Improvement the expression and purification of Loa22: a lipoprotein with OmpA domain from pathogenic *Leptospira* serovars

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**Research Article**

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Abstract

Outer membrane proteins (OMPs) are the main surface antigens of the pathogenic Leptospires. One of the highly conserved outer membrane proteins expressed only by pathogenic Leptospires is Loa22. This study aims is to obtain the optimum conditions for high expression and purification of Loa22 recombinant (rLoa22) protein. Based on evidence of phylogenetic studies, complete coding sequence of loa22 gene was optimized based on codon usage chart and sub-cloned into a pET32a (+) expression vector. BL21 (pLysS) was used as expression host for transformation. The recombinant clones selected on ampicillin plates and subjected to PCR by using pET T7 primers and expression conditions optimized then by adjusting parameters such as culture media, induction time, temperature, and IPTG concentration. SDS-PAGE Analysis showed that the production of rLoa22 protein was at the highest level when post induction incubation, IPTG concentration, and duration of induction were 37°C, 0.1 M and 5 h in 2xTY medium respectively. Due to the soluble nature of the protein, the purification of the rLoa22 protein under native conditions using Ni-NTA pull-down was optimum in one hour binding process at 37°C, five times washing process and elution buffer with a pH 7.4 and a 0.3 M imidazole concentration. Based on the results of this study, optimizing the expression and purification process for over production of rLoa22 protein resulted in the large quantity of pure recombinant antigen that forms the basis for future investigation on the design of rapid diagnostic tests and more effective subunit vaccine candidates for leptospirosis.

1. Introduction

Leptospirosis is a worldwide public health problem affecting both domestic animals and humans. The etiologic agent of the disease is pathogenic spirochetes of the genus Leptospira [1, 2]. Leptospira interrogans constitute the major pathogenic Leptospira species that is responsible for human infection [3]. Leptospira spreads through direct contact with urine or tissues of infected animals, or indirect exposure to contaminated water, soil, or vegetation. [2, 4].

Leptospirosis has a broad geographical distribution but deployed in tropical and subtropical areas with high fall rain. In Iran, the areas along the Caspian Sea have the highest reported prevalence, but there also are case reports in some other parts of Iran [5, 6]. Most common serovars in these areas that cause infection in humans include serovars Icterohaemorrhagiae, and Canicola, in cattle's serovars Hardjo-bovis, Conicola, and Grippotyphosa, in canines serovars Canicola, and Icterohaemorrhagiae, and in rodents serovars Icterohaemorrhagiae, and Grippotyphosa respectively [1, 6, 7].

Accurate diagnosis of leptospirosis due to a wide diversity of clinical manifestations shared with many other febrile diseases depends on the type of diagnostic test [1, 3]. Laboratory diagnosis of leptospirosis usually is accomplished by several different methods including; isolation of the causative leptospures, direct microscopy, molecular and serological methods. Although culture is a golden standard method, but is not effective for the early detection of the disease and also has low sensitivity [1, 5]. Direct microscopy methods including using dark-field or phase contrast microscopy requires expertise and careful
examination\[8\]. Molecular methods employed have included several of PCR-based procedures\[9, 10\]. However, it also require trained experts, and relatively elaborated laboratory outfits\[11\]. Among serological methods, the microscopic agglutination test (MAT) is the gold standard test to diagnose leptospirosis using antigens from live leptospiral serovars to detect specific agglutination antibodies in serum but it is prone to contamination and less sensitive and specific\[5\]. Among serology-based methods, ELISA is a simple, safe, and suitable assay for the examination of a large number of sera samples in diagnoses and seroepidemiological investigations using purified recombinant proteins\[10\].

The importance of control and prevention of leptospirosis, especially in endemic areas, emphasizes the need for the development of rapid and reliable laboratory tests and trying to achieve an effective vaccine. Commercially available leptospirosis vaccines including vaccines based on whole cell bacteria, and lipopolysaccharide (LPS), suffer from several limitations such as serovar-restricted protection, short-term immunity and usually fail to prevent the transmission of the disease. Currently, subunit vaccines based on recombinant proteins have the potential to overcome these limitations \[12, 13\].

Outer membrane proteins (OMPs) are the main surface antigens of the *Leptospira* spp. and in contrast to LPS are thought to be highly conserved. The OMPs play an important in maintaining the cell structure, and attachment to various extra cellular matrix (ECM) components, and are protective immunogens in animal models of the disease \[14, 15\]. Generally, these recombinant antigen-based tests are safe and better alternatives to live cultural antigens for the serodiagnosis of leptospirosis \[16, 17\].

The outer membrane protein A-like protein Loa22 from *Leptospira interrogans* is a 22KDa lipoprotein with an OmpA domain in the C-terminus \[16, 18\]. Several studies have shown that Loa22 is highly conserved and expressed only by pathogenic *Leptospira* during infection and is involved in pathogenesis at both acute and convalescent stages of infection \[15, 16\]. In addition, experimental evidence has proven that Loa22 protein stimulated inflammatory responses and deletion of this protein from pathogenic *Leptospira* attenuated toxicity, while re-expression of the protein revives the virulence \[18, 19\].

In the last decades, recombinant outer membrane proteins have investigated with two different approaches covering their potential in the development of serodiagnosis tests and overcoming restrictions of available leptospirosis vaccines \[12, 20-22\]. Although there are some difficulties concerning to this matter, such as low levels of immune responses and in some cases, expression at only low levels or may not be expressed at all by host cells grown in artificial media\[23, 24\]. On the other hand, the next subject that must also be considered in preparing recombinant proteins as a candidate for the development of rapid and reliable laboratory tests or vaccines is the purification procedure. Therefore the selection of protective leptospiral OMPs and investigation of their desirable expression, purification, and immune responses is essential\[7, 25\].

By achieving the purpose of this study, it is expected to be used as a basis for Loa22 protein expression and purification on a larger scale in the provision of reliable diagnostic tests and to develop investigations into an effective vaccine.
2. Materials And Methods

*Leptospira* serovars and culture conditions

Six serovars including *L. interrogans* serovar Icterohaemorrhagiae, Canicola, Hardjo prajitno, and *L.Kirschneri* serovar Grippotyphosa and two nonpathogenic serovars of *L.bifelxa* obtained from reference laboratory of *Leptospira* of Razi Vaccine and Serum Research Institute, Iran.

The bacteria were inoculated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco, USA) supplemented with *Leptospira* Enrichment under aerobic conditions at 28°C. Bacterial growth within 7-10 days was confirmed by the dark field microscope (Nikon Eclipse, Japan) observation, and then pelleted through centrifugation at 17000 ×g for 20 min.

DNA extraction and PCR

Genomic DNA extracted from resuspended pellets by the phenol-chloroform method. The presence of the *loa22* gene in the pathogenic and nonpathogenic serovars examined by PCR using the upstream primer: 5´- CGGCTTTTGAAAGATCGAATTG-3´ and the downstream primer: 5´- AACACTCTGATACCAAACCCCT-3´ that designed to cover the CDs region of *loa22* gene. The PCR was performed with 50ng of DNA template, 10pM of each primer, and 1x of Taq 2x master mix (amplicon) in a 50 µl final reaction volume. The amplification was carried out with the initial heat denaturation at 95°C for 5 min followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 60°C (annealing), 1 min at 72°C (extension), and the final extension at 72°C for 10 min.

Sequencing and phylogenic studies

The amplified PCR products purified using the High Pure PCR product Purification Kit (Roche, Germany) and sequenced by Sanger dideoxy sequencing technology (ABI3730XL, Macrogen, Korea). DNASTAR Laser gene V.17 software used to assemble sequences generated from forward and reverse primers. In addition to our sequences, fourteen sequences of the *loa22* gene were obtained in a parallel study (accession No OP038304 - OP038317), and 17 sequences were derived from pathogenic *Leptospira* whole genomes registered in the NCBI Database (http://www.ncbi.nlm.nih.gov) by blasting with the only complete CDs sequence of corresponding *loa22* from Grippotyphosa (accession No. KC311551) as a reference (Table).

All sequences of the *loa22* gene in this study compared using the multiple sequence alignment program of MEGA software (version 7.0.26), where some sequences had to be trimmed to allow comparison. The phylogenic tree of the aligned sequences was drawn using the maximum-likelihood (ML) method, using the Tamura 3-parameter model and bootstrap analysis (n=1000) with MEGA7.0 software.
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Table. *Loa22 gene* sequences of *Leptospira* serovars used in this study

Bioinformatic study

Editseq™ software and Expasy-ProtParam used to predict the *loa22* gene-coding region, its amino acid sequence, the amino acid composition, and various other physiochemical properties of Loa22 protein, including theoretical pI, molecular weight, polarity, etc. Based on the evidence of phylogenetic and physiochemical studies, the complete coding sequence of the *loa22* gene was considered in a way to remove the signal peptide as predicted by the online server SMART (http://smart.embl-heidelberg.de/). The coding sequence optimized based on the codon usage chart of *E.coli* as a compatible host for the high-level expression.
The pET32a(+) vector was then designed by insertion of the optimized \textit{loa22} sequence into the Ncol and Xhol restriction sites in the frame with histidine tag sequence at the N-terminal end.

**Protein expression**

The recombinant plasmid transformed into the \textit{E. coli} BL21 (DE3) pLysS using cold CaCl$_2$ treatment followed by heat shock method and was grown in LB medium containing 50μg/mL ampicillin. To confirmation of the positive recombinant clones selected on ampicillin plates and related plasmids were isolated from bacterial cells using the Plasmid Mini extraction kit (Roche, Germany) as described by the manufacturer protocol. Extracted plasmids and positive clones subjected to PCR using pETT7 universal primers.

The expression of recombinant protein optimized by adjusting parameters such as culture media, induction time, temperature, and isopropyl-β-d-thiogalactopyranoside (IPTG, Sigma-Aldrich, Korea) concentration. The best concentrations of inducer were determined by inducing the culture with different concentrations of IPTG. Recombinant colonies were cultured into 10 ml fresh sterile 2xTY (Tryptone 16 g/L, Yeast extract 10 g/L, NaCl containing 50 μg/mL ampicillin and incubated overnight at 37°C with constant shaking at 150 rpm. The following day 400 mL of 2xTY was inoculated with 1mL of preculture and when the growth achieved the mid-log phase (OD$_{600}$ nm, 0.6) the culture was equally divided into four flasks. The cultures then induced by IPTG concentrations (0.1 M, 0.2 M, 0.3 M, and 0.5 M). One ml culture from each of the flasks were centrifuged at 10,000 xg for 10 min to obtain the pellet.

To determine the optimum temperature and induction time, recombinant colonies were cultured into 100ml fresh 2xTY media (3 flasks) containing 50µg/mL ampicillin and incubated at 37, 30 and 22°C with constant agitation (150 rpm) until cells reached OD$_{600}$ nm, 0.6. The cultures induced for up to 20 hours with 0.1M of IPTG. An equal amount of Samples collected in situ at the following time points: 0, 1, 2, 3, 5, and 20 h and stored at -20°C.

To achieve the high yield of the Loa22 protein in \textit{E. coli}, we evaluated three different media formulations; 2xTY medium (16g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl), Terrific broth (TB) complex cultivation medium (12 g/L tryptone, 24 g/L yeast extract 0.04 g/L glycerol, 12.54 g/L KH$_2$PO$_4$, 15 g/L K$_2$HPO$_4$) and LB (10g/L tryptone and 5g/L yeast extract, 5g/L NaCl). The pH of all media adjusted to 7.4 and supplemented with the corresponding antibiotic (ampicillin 50 μg/mL). Overnight culture of \textit{E. coli} BL21 (DE3) pLysS harboring pET32-Loa22 was grown overnight at 37 °C with shaking at 150 rpm. Then 1 mL of the starting culture inoculated into 100 mL of each of the three media. When the growth in OD$_{600}$ reached 0.6, cultures induced with 0.1M IPTG and incubated at 37°C with shaking at 150 rpm. After 5h of incubation, the cell suspensions harvested by centrifugation, washed with ice-cold 1xPBS and the bacterial pellets were stored at -20°C until further processing.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE 12%) performed to evaluate protein expression for each sample collected.
Optimization of purification using Ni-NTA resin

The solubility or insolubility of the expressed recombinant protein was determined by resuspending the bacterial pellet (5h post-induction) in 1xPBS and disrupted by sonication (6 cycles, 1 min each with 1 min of intervals). Phenyl Methyl Sulfonyl Fluoride (PMSF) added to inhibit probable protease activity. The lysate was centrifuged at 10000 xg at 4 °C for 20 min and the production of recombinant protein, either in solubilized or insolubilized form, was analyzed by SDS-PAGE.

Purification of the recombinant protein was performed by Ni-NTA chromatography under the native buffer system, according to Thermo Scientic protocol[26]. Optimization of the protein purification process carried out at the stage of binding, washing, and elution. Therefore, after sedimentation, the supernatant was loaded onto 500 μl Ni-NTA resin and shacked for an hour at 37ºC with constant agitation (200 rpm) to more interaction between His-tag in recombinant proteins and Ni-NTA resin. After the protein was bound, impurities washed using wash buffer(50 mM NaH$_2$PO$_4$, 0.3M NaCl, 0.25M imidazole, adjust pH to 7.4) with repeated variations two, three, and five times. The protein, in the next step, eluted in three steps by gradient concentration of imidazole ranging from 0.05-0.3 M in potassium phosphate buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl). Depending on the structure and the isoelectric point (pI) of the protein, the elution buffer then optimized in terms of pH (4, 6, and 7.4). The Purity of Loa22 protein in each step checked on 12% SDS-PAGE.

Measurement of purified recombinant protein

Total concentration of protein measured using Spectrophotometer (Epoch, BioTek, USA) and colorimetric assay according to Bradford standard method.

Linear standard BSA curve (bovine serum albumin) is created using Microsoft Excel to find out the equation of the line obtained, so that the sample concentration can be calculated. The standard concentrations used in range of 0 to 20 mg/ml.

Dot-blot and western-blotting analysis

Overall, 2μg/mL of 6X his-tagged rLoa22 protein, the E.coli total protein extract not induced with IPTG as a negative control, the extract of the transformed bacteria, and purified recombinant protein were dotted on the nitrocellulose membrane. It was immersed in 5% skim milk and shaken (30 minutes) and washed with PBST (137 Mm NaCl, 2 mM KH$_2$PO$_4$, 10 mM Na$_2$HPO$_4$, 2.7 mM KCl, pH 7.4 with 500µl Tween20) and incubated for 1 hour with HRP conjugated anti-6x His-Tag antibodies (Abcam, USA). The membrane washed with PBST. Color development was detected by Tetramethyl Benzidine (TMB-blotting substrate solution, Sigma Aldrich).

For western blot assay the samples, along with the marker, were run on 12% SDS-PAGE using a discontinuous buffer system and subsequently transferred (0.5 V/cm$^2$ for 2 hour) to the nitrocellulose membrane (Bio-Rad) by the wet blot transfer method in transfer buffer (0.2 M glycine; 24 mM Tris; 10%
methanol, pH 8.3). The membrane immediately placed in blocking buffer (5% BSA in PBS containing 500µl Tween20) for 1 hour at 4°C to block non-specific binding sites and the membrane washed with PBST, three times for 5 min per wash. Afterwards the blot again incubated at 37°C for 2 hour with a HRP conjugated anti-6x His-Tag antibodies, in the blocking buffer. The membrane washed as described above, and Anti-His tagged antibody was probed by adding TMB.

3. Results

DNA isolation and PCR amplification

The amplified fragments by PCR viewed at 1% agarose gel and obtained a single band of 671bp; while it was, absent in saprophyte serovars such as L. biflexa as shown in Figure 1.

Sequencing and phylogenic analysis

The sequences analysis, showed 97-100% similarity among all serovars examined in this study. Based on the results, the loa22 gene of local L.interrogans serovars Hardjo prajitno and Conicola had 100% homology. Local isolates of L. Kirschneri serovar Grippotyphosa were in a close relationship (99.7% identity) with Grippotyphosa isolated in India (KC311551). Similarity between L.interrogans and L.krischneri serovars were up to 97.3%. The alignment of the deduced amino acid sequences of the loa22 genes using MEGA software revealed amino acid substitutions at positions 37 (Ala→Thr), 87 (Ala→Pro), and 165 (Val→Ala), were the only consistent differences between the L.krischneri and L.interrogans serovars respectively.

The phylogenetic tree presentation showed two distinctive clusters. A cluster consisting of three Grippotyphosa serovars belongs to the L. Kirschneri serogroup and 33 serovars belong to other serogroups, which formed a separate cluster (Figure 2).

Bioinformatics study to construct of the loa22

The Loa22 protein sequence contained 199 amino acids with a molecular weight of 21.6 kDa, pI of 8.8, and maximum activity at pH.7 as predicted using the Expasy-translate online application and Editseq software. Bioinformatics analysis predicts that loa22 encodes membrane protein; has a signal peptide with a cleavage site between amino acids 24/25 and has no assigned function.

Finally, based on the physiochemical properties and findings from the phylogenetic studies, the local Loa22 protein pattern was used to design the recombinant vector Loa22-pET32a(+) by insertion of the optimized loa22 sequence into the Ncol and Xhol restriction sites in frame with histidine tag sequence at N-terminal end. General Biosystems, Inc, USA, synthesized the designed gene construct.

Transformation
For over-expression and production, Loa22+pET32a construct transformed into *E. coli* BL21 (DE3) pLysS under the control of the T7 promoter.

The recombinant plasmid purified from the transformed cell and identified based on plasmid size (Figure 3a). Eventually, the results of colony PCR assay with T7 universal primer and growth in presence of ampicillin revealed that recombinant plasmid correctly transformed into the host (Figure 3b).

**Protein expression**

To examine the effect of media on the production of rLoa22 protein, the relative level of expression analyzed using the three different media by SDS-PAGE. The induced culture in the TB medium expressing Loa22 showed the lowest volumetric yield relative to LB and 2xTY medium, although there was no significant difference in growth rate in LB and 2xTY medium. However, the 2xTY medium was found to support the best growth rate and produce more recombinant protein than other media under induced culture conditions (Figure 4b).

The best conditions for the high-level Loa22 expression were achieved at 0.1M IPTG concentration, 37°C(Figure 4c), and 5 hours induction(Figure 4a), although other IPTG concentrations also demonstrated sufficient results (Figure 4d). The cells harboring recombinant lipoproteins in optimum conditions expressed a specific band of approximately 38KDa (pET32; 18.5KDa +Loa22; 19.3KDa) compared with the cultures without IPTG as the control on 12% SDS-PAGE electrophoresis after staining.

**Optimization of purification using Ni-NTA resin**

SDS-PAGE analysis after disrupting the cells with ultrasound showed that the amount of protein in the soluble part was significantly higher. After sonication, the contents centrifuged at 5000×g for 15 min and the recombinant protein in the supernatant purified by Ni-NTA resin as a chromatographic procedure.

The SDS PAGE characterization process indicated that more washing carried out by the wash buffer containing 0.025M imidazole, caused non-specific proteins to be released from the resin. In washing twice there were still some impurities in the presence of non-target protein bands, and washing five times has almost no impurities, although, some protein waste was also observed (Figure 5b). The band thickness and high protein purity increased with the increase in the concentration of imidazole and elution at a concentration of 0.3M gave maximum results (Figure 5a). In addition, the pH of solutions was 7.4, which is a mimic biological condition, and most proteins are stable at this pH (Figure 5c).

**Measurement of purified rLoa22 protein**

The standard BSA concentration used in the range of 0 to 30 mg/ml. The concentration of the recombinant protein in the optimal conditions of purification (0.3M Imidazole, pH 7.4) was about 3.3 mg/ml by Bradford assay, almost consistent with the results of the Nanodrop spectrophotometer (3.1mg/ml).
The *dot blot* and western blotting analysis

The band (38KDa) of interaction with His-tagged recombinant protein and anti-His tagged antibodies in 1:2000 dilution corresponding to pre-stained protein ladder were detected by western blot TMB (Figure 6a and 6b). The dot blot results showed no detectable blot in negative control while rLoa22 observed both in the supernatant fraction of induced *E.coli* cell lysates and purified recombinant protein (Figure 6c).

4. Discussion

Leptospirosis is one of the most widespread zoonosis and public health concerns, particularly in tropical and sub-tropical areas [27]. Leptospirosis is frequently underreported due to a lack of reliable diagnostic tests, variable symptoms, and a wide variety of pathogenic serovars on the other hand its initial symptoms can easily be mistaken for other infectious diseases [3, 9]. Therefore, it is required to develop rapid and effective tests and vaccines for prevention and treatment of leptospirosis. The leptospiral highly conserved OMPs which are expressed on the surface of pathogenic serovars and expressed during infection may have potential immunoprotective capabilities and a beneficial tool for serodiagnosis and vaccine development for leptospirosis [23, 28].

LipL32, Loa22, and Lip41 are the three most abundant known lipoproteins on the surface of *Leptospira*, respectively[28]. Loa22 is also the first genetically described virulence factor in *Leptospira* that confirmed by mutagenesis studies[18]. It is a lipoprotein with the C-terminal OmpA domain located in the outer membrane[15].

The first step in this study was to design a recombinant *loa22 gene* construct based on phylogenetic evidence and homology studies using bioinformatics tools. The resultant nucleotide and amino acid sequences of *loa22* analyzed using the BLAST online program of NCBI, which revealed more than 99% similarity at both levels among pathogenic *Leptospira* serovars. The percentage of homology at the nucleotide and amino acid levels among *L.krischneri* Grippotyphosa serovars were 97.3 and 98.3% respectively with *L. interrogans* serovars. Difference in three amino acids was the reason for the difference in identity *L.krischneri* Grippotyphosa serovars with *L. interrogans* serovars. Generally, the results of multiple sequence alignment for the deduced nucleotide and amino acid sequences of all serovars in the study for the loa22, in congruent with the results of Kaur et al (2014) Balamurugan et al (2021), conferred a high degree of homology and present only in pathogenic serovars of *Leptospira*.

The production of a pure protein in sufficient amounts is the key to its study or use as an antigen for diagnostic or immunization purposes. Today, recombinant protein production in large volumes and their application in genetic engineering have increased. However, high-level production of prokaryotic recombinant proteins in *E. coli* may not be a routine matter; sometimes it is quite challenging [23, 24, 29].

Here, loa22 gene encoded an approximately 38kDa recombinant protein which was confirmed with immune-blotting analysis. Based on our results, deletion of the signal sequence of Loa22 protein located in the transmembrane region resulted in a soluble mature protein, which similar to other studies
supported the theory that the mature protein, without the signal peptide, retained in the soluble fraction [16, 20].

The high percentage of similarity in the Loa22 amino acid sequences among different serovars from Iran and other countries considered to design the recombinant plasmid according a pattern consisting of common pathogenic serovars. Codon optimization strategies attempt to increase the protein of interest expression by altering the codon usage of the gene. Based on the optimization results, the high yield of purified Loa22 protein showed that the optimized gene construct according to the genomic codon usage in the \(E. \text{coli}\), is probably one of the reasons for the higher yield compared to restriction cloning with wild-type sequences. Previously, expression of LipL41 by Golab et al 2020, and LipL21 by Kamalzadeh et al 2021, using a codon optimization approaches have been conducted and have obtained considerable amounts of recombinant protein, which is useful for academic studies and preclinical work [7, 25].

In this study, using the pET32a vector to express the Loa22 along with other parameters, led to the production of a high amount of the recombinant protein (3.3mg/ml) after purification. However, a study by Xin Zhao and colleagues were unable to successfully express the rLoa22 [30]. In two other studies, Loa22 produced through different vectors including pETite with the yield of 5-6mg/L, and pGEX-4T with the yield of 16 mg/L of culture [16, 20].

Since the high concentration of IPTG may have toxic effects and cause the expressing of the protein in an insoluble form, we considered the minimum required concentration for induction. And also reduction of the temperature down to 30°C along with incubation time of 16 h decreased the protein yield compared to the 5 hours post induction at 37°C, which was consistent with findings of Fraser et al. 2017 regarding the reduction of Loa22 expression at 30°C [31]. The represented experimental data showed that the production of rLoa22 in the native state was at the highest level when post-induction incubation, IPTG concentration, and duration of induction were 37ºC, 0.5mM, and 5 h in a 2xTY medium respectively.

According to our results, this protein expressed in native form and found in the flow-through. These results were in agreement with the findings of the other researchers that corroborated that the \(E. \text{coli} \) BL21 (DE) pLysS expression vector was able to express Loa22 protein in a soluble form [5, 16].

Protein purification is a set of processes that usually involves one or more chromatographic steps, depending on the size, physicochemical properties, binding affinity, and biological activity[26]. The presence of the N-terminal His-tag facilitated an efficient Ni-NTA affinity purification, which resulted in a propertyield of soluble purified recombinant protein without contaminants. The optimization of the purification process conducted at the stage of binding, washing, and elution. Increasing the time and repetition of the binding process was expected to increase the binding target protein to Ni-NTA resin. The more washing also there will be produced proteins that have fewer impurities. Imidazole in low concentration in washing solution (25 mM) can reduce nonspecific binding of resin.

Concentration of Imidazole, as a competitive agent for elution of histidine-Tagged proteins due to having a structure similar to the histidine, influences the elution process. In the elution step, it found that
increasing the concentration of imidazole and the pH of the elution buffer around the pI of the protein lead to an increase for protein. In addition, pH adjustment is necessary to keep the protein soluble and not degraded.

Hence, following the thorough utilization of optimization parameters, the purification of the Loa22 recombinant protein under native conditions using Ni-NTA pull-down was optimum in a one-hour binding process at 37°C, five times washing process, and elution buffer with a pH a near-neutral range (7.4) and the 0.3 M imidazole concentration.

5. Conclusion

Based on the results of this study, the high percentage of loa22 gene sequence similarity between Iranian serovars and serovars from other countries proves that this gene is conserved. We used this conservation to design a gene construct based on the loa22 gene that can cover all serovars. Then we optimized the expression and purification process for over production of Loa22 protein. The large quantity of pure recombinant antigen produced by these methods forms the basis for future investigation on the design of rapid diagnostic tests and more effective vaccine for leptospirosis based on Loa22 protein in prompt treatments and prevention and control of leptospirosis.

Declarations

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References


Figures

Figure 1

Agarose gel (1%) electrophoresis showing PCR product of 671bp encoding for loa22 gene in the pathogenic Leptospira. Lane 1. L. interrogans serovar Canicola, Lane 2. L. interrogans serovar Hardjo Lane 3. L. kirschneri serovar Grippotyphosa, Lane 4. L. interrogans serovar Icterohaemorrhagiae, lane 5 and 6: L. Biflexa, Lane 7. Positive control. M. 100bp plus DNA Marker (Thermo scientific, Lithuania).
Figure 2

The phylogenic tree was constructed with nucleotide of the aligned *Loa22* gene sequences using the maximum-likelihood (ML) method, with the Tamura 3-parameter model and bootstrap analysis (n=1000) within MEGA7.
Figure 3

Figure 4. (a) Purified plasmid from transferred cells. (b) Clony-PCR amplification of loa22 from pE32-loa22 plasmid with pET T7 primers, Lane 1. Untransferred cell, Lane 2-5. Transferred cells. M. 100bp plus DNA Marker (Thermo scientific, Lithuania).
Figure 4

(a) SDS-PAGE analysis of expressed Loa22 protein in E. coli (plysS) after incubation at 37 °C. Lane 1. Non-induced cell lysate, lanes 2 to 5. Cells induced with 0.1mM IPTG at 1, 2, 3, and 5 hours after induction, respectively. Lane 6. Cells induced after 20 hours. (b) SDS-PAGE analysis of Loa22 expression in three different culture media. Lane1. LB Broth Lane2. 2xTY medium Lane3. BT Broth. (c) The effect of temperature on Loa22 expression. Lane1. (21°C), Lane2. (30°C) lane 3. (37°C). (d) Lane 1. Non-induced cells, lanes 2 to 5. Cells induced with IPTG at 0.1 to 0.4 mM concentrations. M. Protein Size Marker (Thermo scientific, Lithuania).
Figure 5

(a) Washing step. Lane 1-3. Washing 2, 3, and 5 times, respectively. (b) Effect of concentration of imidazole in 1xPBS with a pH close to a neutral range (7.4). Lane 1-4. Elution with 0.05, 0.1, 0.2, and 0.3M concentration of imidazole, respectively. (c) Effect of buffer pH on the elution. Lane 1 to 3, solution with pH 4, 6, and 7.4 respectively. M. Prestained Protein Size Marker (Thermo scientific, Lithuania).
**Figure 6**

Immunoblotting of recombinant proteins using HRP conjugated anti-6x His-Tag antibodies. (a) Lane 1. The *E.coli* total protein extract not induced with IPTG, Lane 2. The transformed cell lysate (b) Lane 1. Purified recombinant protein (c) Dot-blot analysis of the Loa22 protein, 1. Purified protein, 2. Transformed cell lysate. M. Protein Size Marker (Thermo scientific, Lithuania).