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Characterization of *Leishmania* (*L.*) *amazonensis* oligopeptidase B and its role in macrophage infection

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Research Article

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Abstract

Leishmania spp. are parasitic protozoa that cause leishmaniasis, a disease endemic in 98 countries. *Leishmania* promastigotes are transmitted by the vector and differentiate into amastigotes within phagocytic cells of the vertebrate host. To survive in multiple and hostile environments, the parasite has several virulence factors. Oligopeptidase B (OPB) is a serine peptidase present in prokaryotes, some eukaryotes and some higher plants. It has been considered a virulence factor in trypanosomatids, but only a few studies, performed with Old World species, analysed its role in *Leishmania* virulence or infectivity.

L. (*L.*) *amazonensis* is an important agent of cutaneous leishmaniasis in Brazil. The *L.* (*L.*) *amazonensis* OPB encoding gene has been sequenced and analysed *in silico* but has never been expressed. In this work, we produced recombinant *L.* (*L.*) *amazonensis* OPB and showed that its pH preferences, K_m and inhibition patterns are similar to those reported for *L.* (*L.*) *major* and *L.* (*L.*) *donovani* OPBs. Since *Leishmania* is known to secrete OPB, we performed *in vitro* infection assays using the recombinant enzyme. Our results showed that active OPB increased *in vitro* infection by *L.* (*L.*) *amazonensis* when present before and throughout infection. Our findings suggest that OPB is relevant to *L.* (*L.*) *amazonensis* infection, and that potential drugs acting through OPB will probably be effective for Old and New World *Leishmania* species. OPB inhibitors may eventually be explored for leishmaniasis chemotherapy.

Introduction

Leishmaniasis is an anthropozoonosis considered a public health problem, with a broad clinical spectrum and epidemiological variety. It is caused by parasites of the genus *Leishmania*, transmitted to mammals during the blood meal of female sand flies of the genus *Phlebotomus* or *Lutzomyia* (Kevric *et al.*, 2015). More than 50 species of *Leishmania* have been described, 20 of which cause human disease, most of them grouped into *Leishmania* (*L.*) and *Viania* (*V.*) subgenera (Cupolillo *et al.*, 2000; Akhoundi *et al.*, 2016). The 2 main clinical forms in humans are the cutaneous (CL) and visceral (VL) leishmaniasis. The clinical manifestations and disease severity are mainly determined by the host immune response and by the *Leishmania* species and strains (Podinovskaia and Descoteaux, 2015; Velasquez *et al.*, 2016; de Rezende *et al.*, 2017). According to the World Health Organization (WHO), in 2018, 92 and 83 countries were considered endemic for CL and VL, respectively, and approximately 30 000 new cases of VL and 1 million new cases of CL occurred annually (WHO, 2018). In Brazil, *Leishmania* (*L.*) *amazonensis* is one of the species frequently associated with CL (Silveira *et al.*, 2009).

Leishmania promastigotes develop in the vector and are transmitted to mammals. In the vertebrate host they are phagocytosed by phagocytes, mainly macrophages, and convert into amastigotes (Dostalova and Volf, 2012; Podinovskaia and Descoteaux, 2015). Adaptation to the hostile environment of macrophages relies on parasite virulence factors (Podinovskaia and Descoteaux, 2015). Lipophosphoglycan (LPG) and glycoprotein 63 (GP63) are well-known virulence factors in *Leishmania*, which contribute to the survival of the parasite in the vertebrate host by affecting several processes (Brittingham *et al.*, 1995; Sacks *et al.*, 2000; Chang *et al.*, 2003; Spath *et al.*, 2003; Yao *et al.*, 2003; Svarovska *et al.*, 2010). Many proteases have also been described as virulence factors, degrading proteins and peptides that participate in a range of biological processes involved in the infection (Silva-Almeida *et al.*, 2012). A well-studied example is cysteine protease B (CPB), which induces a Th2 response in mice infected by *L.* (*L.*) *mexicana*, increasing parasite survival (Weinheber *et al.*, 1998; Bennett *et al.*, 2001; Buxbaum *et al.*, 2003; Pollock *et al.*, 2003; Cameron *et al.*, 2004; Mottram *et al.*, 2004; Saravia *et al.*, 2006; Abu-Dayyeh *et al.*, 2008). Oligopeptidase B (OPB) is another protease that may be considered a virulence factor in *Leishmania*.

OPB (MEROPS code S09.010) was first described in *Escherichia coli* as a protease II and was later characterized in *Trypanosoma cruzi* and *T. brucei* as an alkaline protease (Pacaud and Richaud, 1975; Ashall, 1990; Kornblatt *et al.*, 1992). It is classified as a serine peptidase from the prolyl oligopeptidase family (POP) (SC clan, family S9, subfamily A) (Rawlings *et al.*, 2010; Munday *et al.*, 2011), present in prokaryotes, in unicellular eukaryotes such as

trypanosomatids, in plants and fungi. OPB has never been described in any mammalian genome (Motta *et al.*, 2019). It is considered an important virulence factor in *T. cruzi*. Indeed, it is believed that an unknown OPB product secreted by the parasite binds to host cell receptors, triggering the release of Ca^{2+}. The mobilization of Ca^{2+} induces lysosomal exocytosis and disruption of actin filaments that facilitate cell invasion by the parasite (Burleigh and Andrews, 1995; Rodriguez *et al.*, 1995; Burleigh *et al.*, 1997; Caler *et al.*, 1998; Motta *et al.*, 2019).

OPB was annotated in the first *Leishmania* genome reported (Ivens *et al.*, 2005), but was only studied in the genus in 2007 (de Matos Guedes *et al.*, 2007). This study showed that *L.* (*L.*) *amazonensis* OPB was a single copy gene with 2.196 base pairs (bp), encoding a 731 amino acid protein with predicted mass and isoelectric point of 83.52 kDa and 5.61, respectively. The protein lacks a signal peptide at the N-terminal extension, indicating that it is probably synthesized as an active protease (de Matos Guedes *et al.*, 2007). The structure of *L.* (*L.*) *amazonensis* enzyme is composed of 2 distinct domains, an α/β hydrolase fold and 7 blades with β helix domains. OPB amino acid sequences of *L.* (*L.*) *amazonensis*, *L.* (*L.*) *major*, *L.* (*L.*) *chagasi* and *L.* (*V.*) *braziliensis* are conserved, mainly in the catalytic region (de Matos Guedes *et al.*, 2007). Another gene from the OPB group, later named OPB-2, was annotated in the *L.* (*L.*) *major* genome (Ivens *et al.*, 2005) and sequenced in *L.* (*L.*) *amazonensis* (de Matos Guedes *et al.*, 2008). OPB-2 amino acid sequences are conserved among *Leishmania* but display several insertions and deletions and a C-terminal extension domain when compared to OPBs (de Matos Guedes *et al.*, 2008). No enzymatic or functional studies for OBP-2 were reported to date, but comparative modelling indicates that *L.* (*L.*) *amazonensis* OPB and OPB2 have many similarities and may be sensitive to dual inhibitors (Sodero *et al.*, 2017). OPB is secreted by *Leishmania*, as it was found in exosomes shed by *L.* (*L.*) *donovani* and *L.* (*L.*) *major* promastigotes (Silverman *et al.*, 2008, 2010). The protein is present in the soluble fraction of *L.* (*L.*) *major* procyclics, metacyclics and amastigotes (Munday *et al.*, 2011), and higher activity was observed in *L.* (*L.*) *donovani* and *L.* (*L.*) *mexicana* axenic amastigotes compared to promastigotes (Swenerton *et al.*, 2011).

OPB hydrolyses peptide bonds preferentially after a pair of dibasic residues, preferentially with an arginine at the P1 position (Kanatani *et al.*, 1991; Polgar, 2002; Motta *et al.*, 2019). Enzymatic profiles of *Leishmania* OPBs were analysed using recombinant proteins. *L.* (*L.*) *donovani* and *L.* (*L.*) *major* OPBs were produced in *Pichia pastoris* and *Escherichia coli*, respectively. The 2 enzymes showed preference to an Arg at position P1 and similar inhibition profiles employing commercial inhibitors (McLuskey *et al.*, 2010; Swenerton *et al.*, 2011).

Only 2 functional studies on *Leishmania* OPBs have been published, and they are restricted to 2 parasite species. *L.* (*L.*) *major* parasites deficient in OPB showed impaired differentiation into metacyclic promastigotes, decreased infectivity in macrophages *in vitro* (Munday *et al.*, 2011) and generated smaller footpad lesions in mice compared to wild-type (wt) parasites (Munday *et al.*, 2011; Swenerton *et al.*, 2011). *L.* (*L.*) *donovani* promastigotes knockout (ko) for OPB expressed more enolase isoforms and higher abundance of the enzyme on the parasite surface (Swenerton *et al.*, 2011). Since enolase activity was similar between ko and wt parasites, the authors suggested an accumulation of inactive enolase on the surface of ko parasites. They also hypothesized that during differentiation into amastigotes OPB is responsible for removing enolase and plasminogen from the parasite surface (Swenerton *et al.*, 2011).

A study carried out by our group compared the proteome of *L.* (*L.*) *amazonensis* amastigotes derived from BALB/c and BALB/c nude mice lesions and showed that 4 OPB isoforms were more abundant in parasites from BALB/c nude mice (Teixeira *et al.*, 2015). This suggests that T cells or their mediators may be responsible for controlling OPB post-translational modifications (Teixeira *et al.*, 2015).

No study has analysed the profile and the role of OPB from *Leishmania* species from the New World. In the present study, we produced *L.* (*L.*) *amazonensis* recombinant OPB and characterized its enzymatic profile. Both pH preference, V_{max}, K_m and inhibition pattern were characterized. Since OPB was shown to be secreted by *Leishmania* promastigotes, we analysed the effect of the recombinant protein in macrophage infection by this parasite stage. Our data indicate that active soluble OPB plays a role in *in vitro* infection by *L.* (*L.*) *amazonensis*, suggesting that OPB is relevant to *L.* (*L.*) *amazonensis* infection.

Materials and methods

L. (L.) amazonensis promastigotes and axenic amastigotes

Cultures of promastigotes were periodically obtained from BALB/c mice infected with 2×10^6 *L.* (*L.*) *amazonensis* strain LV79 (MPRO/BR/72/M1841) stationary phase promastigotes in the plantar pad of the left hind paw. Promastigotes were maintained in complete medium 199 (see below) at 24°C. Cultures were subcultured weekly to an initial density of 2×10^6 parasites mL^{-1} until the 8th passage.

Axenic amastigotes were obtained as previously described (Miguel *et al.*, 2013). Briefly, 5-day promastigote cultures ($2–3 \times 10^6$ mL^{-1}) were mixed with the same volume of M199 medium supplemented with 0.25% glucose, 0.5% trypticase and 40 mM Na succinate, adjusted to pH 5.5. Cultures were maintained at 24°C for 16 h and at 34°C for 3 days, diluted at a 1:5 ratio and maintained for up to 5 days at 32°C.

Production of recombinant OPB

The OPB gene was amplified from *L.* (*L.*) *amazonensis* genomic DNA using Platinum™ Superfi™ DNA polymerase (Invitrogen) and primers derived from the 5′ and 3′ ends of the *L.* (*L.*) *amazonensis* ORF deposited in TriTryp (LAMA_000147800) (forward: 5′- ATA GAA TTC ATG TCG TCG GGC AAC – 3′ and reverse: 5′- AAT CTC GAG TTA CCT GCG AAC CAG – 3′). The resultant 2.196 kb PCR product was cloned into pJET 1.2/blunt (Life Technologies) and then into the pET28a expression vector. Competent *Escherichia coli* BL21 pGro7 was transformed with pET-28aOPB and 2 clones were confirmed by sequencing. One of them was grown and expression of OPB was induced with 0.1 mM IPTG at 37°C for 4 h.

The culture was centrifuged and the pellet was resuspended in binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 5 mM imidazol) plus 0.4 mg mL^{-1} lysozyme and 1 mM PMSF and kept at −20°C for 16 h. Bacteria were disrupted by sonication at 25% amplitude, centrifuged and the soluble fraction was loaded on a 1 mL Histrap™ HP (GE HealthCare) nickel column previously washed with 10 volumes of H_2O and equilibrated with 50 volumes of binding buffer. Protein was eluted with 20 mM NaH_2PO_4, 0.5 M NaCl containing imidazole from 25 to 500 mM. Imidazole was removed using the Amicon® Ultra 4–30 K filter (Millipore), the protein was reconstituted with 1 mL of phosphate-buffered saline (PBS) + 15% glycerol and quantified by Bradford assay. The purified protein showed undetectable amounts of LPS according to the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Scientific).

Recombinant OPB activity assays at different pHs

Enzyme assays to determine the pH preference were adapted from Swenerton *et al.* (2011). Assays were performed at 25°C in a

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Corning Costar 3603 plate (Corning®) with 0.5 ng (1 μL) of OPB, 5 μM (1 μL) of Z-Arg-Arg-AMC substrate (Sigma-Aldrich) and a gradient of buffers (98 μL) from pH of 3–10 with 0.5 intervals. The buffers used were 0.2 M citrate phosphate (pH 3–7) and 50 mM Tris-HCl (pH 7–10). The fluorescence readings, which result from the product AMC released, were taken every minute for 45 min in POLARstar Omega reader (BMG Labtech) with 390 nm excitation and 480 nm emission. The slope of fluorescence *vs* time lines corresponds to the enzyme activity. The mean activity at each pH was calculated based on 3 linear plots and expressed as relative activity taking the highest mean activity as reference. This triplicate assay was repeated 3 times. Control tests without OPB were carried out to check the substrate spontaneous hydrolysis in different pHs.

Recombinant OPB inhibition assay with commercial inhibitors

The choice of inhibitors was based on the work of Swenerton *et al.* (2011). Recombinant OPB inhibition assays were performed at 25°C in a Corning Costar 3603 plate (Corning®) with 0.5 ng (1 μL) of OPB enzyme, and 5 μM (1 μL) of Z-Arg-Arg-AMC substrate in 50 mM Tris-buffer HCl, pH 8.0 with the following commercial inhibitors: 100 μM Antipain, 100 μM leupeptin, 5 mM EDTA, 1 μM pepstatin A, 10 mM Ca^{2+} and 10 mM Mg^{2+}. The enzyme was pre-incubated with each inhibitor for 10 min in the absence of substrate. After addition of the substrate, fluorescence readings were taken every minute for 45 min in POLARstar Omega reader (BMG Labtech) with 390 nm for excitation and 480 nm for emission. The slope of fluorescence *vs* time lines corresponds to the enzyme activity. The mean activity in the presence of the putative inhibitors was calculated based on 3 linear plots. Then it was expressed as a relative activity taking the activity in the absence of any inhibitor as a reference. This triplicate assay was repeated 2 times.

Calculation of K_m and V_{max}

Enzyme assays were performed with substrate concentrations ranging from 0.8 to 8 μM (in triplicates). Reactions were performed at 25°C in a Corning Costar 3603 plate (Corning®) with OPB enzyme and Z-Arg-Arg-AMC substrate in 50 mM Tris-buffer HCl, pH 8.0. Fluorescence readings were taken every minute for 45 min in POLARstar Omega reader (BMG Labtech) with 390 nm for excitation and 480 nm for emission. The slope of the fluorescence *vs* time lines corresponds to the reaction initial rate (afu min^{-1}; afu, arbitrary fluorescence units). The Michaelis–Menten equation was fitted to [S] *x* rate data resulting in the K_m and V_{max}. Such fitting process was done using the Origin 2019 software.

Anti-OPB serum and Western blot

To obtain anti-OPB serum, BALB/c mice were immunized by the intraperitoneal route with 15 μg of recombinant OPB in 100 μL, emulsified 1:1 with incomplete Freund's adjuvant. A control mouse was inoculated with PBS emulsified with incomplete Freund. Immunization was carried out in 2 steps with an interval of 30 days and serum collection was carried out 60 days after the first immunization. The reactivity of anti-OPB serum was confirmed by enzyme-linked immunosorbent assay (ELISA).

Parasites were lysed by 8 cycles of freeze–thaw at the density of 2×10^9 promastigotes mL^{-1} in PBS with protease inhibitors (800 nM aprotinin, 50 nM bestatin, 1 mM AEBSF-HCl, 15 nM E64, 2 nM leupeptin and 1 nM pepstatin A; Fermentas). Proteins were separated by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (GE Healthcare) in 25 mM Tris buffer, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.2, for 1 h at 5 V using the TE77 equipment (GE Healthcare). The membrane was blocked in PBS 5% skim milk and 0.1% Tween 20 for 1 h and incubated with anti-OPB serum diluted 1:500 in PBS 2.5% skim milk 0.1% Tween 20 for 16 h at 4°C. Then, the membrane was washed 3 times for 10 min with PBS 0.05% Tween 20 and incubated for 1 h with secondary antibodies anti-mouse HRP (from KPL) diluted 1:5000 in PBS 2.5% skim milk and 0.05% Tween 20. Three washes were performed as described and the membrane was incubated with ECL substrate (Amersham™ ECL™ detection systems, GE Healthcare) for 5 min and developed in a ChemiDoc™ XRS (Bio-Rad).

Infection assay

Resident macrophages of the peritoneal cavity were obtained from BALB/c mice. The animals were euthanized and washed with 70% ethanol. The peritoneal cavity was exposed and 5 mL of sterile PBS at 4°C was injected into the cavity, which was massaged for 30 s before recovering the aspirate with a syringe and 21 G needle. The aspirate was centrifuged at 3000 ***g*** for 10 min at 4°C, the supernatant was discarded, and cells resuspended in RPMI. Cells were counted in a Neubauer® chamber and transferred to 48-well plates containing circular glass coverslips. After 2 h at 37°C 5% CO_2, the medium was changed to RPMI supplemented with 10% fetal bovine serum (FBS), 20 μg mL^{-1} gentamicin (from now on called supplemented RPMI) and OPB in different conditions. Plates were incubated overnight at 37°C and 5% CO_2. Alternatively, medullary macrophages were obtained as previously described (Galuppo *et al.*, 2018) and employed in infection assays using the same conditions.

To assess the importance of peptidase activity on infection, we inhibited OPB with 4 mM Pefabloc® (Sigma) for 2 h at 37°C and then used the Amicon® Ultra 4–30 K filter (Millipore) to remove the free inhibitor. To assess the importance of OPB structure and of eventual LPS, aliquots of OPB were incubated at 95°C for 5 min. Recombinant OPB and these 2 controls were incubated at the time of macrophage plating and maintained until the end of the experiment.

Infection was performed with promastigotes at day 4 of culture at a multiplicity of infection (MOI) of 10:1, in supplemented RPMI at 34°C, 5% CO_2 with OPB and controls described above. After 4 h, the medium was changed and the plates incubated for another 20 h at 34°C and 5% CO_2. Cells were fixed with methanol and stained with the Instant Prov Kit dye set (Newprov). Assays were performed in technical triplicates and 100 cells were counted from each cover slip. The percentage of infected macrophages and the number of amastigotes per macrophage were calculated.

Statistical analysis

Statistical analyses were performed by ANOVA test followed by Tukey post-test ($n \geqslant 3$), Sidak's post-test or by Student's *t* test ($n \leqslant 2$). Differences were considered significant for *P* value $\leqslant 0.05$.

Results

Sequencing and production of recombinant OPB

OPB from *L.* (*L.*) *amazonensis* PH8 strain has already been sequenced and studied *in silico* years ago (de Matos Guedes *et al.*, 2007). In this work we cloned and sequenced OPB gene from *L.* (*L.*) *amazonensis* LV79 strain, which is shown in Fig Sup 1 along with its translation. LV79 OPB is similar to the sequence reported for PH8 strain, except for a change in 1 nucleotide, generating a valine instead of an isoleucine at the 558

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position, as depicted in Fig Sup 1. This substitution occurs outside of relevant sites for enzyme activity, such as the catalytic triad and S1 and S2 subsites, as indicated in Fig Sup 2.

The alignment of *L.* (*L.*) *amazonensis* LV79 OPB with sequences from other *Leishmania* species indicated high identity and similarity, as shown in Table 1. Structures of *L.* (*L.*) *amazonensis* and L. (*L.*) *major* OPBs (published by de Matos Guedes *et al.*, 2007 and McLuskey *et al.*, 2010) after *in silico* and crystal studies, respectively, were very similar. Besides, *L.* (*L.*) *amazonensis* and *L.* (*L.*) *major* sequences display the same residues in the catalytic triad and S1 and S2 subsites (Fig Sup 2), suggesting that the 2 enzymes probably have similar enzymatic activities. Apart from these residues, we also observe conservation in residues involved in interactions between the catalytic and the propeller domain, which also determine enzyme activity and specificity. These residues, already cited by McLuskey *et al.* (2010), are E179-R664, which form a salt-bridge that affects substrate binding pocket; E621 and Y499, important for the preference for RR residues; E623, which connects the 2 domains in the active enzyme; D504-R366 and H404-Q506, which connect the 2 domains and probably influence substrate specificity; E538-R302, forming a salt-bridge that is broken when OPB changes conformation and replaced by a bridge between S271 and R534.

Since active recombinant *L.* (*L.*) *major* OPB had been previously produced in a bacterial system (McLuskey *et al.*, 2010), we attempted to produce *L.* (*L.*) *amazonensis* OPB in *Escherichia coli.* The analysis of the samples from the recombinant OPB expression and purification steps is shown in Fig. 1. As can be noted, a his-tagged protein was present mainly in the soluble fraction (Fig. 1A) and was efficiently recovered using nickel columns (Fig. 1B). The size of the protein seems a little larger than expected even considering the addition of his and T7 tags at the N-terminal end, as sometimes happens in SDS-PAGE migration.

Recombinant OPB activity at different pHs and in the presence of inhibitors

The recombinant enzyme was analysed in terms of activity in different pHs and in the presence of commercial inhibitors, following procedures previously employed to study *L.* (*L.*) *major* and *L.* (*L.*) *donovani* OPBs (McLuskey *et al.*, 2010; Swenerton *et al.*, 2011).

Figure 2A shows that activity of *L.* (*L.*) *amazonensis* OPB is highest at pH 9.5 and 10 and very low at pH around 5, like OPBs from other *Leishmania* species. OPB activity was completely inhibited by antipain and leupeptin and diminished with Mg^{2+} (Fig. 2B). Conversely, pepstatin had no effect, while EDTA increased OPB activity.

K_m and V_{max} were determined employing different substrate (Z-Arg-Arg-AMC) concentrations and the same conditions used for assays in the presence of inhibitors. The Michaelis–Menten equation showed a precise fit to the curve obtained with the complete set of data points (correlation coefficient (R) = 0.99), leading to values of $K_m = 2.1 \pm 0.4\,\mu$M and $V_{max} = 1.7 \pm 0.1$ afu min^{-1}.

Table 1. Identity and similarity between amino acid sequences of *L.* (*L.*) *amazonensis* LV79 strain OPB and those from *L.* (*L.*) *mexicana, L.* (*L.*) *donovani*, *L.* (*L.*) *infantum, L.* (*L.*) *major* and *L.* (*V.*) *braziliensis* OPBs

Leishmania spp.	Identity (%)	Similarity (%)
L. (*L.*) *mexicana*	99	99
L. (*L.*) *donovani*	91	95
L. (*L.*) *infantum*	91	95
L. (*L.*) *major*	90	95
L. (*V.*) *braziliensis*	84	92

Presence of OPB in promastigotes and amastigotes of L. (L.) amazonensis

The recombinant protein was used to immunize mice and obtain anti-OPB sera. Sera from 4 mice were tested in ELISA (data not shown), and 1 was selected for use in Western blot of total promastigote and amastigote extracts. Day 2 represents the beginning of the logarithmic phase, day 4 the initial stationary phase and day 6 the late stationary phase. The results shown in Fig. 3 indicate that *L.* (*L.*) *amazonensis* promastigotes from all stages and amastigotes express OPB, and that the endogenous enzyme displays a mass below 98 kDa in SDS-PAGE gels, a little lower than the recombinant OPB but above the expected 85 kDa predicted from OPB amino acid sequence.

Infection of peritoneal macrophages by L. (L.) amazonensis *in the presence of soluble OPB*

The presence of OPB in *L.* (*L.*) *amazonensis* promastigotes (Fig. 3) and the secretion of OPB by promastigotes previously reported (Silverman *et al.*, 2008, 2010) prompted us to analyse whether soluble OPB could affect macrophage infection *in vitro*. Peritoneal macrophages were plated and kept in the presence of recombinant OPB throughout the experiment. Figure 4 shows numbers of infected cells after 24 h. As controls we used Pefabloc® (Sigma)-inhibited OPB and OPB inactivated by boiling at 95°C for 5 min. Pefabloc serves as a control for inactive OPB, while boiled OPB serves as a control for eventual residual LPS, since this procedure denatures proteins but does not destroy LPS. As can be noted in Fig. 4A, the presence of OPB before and during infection increased the percentage of infected

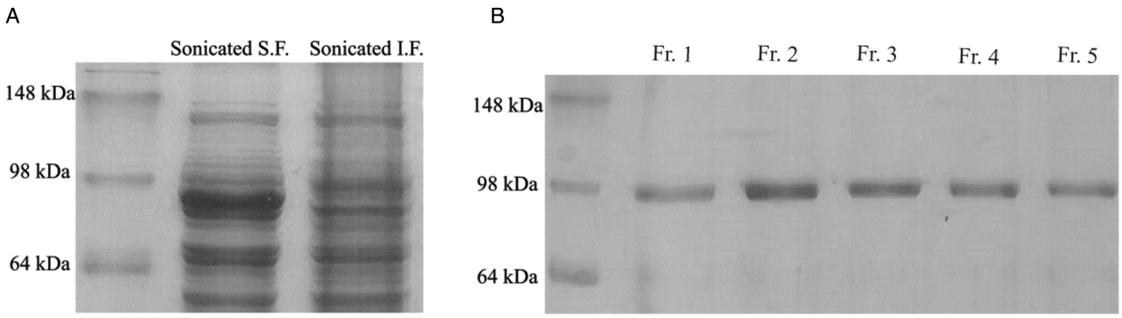

Fig. 1. Recombinant *L.* (*L.*) *amazonensis* OPB expression and purification. (A) 10% SDS-PAGE showing soluble (S.F.) and insoluble (I.F.) fractions of bacterial extracts after sonication. Protein marker: SeeBlue Plus2, Invitrogen. (B) 10% SDS-PAGE showing fractions (Fr.) 1–5 of the soluble fraction of the bacterial lysate eluted from the nickel column. Protein marker: SeeBlue Plus2, Invitrogen.

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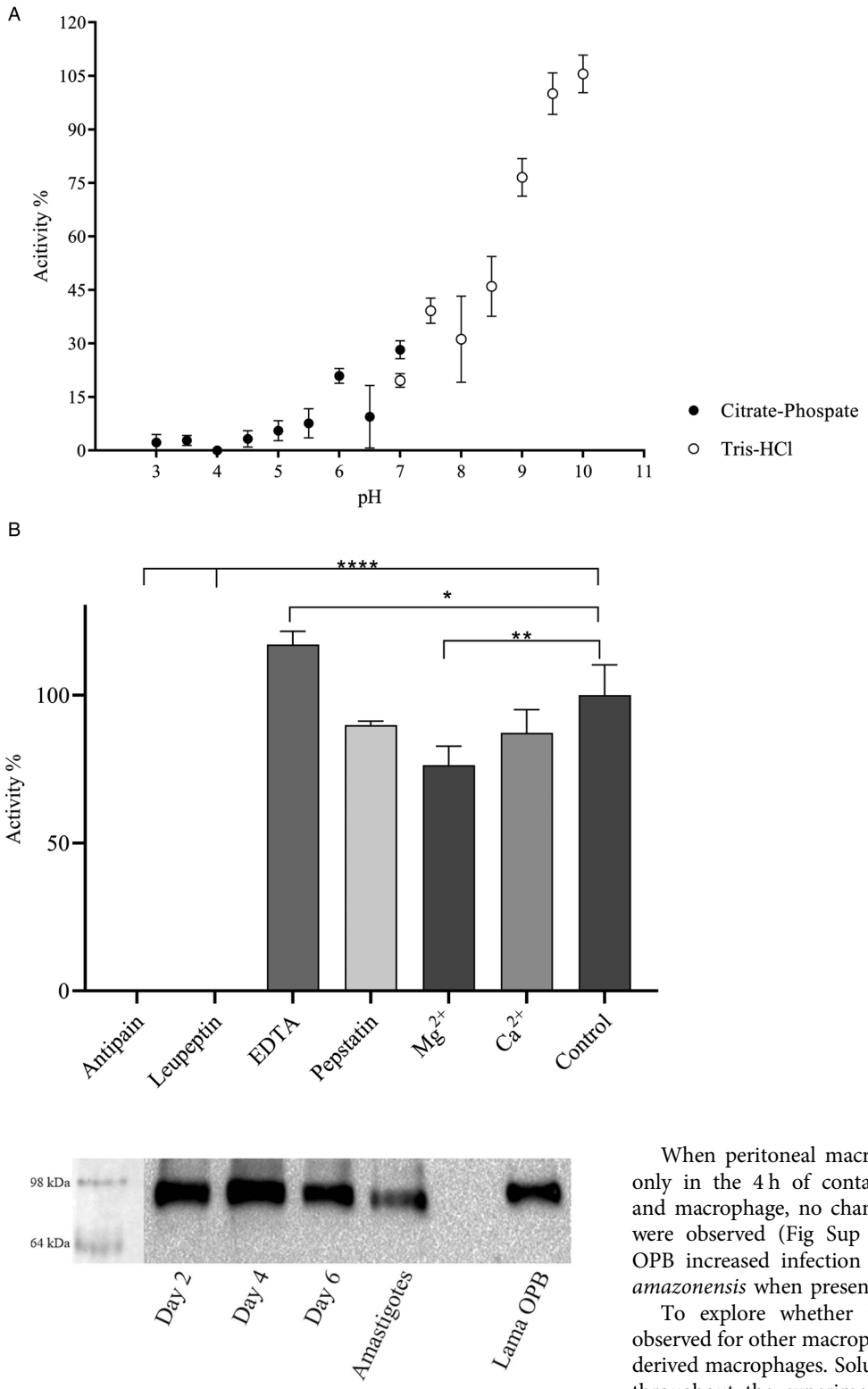

Fig. 2. Characterization of the enzymatic activity of the recombinant OPB from *L.* (*L.*) *amazonensis*. (A) Activity in different pHs. The substrate Z-Arg-Arg-AMC was prepared in 0.2 M citrate-phosphate and 50 mM Tris-HCl buffers. Mean relative activities and standard deviations were calculated based on 3 enzyme assays. For more details see Materials and Methods. Negative relative activities were considered as 0. (B) Activity in the presence of commercial inhibitors and bivalent cations. Inhibitors were pre-incubated with the enzyme for 10 min and the activity was measured by hydrolysis of the substrate Z-Arg-Arg-AMC in a 50 mM Tris-HCl buffer, pH 8.0. Negative relative activities were considered as 0. Mean relative activities and standard deviations were calculated based on 3 enzyme assays. For more details see Materials and Methods. Statistical analysis for one-way ANOVA and Sidak's multiple comparison (*$P < 0.05$; **$P < 0.005$; ****$P < 0.0001$).

Fig. 3. Western blot to assess the presence of OPB in *L.* (*L.*) *amazonensis* promastigotes from days 2, 4 and 6 and amastigotes, in parallel with recombinant (Lama OPB). 20 μg of each total protein extract and 0.5 μg of recombinant protein were probed with the primary anti-OPB serum at a dilution of 1:500 and secondary anti-mouse HRP at a dilution of 1:5000. Protein marker: SeeBlue Plus2, Invitrogen

macrophages compared to control, Pefabloc and boiled conditions. Results with Pefabloc-inhibited OPB and boiled OPB did not differ from the control without OPB, indicating that the active enzyme is necessary for modulation of infection. We observed no differences in the number of intracellular parasites among the 4 conditions (Fig. 4B).

When peritoneal macrophages were exposed to soluble OPB only in the 4 h of contact between *Leishmania* promastigotes and macrophage, no changes in the percentage of infected cells were observed (Fig Sup 3). These results indicate that active OPB increased infection of peritoneal macrophages by *L.* (*L.*) *amazonensis* when present before and during infection.

To explore whether the effect of recombinant OPB was observed for other macrophage types, we employed bone marrow-derived macrophages. Soluble OPB was added during plating and throughout the experiment, using a design similar to the one showed in Fig. 4. Interestingly, no increase in infection was observed in the presence of OPB, even at 200 ng mL^{-1} (Fig. 5A). Similarly to what was observed for peritoneal macrophages, OPB did not affect the number of intracellular parasites (Fig. 5B). These results indicate that OPB did not modulate the infection of medullary macrophages by *L.* (*L.*) *amazonensis*, demonstrating that macrophage types responded differently to OPB.

Discussion

In this paper we described the production of *L.* (*L.*) *amazonensis* recombinant OPB. The sequence obtained for the LV79 strain

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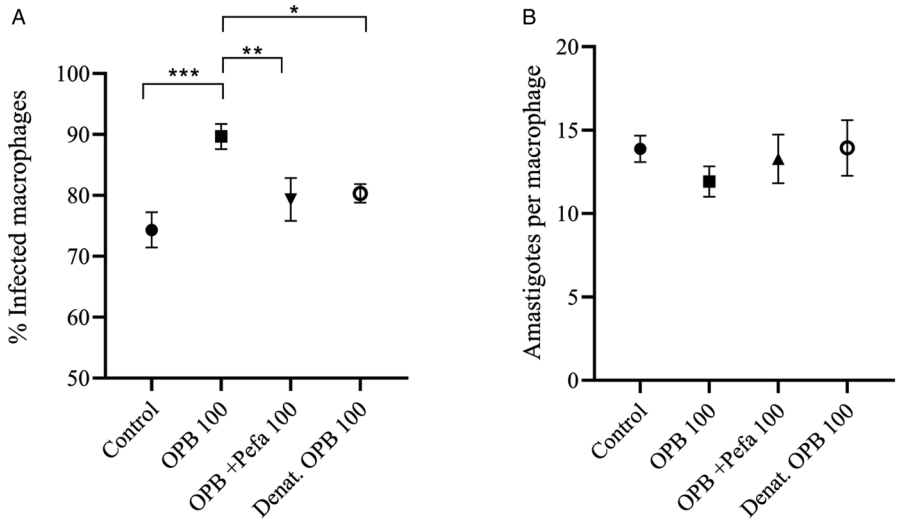

Fig. 4. Infection of peritoneal macrophages with *L.* (*L.*) *amazonensis* in the presence of recombinant OPB (100 ng mL^{-1}), OPB inhibited with Pefabloc® (Sigma) (OPB + Pefa, 100 ng mL^{-1}) and denatured OPB (Denat. OPB, 100 ng mL^{-1}) throughout the experiment. (A) Percentage of infected macrophages. (B) Number of amastigotes per macrophage. Results represented as mean ± standard deviation of 3 technical replicates from a representative experiment of 3 with similar profiles. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons (*$P \leqslant 0.01$; **$P \leqslant 0.005$; ***$P \leqslant 0.0005$).

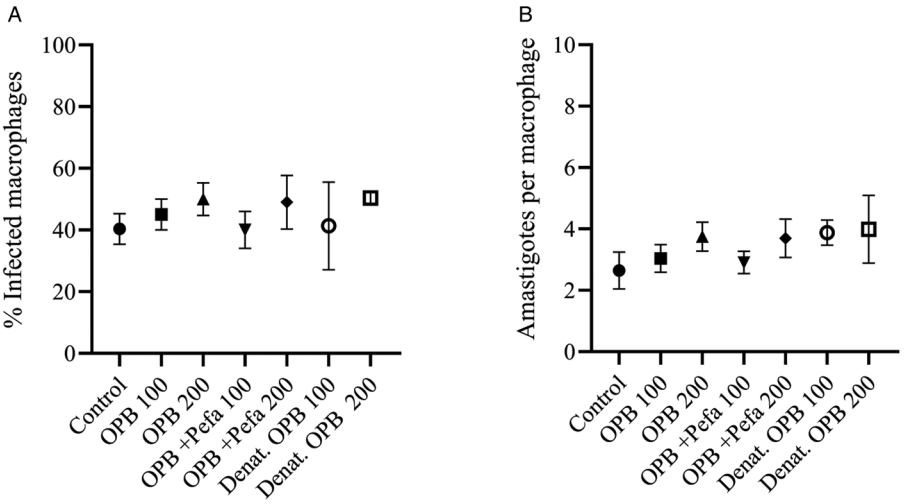

Fig. 5. Infection of MDMOs with *L.* (*L.*) *amazonensis* in the presence of recombinant OPB (100 and 200 ng mL^{-1}), OPB inhibited with Pefabloc® (Sigma) (OPB + Pefa 100 and 200 ng mL^{-1}) and denatured OPB (Denat. OPB, 100 and 200 ng mL^{-1}) throughout the experiment. (A) Percentage of infected macrophages. (B) Number of amastigotes per macrophage. Results of a single experiment, represented as mean ± standard deviation of 3 technical replicates. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons.

(Sup Fig 1) was very similar to the sequence reported for PH8 strain, except for a change in 1 nucleotide.

This recombinant protein showed larger size than the expected, but the similarity in kinetic parameters with other OPBs and purity of the band reinforce its identity as *L.* (*L.*) *amazonensis* OPB. Anti-sera obtained from immunized mice recognized a band of similar size in promastigotes and amastigotes, slightly smaller than the band of the recombinant protein (Fig. 3). Western blots of promastigote lysates obtained from cultures at days 2, 4 and 6 cultures and axenic amastigotes indicated that promastigotes from different stages and amastigotes expressed OPB. This analysis is merely qualitative and OPB levels may vary during differentiation since the protein may be secreted at different levels and we only analysed whole cell lysates. Our findings agree with a previous report showing OPB transcripts in *L.* (*L.*) *amazonensis* procyclic and metacyclic promastigotes (de Matos Guedes *et al.*, 2007).

L. (*L.*) *amazonensis* OPB activity was analysed at different pHs and in the presence of protease inhibitors (Fig. 2). The pH effects on *L.* (*L.*) *amazonensis* OPB activity were similar to those reported for *L.* (*L.*) *major* and *L.* (*L.*) *donovani* OPBs (McLuskey *et al.*, 2010; Swenerton *et al.*, 2011). Interestingly, OPBs optimal pHs are higher than the most neutral milieus where OPB is found – inside promastigotes, secreted in the phlebotomine fly or in the vertebrate host dermis. It is possible that neutral physiological pH keeps the enzyme activity levels low enough not to cause harm to parasite proteins.

Effective inhibitors for *L. amazonensis* OPB were also the same observed for the 2 other species, and K_m values for the substrate Z-Arg-Arg-AMC were similar for *L.* (*L.*) *amazonensis* and *L.* (*L.*) *major* OPBs (2.1 and 0.93 μM, respectively). These findings were expected, since OPB sequence conservation was observed among *Leishmania* species. The lack of OPB in mammals has prompted some groups to search for potential OPB or OPB/OPB2 inhibitors as eventual drugs for leishmaniasis (Goyal *et al.*, 2014; Sodero *et al.*, 2017). The conservation in OPB activity among the 3 species analysed (*L.* (*L.*) *major* and *L.* (*L.*) *donovani* and *L.* (*L.*) *amazonensis*, in this work) suggests that drugs designed for 1 species would probably be effective for others.

Since OPB is present in promastigotes from day 4 cultures (Fig. 3) used in our infection experiments and considering that data from literature showed OPB secretion in *L.* (*L.*) *donovani* and *L.* (*L.*) *major* promastigotes (Silverman *et al.*, 2008, 2010), we evaluated the effect of soluble recombinant OPB in *L.* (*L.*) *amazonensis* macrophage infections. In the first assay we used peritoneal macrophages and included OPB in the medium during the whole experiment. We observed that OPB increased the number of infected macrophages and that this effect was dependent on enzyme structure and activity. A similar experiment including OPB only during parasite–macrophage contact (Fig Sup 3) showed no difference between OPB and control or boiled conditions, indicating that OPB increase in infection depends on its presence throughout infection. Since macrophages are very heterogeneous and exhibit high plasticity (Taylor *et al.*, 2005;

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Ghosn *et al*., 2010; Gordon, 2012; Mills, 2015), we also analysed the effect of OPB on the infection of medullary macrophages. Interestingly, no difference in infection was observed in the presence of OPB throughout the experiment (Fig 5). This was not completely unexpected since peritoneal and medullary macrophages display different receptors or in different abundances (Taylor *et al*., 2005), and these (and other) phenotypic peculiarities may affect their responses to OPB.

We believe this work contributes to a better knowledge of OPB from a New World *Leishmania* species. Our enzymatic analysis suggested that similar inhibitors or drugs may be used to target the enzyme on several *Leishmania* species. The demonstration that recombinant OPB increased macrophage infection by *L*. (*L*.) *amazonensis in vitro* agrees with reports of lower infection for *L*. (*L*.) *major* parasites deficient for OPB, suggesting that the protein affects infection of many and maybe all *Leishmania* species. Future studies will be done to evaluate the effect of OPB deletion in *L*. (*L*.) *amazonensis* infectivity and to decipher the mechanism by which soluble OPB increases macrophage infection.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182022000816.

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Author contributions. GRB and BSS conceived and designed the study, SRM helped designing activity assays. GRB performed experiments. GRB, BSS and SRM performed statistical analyses. GRB and BSS wrote the article.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. All animals were used according to the Brazilian College of Animal Experimentation (CONEP) guidelines, and the protocols were approved by the Institutional Animal Care and Use Committee (CEUA) of the University of São Paulo (protocol number 9829290419/2019). Euthanasia was performed in CO_2 camera.

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