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Refolding of the recombinant protein OmpA70 from *Leptospira interrogans* from inclusion bodies using high hydrostatic pressure and partial characterization of its immunological properties

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ABSTRACT

Leptospira is the etiological agent of leptospirosis, a life-threatening disease that affects human populations worldwide. Available vaccines have demonstrated limited effectiveness, and therapeutic interventions are complicated by the difficulty of establishing an early diagnosis. The genome of *Leptospira* strains was sequenced, and bioinformatic analyses revealed potential vaccine and serodiagnosis candidates. The present work studied OmpA70, a putative outer membrane protein from *Leptospira interrogans* serovar Copenhageni that combines structural features of Loa22, the first genetically defined virulence factor in *Leptospira*, and Lp49, a protein that reacts with sera from early and convalescent patients. Recombinant OmpA was produced in *Escherichia coli* in an insoluble form. Considering the importance of the structural integrity of a protein to confer immune protection, high hydrostatic pressure (HHP) was used to refold OmpA70 aggregated as inclusion bodies. HHP was applied in association with redox-shuffling reagents (oxidized and reduced glutathione) and guanidine hydrochloride or L-arginine. About 40% of the protein was refolded by applying 200 MPa for 16 h in concentrations of L-arginine above 0.4 M. Circular dichroism revealed the presence of secondary structure. OmpA70 has immunogenic and antigenic properties as high antibody titers were seen after immunization with this protein, and sera from infected hamsters reacted with soluble OmpA70.

1. Introduction

Leptospira is the causative agent of leptospirosis, an important zoonotic disease throughout the world. Humans become infected by contact with water or soil containing urine from infected animals. Clinical manifestations range from subclinical infections to potentially lethal forms characterized by jaundice, renal and pulmonary dysfunction (Faine et al., 1999; Levett, 2001; Bharti et al., 2003; Dolhnikoff et al., 2007). There are more than 230 serovars among pathogenic leptospires, which imposes a major limitation on the development of a multi-serovar vaccine based on inactivated whole cells. The available vaccines neither induce long-term protection nor provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. The genome sequencing of pathogenic *Leptospira* strains has lead to the identification of proteins that may play a role in virulence (Ren et al., 2003; Nascimento et al., 2004; Bulach et al., 2006). Outer membrane proteins conserved among pathogenic serovars might be useful for leptospirosis diagnosis as well as for the production of a recombinant vaccine (Gamberini et al., 2005).

The gene LIC10537 encodes for OmpA, a putative outer membrane protein from *Leptospira interrogans* serovar Copenhageni. This protein has a C-terminal OmpA domain and WD40 repeats, combining structural features of two important leptospiral proteins, Loa22 and Lp49. Loa22 has a C-terminal OmpA domain and it is the first genetically defined virulence factor in *Leptospira*. Loa22 is also conserved among pathogenic species (Koizumi and Watanabe, 2003; Yang et al., 2006; Ristow et al., 2007). Lp49 is a membrane-associated protein that is recognized by sera from early and convalescent leptospirosis patients (Neves et al., 2007). Lp49

Abbreviations: HHP, high hydrostatic pressure; IBs, inclusion bodies; GdnHCl, guanidine hydrochloride; OmpA70, 70 kDa leptospiral protein with OmpA domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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has WD40 repeats that are putative protein–protein binding sites, suggesting a possible role in host–pathogen interactions (Giuseppe et al., 2008).

The aim of this work was to obtain the protein OmpA70 to be used in vaccine and diagnosis studies. However, the protein is insoluble when expressed in *Escherichia coli*. The integrity of the native conformation of a vaccine can be extremely important in inducing protective immunity. In infections caused by the spirochete *Borrelia burgdorferi*, for example, immunization with a recombinant soluble form of OspC (outer surface protein C) protected mice in challenge assays while the denatured protein did not, suggesting that the OspC protective epitopes were shaped by protein folding (Gilmore and Mbow, 1999). Considering that OmpA70 was expressed in an insoluble form, refolding procedures were required after inclusion bodies (IBs) isolation.

High hydrostatic pressure (HHP) is emerging as a powerful tool for disaggregation and refolding proteins from insoluble aggregates, including amorphous precipitates, amyloid fibrils and IBs (Randolph et al., 2002; Kim et al., 2006). HHP promoted high recovery of native proteins from aggregates of recombinant human growth hormone, IBs of β-lactamase, and disulfide bond cross-linked aggregates of lysozyme (St John et al., 1999, 2001, 2002).

HHP disaggregates and refolds proteins from aggregates by disfavoring intermolecular hydrophobic and electrostatic interactions, because hydration of hydrophobic and charged residues reduces system volume (van Eldik et al., 1989; Silva and Weber, 1993). In contrast, hydrogen and disulfide bonds are not sensitive to HHP. To facilitate disruption of hydrogen bonding, chaotropes such as guanidine HCl (GdnHCl) can be included in the protein preparation, and redox-shuffling reagents are required to reshuffle non-native disulfide bonds into native ones (St John et al., 2002).

The effect of additives on refolding yield has been widely tested at atmospheric pressure. In this type of approach, IBs are first dissolved in high concentrations of chaotrope (urea or GdnHCl) and the refolding is fostered by subsequently reducing chaotrope concentrations by dilution and/or dialysis (De Bernardez Clark et al., 1999). The yield of soluble protein is often low due to competition between aggregation and refolding (Ho and Middelberg, 2004). Amino acids such as L-arginine have been found to reduce protein aggregation, leading to substantial increase in refolding yield at atmospheric pressure (Yasuda et al., 1998). However, there is still a lack of information about the effects of these additives on refolding under HHP.

In the present study, the effects of HHP on the refolding of the protein OmpA70 from *E. coli* IBs in the presence of a chaotrope (GdnHCl), redox-shuffling reagents (reduced and oxidized glutathione), or an amino acid (L-arginine) were investigated. The refolded protein was purified and its structural integrity analyzed by circular dichroism spectroscopy. The immunogenic and antigenic properties of OmpA70 were also evaluated.

2. Materials and methods

2.1. Accession number, in silico identification and protein characterization

The proteins from *L. interrogans* serovar Copenhageni can be accessed by their genome nomenclature for the gene locus, LIC number. In the present work, the coding sequence LIC10537 (accession 45599670) was selected based on its cellular localization predicted by the PSORTb program (Nakai and Kanehisa, 1991) (URL: http://psort.ims.utokyo.ac.jp/form.html). The presence of the OmpA domain and WD40 repeats was predicted by SMART and PSORTb programs (Letunic et al., 2004; Finn et al., 2006) (URLs: http://smart.embl-heidelberg.de/ and http://pfam.sanger.ac.uk/ respectively).

2.2. Bacterial strains and culture conditions

E. coli DH5α was the cloning host strain and *E. coli* BL21 (DE3) Star [pLysS] (Novagen) was used to express the recombinant protein. Transformed *E. coli* cells were grown in 2YT + Amp (2YT medium with 100 μg/ml of ampicillin).

2.3. Cloning of the gene LIC10537 and construction of the expression plasmid

PCR was used to amplify LIC10537 (OmpA70) using genomic DNA purified from *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 (Nascimento et al., 2004) as the template, with the forward (F) and reverse primers (R) (F: 5′-GGATCC-CATACCCTGCTGACTCTGATC-3′, R: 5′-AAGCTTATCCTTGCACCG-AACACACG-3′) containing BamHI or HindIII restriction sites (underlined) respectively. The PCR product was cloned in pGEM-T Easy plasmid (Promega) and subcloned into the BamHI/HindIII sites of the expression vector pAE (Ramos et al., 2004). This vector allows the expression of proteins fused with six histidine residues at the N-terminus. The construct pAE-LIC10537 was verified by restriction endonuclease analysis and sequenced on an ABI Prism 3100xL sequencer (Seq-Wright, Houston) with the primers T7 forward (5′-TAATACGACTCACTATAGGG-3′) and pRSET reverse (5′-ATGCTAGTTATTGCTCAGCGGTGG-3′).

2.4. Expression and solubility analysis of the recombinant protein OmpA

E. coli BL21 (DE3) Star [pLysS] (Novagen) cells transformed with pAE-LIC10537 were inoculated in 2000 ml of 2YT + Amp medium. The expression of the protein OmpA70 was induced with isopropyl-b-D-thiogalactopyranoside (1 mM) when the optical density at 600 nm reached 0.6, and growth was then continued for 5 h (37 °C, 180 rpm). Bacterial cells were collected by centrifugation at 2500 × *g* for 10 min at 4 °C. A culture not induced was used as a negative control. 10 ml aliquots of the induced and non-induced cultures were collected for solubility analysis. Cell pellets were suspended in 600 μl lysis buffer (100 mM Tris–HCl, pH 7.8, 5 mM EDTA) and subjected to sonication to disrupt the cells. The proteins were separated into soluble and insoluble fractions by centrifugation at 8500 × *g* for 10 min at 4 °C. The supernatant, constituting the soluble protein fraction, was transferred to a fresh tube and the pellet was dispersed in 100 μl of Laemmli buffer. The soluble and insoluble protein fractions were analyzed by SDS-PAGE (Laemmli, 1970) and Western blot.

2.5. Isolation and preparation of inclusion bodies for pressure assays

After induction, bacteria cells were collected by centrifugation and resuspended in 50 ml of lysis buffer. Lysozyme was added to a final concentration of 50 μg/ml. The suspension was incubated at room temperature (RT) for 15 min, disrupted in a French Press (Thermo Spectronic), and centrifuged at 8500 × *g* for 10 min at 4 °C. The insoluble fraction (inclusion bodies – IBs) was washed twice with 30 ml of buffer A (100 mM Tris–HCl, pH 8.0, 5 mM EDTA, containing 0.1% [w/v] sodium deoxycholate), five times with buffer B (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 3 M urea) and once with buffer C (50 mM Tris–HCl, pH 8.0, 1 mM EDTA), being subsequently stored at −20 °C in 10 ml of buffer C (basic refolding buffer) for further assays.

2.6. Formulation of refolding buffers and sample pressurization assays

The IBs suspension was diluted in refolding buffer containing final concentrations of L-arginine, GdnHCl, and oxidized (GSSG) and reduced (GSH) glutathione, as indicated in the figures. 1.0 ml samples of the suspensions were placed into plastic bags that were sealed and placed in other plastic bags that were then vacuum/heat-sealed. The bags were positioned in a pressure vessel with a mixture of water and oil as a pressure-transmitting fluid and high pressure (200 MPa, 2000 bar, or 29,000 psi) was applied for 16 h. After pressure release, the samples were centrifuged at 12,000 × *g* for 15 min. The supernatant was dialyzed against 50 mM Tris–HCl, pH 8.0 buffer and centrifuged (12,000 × *g* for 15 min) to remove insoluble aggregates eventually formed.

2.7. Gel electrophoresis, densitometry analysis and protein quantification

SDS-PAGE analysis (Laemmli, 1970) was carried out on 10% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue R-250. The yield of soluble OmpA70 recovered after pressurization was calculated by densitometry using a Densitometer GS-800 (Bio-rad) coupled to Quantity One software. Total protein content in the samples was determined by the Bradford assay, using bovine serum albumin as standard.

2.8. Purification of the recombinant OmpA70 by nickel affinity chromatography

The refolded OmpA70 was purified on a 1 ml Histrap HP prepacked column (GE Healthcare). Imidazole and NaCl concentrations of the soluble protein were raised to 5 mM and 150 mM respectively. The resin was washed with 10 column volumes (CV) of water and equilibrated with 10 CV of buffer A (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM imidazole). The protein was applied to the column and washed with 10 CV of buffer A. OmpA70 was eluted using an imidazole gradient (10 CV of buffer A with 20 mM, 40 mM, 60 mM, 100 mM, 300 mM or 1 M imidazole). 1 ml fractions were collected and analyzed by SDS-PAGE (Laemmli, 1970). The fractions containing OmpA70 were pooled, dialyzed against buffer B (50 mM Tris–HCl pH 8.0, 150 mM NaCl) and stored at −20 °C.

2.9. Size-exclusion high-performance liquid chromatography (SEC-HPLC)

A Tosohaas (Montgomeryville, PA, USA) G2000 SW column (60 cm × 7.5 mm i.d., particle size 10 μm, pore size 125 Å) coupled to a 7.5 cm × 7.5 mm i.d. SW guard column was used for size-exclusion chromatography. The mobile phase used was 50 mM NaCl, 20 mM sodium phosphate, pH 7.0, at a flow rate of 1.0 ml/min. Sample elution was detected by UV absorbance at 220 nm.

2.10. Circular dichroism spectroscopic studies

Circular dichroism (CD) measurements were acquired using a J-810 Circular Dichroism Spectropolarimeter (Jasco Inc.) coupled to a Peltier Jasco PFD-425S temperature control system. The far-UV spectrum was recorded in the spectral range 190–260 nm and averaged over five scans at 20 °C, using a 1 mm path length quartz cell. The protein concentration was 10 μM in 20 mM Na-phosphate buffer, pH 7.4. Spectral correction was performed by subtracting the blank. Measured ellipticity, θ (mdegree), was converted to molar mean residue ellipticity, $[\theta]$ (degree cm^2 dmol^{-1}).

2.11. Production of OmpA70 anti-serum

Seven female BALB/c mice (18–22 g) were immunized intraperitoneally with 5 μg of OmpA70. Aluminum hydroxide was used as the adjuvant. Two booster injections were given at 2-week intervals. One week after each immunization, the mice were bled from the retro-orbital plexus and the blood was incubated for 1 h at RT. The clot was then removed by centrifugation (2300 × *g* for 3 min at 4 °C) and the serum was collected from the supernatant. The pooled sera were analyzed by ELISA. Negative control mice were injected with PBS plus the adjuvant.

2.12. Analysis of OmpA70 recognition by serum from mice immunized with the protein by enzyme-linked immunosorbent assay (ELISA)

The protein solution (5 ng/μl in PBS pH 7.4) was added to the microtiter plate (Polysorp-NUNC), incubated overnight at 4 °C, and washed with PBS–T (PBS + 0.05% [v/v] Tween-20). The plate was blocked with blocking buffer (PBS–T 10% [w/v] non-fat dry milk) at 37 °C for 3 h and washed again. For OmpA70 detection, dilutions of OmpA70 anti-serum (1:20 to 1:10,960) pre-incubated for 1 h in the absorption solution (blocking buffer containing 25% [v/v] of *E. coli* extract, applied to remove possible antibodies against *E. coli* proteins presented in the sera) were transferred to the plate and incubated at 37 °C for 1 h. After washing, a peroxidase-conjugated anti-mouse IgG (KPL) (1:5000 dilution) was added and incubated for 1 h at 37 °C. The plate was washed again and the reaction was developed by the addition of 100 μl of the revelation solution (0.2 M citrate–phosphate buffer, pH 5.0, containing 0.05% [w/v] o-phenylenediamine and 0.015% [v/v] H_2O_2), followed by incubation for 15 min at 37 °C. The reaction was stopped with 50 μl of 4 M H_2SO_4, and absorbance was measured at 492 nm in an ELISA plate reader (Labsystems).

2.13. Experimental infection of hamsters with L. interrogans

The virulence of *Leptospira* strains was maintained by iterative passages in hamsters (*Mesocricetus auratus*). Ten 4–5-week old male hamsters were infected with 10^6 *L. interrogans* serovar Pomona as previously described (Faine et al., 1999). Before infection, the animals were bled from the retro-orbital plexus and the blood was processed as in item 2.11. After 28 days, the hamsters were bled by cardiac puncture and euthanized under anesthesia. The kidneys were collected and submitted to bacteriological examination. The cultures were observed on a weekly basis for 6 weeks using dark-field microscopy (Faine et al., 1999).

2.14. Analysis of the protein OmpA70 by Western blot

OmpA70 was expressed in fusion with six histidine residues on its N-terminal region. Anti-his antibody (Quiagen) and a specific antibody against OmpA70 were used to characterize the protein by Western blot. *E. coli* extracts from cultures induced or not to express the recombinant protein and the purified OmpA70 were transferred from 10% SDS-PAGE (Laemmli, 1970) to a PVDF membrane. The membrane was incubated overnight at 4 °C with blocking buffer (PBS + 0.05% [v/v] Tween-20 with 10% [w/v] non-fat dry milk). The antibodies used were an anti-his antibody (Quiagen) or an anti-serum produced against OmpA70 (see item 2.11); both were diluted 1:3000 in PBS–T and incubated with the membrane for 1 h. After three washes with PBS–T, the membrane was incubated for 1 h with peroxidase-conjugated anti-mouse IgG (KPL) (1:5000 dilution). Following three PBS–T washes, the bands were revealed by Super Signal West Pico Chemiluminescent Substrate (Pierce).

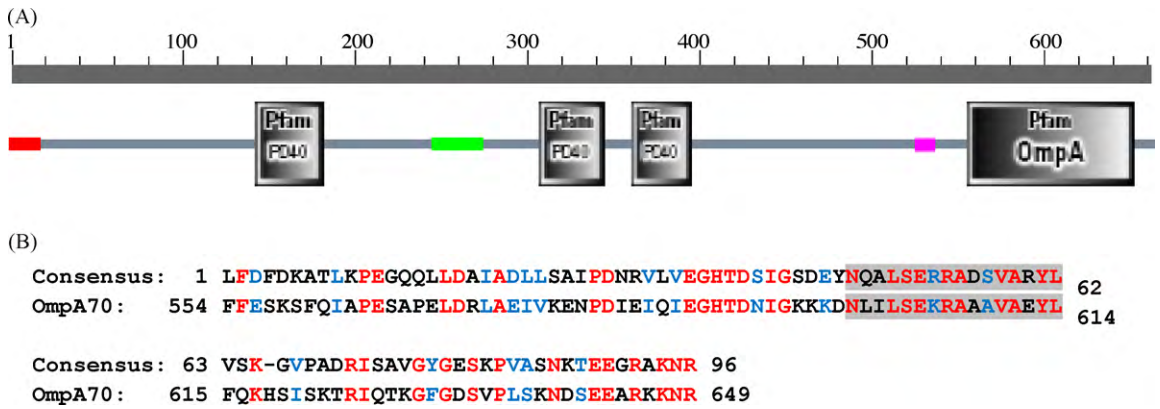

Fig. 1. Schematic representation of the protein OmpA70. (A) In the primary sequence of the protein there is an N-terminal signal peptide (red), three PD40 motifs (Pfam PD40), a coiled-coil region (green), a low complexity region (pink) and a C-terminal OmpA domain (Pfam OmpA). (B) Alignment of the predicted amino acid sequence of the protein OmpA70 and the consensus sequence of the OmpA domain (pfam00691). The numbers are based on the sequence of each protein. Identical, similar and different amino acids are shown in red, blue and black, respectively. The gray sequence indicates the position of the predicted peptidoglycan-associating motif ($NX_2LSX_2RAX_2VX_3L$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The recognition of OmpA70 by serum from hamsters infected with *Leptospira* was also analyzed by Western blot. The protocol employed was the same as described above, with some modifications. The primary antibody used was hamster sera collected before and after 28 days of infection with *L. interrogans* serovar Pomona (1:1000 dilution). The second antibody used was a peroxidase-conjugated anti-hamster IgG (KPL) (1:1000 dilution). The protein LipL32 was used as positive control. LipL32 is the major protein from *Leptospira* and it is expressed during mammalian infection (Haake et al., 2000). Expression and purification of recombinant LipL32 were performed as previously described (Hauk et al., 2005).

3. Results and discussion

3.1. Features of the protein OmpA70

The gene LIC10537 encoding for OmpA70 was identified on chromosome I of the *L. interrogans* serovar Copenhageni genome by analysis of the probable ORFs (Nascimento et al., 2004). The gene consists of 2026 base pairs, encoding a protein with 660 amino acid residues that is predicted to be localized in the bacteria outer membrane. The primary sequence of OmpA70 has an N-terminal signal peptide, three PD40 motifs, a coiled-coil region, a low complexity region, and a C-terminal OmpA domain (Fig. 1A). The amino acid sequence of the OmpA domain has a conserved peptidoglycan binding motif, suggesting a possible role of the protein OmpA70 in the stabilization of the leptospiral outer membrane structure (Fig. 1B). The PD40 motifs contain WD40-like beta propeller repetitions that are found in many families of eukaryotic proteins, and less commonly in prokaryotic organisms. Its structure has repetitions of approximately 40 amino acids with polypeptide chain ending in tryptophan (W) and aspartate (D). The WD repetitions are involved in protein-protein interactions, coordinating the formation of multi-protein complexes (Finn et al., 2006). The alignment of the amino acid sequence of OmpA70 with the consensus sequence of the OmpA domain is shown in Fig. 1B.

3.2. Cloning, expression and solubility analysis of the recombinant protein OmpA70 in E. coli

The expression cassette pAE-LIC10537 was constructed in order to obtain the leptospiral protein OmpA70. The recombinant plasmid was proven to be correct by analysis of the restriction endonuclease digestion and DNA sequencing. To express the protein OmpA70, *E. coli* BL21 (DE3) Star [pLysS] transformed with the construct pAE-LIC10537 was induced with IPTG. Several temperature conditions (20 °C, 30 °C and 37 °C), induction times (1–5 h), and IPTG concentrations (0.1–1 mM) were tested to express the protein in the soluble fraction of *E. coli*. In addition to *E. coli* BL21 (DE3) Star [pLysS], the *E. coli* strains BL21 (DE3) C43 and BL21 (DE3) trxB were also used. However, analysis of the cell extracts by gel electrophoresis demonstrated that OmpA70 was exclusively in the insoluble fraction (IBs), independent of induction time, temperature, inducer concentration, or *E. coli* strain (data not shown). Therefore, in order to maximize the recombinant protein content in the IBs, OmpA70 expression was induced in *E. coli* BL21 (DE3) Star [pLysS] with 1 mM IPTG at 37 °C for 5 h.

In SDS-PAGE of induced and not induced *E. coli* extracts, the band with the expected molecular weight of OmpA70 (76 kDa) appeared in the induced extract (Fig. 2A). In the Western blot, the anti-his antibody recognized a 76 kDa band only in the induced *E. coli* extract (Fig. 2B – 1). Unspecific bands were also detected using this antibody, but these contaminants were not co-purified with OmpA70

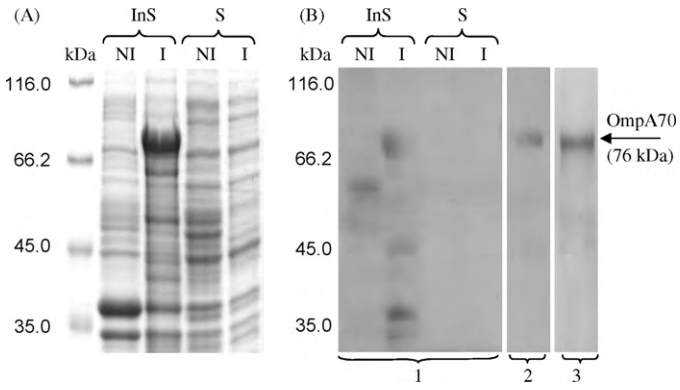

Fig. 2. Expression of the recombinant protein OmpA70 in *E. coli*. (A) Denaturing 10% SDS-PAGE analysis of soluble (S) and insoluble (InS) fractions of *E. coli* cells extracts from cultures that were induced (I) or not (NI) to express the protein OmpA70. (kDa) Unstained protein molecular weight marker (Fermentas). The gel was stained with Coomassie Blue R-250 (Pierce). The protein (76 kDa) was expressed in the insoluble fraction. (B) Western blot analysis with anti-his antibody of (1) *E. coli* induced and not induced cell extracts (same samples shown in A) and (2) purified OmpA70 obtained by affinity chromatography. (3) Analysis of the purified OmpA70 with specific antibody against the protein. The arrow indicates the band with expected molecular weight of OmpA70 (76 kDa). The recombinant OmpA70 was recognized by anti-his antibody in the induced *E. coli* extract and in its purified form. The purified OmpA70 also reacts with the specific antibody, confirming the expression of the recombinant protein.

as only the 76 kDa band was recognized by the anti-his antibody in the purified preparation (Fig. 2B – 2). Additionally, specific sera against the recombinant protein also reacted with OmpA70 (Fig. 2B – 3).

3.3. Solubilization and refolding of OmpA70 from E. coli IBs

High-level expression of recombinant proteins in *E. coli* frequently leads to the formation of insoluble protein aggregates, termed inclusion bodies (IBs). In order to achieve biologically active proteins from IBs, a refolding step is required. Refolding is commonly performed after solubilization in denaturing solutions, followed by dilution or dialysis. Our attempts to refold OmpA70 IBs by pulsed dilution after solubilization with 8 M urea or by dialysis after purification by metal affinity chromatography all failed (data not shown). Therefore, high hydrostatic pressure (HHP) was applied to solubilize and refold OmpA70 from IBs.

Proteins embedded in IBs possess secondary (Ami et al., 2006) and even tertiary structures (Garcia-Fruitos et al., 2007) similar to their native structure. HHP (200 MPa) has the advantage of solubilizing proteins under relatively mild conditions, compared to elevated concentrations of chaotropes, allowing retention of native-like structures and improving the yield of bioactive protein during refolding. HHP promotes protein solubilization by decreasing the hydrophobic and electrostatic interactions involved in aggregation (Qoronfleh et al., 2007). As in traditional refolding procedures, HHP refolding requires standardization and optimization.

The protein OmpA70 was pressurized (200 MPa) for 16 h in solutions containing different concentrations of guanidine hydrochloride (GndHCl) or L-arginine, both with and without redox-shuffling agents (reduced and oxidized glutathione, GSH and GSSG). Concentrations of GndHCl less than 2 M are widely employed in HHP refolding trials (St John et al., 1999, 2001, 2002). However, the protein OmpA70 was not successfully refolded by HPP in refolding buffer containing 0.25 M, 0.5 M, 0.75 M, 1.0 M or 2.0 M GndHCl, or without this reagent (data not shown).

Despite the fact that L-arginine is a widely used additive for protein refolding assays in general, only one article in the literature described the use of HHP with L-arginine for refolding *E. coli* IBs (Lee et al., 2006). In our experiments, solubilization and refolding of OmpA70 were achieved by pressurization at L-arginine concentrations above 0.4 M. About 40% of the protein was recovered in a soluble form, as determined by densitometry analysis (Table 1).

Table 1
Soluble protein OmpA70 obtained by pressurization with increasing L-arginine concentrations in the presence or not of oxidized and reduced gluthatione (GSSG:GSH).

Pressurization conditions[a]	Density (AU)[b]	% soluble protein[c]
1. Non-pressurized	123.8 ± 8.7	–
2. 0.2 M L-arginine + GSSG:GSH	5.8 ± 0.3	4.7
3. 0.4 M L-arginine + GSSG:GSH	35.3 ± 1.8	28.5
4. 0.6 M L-arginine + GSSG:GSH	45.4 ± 2.0	36.7
5. 0.8 M L-arginine + GSSG:GSH	44.1 ± 2.0	35.6
6. Non-pressurized	131.7 ± 13.3	–
7. 0.4 M L-arginine	47.1 ± 4.7	35.8
8. 0.6 M L-arginine	57.3 ± 5.5	43.5
9. 0.8 M L-arginine	52.0 ± 5.5	39.5
10. 1.0 M L-arginine	47.2 ± 3.7	35.8

[a] Soluble fractions obtained after pressurization and dialysis were subjected to SDS-PAGE and densitometry analysis considering the band correspondent to the expected size of OmpA70 (76 kDa).
[b] The density of the bands are represented in arbitrary units (AU) as measured by the analysis software. The numbers correspond to the median of three independent experiments (median ± standard deviation).
[c] The percentage of soluble OmpA70 obtained by HHP was calculated considering the non pressurized sample as 100%.

Neither pressurization nor L-arginine alone were able to promote protein refolding. The redox-shuffling agents did not interfere with the solubilization and refolding of the protein OmpA70 by HHP (Table 1), which possibly occurs due to the presence of only two cysteines in the primary sequence of the protein.

L-Arginine acts as a protein aggregation suppressor. The affinity of the L-arginine for the p-electron cloud of aromatic amino acids is at least one factor contributing to its unique effects on proteins (Kita et al., 1994). It has been proposed that L-arginine binds to partially folded or denatured proteins, inhibiting non-native hydrophobic interactions like chaperones, and therefore decreasing aggregation (Arakawa and Tsumoto, 2003; Ishibashi et al., 2005; Chura-Chambi et al., 2008). Another possible role of L-arginine in the suppression of aggregation is explained by a shift from attractive to repulsive intermolecular interactions (Ho et al., 2003). Additionally, L-arginine could slow down the refolding rate, increasing the stability of the unfolded state and of intermediates (Chen et al., 2009). The mechanisms proposed above are likely operative during HHP treatment.

3.4. Purification of the solubilized OmpA70 and analysis by SEC-HPLC and circular dichroism

An 8 ml volume of IBs suspension was pressurized in refolding buffer containing 0.6 M L-arginine. The protein OmpA70 solubilized and refolded by HHP was purified by metal affinity chromatography. The protein was eluted from the column in buffer containing 300 mM imidazole (Fig. 3).

The soluble fraction obtained after IBs pressurization and dialysis was applied to a SEC–HPLC column. The chromatogram shown in Fig. 4A has a main peak with a retention time (t_R) of 19.8 min that represents a monomer of OmpA70, since its t_R is superior to the t_R of monomeric form of bovine serum albumin (MW = 67 kDa, t_R = 13.5) and no other peaks are seen with lower retention times. The structural integrity of the purified protein was analyzed by circular dichroism spectroscopy (CD). The far-UV CD spectrum of the recombinant OmpA70 (Fig. 4B) indicates the presence of substantial secondary structure content, suggesting that the purified protein is likely to be folded and functionally active.

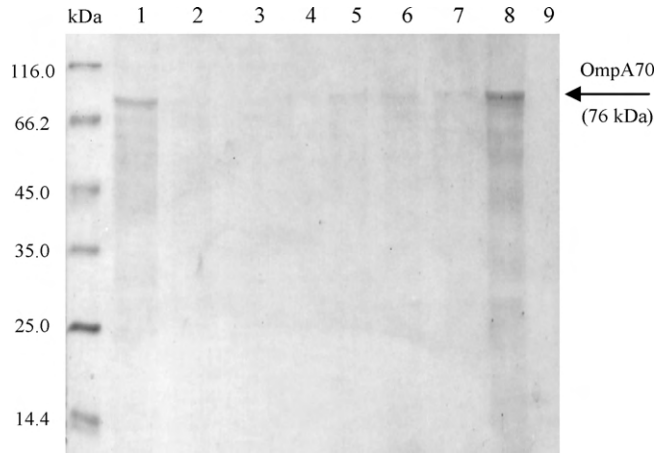

Fig. 3. Purification by metal affinity chromatography of the protein OmpA70 refolded by pressurization. Lane 1: protein solubilized by HHP. Lane 2: flow through. Lane 3: wash with binding buffer. Lanes 4–9: elution buffers with increasing imidazole concentrations (20 mM, 40 mM, 60 mM, 100 mM, 300 mM and 1 M, samples from the first fraction of 1 ml from each solution). (kDa) Unstained protein molecular weight marker (Fermentas). The protein was eluted in buffer containing 300 mM imidazole. The arrow indicates the band with expected molecular weight of OmpA70 (76 kDa).

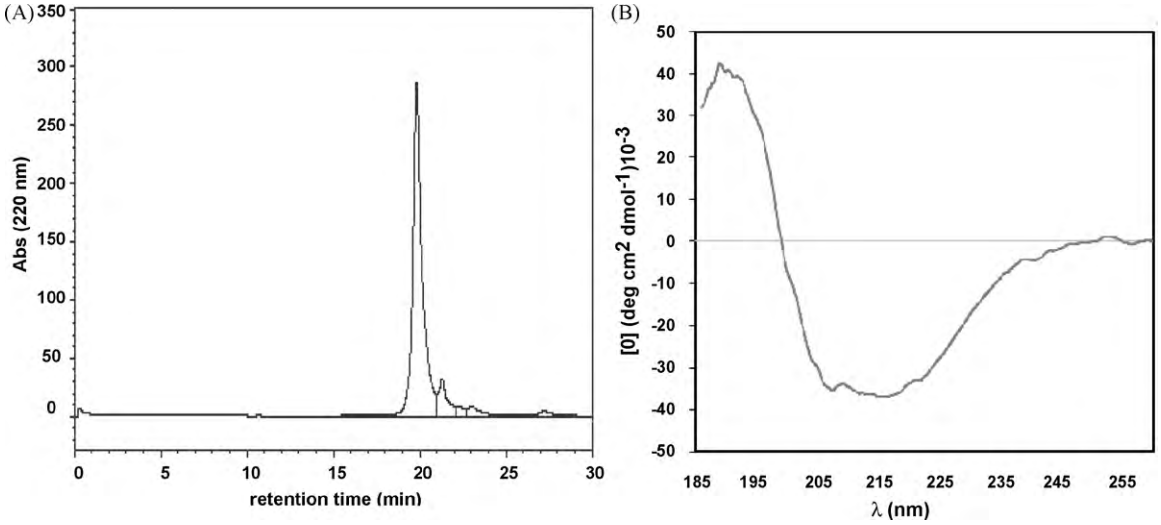

Fig. 4. Characterization of OmpA70. (A) SEC-HPLC analysis of OmpA70. The soluble fraction obtained after IBs pressurization and dialysis was applied to HPLC G2000 SW size-exclusion column. The chromatogram shows a main peak with retention time of 19.8 min. There are no peaks in lower retention times, indicating that OmpA70 is in monomeric state. (B) Circular dichroism spectrum of the recombinant OmpA70. The spectrum was recorded in the wavelength range of 190–260 nm and it is an average of five scans at 20 °C. The measured ellipticity, θ (mdegree), was converted to molar mean residue ellipticity, $[\theta]$ (degree cm^2 dmol^{-1}). Protein concentration was 10 μM in 20 mM Na-phosphate buffer, pH 7.4. The spectrum indicates that the protein has a regular secondary structure, suggesting that the purified protein is likely to be folded.

3.5. Immunogenic and antigenic properties of refolded OmpA70

The protein OmpA70 was recognized by specific IgG antibodies present in the serum of mice immunized with the protein that was solubilized and refolded by pressurization in ELISA assay. IgG titers against the refolded protein were raised upon immunization (Fig. 5A), demonstrating that OmpA70 is highly immunogenic. Currently available vaccines against leptospirosis are bacterins of whole inactivated leptospires or membrane preparations. These vaccines do not confer a T cell dependent response, thus requiring annual revaccinations. A recombinant protein vaccine that could stimulate the production of conformational and protective antibodies, combined with the induction of a T cell dependent response, would be an important achievement for leptospirosis prophylaxis. In this context, the fact the protein OmpA70 was highly immunogenic encourages further investigation of its protective capacity in challenge assays with virulent leptospires. A preliminary assessment of the diagnostic potential of the protein OmpA70 was performed by Western blot with sera from hamsters experimentally infected with *L. interrogans* serovar Pomona. Leptospires were found in the kidneys of all animals, confirming infection by this spirochete. The protein OmpA70 was recognized by IgG antibodies in the sera collected from the hamsters 28 days after infection (Fig. 5B). Rapid and efficient diagnostic tests are not available for

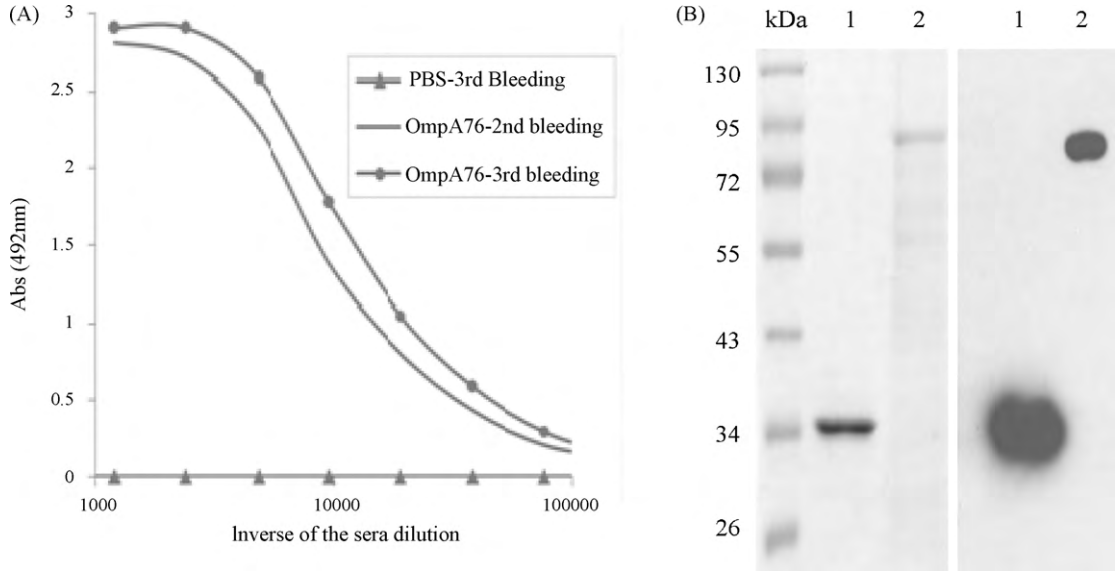

Fig. 5. Immunogenic and antigenic properties of refolded OmpA70. (A) Analysis of the reactivity of OmpA70 with sera from mice immunized with OmpA70, using aluminum hydroxide as adjuvant. Two booster injections were given at 2-week intervals. Negative control mice were injected with PBS and adjuvant. OmpA70 was highly immunogenic, presenting elevated IgG titers after the second and third immunization, analyzed by ELISA. The graphic shows the relation between the absorbance (492 nm) and the inverse of the sera dilution. (B) Reactivity of OmpA70 with sera from hamsters infected with *Leptospira interrogans* serovar Pomona. All the animals were positive for the isolation of leptospires from the kidneys confirming the infection. The hamsters were bled before and 28 days after infection. SDS-PAGE of the purified proteins (left) and Western blot analysis (right). Lane 1: LipL32 (positive control, major protein from *Leptospira*). Lane 2: OmpA70. (kDa) Prestained molecular weight marker (Fermentas). Both proteins did not react with sera collected before the infection (data not shown). OmpA70 is immunogenic and antigenic since the protein raised antibodies titers upon immunization and reacts with sera from infected hamsters.

use in human and veterinary leptospirosis (Levett, 2001; Faine et al., 1999). The fact that the protein OmpA70 reacts with infected hamster sera not only demonstrates its antigenic properties, but also suggests that this protein can be expressed during the course of infection. OmpA70 may therefore serve as the basis for developing a recombinant protein-based serologic test.

4. Conclusions

We described in this work the production of the protein OmpA70 from *L. interrogans* serovar Copenhageni. The recombinant protein expressed as inclusion bodies in *E. coli* was successfully solubilized and refolded by the association of high hydrostatic pressure and L-arginine. To our knowledge, this is the first vaccine and diagnosis candidate obtained by pressurization. Additionally, L-arginine proved to be a useful additive and should be considered when screening for optimal refolding under traditional as well as pressurization conditions. A preliminary characterization of the immunological properties of OmpA70 demonstrated that the protein is immunogenic and antigenic. Future investigations will focus on the effectiveness of the protein as a recombinant vaccine, and challenge assays with virulent leptospires in hamster model of leptospirosis are currently underway.

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