organisms, cysteine proteases have been described as responsible for hemoglobin digestion in the gut of *Schistosoma mansoni* (Brady et al., 1999) and in the vacuole of the *Plasmodium falciparum* (Salas et al., 1995). In the *R. microplus* tick, cathepsin L-like enzymes have been found in the midgut and eggs, which suggests the involvement of these enzymes in meal digestion (Renard et al., 2002) and in vitellin degradation (Estrela et al., 2007; Seixas et al., 2003), respectively.

Previously, Renard et al. (2000) cloned and expressed an *R. microplus* cathepsin L-like enzyme named BmCL1. The recombinant enzyme was able to hydrolyze natural substrates such as tick vitellin, bovine hemoglobin and gelatin at acidic pH. Moreover, it was shown that the BmCL1 transcript is expressed in partially engorged tick females, and that the enzyme seems to be localized in secretory gut cells. Taking all these observations together, the authors postulated that BmCL1 may be involved in bovine hemoglobin degradation in the *R. microplus* gut (Renard et al., 2002). Recently, it was shown that bovine hemoglobin degradation by BmCL1 generates potential antimicrobial peptides based on human and bovine hemocidins (Cruz et al., 2010).

Various methods have been used to identify drug targets for parasite control (Canales et al., 2009), among which combinatorial mutated peptides or proteins displayed on filamentous bacteriophage surface, which is known as phage display system, have evolved as an important tool to study protein– or peptide–protein interactions (Smith, 1985). Phage display libraries have been used to select specific protease inhibitors (Campos et al., 2004; Markland et al., 1996a, 1996b; Roberts et al., 1992; Tanaka et al., 1999), to increase the anticoagulant activity of proteins (Maun et al., 2003; Yang et al., 2002), and to select specific antibodies (Barbas et al., 1991; Marks et al., 1991). In addition, peptide phage display libraries have also been successfully employed to define enzyme substrates (Deperthes, 2002; Matthews and Wells, 1993).

In the present work, we cloned and highly expressed the active BmCL1 (rBmCL1) enzyme using the *Pichia pastoris* system. The purified rBmCL1 enzyme was used in biochemical characterization and in substrate specificity investigation using a peptide phage display library.

2. Materials and methods

2.1. Materials

The pPIC9 vector and *P. pastoris* strain GS115 were purchased from Invitrogen Corporation (Carlsbad, CA, USA) and used following the supplier's instructions. Media components were from Difco (Detroit, MI, USA). Restriction endonucleases and T4 DNA ligase were obtained from Fermentas (Beverly, Vilnius, Lithuania). Taq DNA polymerase was obtained from Promega Corporation (Madison, WI, USA); Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA). DNA sequencing was performed using DYEnamic™ ET Dye Sequencing kit from Amersham Little Chalfont (Buckinghamshire, England, UK) on an ABI377 sequencer from Applied Biosystems (Foster City, CA, USA). Amicon Ultra 5000 MWCO membrane was from Millipore (Billerica, MA, USA), and the HiTrap SP column was from Amersham Biosciences (Piscataway, NJ, USA). The substrates: Bz-Phe-Arg-pNa, Z-FR-MCA, Z-RR-MCA (MCA = methyl-7-aminocoumarin amide) and the inhibitor E-64 ([trans-epoxy-succinyl-L-leucylamido-(4-guanidino)butane]) were purchased from Sigma (St. Louis, MO, USA). The substrate Z-LR-MCA was from Novabiochem (Darmstadt, Germany).

2.2. Transcription analysis of BmCL1 by PCR

The cDNA sequence encoding *BmCL1* was amplified using cDNA prepared of mRNA from ovary, fat body, salivary gland, gut, and haemocytes of *R. microplus* engorged adult females. The PCR was performed using two specific primers, the sense primer *BmCL1*fw (5′-GTATCTCTCGAGAAAAGATCTCAAGAAATCCTACGCACC-3′) and the antisense primer *BmCL1*rv (5′-CCCGTGCGGCCGCTTAGACGAGGGGGTAGC-3′). The PCR was performed in 50 μL reaction volume containing 1 μL of cDNA sample, 25 pmol of each primer, 100 μM dNTPs, 1.5 mM $MgCl_2$, and 2.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). The control of DNA amplification was made using 25 pmol of *R. microplus* actin specific primers: Actf (5′-TCCTCGTCCCTGGAGAAGTCGTAC-3′) and Actr (5′-CCACCGATCCAGACCGAGTACTTC-3′). PCR conditions were: 94 °C for 5 min; 25 cycles (94 °C–40 s, 62 °C–40 s, 72 °C–1 min), 72 °C for 5 min.

2.3. BmCL1 DNA fragment amplification and cloning into pPIC9 vector

The DNA fragment encoding the *BmCL1* gene was amplified by PCR using a plasmid construct containing the *BmCL1* coding sequence in vector pMAL-p as template (Renard et al., 2000). The PCR was performed using a primer set constructed based on the *BmCL1* coding region sequence, introducing restriction sites for Xho I and Not I enzymes at 5′ and 3′ ends, respectively. The sense oligonucleotide was: 5′-CTC GAG GTA TCT CTC GAG AAA AGA TCT CAA GAA ATC CTA CGC ACC-3′, while the antisense was: 5′-GC GGC CGC CCC GTG CGG CCG CTT AGA CGA GGG GGT AGT-3′. PCR reaction conditions were conducted in a final volume of 50 μL, 1.5 mM $MgCl_2$, 100 μM dNTPs, 10 pM of each primer, 5 U of Taq DNA polymerase, and its corresponding buffer (100 mM Tris–HCl pH 8.8, 500 mM KCl, and 0.8% (v/v) Nonidet P40). The PCR parameters were: pre-denaturation at 94 °C for 2 min; 30 cycles of (94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min); followed by 5 min at 72 °C. *BmCL1* DNA fragment band was separated by agarose gel electrophoresis 1% (w/v) and purified from agarose gel using QIAquick Gel Extraction kit from QIAGEN (Hilden, Germany). Purified DNA fragment was digested with Xho I and Not I restriction enzymes, followed by ligation to pPIC9 vector, which was previously digested with the same enzymes, generating the *BmCL1*-pPIC9 construction. *Escherichia coli* DH5α was transformed using the construction and plated on Lenox Broth (LB) agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) containing 200 μg/mL ampicillin. Correct