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# The recombinant prepro region of TvCP4 is an inhibitor of cathepsin L-like cysteine proteinases of *Trichomonas vaginalis* that inhibits trichomonal haemolysis

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ABSTRACT

*Trichomonas vaginalis* expresses multiple proteinases, mainly of the cysteine type (CPs). A cathepsin L-like 34 kDa CP, designated TvCP4, is synthesized as a 305-amino-acid precursor protein. TvCP4 contains the prepro fragment and the catalytic triad that is typical of the papain-like CP family of clan CA. The aim of this work was to determine the function of the recombinant TvCP4 prepro region (ppTvCP4r) as a specific inhibitor of CPs. We cloned, expressed, and purified the recombinant TvCP4 prepro region. The conformation of the purified and refolded ppTvCP4r polypeptide was verified by circular dichroism spectroscopy and fluorescence emission spectra. The inhibitory effect of ppTvCP4r was tested on protease-resistant extracts from *T. vaginalis* using fluorogenic substrates for cathepsin L and legumain CPs. In 1-D zymograms, the inhibitory effect of ppTvCP4r on trichomonad CP proteolytic activity was observed in the ~97, 65, 39, and 30 kDa regions. By using 2-D zymograms and mass spectrometry, several of the CPs inhibited by ppTvCP4r were identified. A clear reduction in the proteolytic activity of several cathepsin L-like protein spots (TvCP2, TvCP4, TvCP4-like, and TvCP39) was observed compared with the control zymogram. Moreover, pretreatment of live parasites with ppTvCP4r inhibited trichomonal haemolysis in a concentration dependent manner. These results confirm that the recombinant ppTvCP4 is a specific inhibitor of the proteolytic activity of cathepsin L-like *T. vaginalis* CPs that is useful for inhibiting virulence properties depending on clan CA papain-like CPs.



## 1. Introduction

The cysteine proteinases (CPs) of parasitic organisms are divided into two main clans: CA and CD. CPs of clan CA, such as cathepsins B, L, K, M, O, and S, are the best characterized in parasites such as *Entamoeba*, *Fasciola*, *Leishmania*, *Trichomonas*, and *Trypanosoma*. CPs of clan CD include legumain of family C13. Although most of these CPs appear to function primarily as lysosomal enzymes, they also play additional roles that differ from those of the host enzymes (Sajid and McKerrow, 2002).

The CPs of clan CA are synthesized as inactive precursors, formed by a prepro region and a mature enzyme or catalytic domain. The catalytic domain has the highest level of sequence conservation and proteolytic activity. The activation process of these enzymes involves the removal of the prepro region, which functions as an

*Abbreviations:* α-His, anti-histidine tag; AE-like, asparaginyl endopeptidase-like; CBB, Coomassie Brilliant Blue; CNCD, Centro Nacional de Clínica de Displasias; CPs, cysteine proteinases; ESI-LC–MS/MS, electrospray ionization-liquid chromatography–tandem mass spectrometry; IEF, isoelectric focusing; MALDI-MS, matrix assisted laser desorption/ionization-mass spectrometry; IPG, immobilized pH gradient; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate buffered saline; ppTvCP4, TvCP4 prepro region; ppTvCP4r, recombinant ppTvCP4; TvCP1, *T. vaginalis* CP1; TvCP2, *T. vaginalis* CP2; TvCP3, *T. vaginalis* CP3; TvCP4, *T. vaginalis* CP4; TvCP4-like, *T. vaginalis* CP4-like; TvCP12, *T. vaginalis* CP12; TvCP39, *T. vaginalis* cytotoxic 39 kDa CP; TvCPT, *T. vaginalis* CPT; TvLEGU-1, *T. vaginalis* legumain-1 CP; TvPRE, trichomonad protease-resistant extract; WB, western blot.

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inhibitor of the mature enzyme, *via* changes in the pH or redox conditions. This gives rise to the active form (mature enzyme) (Rzychon et al., 2004; Kominami et al., 1988). The most remarkable feature of the prepro region of these CPs is its role as an inhibitor of their proteolytic activity.

The cathepsin L precursor must be processed either autocatalytically or with the aid of processing enzymes to become active (Turk et al., 2012). Activation takes place *via* limited intramolecular and intermolecular proteolysis, which cleaves off the inhibitory prepro region (Wiederanders et al., 2003). Moreover, in addition to CP inhibitory activity (Yamamoto et al., 2002), other diverse functions have been attributed to the prepro region of CP precursors *in vivo* and *in vitro*. For example, the prepro region has been shown to be involved in the proper folding of the newly synthesized enzyme (Tao et al., 1994; Yamamoto et al., 1999), the stabilization of the enzyme under denaturing conditions at neutral to alkaline pH (Turk et al., 1993; Mach et al., 1994), the mediation of CP membrane association, and the transport of CPs to lysosomes (McIntyre et al., 1994; Cuozzo et al., 1995). Because the cathepsin L prepro region can fold independently, the existence of novel inhibitory proteins derived from the CP prepro region has been postulated (Jerala et al., 1998).

*Trichomonas vaginalis* is a flagellated protozoan parasite found in the human urogenital tract that is responsible for human trichomoniasis, which is one of the most common, non-viral sexually transmitted infections (STIs) worldwide (Figueroa-Angulo et al., 2012). *T. vaginalis* possesses an extremely complex degradome, and half of its proteolytic activity is conferred by CPs (Carlton et al., 2007). A total of 48 genes encode for papain-like CPs, and 10 genes encode for legumain-like CPs. A number of these CPs have been characterized as virulence factors. Ramón-Luing et al. (2010) used MALDI-MS analysis to identify nine CPs—seven cathepsin L-like CPs (TvCP1, TvCP2, TvCP3, TvCP4, TvCP4-like, TvCP12, and TvCPT) and two asparaginyl endopeptidase-like (AE-like) or legumain-like CPs (TvLEGU-1 and an uncharacterized AE-like CP)—in the active degradome of *T. vaginalis* obtained from a protease-resistant extract (TvPRE).

The *T. vaginalis tvcp4* gene encodes for a 34 kDa cathepsin L-like CP precursor, TvCP4. TvCP4 is upregulated by iron, localized at the surface of the parasite, and participates in the haemolysis caused by *T. vaginalis* (Solano-González et al., 2007; Cárdenas-Guerra et al., 2013). It is one of the most immunogenic trichomonad CPs and is found in vaginal secretions of patients with trichomoniasis (Ramón-Luing et al., 2010; Cárdenas-Guerra et al., 2013). The precursor TvCP4 contains a prepro region (1–86 aa residues) and a catalytic domain (C25, H159, N175) typical of the papain superfamily with a high level of homology to cathepsin L (Solano-González et al., 2007).

The goal of this work was to determine the inhibitory effect of the recombinant TvCP4 prepro (ppTvCP4r) region against *T. vaginalis* CP proteolytic activity and virulence. Our results show that ppTvCP4r is a specific inhibitor of the proteolytic activity of several cathepsin L-like CPs of *T. vaginalis*, some of them involved in virulence properties such as haemolysis.

## 2. Materials and methods

### 2.1. Parasite culture

*T. vaginalis* parasites from the fresh clinical isolate CNCD 147 were used in this study for all experiments (Alvarez-Sánchez et al., 2000). Trichomonad cultures were maintained for up to two weeks by daily passage in trypticase–yeast extract–maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (HIHS) at 37 °C (Diamond, 1957). Only organisms at mid-logarithmic phase were used for the assays.

### 2.2. Cloning and expression of the recombinant TvCP4 prepro region (ppTvCP4r)

A fragment (258 bp) of the *tvcp4* gene (GenBank accession number AY679763) encoding for the prepro region of TvCP4 (1–86 aa residues) was amplified by PCR using a plasmid containing the complete *tvcp4* gene as a template (Solano-González et al., 2007). The specific primers 5′-GAGCTTGGATCCGAATACCAGACACGACTTGGC-3′ and 5′-GAGCTTAAGCTTTTAAGCGTTGGACTTGACGGCCTT-3′ were used as sense and antisense primers, respectively. The restriction sites used were *Bam*HI (sense) and *Hind*III (antisense), which are underlined in the above sequences. The amplicon encoding for the ppTvCP4 region was directionally cloned into the pCold I prokaryotic expression vector (Takara Bio Inc., Mountain View, CA, USA), as recommended by the manufacturer. Prior to bacterial expression, candidate clones were sequenced using an Applied Biosystems AB1377 Automatic Sequencer (UNAM, Institute of Cell Physiology) to select the clones containing the correct insert. Recombinant protein expression in the *Escherichia coli* BL21 (DE3) strain was induced using 1 mM IPTG for 16 h at 37 °C and analyzed by SDS-PAGE and western blot (WB) assays. The WB assays were performed by using an anti-histidine monoclonal antibody (α-His) as the primary antibody and a peroxidase-conjugated goat anti-mouse polyclonal secondary antibody (Invitrogen-Gibco, Carlsbad, CA, USA) at a 1:3000 dilution. The bacteria (1 g of cells) were lysed in 10 ml of cell lysis buffer (20 mM Tris–HCl [pH 8.0], 20 mM NaCl, 5 mM DTT, 1 mM PMSF, 1 mM DNAse, and 1 mg/ml lysozyme) and incubated for 45 min at 4 °C. Additionally, three freezing and thawing cycles were performed. To separate the soluble and insoluble fractions, the lysate was centrifuged at 16,000 × *g* for 15 min at 4 °C and analyzed by 12% SDS-PAGE. Inclusion bodies were washed four times with washing buffer (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl, 2% Triton X-100, and 2 M urea). Three additional washes were then performed using the same buffer without urea. Inclusion bodies were solubilized for 16 h at room temperature in binding buffer (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl, 5 mM imidazole, and 8 M urea). The suspension was centrifuged at 16,000 × *g* for 30 min to remove insoluble material, and proteins were analyzed by SDS-PAGE.

### 2.3. Purification and refolding of the recombinant ppTvCP4r protein by nickel affinity chromatography

The recombinant ppTvCP4r protein was purified on a 1.5 cm × 15 cm low-pressure chromatographic column packed with 10 ml of Profinity IMAC Ni-Charged Resin (Bio-Rad, CA, USA) using a low-pressure chromatographic system (Bio-Rad BioLogic LP). The column was washed with 50 ml of deionized water and equilibrated using 30 ml of binding buffer at a flow rate of 1 ml/min. A sample of solubilized inclusion bodies was applied to the Ni-affinity column and washed with 40 ml of binding buffer. The protein was refolded into the column by diluting the urea concentration in two steps: first, the column was washed with 40 ml of washing buffer A (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl, 20 mM imidazole, and 4 M urea); next, a 50 ml linear gradient to 100% of washing buffer B (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl, 20 mM imidazole, and 1 M urea) was applied. Then, affinity-bound protein was eluted with 15 ml of elution buffer (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl, 0.5 M imidazole, and 1 M urea). Fractions (1 ml) were collected and analyzed by SDS-PAGE. Fractions containing the purified and refolded ppTvCP4r protein were pooled and stored at 4 °C.

Previous to any functional assays, buffer of purified protein was exchanged to PBS by using a PD10 desalting column (GE Healthcare, CA, USA) following a procedure recommended by the provider. Briefly, a 2.5 ml of the purified and refolded ppTvCP4r protein

(0.1–0.2 mg/ml) was applied to a PD10 column pre-equilibrated with PBS (pH 7.4) then, protein was eluted with 3.5 ml of PBS, and protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce), following the manufacturer's recommendations using bovine serum albumin (BSA) as standard. To corroborate that refolded ppTvCP4r acquired a native-like functional conformation circular dichroism (CD) and intrinsic emission fluorescence spectra were obtained. Additionally, the aggregation state of the refolded ppTvCP4r was estimated by a gel-filtration chromatography.

### 2.4. Circular dichroism (CD) spectroscopy

CD measurements were performed in a JASCO J-815 spectropolarimeter (Jasco Inc., Easton, MD) equipped with a PFD-425S Peltier-type cell holder for temperature control and magnetic stirring. CD spectra of the refolded ppTvCP4r protein and temperature-denatured ppTvCP4r protein as an unfolded control was recorded at a concentration of 10 μg/ml from 200 to 250 nm at 0.5-nm intervals with a spectral bandwidth of 1 nm and a 4 s integration time; 0.1 cm path-length cells were used. Ellipticity is reported as the mean residue ellipticity [$\theta$], (deg cm$^2$/dmol). To estimate secondary structure of the native-like ppTvCP4, a 3D structure model of the native-like precursor of TvCP4 was obtained by using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Roy et al., 2010). This model was visualized with the PyMOL Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC, USA).

### 2.5. Fluorescence emission spectra

Steady-state emission fluorescence measurements were performed using a Fluoromax-3 spectrofluorometer (Horiba, Japan) with a temperature-controlled cell holder in 1 cm path length quartz cuvettes. Emission spectra of the refolded ppTvCP4r protein in PBS with 8 M, 1 M, and without urea were recorded between 310 and 400 nm with a fixed excitation wavelength of 290 nm at 25 °C. All spectra represent the average of three scans and were corrected by subtraction of the corresponding blank spectrum. The emission spectra were normalized. The emission fluorescence intensity was reported as arbitrary units and the fluorescence-emission spectral center of mass (SCM) of each spectrum was estimated (Ferrão-Gonzales et al., 2000).

### 2.6. Gel filtration chromatography of ppTvCP4r

To determine the aggregation state of ppTvCP4r after its purification and refolding in the Ni-affinity column and after the exchange of PBS before functional assays, samples of ppTvCP4r were conditioned using a PD10 column equilibrated with 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 1 M urea or PBS (pH 7.4) without urea. Then, 1 ml samples were loaded into a gel filtration column (2.5 cm × 30 cm) packed with 45 ml of Sephacryl-HR 100 (GE Healthcare) previously equilibrated with buffer A (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) and resolved at a flow rate of 1 ml/min by using a NGC Q10 chromatographic system (Bio-Rad). The column was calibrated with gel filtration standards containing Thyroglobulin (670,000), γ-globulin (158,000), Ovalbumin (44,000), Myoglobin (17,000), and Vitamin B12 (1350) (Bio-Rad). The elution volume ($V_e$) of the Ovalbumin, Myoglobin, and Vitamin B12 were used to obtain the calibration curve by plotting the Log MW *vs* $K_{va}$. The elution volume of the first peak (Thyroglobulin and γ-globulin) was taken as the void volume ($V_o$) to estimate $K_{va}$.

### 2.7. Enzyme inhibition assays

All enzyme inhibition assays were performed at 25 °C in a FluroMax-3 spectrofluorometer (Horiba Scientific) with 355 and 460 nm excitation and emission wavelengths, respectively. The inhibition kinetics of ppTvCP4r (0.0, 0.05, 0.15, 0.3, and 0.9 μM) over a protease-resistant extract from *T. vaginalis* (TvPRE) that is rich in active CPs (Alvarez-Sánchez et al., 2000) was measured using a fluorogenic substrate (Z-Phe-Arg-AMC) (Peptide Institute Inc.) specific for cathepsin L CPs to obtain the ppTvCP4r dissociation constant ($K_i$) for inhibition of CPs proteolytic activity. Comparative inhibition of peptidase activity by ppTvCP4r was evaluated using the TvPRE (0.216 μM), ppTvCP4r (0.9 μM) with 5 mM β-mercaptoethanol, and 3.33 μM Z-Phe-Arg-AMC or Z-Ala-Ala-Asn-AMC, fluorogenic substrates specific for cathepsin L or legumain CPs, respectively. The reaction was initiated by the addition of the fluorogenic substrate, and the increase in fluorescence due to the release of amino methyl coumarin (AMC) was measured. Under the experimental conditions used, progress curves for the inhibition of cathepsin L-like and legumain-like CPs were linear. The experiments were performed at least three times with similar results. The ppTvCP4r $K_i$ was determined by measuring the steady state rate of substrate hydrolysis ($\upsilon$) in the presence of varying concentrations of the inhibitor at a fixed substrate concentration. The $K_i$ values were calculated from Eq. (1) (Beynon and Bond, 2001)

$$\frac{\upsilon_o}{\upsilon_i} - 1 = \frac{I}{K_i} \quad (1)$$

### 2.8. Statistical analysis

Statistically significant differences between the means were determined by analysis of variance (ANOVA) using GraphPad Prism 5.0. The data were analyzed by one-way ANOVA using the Bonferroni method; all pairs of columns in Figs. 3 and 6 were compared ($P < 0.001$). The scores showing statistically significant differences are indicated with asterisks in the figure. The corresponding $P$ values are indicated in the figure legend.

### 2.9. Protein preparation, 1-D gel electrophoresis, and 2-D gel electrophoresis

A TvPRE with proteolytic activity was obtained from a clarified detergent extract of $2 \times 10^7$ parasites preincubated in the presence or absence of ppTvCP4r for 20 min at 4 °C, centrifuged to remove the inhibitor, lysed, and analyzed using 1-D substrate SDS-PAGE on 12% polyacrylamide gels copolymerized with 0.2% gelatin (Bio-Rad). After electrophoresis, proteinases were renatured with 2.5% Triton X-100 and activated with 100 mM sodium acetate buffer (pH 4.5) containing 0.1% β-mercaptoethanol for 12 h at 37 °C, as previously reported (Alvarez-Sánchez et al., 2000; Hernández-Gutiérrez et al., 2004), and stained with Coomassie Brilliant Blue (CBB). Proteolytic activity can be visualized as white bands or spots against a dark background. The experiments were performed at least three times with similar results.

2-D gel electrophoresis was performed as recently described (Ramón-Luing et al., 2011). Briefly, $6 \times 10^7$ parasites were pretreated in the presence or absence of ppTvCP4r for 20 min at 4 °C, centrifuged to remove the inhibitor, and lysed in the 2-D rehydration/sample buffer (Bio-Rad). After centrifugation, the supernatant was loaded onto ReadyStrip immobilized pH gradient (IPG) strips (7 cm, linear pH gradient from 4 to 7; Bio-Rad) and actively rehydrated for 16 h at 4 °C. Isoelectric focusing (IEF) of the proteins was performed with the Protean IEF Cell (Bio-Rad) in three steps: 250 V for 20 min, 4000 V for 3 h, and 4000 V with a gradual increase up to 10,000 Vh. After IEF, the strips were equilibrated for reduction

in equilibration buffer I (Bio-Rad) for 10 min at room temperature. The IPG strips were subjected to 2-D electrophoresis *via* SDS-PAGE. To accomplish this, the IPG strips were loaded onto 12% polyacrylamide (37.5:1 crosslinker ratio) gels (8.0 cm × 7.3 cm) and sealed with overlay agarose (Bio-Rad). The protein gels were silver-stained according to Shevchenko et al. (1996). For the 2-D zymograms, the reduced IPG strips were loaded onto 12% polyacrylamide gels copolymerized with 0.2% gelatin (Bio-Rad). After electrophoresis, the proteinases were renatured, as described above for the 1-D gels. The experiments were performed at least three times, with similar results. Images from 2-D gels were obtained with the Molecular Imager ChemiDoc XRS System using the Quantity One software (version 4.6.0) and analyzed with the PDQuest Advanced software (version 7.4.0) (Bio-Rad).

### 2.10. Mass analysis by MALDI-MS and ESI-LC–MS/MS

Identification of protein spots was performed at the Protein Unit of Columbia University (New York, USA). Protein spots of interest were manually excised from silver-stained gels, distained according to Gharahdaghi et al. (1999) and prepared for in-gel digestion with 0.020 mg of modified trypsin and 0.1 mg of endoproteinase Lys-C (sequencing grade, Roche Molecular Biochemicals) in 13–15 ml of 0.025 M Tris–HCl (pH 8.5) for 16 h at 32 °C. The resulting peptides were extracted with 50 ml of a 50% ACN/2% TFA solution, and the combined extracts were dried and resuspended in the matrix solution containing CHCA. Angiotensin and ACTH 7–38 peptides were used as internal standards. MALDI-MS analysis was performed on the digests with a Voyager DE Pro mass spectrometer operated in linear mode (Applied Biosystems). For the identification of proteins by PMF, the molecular mass of each tryptic fragment was searched against the National Center for Biotechnology nonredundant database using the MASCOT program (http://matrixscience.com).

To obtain partial peptide sequences, after in-gel digestion of protein spots as described above, peptide extracts were reduced to ~10 ml, and ESI-LC–MS/MS was performed using a Micromass hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source. The capillary voltage was set according to the mass and charge of the ion, from 14 to 50 eV. Chromatography was performed on an LC Packing HPLC with a C18 PepMap column; a linear ACN gradient (flow rate of 200 nl/min) was employed. Raw data files were processed with the MassLynx ProteinLynx software, and .pkl files were submitted for searching against the National Center for Biotechnology nonredundant database using the MASCOT algorithm.

### 2.11. Database search

MASCOT searches of the tryptic peptides were performed, considering monoisotopic mass values, the possible oxidation of methionine residues, and carbamidomethylation at cysteine residues as variable modifications. A maximum of one missed tryptic cleavage per protein was allowed, and no taxonomic restrictions were considered in the database search. A mass accuracy of 100 ppm was used for MS, and 0.6 Da was used for the MS/MS Ion Search program. MASCOT scores of 56 and above were considered significant ($P < 0.05$) for positive identification.

### 2.12. Haemolysis assays

Haemolysis assays were performed using a previous method (Cárdenas-Guerra et al., 2013). Parasites grown in normal conditions were washed with PBS and suspended ($2 \times 10^6$ organisms/ml) in PBS-0.5% maltose (PBS-M), pH 5.8. Group $O^+$ erythrocytes were obtained from a healthy human donor (volunteer student), immediately diluted five-fold in PBS-M (pH 5.8), and washed three times in the same buffer. The experiments were performed with fresh blood. The parasites ($2 \times 10^6$) were incubated with erythrocytes ($60 \times 10^6$; 1:30 ratio) in a 7 ml volume for 16 h at 37 °C with gently waving motion to keep the cells in suspension, centrifuged at 900 × *g*, and the hemoglobin released in the supernatant was quantified by spectrophotometric analysis at 546 nm. In another experiment, prior to the interaction with erythrocytes, the parasites were incubated with 100 μM E-64 or with (0.05, 0.15, 0.3, and 0.9 μM) of the ppTvCP4r for 20 min at 4 °C. The parasites were then washed in PBS, and their haemolytic ability was evaluated. Control parasites were incubated without ppTvCP4r. As a negative and baseline controls, we incubated only erythrocytes in PBS-M (pH 5.8), for 16 h at 37 °C. The assays were performed in triplicate, and the experiments were performed at least three times with similar results.

### 2.13. Western blot (WB) assays

The WB assays were performed as previously described. ppTvCP4r protein separated using 1-D SDS-PAGE on 15% polyacrylamide gel was transferred onto nitrocellulose (NC) membranes (Bio-Rad), blocked with 5% non-fat dried milk, and incubated with sera from patients with trichomoniasis confirmed by *in vitro* culture, Tv (+) or with other vaginitis Tv (−) (Supplementary Table S1) at 1:1000 dilution for 18 h at 4 °C. After incubation for 2 h at room temperature with a peroxidase-conjugated goat anti-human secondary antibody (1:3000 dilution; Bio-Rad), the NC membranes were washed and developed using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific-Pierce, Rockford, IL, USA). Images were captured with the ChemiDoc XRS System (Bio-Rad) and analyzed by using the Quantity One software (Bio-Rad).

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.12.001.

## 3. Results

### 3.1. Expression, purification, and refolding of ppTvCP4r

The TvCP4 prepro region was overexpressed in the bacterial extracts after IPTG induction compared with extracts without IPTG as a ~13 kDa protein as observed in CBB-stained gels, ~9.5 kDa corresponds to the TvCP4 prepro region and ~3.5 kDa to the N-terminal polyhistidine tag (Fig. 1A, lanes 1 and 2). In WB, the α-His antibody detected a strong band of ~13 kDa after IPTG induction (Fig. 1A, lane 3). Analysis of the soluble and insoluble (inclusion bodies) fractions indicated that ppTvCP4r was expressed only as an insoluble protein and it was solubilized from inclusion bodies using 8 M urea (Fig. 1B, lanes 1 and 2). The denatured recombinant polypeptide was refolded in the Ni-affinity column by decreasing the urea concentration from 8 M to 4 M in one washing step, from 4 M to 1 M by applying a linear gradient, and then the protein was eluted (Fig. 1B, lane 3). Purified and refolded ppTvCP4r stored a 4 °C was stable up to two months under these conditions. However, after exchanging the refolded ppTvCP4r to PBS buffer the protein solution ≤0.15 mg/ml was stable up to 4 h at 4 °C or room temperature (Supplementary Fig. S1). Interestingly, unfolded protein samples (8 M or 4 M urea) exhibited similar profile than refolded protein, but a small fraction (10%) of the protein eluted in the void volume (data not shown), indicating that ppTvCP4r can be also refolded by using gel filtration chromatography as we have previously reported for the EhCP112 of *Entamoeba histolytica* (Quintas-Granados et al., 2009). However, some of the protein was

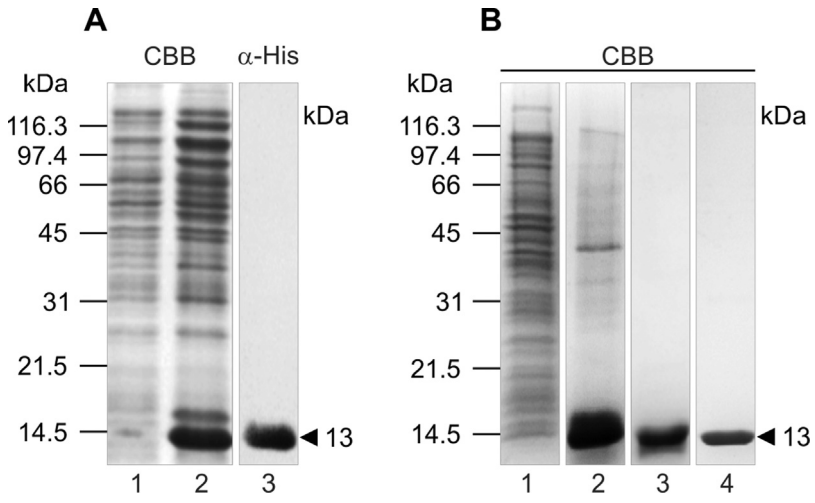


**Fig. 1.** Expression of the recombinant TvCP4 prepro region (ppTvCP4r). (A) Coomassie Brilliant Blue (CBB)-stained 12% SDS-PAGE of *Escherichia coli* cell extracts harboring the pCold I-TvCP4 prepro region construct before (lane 1) and after (lane 2) IPTG induction. A duplicate of the sample in lane 2 was transferred onto a nitrocellulose membrane and incubated with the α-His antibody (lane 3). (B) CBB 12% SDS-PAGE of soluble (lane 1) and insoluble (lane 2) fractions, the nickel affinity-purified and refolded recombinant ppTvCP4r protein in 1 M urea (lane 3), and purified ppTvCP4r in the absence of urea (lane 4). The lines on the left side indicate the molecular weight markers in kilodaltons. The arrowhead shows the molecular weight of ppTvCP4r.

not properly refolded and eluted as an aggregated species in the void volume.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.12.001.

To avoid this aggregation problem, previous to each functional assay the ppTvCP4r storage buffer was exchanged to PBS in a PD10 column (Fig. 1B, lane 4) at a concentration ≤0.15 mg/ml and used immediately.

To determine whether ppTvCP4r used in the functional assays acquired a properly folded conformation a CD spectroscopy analysis was performed. The CD spectrum of the refolded ppTvCP4r exhibited characteristics of a protein rich in alpha-helices (Fig. 2A) as compared with the CD spectrum of temperature-denatured protein. This spectrum is consistent with the alpha-helix fold of ppTvCP4r (blue) shown in the 3D molecular model of TvCP4 precursor (Fig. 2D). Such a residual structure might well include about 17% of alpha-helix, according to the analysis of the difference spectrum by means of the SELCON algorithm in the web DichroWeb (Sreerama et al., 1999). These results are consistent with conformational changes observed in the intrinsic fluorescence emission spectra (Fig. 2B). In the presence of 8 M urea (red line), ppTvCP4r exhibited a fluorescence-emission spectral center of mass (SCM) of 357 ± 1 nm that was clearly blue-shifted to SCM of 352 ± 1 nm in the refolded ppTvCP4r at 1 M urea (blue dashed line) or in the absence of urea (black line) (Fig. 2B). Additionally, a gel filtration elution profile of ppTvCP4r (with or without 1 M urea) shows that purified and refolded ppTvCP4r eluted as a single peak with an apparent size of 9–10 kDa, suggesting that ppTvCP4r used in functional assays was present as a monomer (Fig. 2C).

These results strongly suggest that under the experimental conditions used in the inhibition assays, refolded ppTvCP4r has a native-like conformation that is functional as an exogenous CP inhibitor.

### 3.2. The recombinant prepro region TvCP4 inhibits trichomonad CPs of clan CA but not those of clan CD

To determine whether ppTvCP4r function as an inhibitor of *T. vaginalis* CP proteolytic activity, we performed proteolytic activity inhibition assays with specific fluorogenic substrates for cathepsin L (Z-Phe-Arg-MCA) or legumain (Z-Ala-Ala-Asn-MCA) and a trichomonad extract (TvPRE) as a source of active CPs. The specific inhibitors E-64 and TLCK were used as controls. Fig. 3 shows that ppTvCP4r inhibited the proteolytic activity of cathepsin L-like CPs present in the trichomonad extract (TvPRE) in a concentration-dependent manner (Fig. 3A) with an estimated, $K_i$ of 0.13 μM. This result is consistent with the fact that TvCP4 is a cathepsin L-like CP. Fig. 3B shows that at the maximum concentration tested (0.9 μM), ppTvCP4r inhibited >95% of the total cathepsin L-like proteolytic activity present in the trichomonad extract, which is similar to the result obtained with 100 μM E-64 (~98%), a specific inhibitor of Clan CA CPs. However, no significant trichomonad proteolytic activity inhibition was observed with ppTvCP4r (~10%) or E-64 (<20%) when a specific fluorogenic substrate for legumain was used; whereas 1 mM TLCK, an inhibitor of clan CA and clan CD CPs, caused ~85% inhibition (Fig. 3C). These results show that ppTvCP4r is a specific inhibitor of cathepsin L-like (clan CA) but not legumain (clan CD) trichomonad CPs.

### 3.3. Inhibitory effect of ppTvCP4r on the proteolytic activity of T. vaginalis surface proteases

Due to the surface localization of TvCP4, we analyzed whether ppTvCP4r could inhibit CPs localized at the surface of *T. vaginalis* using live parasites that interacted with ppTvCP4r before lysis. Proteolytic activity was analyzed in 1-D zymograms. Fig. 4A shows a reduction in the proteolytic activity of ~97, 65, 39, and 30 kDa regions after treatment with ppTvCP4r. These data suggest that ppTvCP4r can inhibit several CPs localized at the surface of *T. vaginalis* that may share sequence homology with TvCP4 and participate in the virulence of *T. vaginalis.*

### 3.4. ppTvCP4r inhibits cathepsin L-like CPs from T. vaginalis similar to TvCP4

To identify the surface CPs inhibited by ppTvCP4r, we also performed 2-D zymograms of TvPRE from treated and untreated parasites to obtain the spot profile of active proteinases in *T. vaginalis*. In the 2-D zymogram of TvPRE from untreated parasites, which was used as a control, at least 15 distinct proteolytic spots were detected under these conditions, with a molecular weight range from 97 to 30 kDa and a p*I* range from 4.0 to 6.5 (Fig. 4B). In the 2-D zymogram of TvPRE from ppTvCP4r-treated parasites,

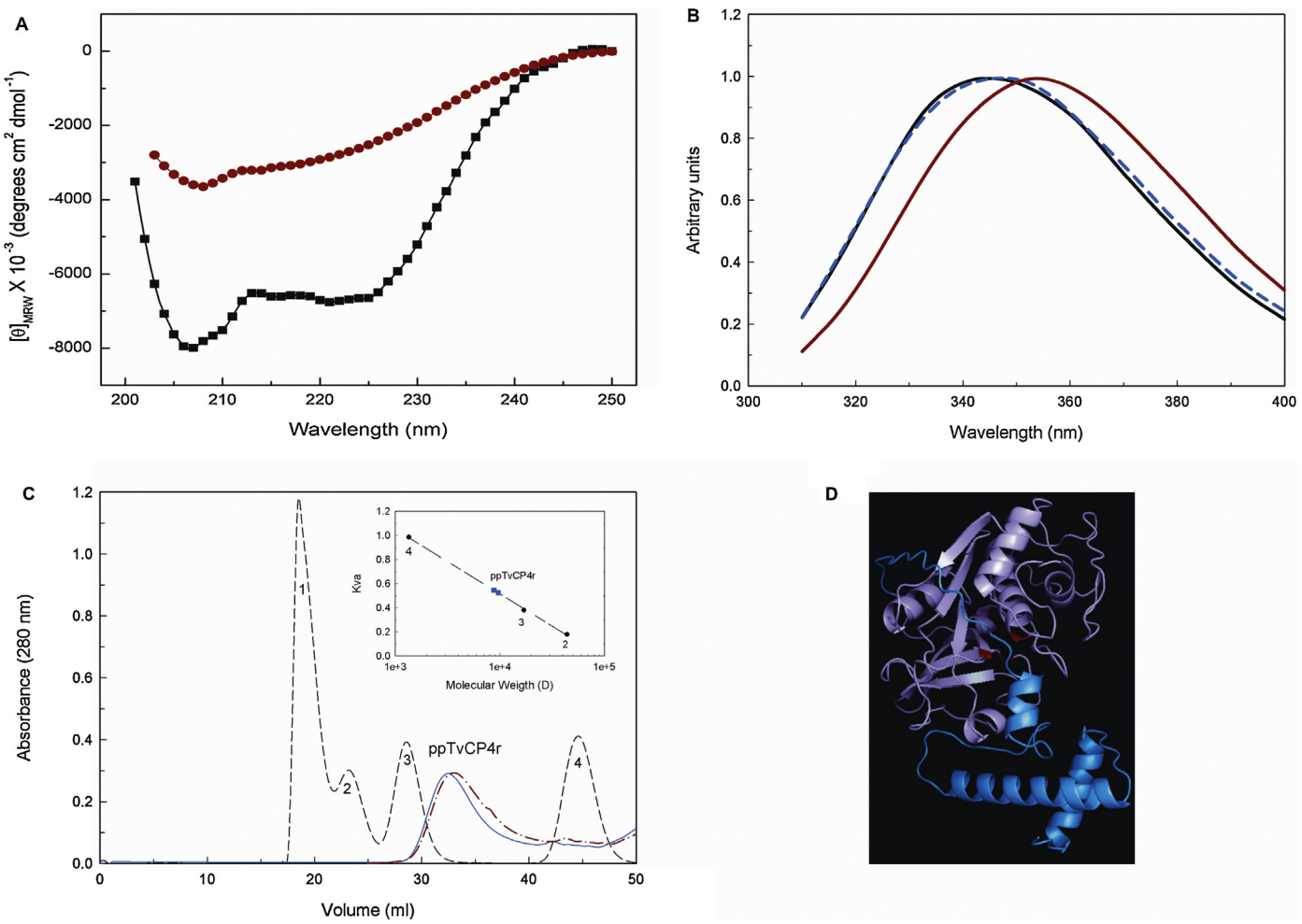


**Fig. 2.** Analysis of the secondary structure of ppTvCP4r. (A) Circular dichroism spectra of refolded (■) or temperature-denatured (•) ppTvCP4r at a concentration of 10 μg/ml in PBS buffer (pH 7.4). (B) Fluorescence emission spectra of ppTvCP4r in the presence of PBS with 8 M urea (red line), 1 M urea (dashed blue line) and in the absence of urea (black line). (C) Gel filtration chromatographic profiles of refolded ppTvCP4r. A gel filtration column packed with Sephacryl-HR 100 (GE Healthcare) previously equilibrated with buffer A (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) and resolved at a flow rate of 1 ml/min by using a NGC Q10 chromatographic system (Bio-Rad). The column was calibrated with gel filtration standards containing (1) Thyroglobulin (670,000), (2) γ-globulin (158,000), (3) Ovalbumin (44,000), (4) Myoglobin (17,000), and Vitamin B12 (1350) (Bio-Rad). The elution volume ($V_e$) of the Ovalbumin, Myoglobin, and Vitamin B12 were used to obtain the calibration curve by plotting the Log MW *vs* $K_{va}$ (inset). The elution volume of the first peak (thyroglobulin and γ-globulin) was taken as the void volume ($V_o$) to estimate $K_{va}$. (D) 3-D molecular model of TvCP4 precursor showing the prepro region in blue, the mature enzyme in purple, and the catalytic site residues are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the proteolytic activity of several spots in the 39–65 kDa region was inhibited (Fig. 4C), while the activity of the spots in the 30 kDa region was slightly affected.

Four spots that were inhibited (TvCP2, TvCP4, TvCP4-like, and TvCP39) and two spots that were not affected (TvLEGU-1 and AE-like CP) by ppTvCP4r treatment were identified by ESI-LC–MS/MS (Fig. 4D; Table 1; Supplementary Table S2). Four to six peptide sequences were obtained from the six protein spots (Supplementary Table S3). The four CPs that were inhibited by ppTvCP4r are cathepsin L-like CPs of clan CA with high (72 and >97%) sequence identity (Fig. 5) to TvCP4; whereas those that were not affected correspond to legumain-like CPs of clan CD (Table 1). These data show that the recombinant prepro region of TvCP4 only inhibits cathepsin L-like CPs similar to TvCP4.

**Table 1**
Protein spots inhibited or not by the recombinant TvCP4 prepro region (ppTvCP4r) in the zymograms (Fig. 4) and identified by ESI-LC–MS/MS.

| CP | Accession no. | Spot no. | Clan | Family | CP subfamily[b] | TvCP compared with TvCP4 Identity (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Mature enzyme | Prepro region |
| TvCP2 | gi\|452294 | 1 | CA | C1 | Cathepsin L-like | 67 | 72 |
| TvCP4 | gi\|56567186 | 1 | CA | C1 | Cathepsin L-like | 100 | 100 |
| TvCP4-like | gi\|123438675 | 2 | CA | C1 | Cathepsin L-like | 97 | 97 |
| TvCP39 | gi\|123457373 | 3 | CA | C1 | Cathepsin L-like | 91 | 97 |
| TvLEGU-1 | gi\|39573850 | 4 | CD | C13 | AE-like | – | – |
| AE-like[a] | gi\|123408789 | 5 | CD | C13 | AE-like | – | – |

[a] AE-like, asparaginyl endopeptidase-like cysteine proteinase of family C13 of clan CD (Ramón-Luing et al., 2010).
[b] Classification of CPs that has been reported in the *T. vaginalis* genome (Carlton et al., 2007) and in the literature (Sommer et al., 2005; Solano-González et al., 2007; León-Félix et al., 2004; Mallinson et al., 1994; De Jesus et al., 2009; Huang et al., 2009).

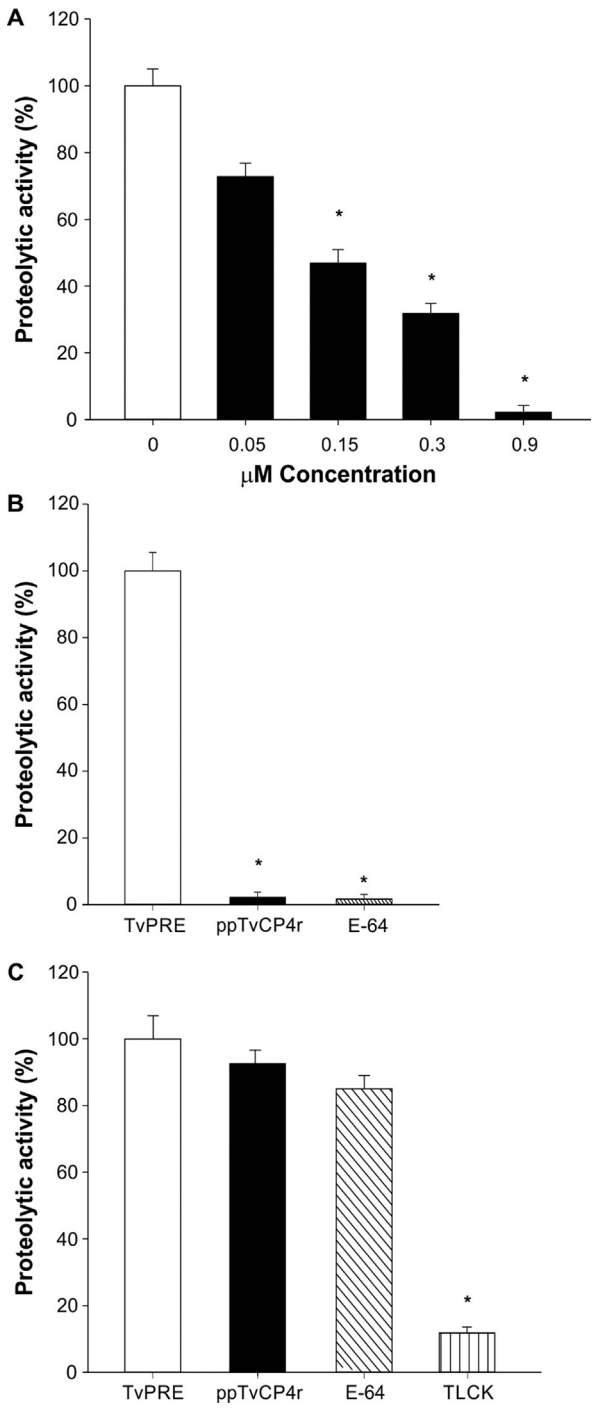


**Fig. 3.** Inhibition of the proteolytic activity of trichomonad cathepsin L-like CPs by ppTvCP4r. The proteolytic activity of a protease-resistant extract from *T. vaginalis* (TvPRE) (white bars) was used without treatment as a control for the total proteolytic activity in the presence of specific fluorogenic substrates for cathepsin L (Z-Phe-Arg-MCA) (A and B) and legumain (Z-Ala-Ala-Asn-MCA) (C). (A) Percentage of cathepsin L-like proteolytic activity in the trichomonad extracts, as measured using the specific fluorogenic substrate Z-Phe-Arg-MCA, in the presence of different concentrations (0.0, 0.05, 0.15, 0.3, 0.9 μM) of recombinant ppTvCP4r (black bars). (B) Percentage of cathepsin L-like proteolytic activity in the trichomonad extracts, as measured using the specific fluorogenic substrate Z-Phe-Arg-MCA, in the presence of 0.9 μM ppTvCP4r (black bar) and 100 μM E-64 (hatched bar). (C) Percentage of legumain-like proteolytic activity in the trichomonad extracts, as measured using the specific fluorogenic substrate Z-Ala-Ala-Asn-MCA, in the presence of 0.9 μM ppTvCP4r (black bar), 100 μM E-64 (hatched bar), or 1 mM TLCK (vertically hatched bar). Significant differences ($P < 0.001$) were found for 0.15, 0.3, and 0.9 μM ppTvCP4r; 100 μM E-64; and 1 mM TLCK, as indicated with asterisks, when compared with the other conditions. The error bars indicate the average of three experiments plus the SEM.

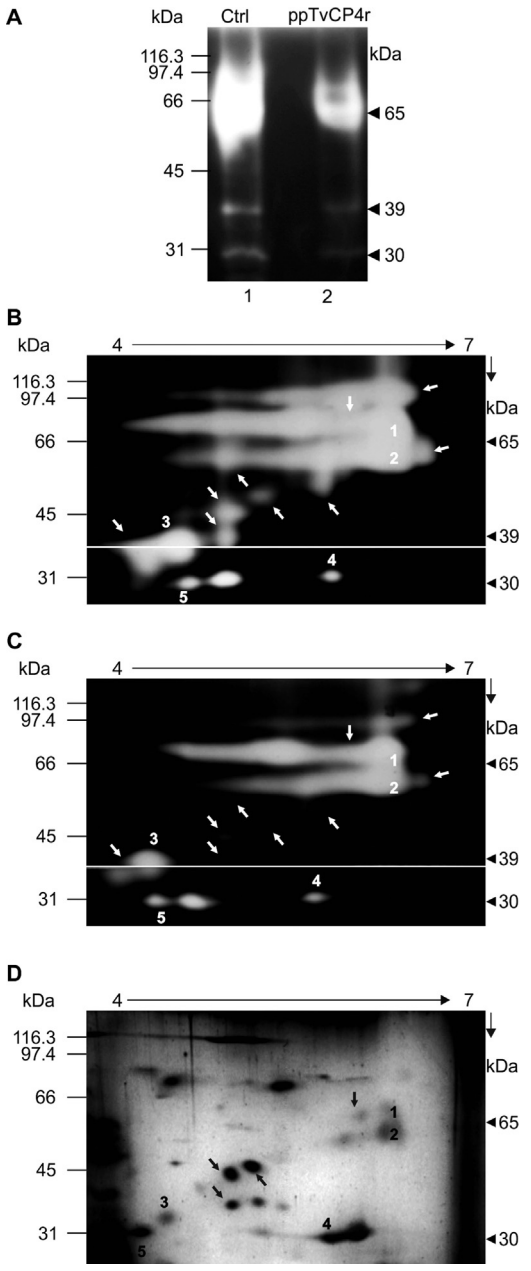


**Fig. 4.** Recombinant ppTvCP4 inhibits CPs from *T. vaginalis*. 1-D and 2-D zymograms of the proteolytic activity of TvPRE obtained from live parasites pretreated with ppTvCP4r. (A) 1-D zymogram of the proteolytic activity patterns of TvPRE from parasites without (lane 1) or with (lane 2) ppTvCP4r treatment before lysis. (B) 2-D zymograms of the proteolytic activity of TvPRE from parasites without ppTvCP4r treatment before lysis. (C) 2-D zymograms of the proteolytic activity of TvPRE from parasites with ppTvCP4r treatment before lysis. (D) Silver-stained TvPRE pattern in 2-D 12% gels used as a control degradome pattern used to pick the spots for MS analysis. Arrows indicate the spots that were inhibited by ppTvCP4r. The numbers indicate the spots that were identified by MS/MS analysis. Molecular weight markers are shown in kilodaltons. Arrowheads indicate the molecular weights of the proteolytic activity bands and spots in kDa. Before activation, the 2-D zymograms were cut to separate the gels in two sections: low and high molecular weight CPs, since the 30 kDa region requires longer activation time than the rest of the zymogram to fully develop the proteolytic activity.

Supplementary Tables S2 and S3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.12.001.

### 3.5. Inhibitory effect of ppTvCP4r in haemolysis

To determine whether the treatment of parasites with ppTvCP4r may affect the virulence of *T. vaginalis*, we investigated the

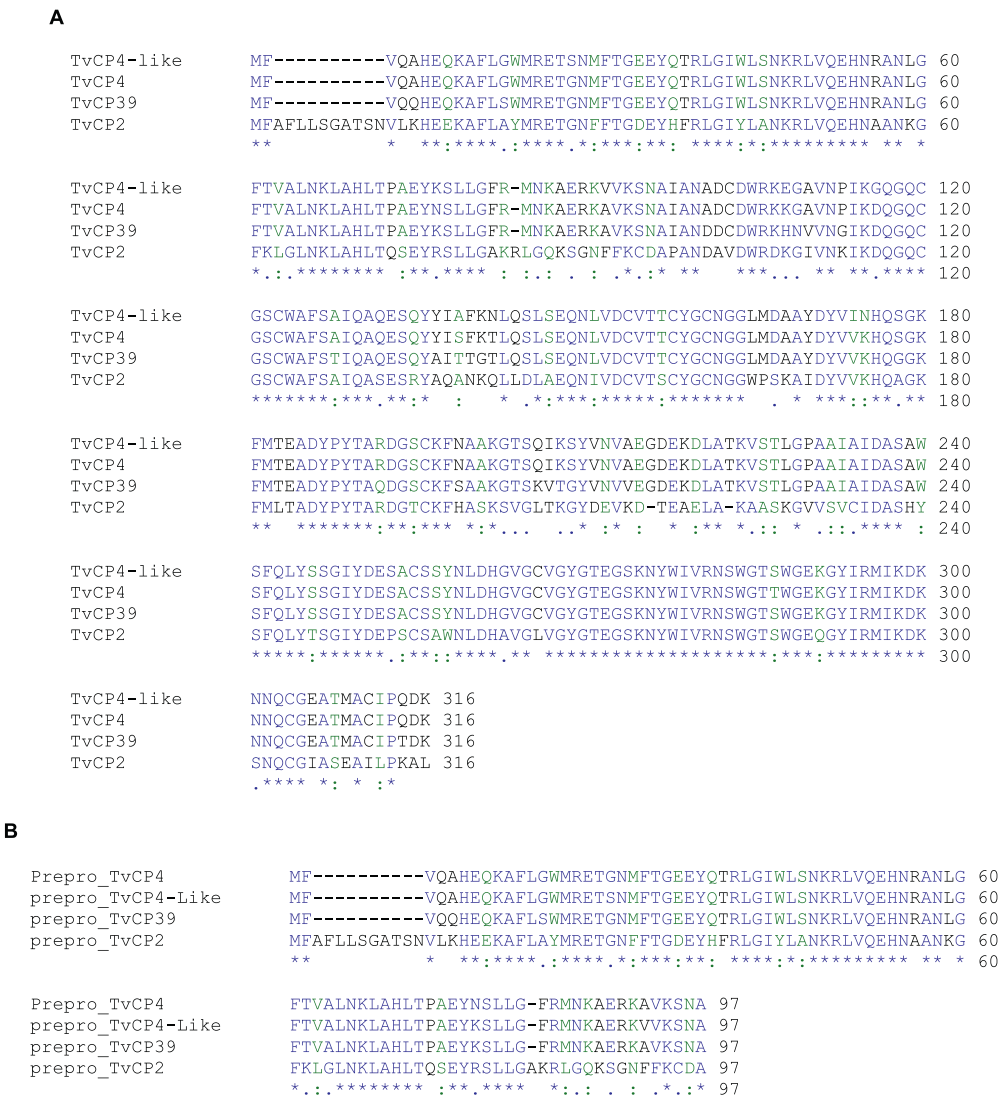


**Fig. 5.** Multiple amino acid sequence alignment of *T. vaginalis* CPs from clan CA that were inhibited by ppTvCP4r. (A) Alignment of complete sequence of CPs. (B) Alignment of prepro regions of CPs identified by MS/MS from Fig. 4. The analysis was performed with the CLUSTALW (1.81) program.

inhibitory effect of the ppTvCP4r against haemolysis, since the cognate CP (TvCP4) is involved in haemolysis (Cárdenas-Guerra et al., 2013). Fig. 6A shows that pretreatment of live parasites with increasing concentrations of ppTvCP4r (0.05, 0.15, 0.3, 0.9 μM) inhibited up to 55% trichomonal haemolysis as compared with untreated parasites that was taken as 100% erythrocyte lysis or E-64 treated parasites that showed ~80% inhibition of the trichomonal haemolysis, a papain-specific CP inhibitor used as a positive control of the participation of CPs in haemolysis (Dailey et al., 1990). These data show that this recombinant exogenous CP inhibitor is a useful tool to study the participation of cathepsin L-like CP in the biology of other organisms as virulence factors.

In addition, we checked for the presence of this TvCP4 prepro region as an individual protein during infection by searching for a ~9.5 kDa protein in vaginal washes from trichomoniasis patients using a polyclonal antibody produced against the ppTvCP4r protein. However, our results were negative probably because this protein is degraded as soon as it is cleavage off from the precursor protein during the activation process (Turk et al., 2012). Instead, we searched for the presence of antibodies produced during infection against the prepro region of TvCP4 that can react with the ppTvCP4r protein used as antigen. To our surprise, only the sera from trichomoniasis patients reacted with it as compared with sera from patients with other vaginitis (Fig. 6B). However, not all trichomoniasis patient sera (4/6) showed detection of a ppTvCP4r clear band. Our data show as an indirect evidence that this region is expressed during infection and is immunogenic. However, we could not discard that this reactivity could be due to the prepro region that is still in the cognate precursor TvCP4 protein.

## 4. Discussion

The cathepsin L CPs are synthesized as inactive precursors that contain a prepro region and a mature enzyme or catalytic domain that possesses proteolytic activity. During precursor activation, the prepro region, which acts as an inhibitor of the mature enzyme, must be removed by an autocatalytic reaction (by changing the pH or redox conditions) or with the aid of another enzyme to produce the active form (mature enzyme) (Rzychon et al., 2004; Kominami et al., 1988). Of the multiple tasks performed *in vivo* by the cathepsin L prepro region, the most remarkable is its ability to inhibit the proteolytic activity of its cognate CP. Thus, in this work, we determined whether the recombinant TvCP4 prepro region has the ability to inhibit the proteolytic activity of certain CPs in *T. vaginalis* as an exogenous inhibitor and affect some of its virulence properties in which the CPs are involved. Our results show that the recombinant

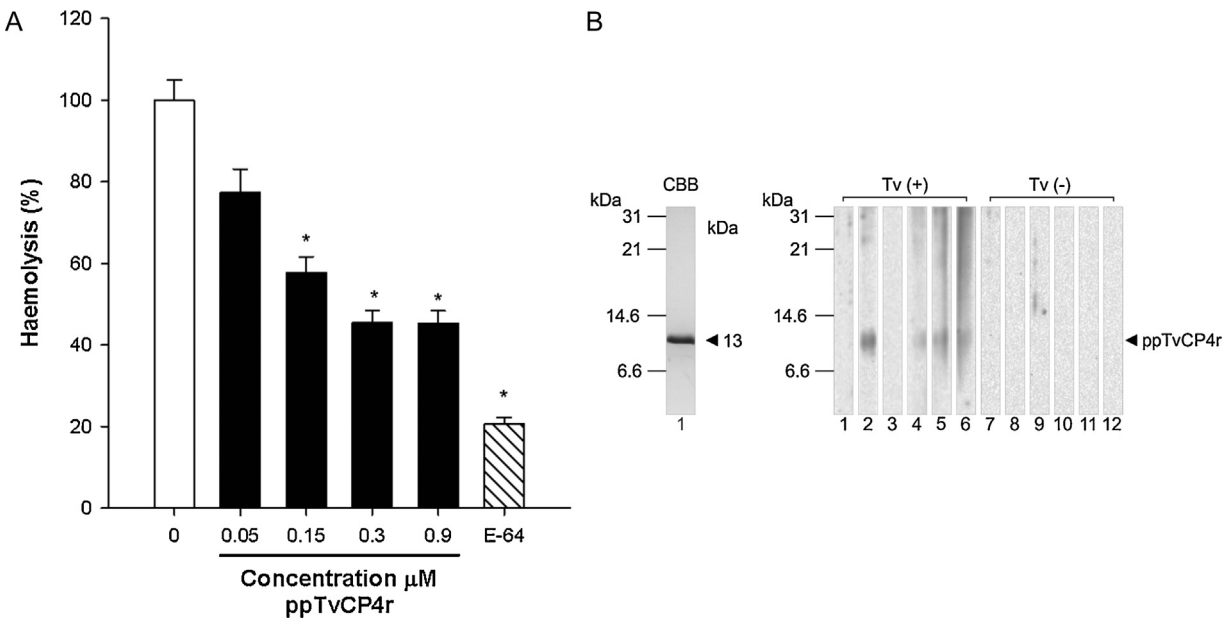


**Fig. 6.** Recombinant ppTvCP4 inhibits *T. vaginalis* haemolysis. (A) Percent of the haemolytic activity of parasites for human erythrocytes under acid conditions in the absence (white bar) or in the presence of different concentrations (0.0, 0.05, 0.15, 0.3, 0.9 μM) of exogenous ppTvCP4r inhibitor (black bars), or specific CP inhibitor E-64 (100 μM) as a positive control (hatched bar). Total lysis of human erythrocytes by trichomonads without ppTvCP4r treatment was used as the positive control and arbitrarily taken as 100% haemolysis (white bars). Significant differences ($P < 0.001$) were found for 0.15–0.9 μM ppTvCP4r or E-64 treatment, as marked with asterisks, when compared with 100% haemolysis. The error bars indicate the average of three experiments plus the SEM. (B) Western blot assay using the sera from patients with clinical trichomoniasis that had been confirmed using *in vitro* culture Tv (+) (lanes 1–6) or from patients with other types of vaginitis Tv (−) (lanes 7–12) (Supplementary Table S1) were used at 1:1000 dilution against the recombinant ppTvCP4r protein as antigen separated on 15% SDS-PAGE gel and transferred onto NC membranes. The lines on the left side of each panel indicate the molecular weight markers in kilodaltons.

ppTvCP4 is an inhibitor of cathepsin L-like but not legumain-like CPs of *T. vaginalis* capable of reducing trichomonal haemolysis in a concentration dependent manner of ppTvCP4r pretreated parasites.

The theoretical model of the structure of the TvCP4 prepro region is very similar to the typical structure of procathepsin L prepro regions (Coulombe et al., 1996; Jerala et al., 1998). The ppTvCP4 region has a compact domain composed of three alpha-helices and also has a less ordered chain in an extended conformation across the active site cleft, which inhibits enzyme activity by sterically preventing the substrate from accessing the active site. Moreover, in the helical domain, ppTvCP4 has a hydrophobic core containing two tryptophans that can be used as a convenient spectroscopic probe for investigating its conformation by CD (Carmona et al., 1996) and fluorescence emission spectra.

The CD spectrum is typical of a protein rich in alpha-helices and, as expected, quite similar to the CD spectra of the propeptide of human cathepsin L (Jerala et al., 1998) in contrast to the temperature-denatured ppTvCP4r CD spectra. These results together with the fluorescence emission spectra show that the refolded recombinant ppTvCP4 acquired a stable and functional conformation as an exogenous cathepsin L-like CP inhibitor (Figs. 2 and 3).

It is well know that prepro regions of papain-like CPs are inhibitors of their cognate enzymes, exhibiting high selectivity for them (Fox et al., 1992; Taylor et al., 1995; Carmona et al., 1996; Volkel et al., 1996; Maubach et al., 1997) and indeed, ppTvCP4r inhibited cathepsin L-like but not legumain-like trichomonad CPs, as expected. Additionally, legumains lack a prepro region and are processed at both the amino and carboxyl termini to become active enzymes (Mottram et al., 2003).

Interestingly, pretreatment of live parasites with exogenous ppTvCP4r inhibited CPs localized at the surface of *T. vaginalis*, such as TvCP4 and other CPs in the ~65, 39, and 30 kDa regions with high identity to TvCP4 that affected the trichomonal virulence by reducing the levels of trichomonal haemolysis in a concentration dependent manner (Fig. 6). Noteworthy to mention that, although ppTvCP4r at 0.9 μM abolished the cathepsin L-like proteolytic activity in the TvPRE (Fig. 3B) the maximum haemolysis inhibition with ppTvCP4r was observed at 0.3 μM, up to 55% and three-fold more inhibitor (0.9 μM) no greater inhibition was obtained (Fig. 6A). This could suggest that at least half of the haemolytic activity of *T. vaginalis* is due to the cathepsin L-like TvCP4 and support that other virulence factors such as a non-secreted 30 kDa CP that degrades spectrin (Fiori et al., 1997, 1999), pore-forming proteins (Fiori et al., 1996) and phospholipase A1 and A2 (Vargas-Villarreal et al., 2005) are also participating in haemolysis, another multifactorial virulence property of *T. vaginalis*. Interestingly, the inhibitory effect of E-64 (80%) in haemolysis was more drastic than with ppTvCP4r (55%). This could suggest that E-64 inhibits both CPs (TvCP4 and the non-secreted 30 kDa CP) whereas ppTvCP4r inhibits only TvCP4.

These data are consistent with the use of exogenous CP inhibitors to identify the involvement of CPs in trichomonal virulence (Arroyo and Alderete, 1989, 1995; Alvarez-Sánchez et al., 2000; Mendoza-López et al., 2000; Hernández-Gutiérrez et al., 2003, 2004). Thus, the ppTvCP4r inhibitor could also affect other trichomonal virulence properties such as cytoadherence, cytotoxicity, and induction of apoptosis in human cells in which cathepsin L-like CPs are involved (Arroyo and Alderete, 1989, 1995; Alvarez-Sánchez et al., 2000; Mendoza-López et al., 2000; Hernández-Gutiérrez et al., 2003, 2004; Ramón-Luing et al., 2011; Sommer et al., 2005; Kummer et al., 2008).

Results of the recombinant ppTvCP4r protein as a CP inhibitor are also consistent with the behavior of the recombinant trichomonad endogenous cystatin-like inhibitor, TC-2r recently described (Puente-Rivera et al., 2014). The recombinant TC-2r protein inhibited trichomonad proteolytic activity of cathepsin L-like

CPs in a concentration dependent manner and some spots with in the zymograms. Furthermore, pretreatment of live parasites with TC-2r inhibited trichomonal cytotoxicity, since one of the major target proteinases that is associated with, TvCP39, is involved in cellular damage, another surface CP of *T. vaginalis* (Puente-Rivera et al., 2014; Ramón-Luing et al., 2011).

Up to now, we have been unable to demonstrate the processing steps of the precursor TvCP4r to obtain a mature enzyme and to recover the cleavage peptides to identify the aa where the cleavage occurs to release the prepro region, as previously shown with EhCP112 (Quintas-Granados et al., 2009). Although, we could not detect the native preproTvCP4 after cleavage from the precursor TvCP4 during activation neither in culture supernatants, excretion-secretion material from *in vitro* secretion assays, or vaginal washes from trichomoniasis patients (data not shown), possible because after cleavage these peptides are immediately degraded (Turk et al., 2012). The presence of antibodies that recognized the TvCP4 prepro region in trichomoniasis patient sera (Fig. 6B) allowed us to show that this region is expressed during trichomonal infection and is immunogenic as the mature TvCP4 that has been considered as a potential biomarker for trichomoniasis (Ramón-Luing et al., 2010). However, we could not discard that this reactivity occurs when this region is still part of the precursor TvCP4 protein or that the antibodies generated against the native precursor are recognizing this recombinant fragment. Our results are consistent to those of the rCsCatL-propeptide reported by Li et al. (2012), which is a valuable candidate for specific IgG4 detection in sera from clonorchiasis patients. The cognate cathepsin L-like CP (CsCatL) was also considered a potential candidate for diagnosis (Yoo et al., 2011).

In 2-D zymograms of TvPRE using ppTvCP4r-treated parasites, we identified a number of the surface CPs previously identified in the degradome of *T. vaginalis* by mass spectrometry (Ramón-Luing et al., 2010). The nature of the inhibited CPs compared with the unaffected CPs indicated that ppTvCP4r is a specific inhibitor of cathepsin L-like *T. vaginalis* CPs that belong to clan CA. Although TvCP2, TvCP4-like, and TvCP39 share ~72% to 97% amino acid sequence identity within the mature portion of TvCP4, increased identity can be observed in their prepro region sequences (Fig. 5). Residues that are highly conserved play important roles in maintaining the globular fold of the N-terminal domain of the prepro region. However, it is important to consider that the selectivity is, in fact, different in each prepro region. The prepro regions exhibit the highest inhibition selectivity for the enzymes from which they originate, and the selectivity correlates with the degree of similarity in prepro region sequences of the target proteinases (Mach et al., 1994; Yamamoto et al., 2002). This aspect is important in the study of ppTvCP4r as an exogenous inhibitor because it has been suggested that the proteinases from *T. vaginalis* may be the result of gene duplication and mutations derived from a single TvCP ancestor (Jia et al., 2008), which appears to be TvCP4. Therefore, *in vitro* ppTvCP4r could inhibit the *T. vaginalis* CPs that are similar to TvCP4. These results are consistent with those observed with C1A cysteine peptidases of *Trypanosoma cruzi* and *Plasmodium falciparum* that were inhibited by their cognate prepro regions (Lalmanach et al., 1998; Pandey et al., 2009). The prepro regions of C1A CPs have been shown to be tight-binding inhibitors of their cognate enzymes; however, they are also inhibitors of related peptidases (Wiederanders et al., 2003). Thus, to fully address the selectivity of prepro region inhibition, both intraspecific and interspecific inhibitory effects must be considered (Wiederanders, 2003). The selectivity of prepro region inhibition is a crucial feature that must be addressed before propeptides can be used as biotechnological tools. Our finding show that the recombinant TvCP4 prepro region from *T. vaginalis* is an exogenous inhibitor of CPs with high aa sequence identity that belong to clan C1A and could be useful to identify and characterize cathepsin L-like CPs as virulence factors in other pathogens.

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