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Expression and substrate specificity of a recombinant cysteine proteinase B of *Leishmania braziliensis*☆

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ABSTRACT

The cysteine proteinase B of *Leishmania* parasites is an important virulence factor. In this study we have expressed, isolated and characterized for the first time a recombinant CPB from *Leishmania braziliensis*, the causative agent of mucocutaneous leishmaniosis. The mature region of the recombinant CPB shares a high percentage identity with its *Leishmania mexicana* CPB2.8 (rCPB2.8ΔCTE) counterpart (76.36%) and has identical amino acid residues at the S_1, catalytic triad and S'_1 subsites. Nevertheless, when the kinetics of substrate hydrolysis was measured using a combinatorial library of internally quenched fluorescent peptides based upon the lead sequence Abz-KLRSSKQ-EDDnp, significant differences were obtained. These results suggest that the differences in substrate utilization observed between the *L. mexicana* and *L. braziliensis* CPs must be related to amino acid modifications outside the core of the active site cleft. Moreover, a potent inhibitor with Pro at P1 and high affinity for *L. braziliensis* recombinant CPB showed less affinity to *L. mexicana* CPB 2.8, which preferred Phe, Leu, and Asn at the same position.

1. Introduction

Leishmaniosis is a disease caused by obligated intracellular parasites from the genus *Leishmania*. In South American countries the most prevalent and widespread species belong to the subgenus Viannia, such as *Leishmania braziliensis*. In Peru, leishmaniosis is the second most important parasitic disease after malaria, due to its wide geographical distribution and to the years of disability following the disease [1]. Two clinical forms of leishmaniosis have been reported in Peru: cutaneous or 'uta', caused by *Leishmania peruviana*, and mucocutaneous or 'espundia', caused by *L. braziliensis* [2].

Chemotherapy with antimonial drugs is critically important in reducing the burden of leishmaniasis, however, the increasing resistance to the pentavalent antimony, SbV, is a major concern for both visceral and mucocutaneous leishmaniosis [1]. Thus, there is a need for new drugs that target the biochemical pathways that are involved in the parasite's adaptation and survival within the vertebrate host.

Cysteine proteinases (CPs) belong to one of the four major groups of proteolytic enzymes produced by a variety of organisms including *Leishmania* [3]. *Leishmania* species possess at least three classes of Clan CA, family C1 CPs denoted as types I, II and III. Type

Abbreviations: CPB, cysteine proteinase class B; CPB2.3, cysteine proteinase derived from the 2.3 kb DNA fragment; CPB2.3ΔCTE, CPB2.3 lacking the C-terminal extension; Abz, *ortho*-aminobenzoic acid; EDDnp, *N*-(ethylenediamine)-2,4-dinitrophenyl amide; Fmoc, fluoren-9-ylmethoxycarbonyl; IPTG, isopropyl-β-D-thiogalactoside; SbV, pentavalent antimony.

☆ *Note:* Nucleotide sequence GenBank accession no. AAV97878.

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II CP, or CPA, is a cathepsin-L-like proteinase reported in *Leishmania major*, *Leishmania mexicana* and *Leishmania infantum* [4–6], and is encoded by a single copy gene lacking the C-terminal extension [7]. Type III, or CPC, is a cathepsin-B-like proteinase reported in *L. mexicana* and *L. major*, also encoded by a single copy gene [3,7]. Finally, type I CP, or CPB, is a cathepsin-L-like proteinase encoded by genes that occur in multicopy tandem arrays, in both *L. major* and *L. mexicana* [4,8]. Furthermore, they distinguish themselves from the other CPs due to their unusual 100 amino acid C-terminal extension that is frequently glycosylated [9].

Several reports have shown that the expression of CPs in *L. mexicana* is stage-specific. CPs are predominantly expressed and activated in the amastigote stage, and to a lesser extent in promastigotes [8,9]. Moreover, previous studies showed that *CPB* gene deletions resulted in reduced in vitro virulence by *L. mexicana* [10] and that reinsertion of multiple *CPB* genes restored virulence for Balb/c mice, indicating that CPB could be considered a virulence factor [11]. Similar results were obtained when several CP inhibitors were used [12]. Furthermore, CPB influenced Th1 and Th2 responses, contributing to lesion progress in experimental animal models [13]. These findings strongly support a crucial role for CPs in the survival of the parasite within the mammalian host, and CPs should be taken into consideration as possible drug targets [14].

At present, there have been no reports on *L. braziliensis CPB* gene organization or gene sequence to allow comparative studies with other *Leishmania* CPBs. Here we report the first cloning, sequencing and expression of a recombinant CPB from *L. braziliensis* (CPB2.3ΔCTE). Furthermore, the S_1–S_3 and S'_1 and S'_2 subsite specificities were investigated using a combinatorial library of intramolecularly quenched fluorescence peptides derived from the lead sequence Abz-KLRSSKQ-EDDnp. We also studied the carboxydipeptidase activity of CPB2.3ΔCTE using the peptides Abz-FRAK(Dnp)-NH_2 and Abz-FRAK(Dnp)-OH. Importantly, this study has revealed specific peptide inhibitory sequences, which could provide useful information for the design of compounds with leishmanicidal activity.

2. Materials and methods

2.1. Isolation of a CPB gene from L. (V.) braziliensis

In the Laboratory of Molecular Biology of Trypanosomatids at the Instituto de Medicina Tropical "Alexander von Humboldt" (IMTAvH), we characterized the genes that encode for *Leishmania* (*V.*) *braziliensis* CPB Cosmid 283, which contains the *CPB* genes from *L. braziliensis* International Standard Reference Strain M2903. Restriction digestion of this cosmid using XhoI generated three DNA fragments (2.9, 2.3 and 1.8 kb) that hybridized with a specific probe for *L. mexicana* CPB. These fragments were sub-cloned into XhoI site of pBluescript SK II, producing two recombinant clones: AZ41 that contained the 2.9 kb fragment and AZ13 that contained the 2.3 kb fragment.

2.2. Subcloning and gene expression of L. braziliensis cysteine proteinase CPB2.3ΔCTE in Escherichia coli

The strategy was similar to that described by Sanderson et al. [15]. Primers were designed for PCR amplification of a truncated version of CPB2.3 lacking the C-terminal extension (CPB2.3ΔCTE). Restriction sites (underlined) were added at the 5′ ends of each primer to allow subsequent ligation of the amplification product to pQE80L expression vector (Qiagen). Primer forward, CYS5, 5′-GGATCCGCTGGCGTGCCTG C-3′; and primer reverse, CYS6, 5′-AAGCTTACTGCCG GACGTCTGTGC-3′. The PCR reaction was performed in 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM dNTPs master mix (Eurogentec), using 9 pmol of both primers, 0.5 ng 2.3 kb purified DNA fragment and 1.5 U Amplitaq (PerkinElmer). The reaction was as follows: 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s (denaturation), 54 °C for 1 min (annealing), and 72 °C for 1 min (extension); and a 10 min final extension step at 72 °C. PCR products were purified with Ciclo Pure Gel Extraction Kit (Amresco) and cloned into pGEM-T plasmid (Promega), generating the recombinant plasmid pGEM-2-10. CPB2.3ΔCTE was excised with BamHI and HindIII restriction enzymes. The resulting fragment was sub-cloned into the pQE80L expression vector, subsequent to digestion with the same enzymes. This recombinant vector, named pQ-CPB, encodes for a fusion protein containing a 6× His tag at the N-terminus and lacking the C-terminal extension. *E. coli* TOP10 F′ strain was used for the expression of this recombinant protein. A 1.5-ml overnight culture of *E. coli* previously transformed with pQ-CPB was grown at 37 °C in 150 ml of 2 × YT media supplemented with 100 μg/ml ampicillin (Sigma) and 10 μg/ml tetracycline (Sigma), until an OD_{600} of 0.6–0.8 was reached. The optimum conditions for expression were determined by varying the IPTG concentration, the length of induction and the growth temperature. The culture was induced with 0.1 mM IPTG and grown further at 37 °C for 2–3 h with vigorous shaking. Cells were pelleted at 3500 × *g* for 20 min, and stored at −70 °C overnight.

2.3. Sequence analysis

To determine whether this DNA fragment (GenBank accession no. AAV97878) encoded a type I cysteine proteinase, the nucleotide sequence was translated using Expasy Translate Tools (http://us.expasy.org/tools/dna.html), and the protein sequence and it was compared with those from *Leishmania pifanoi*, *L. mexicana*, *Leishmania donovani*, *L. infantum* and *L. major* species, available in the GenBank™ database of the US National Center for Biology Information. A multiple sequence alignment was done with CLUSTAL W (1.82).

2.4. Purification and activation of L. braziliensis CPB2.3ΔCTE from E. coli

The method reported by Rafati et al. [4] for purification and enzyme activation was followed with slight modifications. The bacterial pellet was dissolved in 4 ml buffer 1 (6 M guanidine, 20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 0.1% Nonidet-P40), incubated for 2 h at room temperature (RT) and disrupted by sonication with 5 cycles of 25 pulses (1 s each at 60 Hz, Misonic Sonicator 3000, Farmingdale, NY). After centrifugation at 20,000 × *g* at RT for 10 min, the supernatant was added to 1 ml Ni-NTA-agarose resin (Qiagen) and vigorously mixed on a gyratory plate for 45 min. Then, the slurry was packed in a plastic column and a 1.5-ml supernatant aliquot ('overflow') was collected. The Ni-NTA column was washed twice with 4 ml buffer 2 (6 M urea, 20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 10 mM imidazole) and 1.5-ml samples were collected each time. The column was then washed four times with 4 ml buffer 3 (20 mM Tris–HCl, pH 7.9, 150 mM NaCl) to remove any trace of urea, and 1.5-ml aliquots were collected each time. Finally, the active recombinant CPB2.3ΔCTE (rCPB2.3ΔCTE) was eluted by washing the column four times with 0.5 ml buffer 4 (20 mM Tris–HCl, pH 4.9, 150 mM NaCl, 100 mM EDTA) and concentrated by ultrafiltration with an Ultrafree-15 device (molecular weight cut-off 5000 Da, Millipore). The method of Bradford [16] was used in all purification steps to assess total protein concentration. The concentration of rCPB2.3ΔCTE stock solution (427 nM) was determined by active site titration with human cystatin C, which was a generous gift