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Expression and purification of the non-tagged LipL32 of pathogenic *Leptospira*

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Abstract

Leptospirosis is a reemerging infectious disease and the most disseminated zoonosis worldwide. A leptospiral surface protein, LipL32, only occurs in pathogenic *Leptospira*, and is the most abundant protein on the bacterial surface, being described as an important factor in host immunogenic response and also in bacterial infection. We describe here an alternative and simple purification protocol for non-tagged recombinant LipL32. The recombinant $LipL32_{21-272}$ was expressed in *Escherichia coli* without His-tag or any other tag used to facilitate recombinant protein purification. The recombinant protein was expressed in the soluble form, and the purification was based on ion exchange (anionic and cationic) and hydrophobic interactions. The final purification yielded 3 mg soluble $LipL32_{21-272}$ per liter of the induced culture. Antiserum produced against the recombinant protein was effective to detect native LipL32 from cell extracts of several *Leptospira* serovars. The purified recombinant $LipL32_{21-272}$ produced by this protocol can be used for structural, biochemical and functional studies and avoids the risk of possible interactions and interferences of the tags commonly used as well as the time consuming and almost always inefficient methods to cleave these tags when a tag-free LipL32 is needed. Non-tagged LipL32 may represent an alternative antigen for biochemical studies, for serodiagnosis and for the development of a vaccine against leptospirosis.

Key words: Antigen; LipL32; Pathogenic *Leptospira*; Non-tagged protein purification; Diagnosis; Vaccine

Introduction

Leptospirosis is a zoonosis caused by the genus *Leptospira*. Humans and domestic animals are most commonly infected through contact with the urine of carrier animals, either directly or via contaminated water or soil. In humans, this disease is characterized by fever, renal and hepatic insufficiency and pulmonary manifestations (1). The most abundant antigen found in the leptospiral total protein profile is LipL32, displaying around 40,000 copies/cell (2). This lipoprotein is highly conserved among pathogenic *Leptospira* species (3), but no orthologs were observed in the saprophytic non-pathogenic *Leptospira biflexa* (4). Expressed at high levels both during cultivation and natural infection, LipL32 was shown to be surface exposed and highly immunogenic (3). This lipoprotein is also involved in the pathogenesis of tubulointerstitial nephritis that is the main renal manifestation. Pathogenic leptospires accumulate mostly in the proximal tubules and induce the expression of inflammatory molecules, part of this response being mediated by LipL32 (5). Considering these remarkable findings, many strategies have used LipL32 as a target for vaccine development and diagnosis (for a review, see Ref. 6). Studies performed with the C-terminal and intermediate domains of LipL32 have shown that they are recognized by sera of patients. For instance, an IgM response was detected exclusively against the LipL32 C-terminus in both the acute and convalescent phases of illness (7). Moreover, the capacity of LipL32 to interact with extracellular matrix components such as laminin (8), collagen type IV and plasma fibronectin (7) was also described. A dose-dependent and specific binding of LipL32 to collagen IV and plasma fibronectin was observed, and interestingly, the binding capacity could be attributed to the C-terminal portion of this molecule. Both heparin and gelatin could inhibit LipL32 binding to fibronectin in a concentration-dependent manner, indicating that the 30-kDa heparin- and the 45-kDa gelatin-binding domains of fibronectin are involved in this interaction (7,8). Surprisingly, studies using the *lip*L32 mutant have shown that it does not play a role in either

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the acute or chronic models of infection (9). While these data do not suggest a role in pathogenesis, a function for LipL32 has not been demonstrated. In the present study, we describe the cloning and purification of LipL32$_{21-272}$ (LipL32 containing amino acids 21 to 272) without His-tag or any other tag commonly used to facilitate recombinant protein purification.

Material and Methods

Bacterial strains, plasmids, and culture conditions

Leptospiral strains were obtained from the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), Brazil. *E. coli* DH5α was used as the cloning host strain and *E. coli* BL21 (SI) (Gibco/BRL, USA) as the host strain for efficient overproduction of cloned gene products using NaCl as the inducer (10). Recombinant *E. coli* BL21 (SI) cells were grown on 2YT ON (16 g/L tryptone and 10 g/L yeast extract) medium supplemented with ampicillin (100 µg/mL) before induction.

Cloning steps and screening of the recombinant plasmids

To avoid misfolding and aggregation of the recombinant protein, the region coding for the hydrophobic signal peptide (amino acids 1 to 20) was not included in the cloned fragment. PCR was used to amplify the gene fragment coding for LipL32$_{21-272}$ using a genomic DNA purified from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 isolated from a patient in Salvador, Brazil (ATCC No. BAA-1198) (11), as template, with the forward primer (5'-<u>CTC GAGCATATG</u>GGGTGCTTTCGGTGGTCTG-3') containing *Xho*I and *Nde*I restriction endonuclease sites (underlined) and the reverse primer (5'-<u>AAGCTT</u>ACTTAGTCGCGT CAGAAGC-3') containing a *Hin*dIII (underlined) restriction site. Plasmid pGEM-T easy (Promega, USA) was used to clone the amplified gene. The resulting 759-bp fragment was cloned into *Nde*I/*Hin*dIII sites in the T7 promoter-based expression plasmid pAE (12). *E. coli* DH5α competent cells were transformed with the ligation product. Recombinant colonies were analyzed by a protocol based on the size of the plasmid (13). Positive clones were investigated for the presence of the correct insert by DNA sequencing.

Expression in *E. coli*

E. coli BL21 (SI) competent cells (Gibco/BRL) were transformed with the pAE-*lip*L32 and grown overnight at 30°C. In this strain, the expression of T7 RNA polymerase is under the control of the *E. coli* osmotically inducible promoter *proU* (10). The transformed *E. coli* BL21 (SI) was cultured in 1 L 2YT ON medium containing ampicillin and grown until the optical density reached 0.6 at 600 nm. The expression of the recombinant protein was induced with 0.3 M NaCl, followed by culture for an additional 3 h at 30°C. The cells were collected by centrifugation and resuspended in 100 mL 20 mM triethanolamine, pH 7.8, and lysed in a French press (Thermo Spectronic, USA) at 30,000 psi. The soluble and insoluble fractions were isolated by centrifugation at 8400 *g* for 10 min.

Purification of the recombinant LipL32$_{21-272}$

The soluble fraction recovered from the centrifugation step described above was applied to a column (1 cm in diameter) containing 5 mL Q-Sepharose Fast Flow resin (GE HealthCare, USA) previously equilibrated with 20 mM triethanolamine, pH 7.8. The unbound fraction containing LipL32$_{21-272}$ was harvested and then applied to a column of similar dimension containing SP-Sepharose Fast Flow resin (GE Healthcare). After adsorption of LipL32$_{21-272}$ protein to the cationic resin, the preparation was washed with 10 column volumes of 20 mM triethanolamine, pH 7.8, containing 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, and 2.0 M NaCl, respectively. Fractions were analyzed by 15% SDS-PAGE to detect the presence of LipL32$_{21-272}$. Samples containing LipL32$_{21-272}$ were pooled, NaCl was added to a final concentration of 3.0 M and this mixture was then adsorbed to a Phenyl-Sepharose Fast Flow column (GE HealthCare) previously equilibrated with 20 mM triethanolamine, pH 7.8, and 3.0 M NaCl. After the adsorption of the recombinant protein, the resin was washed with 10 column volumes of 20 mM triethanolamine, pH 7.8, containing 2.5, 2.0, 1.5, 1.0, 0.7, 0.5, 0.3, and 0.1 M NaCl, respectively, and finally, 20 mM triethanolamine, pH 7.8, and H$_2$O. The chromatographic steps were carried out with a peristaltic pump (P-1, GE HealthCare). The eluted fractions were analyzed by 15% SDS-PAGE. Proteins were pooled from selected fractions, dialyzed with three changes, each consisting of 2 L 20 mM triethanolamine, pH 7.8, every 18 h, and considered to be the final LipL32$_{21-272}$ preparation.

Production of LipL32$_{21-272}$ antiserum

Five- to eight-week-old female BALB/c mice were immunized intraperitoneally with 10 µg purified LipL32$_{21-272}$ and 100 µg aluminum ion (added in the form of Al(OH)$_3$). The immunizations were performed over a period of 4 weeks, with booster doses at every week. Mice were bled by the retrorbital plexus and blood was incubated for 30 min at 37°C. The clot was then removed by centrifugation and the serum was collected from the supernatant.

Western blot

Leptospira extracts were fractionated by 15% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was incubated with 10% (w/v) non-fat dried milk in 0.05% Tween 20/phosphate-buffered saline (PBS-T) and after three washes with PBS-T for 10 min, it was further incubated with mouse anti-recombinant LipL32$_{21-272}$ serum in 5% non-fat dried milk-PBS-T for 1 h. Following the repetition of the PBS-T wash as described above, the membrane was incubated with a proper dilution of goat

anti-mouse IgG peroxidase conjugate (Sigma) in 5% non-fat dried milk-PBS-T, washed, and developed with ECL reagent (GE HealthCare).

Results

Cloning, expression and purification of recombinant LipL32$_{21-272}$

The gene fragment coding for LipL32$_{21-272}$ (3,14) was amplified by PCR and cloned in the *E. coli* expression vector pAE (12). The resulting plasmid pAE-*lip*L32 codes for the expression of LipL32$_{21-272}$ with an additional Met at the N-terminus of the molecule (we will refer to this molecule as LipL32$_{21-272}$). Recombinant LipL32$_{21-272}$ was expressed in *E. coli* BL21 (SI) after induction by 0.3 M NaCl. The protein was expressed both in the soluble and in the insoluble fractions (data not shown). We found equal levels of LipL32$_{21-272}$ in the inclusion bodies and in the soluble fraction. We preferred to purify LipL32$_{21-272}$ from the soluble fraction, since this material is likely to be in the native and correct conformation. The soluble LipL32$_{21-272}$ was purified in three chromatographic steps (Figure 1A). In the first step, the soluble fraction was adsorbed onto a Q-Sepharose column. The flow through containing LipL32$_{21-272}$ (Figure 1B) was further purified by SP-Sepharose and the protein eluted in 0.1 M NaCl, 20 mM triethanolamine, pH 7.8 (fraction P1, Figure 1B). In the third step, the sample containing LipL32$_{21-272}$ eluted from the cationic chromatography was adjusted to 3 M NaCl and finally purified by Phenyl-Sepharose (fraction P2, Figure 1C). The recombinant LipL32$_{21-272}$ was eluted in 0.5, 0.3, 0.1 M NaCl in 20 mM triethanolamine, pH 7.8, and 20 mM triethanolamine, pH 7.8, and H$_2$O. The samples enriched with LipL32$_{21-272}$ were those eluted with 0.5, 0.3, and 0.1 M NaCl (fraction P2, Figure 1C). These fractions were dialyzed in three steps with 2 L 20 mM triethanolamine, pH 7.8, and concentrated to 10 mL using an Amicon ultrafiltration system with a 10-kDa cut-off membrane (Figure 1D). The final purification yield considering fraction P2 was approximately 3 mg/L culture

Table 1. Purification yield of LipL32$_{21-272}$.

Sample	Recovery (LipL32, mg/L)	Yield (% LipL32)	Homogeneity (%)
Crude extract	35	100	30
Flow through (Q-Sepharose)	30	85	42
P1 (S-Sepharose)	25	71	47
P2 (Phenyl-Sepharose)	3	8	98

The relative amount of LipL32$_{21-272}$ in the fractions was estimated from densitometric analysis of the SDS-PAGE gels shown in Figure 1B and C. LipL32$_{21-272}$ recovery was determined by submitting known quantities of bovine serum albumin to SDS-PAGE gels followed by densitometric analysis. Homogeneity was determined by SDS-PAGE. The data reported are the results of one representative preparation.

and more than 95% pure (Table 1), suitable for crystallographic and tertiary structural studies (15,16).

Production of LipL32$_{21-272}$ antiserum

The purified LipL32$_{21-272}$ was inoculated in female BALB/c mice for the production of antiserum against LipL32. The antiserum obtained was able to recognize the recombinant LipL32$_{21-272}$ in Western blot (Figure 1E, lane 11). Moreover, a conserved 32-kDa band corresponding to the expected size of LipL32 was also recognized in the extracts of a large panel of pathogenic *Leptospira* (*L. interrogans* serovars Canicola, Pyrogenes, Pomona, Autumnalis, Hardjo, Brastilava, Copenhageni, and Icterohaemorrhagiae, and *L. kirschneri* serovar Grippotyphosa), whereas no reaction was observed in the extract of the non-pathogenic saprophytic *L. biflexa* serovar Patoc (Figure 1E).
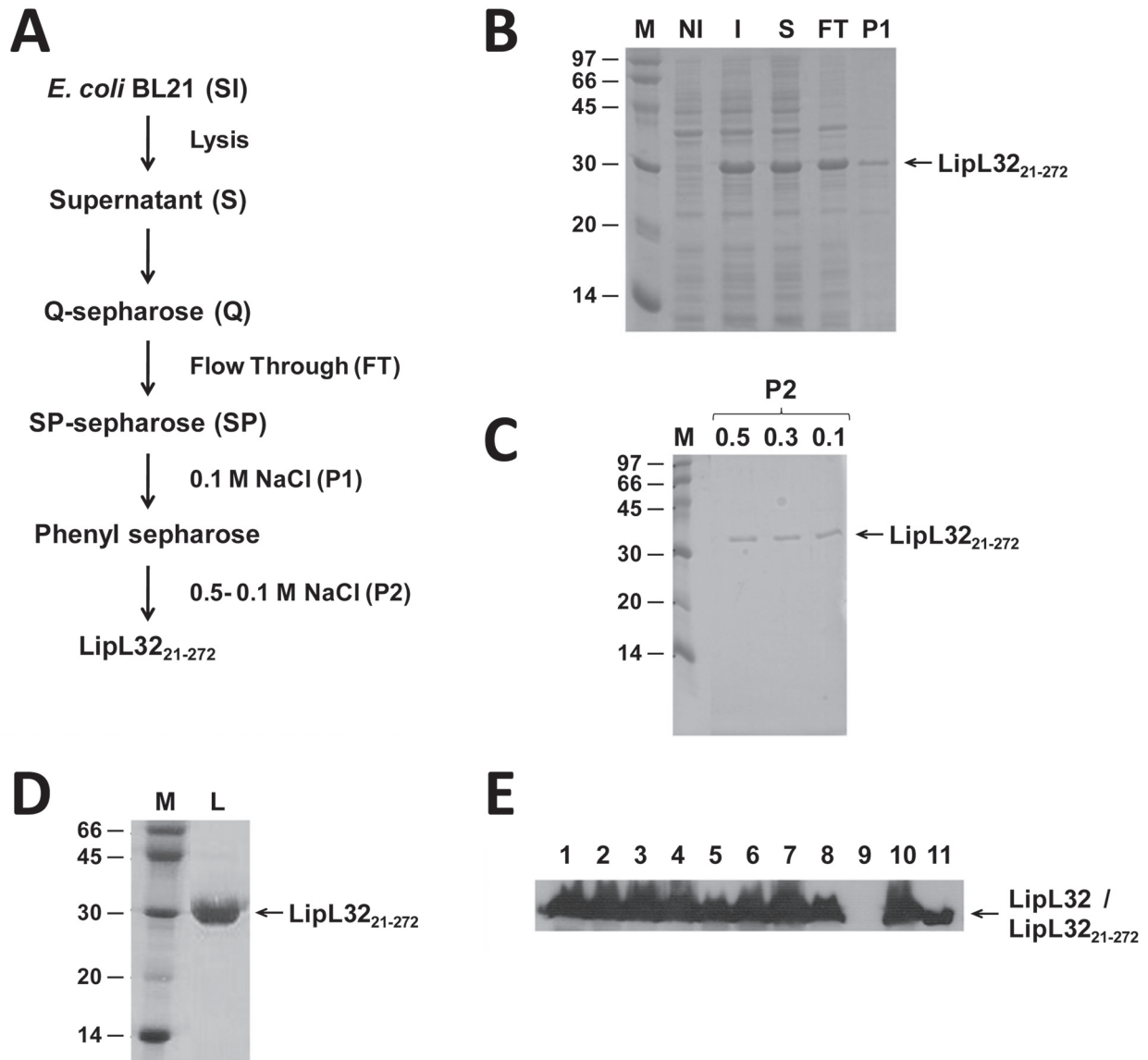
Discussion

Affinity tags are efficient tools for protein purification since they facilitate the derivation of large amounts of highly homogenous proteins from heterologous expression systems (17). His-tags are the most widely used affinity tags, but several others are also widely used such as the maltose-binding protein, glutathione-*S*-transferase, chitin-binding domain, and elastin-like polypeptide tag. His-tag binding to metal is not affected by urea solutions used to solubilize proteins expressed in inclusion bodies in contrast to the other tags that require non-denaturing conditions to be functional. Affinity tags may have a positive or negative effect on the biochemical properties of the target protein (17). Examples of positive effects are protein yield improvement, protein refolding facilitation, proteolysis prevention, preservation of fusion protein antigenicity, and increased solubility. Most of the negative effects are related to changes in protein conformation, lower protein yields, inhibition of enzyme activity, alteration of biological activity, undesired flexibility in structural analysis, and toxicity (17). Due to unpredictable changes that adding a tag would introduce in a protein, we obtained LipL32$_{21-272}$ without any tags. The purification of LipL32$_{21-272}$ was based on ion exchange (both anionic and cationic) and hydrophobic interaction chromatography. For structural studies, an additional concentration step is necessary. LipL32 is considered to be an important antigen in leptospirosis (3,16,18). It is highly immunogenic and expressed on the surface of pathogenic *Leptospira* sp (2,3,7,8). However, the exact biological role of this protein in leptospirosis or in *Leptospira* biology is unknown. Since LipL32 is well conserved in all pathogenic species tested to date, but absent in free-living *L. biflexa* serovar Patoc (3,4) (Figure 1E), it was suggested that LipL32 could be involved in leptospiral pathogenicity. However, an *L. interrogans* mutant

Figure 1. Expression and purification of LipL32$_{21-272}$ and antiserum recognition of native LipL32. *Panel A*, Flowchart showing the three main steps of LipL32$_{21-272}$ purification. SI = *Escherichia coli* BL21; S = supernatant; Q = anion exchange; FT = flow through - unbound proteins; SP = cation exchange; P1 = 0.1 M NaCl - fraction containing LipL32$_{21-272}$ eluted with 0.1 M NaCl; P2 = purified LipL32$_{21-272}$. *Panel B*, SDS-PAGE of LipL32$_{21-272}$ purification shown in *Panel A*. M = Molecular marker; NI = non-induced *E. coli* BL21 (SI); I = *E. coli* BL21 (SI) induced with NaCl; S = supernatant; FT = flow through - unbound proteins; P1 = 0.1 M NaCl - fraction containing LipL32$_{21-272}$ eluted with 0.1 M NaCl. *Panel C*, SDS-PAGE of LipL32$_{21-272}$ purified from Phenyl-Sepharose. M = Molecular marker; P2 = purified LipL32$_{21-272}$ after elution with 0.5, 0.3, and 0.1 M NaCl using Phenyl-Sepharose. *Panel D*, SDS-PAGE showing the purified LipL32$_{21-272}$. M = Molecular marker; L = LipL32$_{21-272}$ after membrane concentration (cut off 10 kDa). *Panel E*, Western-blot showing that anti-non-tagged LipL32$_{21-272}$ antiserum recognizes native LipL32 from cell extracts of different *Leptospira* serovars. *Lane 1*, Whole-cell extracts of *L. interrogans* serovars Icterohaemorrhagiae; *lane 2*, Copenhageni; *lane 3*, Bratislava; *lane 4*, Hardjo; *lane 5*, Autumnalis; *lane 6*, Pomona; *lane 7*, Pyrogenes; *lane 8*, Canicola, and *lane 10*, *L. kirschneri* serovar Grippotyphosa. Serum also recognized the recombinant protein (lane 11) but failed to react with the whole-cell extract of non-pathogenic *L. biflexa* serovar Patoc (lane 9).

lacking functional LipL32 did not show any detectable loss in the ability to infect and colonize hosts or even to survive in water, making this a paradoxical protein (9).

Structural studies have shown a tertiary structure related to jelly roll fold proteins (16,18). Indeed, in both structural studies, LipL32 was crystallized without His-tag. In the first attempt to crystallize LipL32, the protein was obtained with a His-tag that was further cleaved out using the V8 staphylococcus protease. The removal of the His-tag was necessary to obtain suitable LipL32 for high-quality structural studies. The purification method used by Vivian et al. (18) included several steps: Nickel-Sepharose followed by gel-filtration chromatography (S75 16/60), a Mono Q50/50 anion-exchange column and the unbound fraction containing pure LipL32 was proteolyzed to the V8 protease and finally reapplied to the S75 16/60 column. However, problems may be encountered during the cleavage step, including low yield, precipitation of the target protein, tedious optimization of cleavage conditions, high cost of proteases or failure to recover the active or structurally intact protein (19). In addition, some biochemical features of LipL32 may be better explored using a non-tagged protein to avoid possible interferences. We proposed here a simple method to purify homogenous non-tagged LipL32. A partial but not detailed description of this protocol has been published (15,16). Studies performed by our group using this non-tagged LipL32 purified by ion exchange (anionic and cationic) and hydrophobic interactions showed that LipL32 binds to calcium in a specific manner but not to zinc or copper ions. This kind of study would not be possible with a His-tagged LipL32. Besides, the purified non-tagged LipL32 yielded high-quality crystals suitable for diffraction data and three-dimensional structure determination (16) and may also represent an alternative antigen for serodiagnosis and vaccine development for leptospirosis, although His-tagged LipL32 have proved so far to be highly immunogenic as well (20). Overall, we believe that a description of a method for obtaining purified non-tagged LipL32 may be useful for researchers in the field, since this is the most abundant protein on the leptospiral surface.

Acknowledgments

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