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Leptospira interrogans thermolysin refolded at high pressure and alkaline pH displays proteolytic activity against complement C3

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ABSTRACT

Enzymes from the thermolysin family are crucial factors in the pathogenesis of several diseases caused by bacteria and are potential targets for therapeutic interventions. Thermolysin encoded by the gene LIC13322 of the causative agent of leptospirosis, *Leptospira interrogans*, was shown to cleave proteins from the Complement System. However, the production of this recombinant protein using traditional refolding processes with high levels of denaturing reagents for thermolysin inclusion bodies (TL-IBs) solubilization results in poor recovery and low proteolytic activity probably due to improper refolding of the protein. Based on the assumption that leptospiral proteases play a crucial role during infection, the aim of this work was to obtain a functional recombinant thermolysin for future studies on the role of these metalloproteases on leptospiral infection. The association of high hydrostatic pressure (HHP) and alkaline pH was utilized for thermolysin refolding. Incubation of a suspension of TL-IBs at HHP and a pH of 11.0 is non-denaturing but effective for thermolysin solubilization. Soluble protein does not reaggregate by dialysis to pH 8.0. A volumetric yield of 46 mg thermolysin/L of bacterial culture and a yield of near 100% in relation to the total thermolysin present in TL-IBs were obtained. SEC-purified thermolysin suffers fragmentation, likely due to autoproteolysis and presents proteolytic activity against complement C3 α-chain, possibly by a generation of a C3b-like molecule. The proteolytic activity of thermolysin against C3 was time and dose-dependent. The experience gained in this study shall help to establish efficient HHP-based processes for refolding of bioactive proteins from IBs.

1. Introduction

Leptospirosis, caused by pathogenic bacteria of the genus *Leptospira*, is an important medical and veterinary problem and is presumed to be the most widespread zoonosis in the world. Leptospires are currently classified in more than 300 serovars grouped in 22 species. Phylogenetic analyses allowed grouping of *Leptospira* species into three distinct clades that vary in pathogenicity. They include ten pathogenic species, five intermediate species that cause mild clinical manifestations and seven saprophytes [1]. These spirochetes produce several proteases potentially able of causing tissue damage. We have demonstrated that proteins secreted by pathogenic *Leptospira* strains degrade complement proteins, potentially contributing to immune evasion [2]. Analysis of the available *Leptospira* genomes allowed identification of four genes coding for thermolysins that are present only in the pathogenic species [1,3]. The one encoded by LIC13322 was shown to be secreted and displayed proteolytic activity against complement C3 and C6 [2,4]. Thermolysin-like metalloproteases secreted by different pathogens are known to degrade various host substrates [5,6]. So, it is plausible to assume that *Leptospira* thermolysins may also target an array of molecules, thus contributing to bacterial dissemination and survival within the host. However, further studies to fully address the role of these metalloproteases on the invasion and immune evasion processes will require an efficient production of these proteolytic enzymes.

Abbreviations: GdnHCl, guanidine hydrochloride; HHP, high hydrostatic pressure; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani broth; LS, light scattering; TL-IB, inclusion bodies of thermolysin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography.

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According to our previous published data, the refolding of thermolysin from inclusion bodies using conventional protocols that require large amounts of denaturing reagents produced low levels of protein that presented low enzymatic activity [2].

The production of bioactive proteins for functional studies is extremely important for scientists working in different fields. However, many of the proteins of relevant biomedical value are found at low concentration in their native sources and therefore their production using alternative systems is a necessity. Genetically modified bacteria *Escherichia coli* are extremely efficient hosts for the large-scale production of heterologous proteins [7]. The obtainment of soluble recombinant proteins in the cytoplasm of *E. coli* is generally chosen as the favorite form of production since these molecules usually acquire the native state without the need for further processing. However, it has been described that approximately 70% of proteins from eukaryotic organisms have the tendency to form intermolecular bonds at the cost of intramolecular contacts characteristic of native proteins and also tend to aggregate and accumulate as insoluble inclusion bodies (IBs) in the cytoplasm of *E. coli*, especially when the level of expression of the recombinant protein is high [8,9]. Nonetheless, there are advantages regarding the production of recombinant proteins as IBs, and one example is the facility of separation of the insoluble aggregates from the soluble fraction of the bacterial cytoplasm enabling the production of IBs with low levels of contaminants and reducing the need of purification steps. Another advantage in the case that the target protein suffers from autoproteolysis is its protection in an inactive state as IBs until refolding. However, the production of biologically active heterologous proteins from IB requires the setup of an efficient refolding process. Establishing a simple and efficient protocol for the refolding of proteins in IB is a difficult task [10]. Traditional refolding processes use high levels of denaturing reagents for solubilization, but removal of these reagents frequently cause reaggregation and poor recovery of bioactive proteins [11]. As an alternative to these methods, the native-like secondary structures of proteins can be usefully maintained if the solubilization step does not completely unfold the proteins in IBs.

High hydrostatic pressure (HHP) favors protein states with lower volumes and minimal cavity spaces in response to the need to decrease the volume of the system. In HHP and aqueous solution, water penetrates the proteins, leading to an increased exposure of hydrophobic groups to solvent and increased protein ionization. Relatively high pressure (> 10 kbar) is required for the denaturation of proteins [12], whereas moderate pressures (1–3 kbar) have been reported to be effective in dissociating protein oligomers and aggregates at relatively non-denaturing conditions [12,13]. Application of HHP associated with low levels of denaturing reagents such as guanidine hydrochloride (GdnHCl) has been described as an alternative of solubilization of IBs for subsequent refolding [14,15].

Alkaline pH solubilizes protein aggregates by electrostatic repulsion [16]. Another possibility that has been described for solubilization of IBs and subsequent refolding is the utilization of high pH, also in association to low levels of chaotrope reagents [17,18].

The aim of this study was to obtain high levels of enzymatically active LIC13322-encoded thermolysin from inclusion bodies (TL-IB) produced by *E. coli*. We have demonstrated that the association of high pressure and alkaline pH is a non-denaturing condition for solubilization of TL-IBs. Subsequent refolding of thermolysin to an active form was obtained by dialysis at pH 8.0 and purification at size exclusion chromatography (SEC). Purified thermolysin was shown to be fragmented, likely by autoproteolysis, and to cleave the C3 molecule of the complement system in a dose- and time-dependent fashion.

2. Material and methods

2.1. Proteins and antibodies

Complement C3 protein purified from human plasma was purchased from Complement Technology. Goat anti-human C3 and anti-goat IgG conjugated with peroxidase were purchased from Sigma-Aldrich.

2.2. Cloning and expression of Leptospira interrogans thermolysin

Thermolysin encoded by LIC13322 has a signal peptide for secretion, a fungalin/thermolysin propeptide (FTP) domain, a propeptide (PepSY) domain, and a catalytic domain M4 that includes peptidase_M4 and peptidase_M4_C. Cloning and expression of the prothermolysin fragment PepSY-M4 presenting 74 kDa in *E. coli*, were performed as described elsewhere [2,19].

2.3. Leptospira strains and culture supernatants

Leptospira interrogans serovar Kennewicki strain Fromm (LPF) and *Leptospira biflexa* serovar Patoc strain Patoc I (Patoc) were cultivated for 7 days at 29 °C under aerobic conditions in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium base (Difco) supplemented with heat-inactivated rabbit serum (10%), L-asparagine (0.015%), sodium piruvate (0.001%), calcium chloride (0.001%) and magnesium chloride (0.001%), as described [19]. Virulence of the strain LPF is regularly maintained by iterative passages in hamsters. Both strains were kindly provided by Prof. Marcos Heinemann from the School of Veterinary Medicine and Animal Sciences (FMVZ), University of São Paulo. Culture supernatants containing leptospiral secreted proteins were obtained according to Fraga et al. 2014 [2]. Briefly, leptospires were washed twice in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4), and 1×10^9 bacteria were incubated at 37 °C for 4 h. The supernatants were collected after centrifugation, filtrated, and used in the C3 cleavage assays described below.

2.4. Treatment of TL-IBs at HHP

Suspensions of thermolysin IB (TL-IBs) were diluted in refolding buffer (50 mM Tris HCl buffer containing 1 mM EDTA for pH 7.0–9.0 or 50 mM CAPS buffer containing 1 mM EDTA for pH 10.0–12.0) and placed into plastic bags, which were sealed and all the samples were placed into a larger plastic bag that was vacuum/heat-sealed. The bags were placed into a pressure vessel (R4-6-40, High Pressure Equipment) and pressure (2.4 kbar) was applied using oil as pressure-transmitting fluid. After 90 min, the samples were decompressed to 0.4 kbar and maintained in that condition for 14 h 30 min before complete decompression to 1 bar. For determinations of fluorescence and of light-scattering (LS) at HHP, a round quartz cuvette filled with the suspension of TL-IBs and sealed with flexible polyethylene cap was placed into a high-pressure cell (ISS Inc, Champaingn, IL, USA) equipped with 3 optical sapphire windows connected to a pressure generator. Water was utilized as pressure-transmitting fluid. The high-pressure cell was placed inside a spectrofluorometer for light scattering and fluorescence determinations.

2.5. Fluorescence and light-scattering (LS) measurements

Fluorescence and Light Scattering (LS) were collected on a Cary Eclipse spectrofluorometer (Varian). Cuvettes of 1 cm path were used for determinations performed at atmospheric pressure. For determinations at HHP, round quartz cuvettes with caps were used.

The LS measurements were accomplished at 320 nm, at an angle of 90° relative to the incident light, and emission were recorded from 315 to 325 nm. Tryptophan emission fluorescence measurements were carried out with the excitation wavelength at 290 nm. The emission fluorescence spectra were collected between 300 and 400 nm, using a response time of 1 s and a scan speed of 240 nm/min.

2.6. Size exclusion chromatography (SEC)

Superdex 200 10/300 column (GE Healthcare Life Sciences) coupled to an AKTA system (GE Biosciences) was used for analysis/purification of the supernatants of TL-IBs that had been subjected to HHP. The buffers utilized for elution were 50 mM sodium phosphate buffer at a pH of 8.0 or 50 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer at a pH of 10.0 with a flow rate of 0.7 mL/min. The fractions eluted were frozen and analyzed afterwards.

2.7. Assessment of thermolysin proteolytic activity against C3

Recombinant thermolysin and *Leptospira* culture supernatants (0.5 μg of total secreted proteins) were incubated with purified C3 (0.5 μg) at 37 °C. Thermolysin doses and incubation times are indicated in the figure legends. Thermolysin proteolytic activity was inhibited by pre-incubating this metalloprotease (2 μg) with

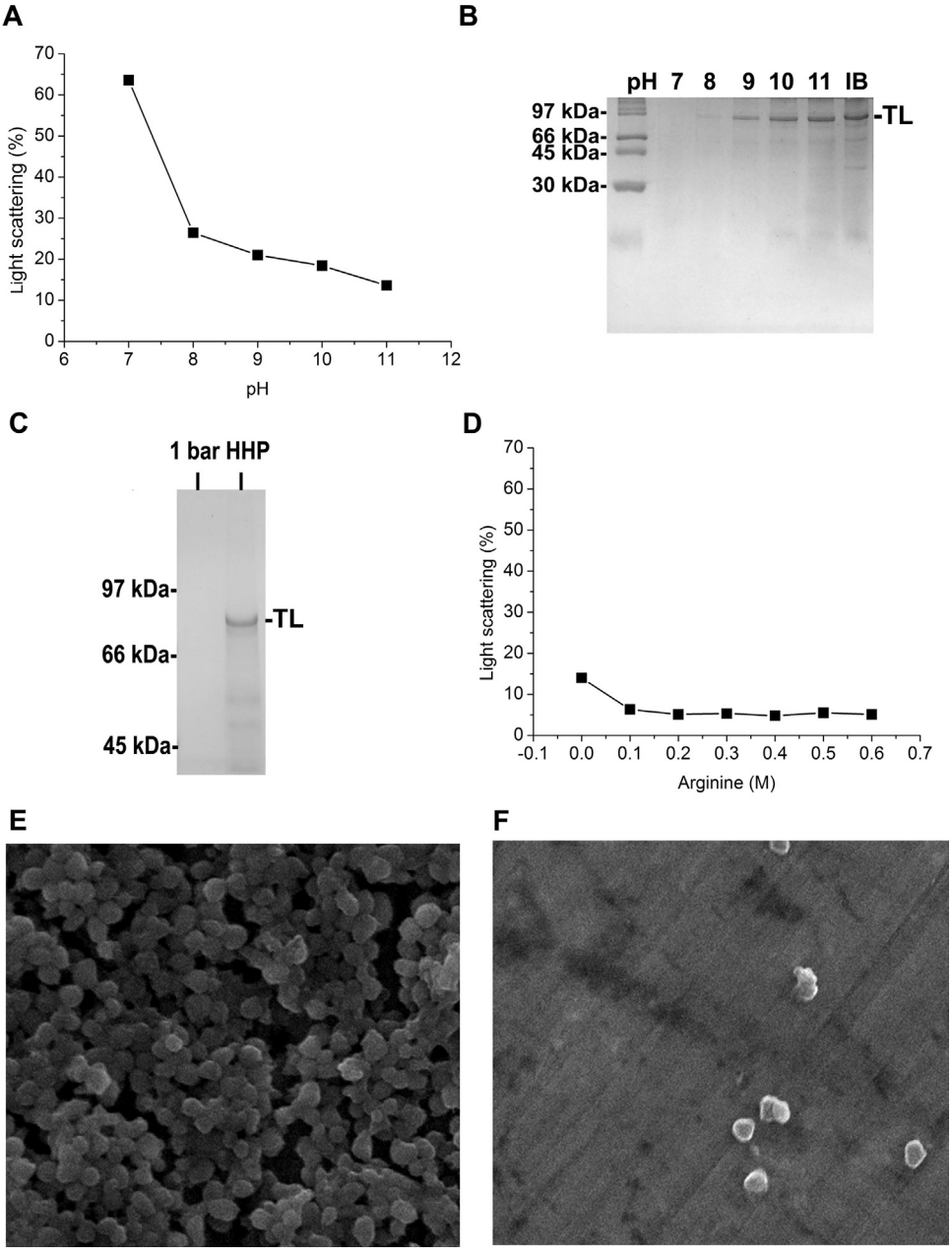

Fig. 1. Application of HHP at alkaline pH induces dissociation of TL-IBs. Suspensions of TL-IBs were subjected to 2.4 kbar/0.4 kbar at 20 °C. A, LS vs pH in 50 mM Tris HCl (pH of 7.0–9.0) or 50 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) (pHs of 10.0 and 11.0) containing 1 mM EDTA; B, soluble fractions of the suspensions of TL-IB subjected to HHP analyzed by SDS-PAGE and the same volume of a suspension of TL-IB maintained at 1 bar (indicated as IB); C, soluble fractions of suspensions of TL-IB subjected to 1 bar or to HHP for 16 h analyzed by SDS-PAGE; D, curve of LS vs arg in 50 mM CAPS, pH 11.0 containing 1 mM EDTA. The LS was measured after complete decompression. The area of LS peak at atmospheric pressure and pH 7.0 before compression (LS_0) was set as 100%. The samples were boiled and reduced before application in SDS-PAGE; E, scanning electron microscopy of TL-IB and F, scanning electron microscopy of insoluble fraction of suspensions of TL-IB subjected to HHP and a pH of 11.0 (5000x magnification).

5 mmol/L 1,10-phenanthroline for 30 min. C3 cleavage products were analyzed by Western blot. Samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 10% skimmed milk in PBS-T, cleavage fragments were detected by goat anti-human C3 polyclonal antibodies (1:5000), followed by peroxidase-conjugated anti-goat IgG (1:10,000). Positive signals were detected by enhanced chemiluminescence (West Pico, Pierce).

2.8. Scanning electron microscopy (SEM)

SEM was performed by drying water-dialyzed TL-IBs or the same volume of insoluble fraction of suspensions that were subjected to HHP at a pH of 11.0. Suspensions were applied onto Philips stubs that were dried and then gold sputter coated. The samples were photographed using a Philips XL-30 scanning electron microscope.

3. Results and discussion

3.1. Solubilization of IB of thermolysin (TL-IB) at HHP

In the present study, we determined the range of pH that in association to HHP, could efficiently solubilize thermolysin inclusion bodies (TL-IBs). We have previously shown that 2.4 kbar induces solubilization of IBs and that incubation at 0.4 kbar is a condition in which protein folding may occur but reaggregation can be inhibited [20]. Therefore, in order to obtain TL-IB solubilization, suspensions were submitted to 2.4 kbar for 90 min, decompressed to 0.4 kbar and incubated for 14 h 30 min (2.4 kbar / 0.4 kbar) for thermolysin folding before decompression to atmospheric pressure. Solubilization of TL-IBs was determined by the reduction in light scattering (LS) values at visible light wavelength. The application of HHP in association with alkaline pH resulted in the solubilization of the TL-IBs. A reduction of the value of LS to less than 20% was obtained by the application of HHP in association to alkaline pH of 10.0 and 11.0 (Fig. 1A). A high yield of soluble thermolysin was obtained by application of HHP to suspensions of IBs at pH of 10.0 and 11.0, as shown by the analysis of the soluble fractions of the pH curve by SDS-PAGE (Fig. 1B). Incubation of the suspension of TL-IB for the same period of time (16 h) at 1 bar and pH of 11.0, however, did not render soluble thermolysin (Fig. 1C). The amino acid arginine (0.1–1 M) is customarily included in solvents used for refolding of proteins because of its effects of suppression of protein aggregation. There is similarity between the effect of arginine and GdnHCl on protein unfolding, although the amino acid has lower denaturing effect [21,22]. To determine if the presence of arginine could improve the yield of soluble thermolysin, we utilized this amino acid during compression of the TL-IBs. In fact, the presence of arginine, even at the lowest concentration evaluated (0.1 M), and at a pH of 11.0 promoted a further decay in the LS values to less than 7% (Fig. 1D). TL-IBs that can be seen in a scanning electron microscopy photography (Fig. 1E) were almost fully solubilized by treatment at HHP and a pH of 11.0 (Fig. 1F).

3.2. Determination of unfolding of thermolysin at HHP

We considered that solubilization under mild conditions would possibly avoid the loss of secondary and tertiary structures similar to native ones that are frequently present in IBs. Therefore, in order to use non-denaturing conditions for TL-IB solubilization, we monitored the level of thermolysin unfolding at HHP. The prothermolysin PepSY-M4 encoded by LIC13322 presents 13 residues of Trp. Conformational alterations of this protease in IB by application of HHP were monitored by determination of shifts of the intrinsic fluorescence of Trp. The maximum value of fluorescence intensity (λ maximum) of TL-IBs suspension at a pH of 7.0 and that had not been subjected to HHP is 339.5 nm. The sample incubated in a fully denaturing condition (6 M guanidine) presents a shift of 15.8 nm of fluorescence to a λ maximum of 355.3 nm, indicating the total exposure of the Trp residues to the aqueous solvent and the complete thermolysin unfolding (Fig. 2A). The fluorescence spectra of suspensions of TL-IBs during incubation at HHP were monitored using a high-pressure cell with quartz windows adapted to a spectrofluorometer. Suspensions of TL-IBs were subjected to increasing pressures (0.5–2.5 kbar), maintained for 15 min in each condition, followed by incubation at 2.4 kbar/0.4 kbar (Fig. 2B). The λ maximum of fluorescence was shifted to red by the increment of pressure levels, indicating the unfolding of the protein. The shift was higher at a pH of 11.0 in the presence of 0.1 M arginine, reaching a λ maximum at 2.5 kbar (355.3 nm). However, incubation at 0.5 kbar led to a decay of the λ maximum fluorescence wavelength, which was further reduced by incubation at atmospheric pressure. The results show that the thermolysin was unfolded by application of HHP, but that the unfolding is almost totally reverted by incubation at atmospheric pressure.

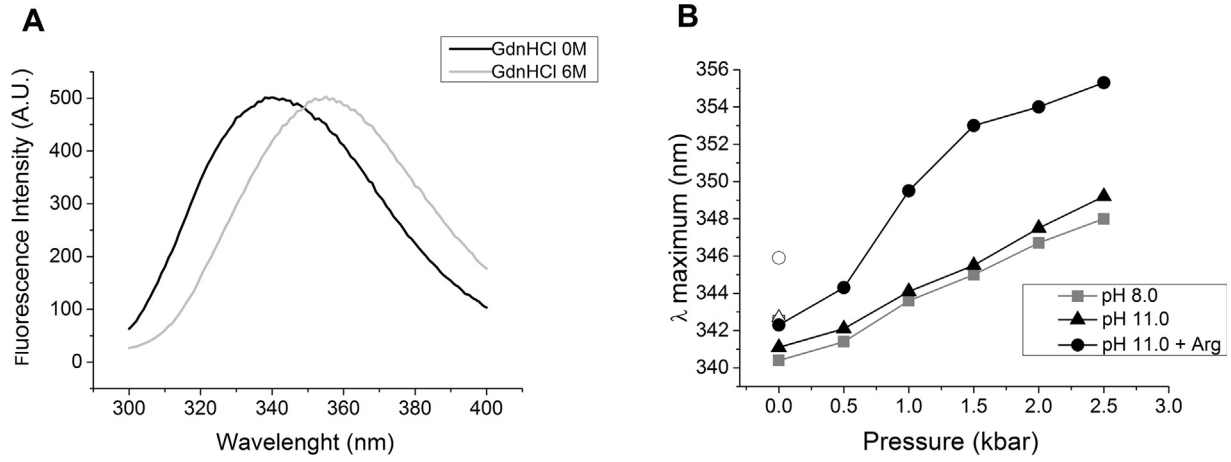

Fig. 2. Application of HHP induced partially reversible changes in the maximum of intrinsic fluorescence of TL-IBs. A, Intrinsic fluorescence of thermolysin in the absence or in the presence of 6 M guanidine hydrochloride; B, Effect of pressure on the λ maximum intrinsic fluorescence of TL-IBs. The suspensions were subjected to increasing pressure levels and incubated for 15 min before fluorescence determination. Open signals indicate the maximal fluorescence obtained 2 h after complete decompression. For fluorescence determination, excitation was performed at 290 nm and the emission was measured between 300 and 400 nm.

We analyzed alterations of the fluorescence spectra of suspensions of TL-IBs subjected to HHP in the same conditions shown in Fig. 1. The protein that was subjected to HHP and pH 11.0 presents a λ maximum shift of 3.0 nm to higher wavelength (342.5 nm), indicating small degree of exposure of the Trp to the aqueous solvent and only partial protein unfolding (Fig. 3A). The presence of 0.3 M arginine promoted a shift in the λ maximum of 10.4 nm (to 349.9 nm) (Fig. 3B), indicating that the protein was not fully unfolded by application of HHP in the presence of this amino acid.

3.3. Refolding and purification of thermolysin

To evaluate whether the HHP-solubilized thermolysin does undergo significant reaggregation after lowering the pH to a near-physiological value, we subjected suspensions to 2.4 kbar / 0.4 kbar at a pH of 11.0 and after decompression the supernatants were subjected to an overnight dialysis against buffer at a pH of 11.0 or at a pH of 8.0 and the supernatants and the insoluble pellets were analyzed by SDS-PAGE. No bands corresponding to thermolysin could be detected in the insoluble fractions of the samples, which indicate that the thermolysin did not reaggregate to insoluble condition (Fig. 4A). The concentrations of thermolysin in the sample that was not dialyzed and in the one that was dialyzed at a pH of 11.0 are similar. However, the band corresponding to thermolysin of the sample that was dialyzed at a pH of 8.0 had lost 72% intensity. Our supposition is that the thermolysin at a pH near the physiologic one (8.0) suffers from autoproteolysis. Therefore, to avoid thermolysin autoproteolysis, quick dialysis (3 h dialysis with 3 buffer changes at 4 °C) using buffers at pHs of 8.0 or of 10.0 were performed on the supernatants of the suspensions of TL-IB subjected to HHP at a pH of 11.0. This procedure was effective to avoid thermolysin degradation during dialysis. The concentrations of soluble thermolysin in the supernatants of the products of dialysis at a pH of 8.0 and of 10.0 are similar to the concentration of this protein in the same volume of the non-treated suspensions of TL-IB in the SDS-PAGE (Fig. 4B). These results show that the yield of thermolysin solubilization using high pressure at a pH of 11.0, followed by dialysis at a pH of 8.0 is near to 100%, which corresponds to 46 mg of soluble thermolysin from 1 L of bacterial culture. The supernatants of the suspensions of TL-IB subjected to HHP at a pH of 11.0 in the absence or in the presence of arginine (0.4 M) were also analyzed by SEC using the same buffers that were used for dialysis. The chromatogram of thermolysin that was dialysed and eluted in the buffer at the pH of 8.0 presented a peak at the volume of 12.1 mL (Fig. 4C). According to the linear regression line: $Y=-34.7\,X+494.7$ ($N=4$, $R^2=0.9557$), obtained for the molecular weight calibration of this column in the range of 44.3 kDa to 132.9 kDa, a volume of elution of 12.1 mL corresponds to a protein with a molecular weight of 74.8 kDa, that can therefore be assigned as monomeric full-length thermolysin, that presents 74.0 kDa. The protein that was dialyzed and analyzed on SEC at a pH of 10.0 eluted in a volume of 10.2 mL, which corresponds to a molecular weight of 140.7 kDa, which is likely a dimer of thermolysin, that would present 148 kDa. At a pH of 11.0 the protein presents the same behavior (data not shown). These results indicate that thermolysin dimers are formed by solubilization of the aggregates during incubation at HHP and alkaline pH and that lowering the pH to 8.0 promotes their dissociation into monomers.

It has been described that thermolysins present autoproteolysis and that the presence of the propeptides can be inhibitors of their own catalytic domain [23,24]. PepSY-thermolysin (74 kDa) is found in the supernatants of TL-IBs that were subjected to HHP at a pH of 11.0 and dialyzed. SEC-purified thermolysin also present another band with slightly higher migration pattern than the 74-kDa band in SDS-PAGE, suggesting that purified thermolysin was autoprocessed, possibly losing the propeptide (Fig. 4D). Thermolysin that were SEC-purified (peaks at 10.0 mL and at 12.1 mL in Fig. 4C) are more fragmented than non-purified protein (Fig. 4E). Therefore, it is reasonable to assume that the presence of the propeptide or other factors supposed to inhibit thermolysin autocatalysis may have been removed after SEC procedure, allowing autocatalysis to occur, thus generating smaller molecular weight fragments. In order to assess functionality of the thermolysin produced under the above-mentioned conditions, proteolytic activity against purified C3 was evaluated. Non-purified and SEC-purified thermolysin (Fig. 4E) were incubated with C3 for 5 h and C3 fragments were analyzed by Western blot with anti-human C3. Native C3 is formed by two chains: C3 α chain (110 kDa) and C3 β chain (70 kDa). A band of approximately 100 kDa corresponding to C3 α′ chain, and of an intact C3 β chain (70 kDa) were observed (Fig. 4F), suggesting the generation of a C3b-like fragment. Regardless of the pH, unpurified thermolysin did not present proteolytic activity against C3, however samples subjected to SEC presented activity. It is reasonable to suppose that autocalysis generates active thermolysin fragments thus enhancing C3-mediated cleavage. Samples dialyzed in a buffer at pH 8.0 were more active than those dialyzed at pH 10.0, what could be eventually attributed to a higher activity of the monomers of thermolysin at this particular pH. The fact that thermolysin solubilized at HHP and alkaline pH present activity shows that the protein is refolded. The presence of arginine during the application of HHP did not improve the yield, as shown in Fig. 4C, nor activity of thermolysin, as shown in Fig. 4F.

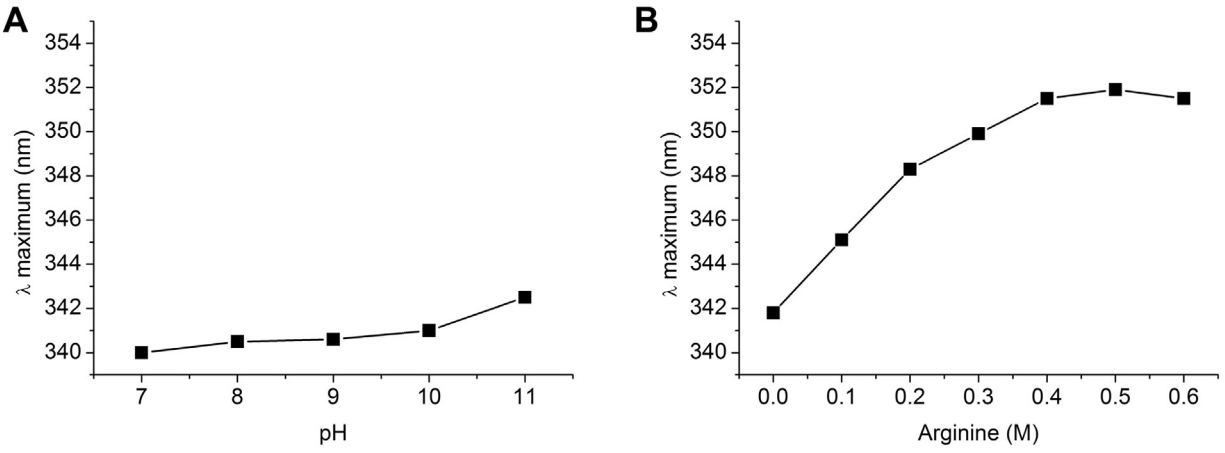

Fig. 3. HHP, alkaline pH and arginine induced partial unfolding of TL in TL-IBs. Suspensions of TL-IBs were subjected to 2.4 kbar/0.4 kbar at 20 °C. **A**, pH vs λ maximum; B, arginine vs λ maximum. The fluorescence was measured after complete decompression. The excitation was set to 290 nm, and emission was collected from 300 to 400 nm.

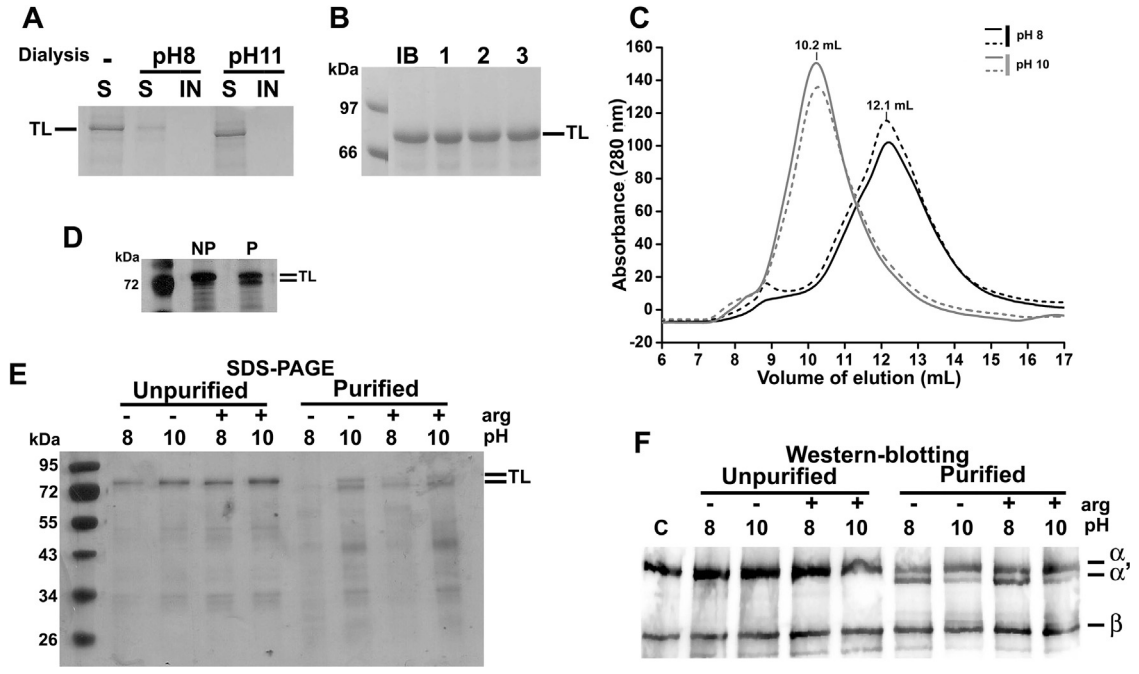

Fig. 4. Analysis of the thermolysin solubilized from TL-IB by application of HHP and pH of 11.0. TL-IB suspensions were subjected to HHP at a pH of 11.0 in the absence or in the presence of arg and the supernatants were dialysed. A, SDS-PAGE analysis of the soluble (S) and insoluble (IN) fractions of the overnight dialysis; B, SDS-PAGE analysis of the TL-IB and of soluble fractions after application of HHP/3 h dialysis. Column 1: soluble fraction without dialysis, column 2: soluble fraction after dialysis at pH 10.0, column 3: soluble fraction after dialysis at pH 8.0; C, purification in SEC (Superdex 200 10/300) of the supernatants of the product of dialysis at pH 8.0 or of 10.0 using the same pH as in the dialysis. The chromatograms of the samples that were subjected at HHP in the absence of arg are indicated as straight lines and the samples that were subjected at HHP in the presence of arg are indicated as dashed lines; D, SDS-PAGE analysis of non-purified (NP) or SEC-purified (P) thermolysin; E, SDS-PAGE analysis of non-purified or purified thermolysin as shown in C. At a pH of 10, the peak eluted at a volume of 10.2 mL and at a pH of 8.0 the peak eluted at 12.1 mL. Concentration of thermolysin was normalized; F, Western blotting analysis of the cleavage of C3 by non-purified or purified thermolysin as shown in C. TL: thermolysin.

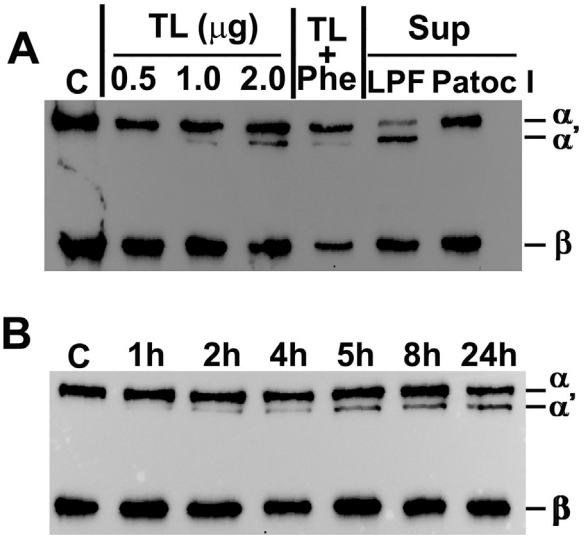

Fig. 5. Recombinant thermolysin cleaves C3 in a dose- and time-dependent fashion. (A) Purified C3 (0.5 μg) was incubated in PBS (C), with 0.5–2.0 μg of thermolysin (TL) or with 2.0 μg thermolysin pre-incubated with 1,10 phenanthroline (Phe). C3 was also incubated with 0.5 μg of total secreted proteins (sup) by *Leptospira interrogans* (LPF) or 0.5 μg of total secreted proteins by *Leptospira biflexa* (Patoc I). The samples were incubated at 37 °C for 5 h. (B) Purified C3 (0.5 μg) was incubated with 2.0 μg of thermolysin for 1 h, 2 h, 4 h, 5 h, 8 h or 24 h. Cleavages were analyzed by Western blot with anti-human C3 polyclonal antibody. The cleavage product of C3 α-chain is indicated.

3.4. Catalytic activity of thermolysin against C3

To further assess thermolysin activity against C3, different amounts of the recombinant protein were incubated with C3 for 5 h. A dose-dependent degradation of the C3 α-chain was observed, and cleavage was more pronounced in the presence of 2 μg of recombinant thermolysin (Fig. 5A, lanes 2–4). As expected, proteolytic activity was almost completely abolished by pre-incubating thermolysin with the Zn(2+)-chelating metalloprotease inhibitor 1,10 phenanthroline (Fig. 5A, lane 5). As native thermolysin (s) has(ve) been detected in the culture supernatants of several pathogenic *Leptospira* strains, but are not produced by saprophytic *Leptospira* strains [2], the supernantants of the virulent *L. interrogans* strain LPF and of the saprophytic *L. biflexa* strain Patoc I were included as positive and negative controls, respectively. Accordingly, no degradation was observed upon incubation with the Patoc I supernatant, but cleavage of C3 α-chain occurred both in the presence of refolded thermolysin and LPF supernantant (Fig. 5A, lanes 6 and 7). As the proteolytic activity against C3 was more efficient in the presence of the bacterial supernatant it is reasonable to assume that our recombinant thermolysin is not fully active or, alternatively, pathogenic *Leptospira* secrete other proteases capable of cleaving C3. Further studies will be required to address this issue.

Cleavage of C3 by recombinant thermolysin was also time-dependent. The C3b-like fragment deriving from the C3 α-chain can be barely detected after 1 h of incubation, but is fully visible after prolonged incubation times, such as 24 h (Fig. 5B).

4. Conclusions

Our study established an efficient method of production of enzymatically active thermolysin by refolding the protein expressed as inclusion bodies (IB) in *Escherichia coli*.

The application of HHP is a relatively mild condition to solubilize aggregated proteins in comparison to the methods that utilize high concentrations of denaturing agents [25]. We have previously shown that application of high pressure (2–3 kbar)

during 30–90 min solubilized the IBs of an enhanced form of green fluorescent protein (eGFP), but that the refolding of this protein did not occur at this pressure level. It was shown, however, that the refolding of eGFP occurs at lower pressure levels (0.35-0.70 kbar) [20]. The yield of eGFP refolding was improved by incubation at 0.35-0.7 kbar in comparison to the condition of direct decompression from 2.4 kbar possibly because at this pressure level there is inhibition of reaggregation, which is a usual problem of refolding processes [20,26], while folding of native eGFP occurs. The same scheme of compression/decompression was used in the present study for refolding of thermolysin, with the objective of avoid reaggregation and obtain high yield of a functional protease.

An unusual type of analysis for protein refolding methods was performed, a thorough analysis of the minimum conditions required for the solubilization of thermolysin. Our results have shown that the innovative association of high pressure and alkaline pH was very efficient for solubilization of TL-IB aggregates. However, unlike the traditional method of solubilization of IBs, the use of high concentrations of chaotropic agents, the unfolding of thermolysin promoted by the association of high pressure and alkaline pH was very low. The small degree of unfolding of thermolysin is likely the reason for the absence of protein re-aggregation when the two factors that promote solubilization were removed.

The whole process is extremely rapid and simple, based on the application of HHP to a suspension of TL-IBs to obtain the soluble protein, followed by a 3 h-dialysis to a lower pH and purification using SEC. No reaggregation was observed upon dialysis and the yield of soluble full-length thermolysin was of 100% in comparison to the protein present in IB. The volumetric yield was high, of 46 mg/L of bacterial culture. Thermolysin fragments that present enzymatic activity toward C3 were generated after SEC purification, thus indicating that the purification step is crucial for protein activity. Although less pronounced, proteolytic activity of purified thermolysin generated a similar C3 cleavage profile compared to that observed in the presence of the culture supernatant of *L. interrogans* strain LPF, a pathogenic strain known to secrete thermolysin(s), thus indicating the correct folding of the protein to a biologically active form. Considering that enzymes from the thermolysin family are crucial factors in the pathogenesis of several diseases caused by bacteria and are potential targets for therapeutic interventions, further studies aiming to extend our current knowledge on these *Leptospira* metalloproteases in the invasion and immune evasion processes will be conducted. The knowledge acquired in the current study will certainly help producing other leptospiral proteases whose functional characterization totally depends on proper solubilization and folding.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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