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OPTIMIZING FACTORS FOR THE EFFICIENT EXPRESSION AND PURIFICATION OF SPAO AND LAMB FROM *Salmonella typhi*

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Abstract

We optimized the expression and purification of outer membrane proteins SpaO and LamB from *Salmonella typhi*. We investigated various factors in the expression and purification processes, including the use of isopropyl β-d-1 thiogalactopyranoside (IPTG), imidazole, and urea. First, PCR amplification was carried out on SpaO and LamB genes. The genes were then cloned in pTZ57R/T, and then expressed in pET28a vector and transformed into *Escherichia coli* BL21 (DE3). Gene insertion was confirmed by enzymatic digestion with *Nde*I and *Xho*I. Inclusion bodies expressing recombinant SpaO and LamB were induced with 200 and 400 µL 0.5 mM IPTG, respectively. The formed protein inclusion bodies were then isolated from the pellet and solubilized in IB buffer containing 8 M urea for SpaO and 6 M urea for LamB. Proteins were refolded by dialysis in 3M urea. Purified proteins with nickel-nitrilotriacetic acid affinity chromatography and eluted with buffer containing 250 mM imidazole for SpaO and 150 mM imidazole for LamB. The protein expression profiles were analyzed by SDS-PAGE, which identified the 33 and 49 kDa bands corresponding to rSpaO and rLamB. Western blotting Purification was carried out by nickel affinity resin with 250 mM and 150 mM imidazole for rSpaO and rLamB and refolded through stepwise dialysis with anti-His tag antibodies confirmed their expression. These optimized methods can be used to generate recombinant proteins for the development of future vaccines.

Keywords: Expression. Outer Membrane Protein. Purification. *Salmonella typhi*. Typhoid.

1. Introduction

Outer membrane proteins (OMPs) present on the surface of Gram-negative bacteria are quickly recognized as extracellular foreign particles through the host immune system, leading to an immune response against the bacterial pathogen (Osman et al. 2014). OMPs that are highly immunogenic against several bacterial species, including *Edwardsiella tarda, Neisseria meningitides, Chlamydia trachomatis, and Aeromonas hydrophila,* have been used as vaccine candidates (Yadav et al. 2014). *Salmonella typhi* (*S. typhi*) is an obligate human pathogen, which causes typhoid fever that continues to be a main health problem

particularly in developing countries (Pang et al. 1998). It has been revealed that *Salmonella* OMPs are involved in provoking a protective immune response (Isibasi et al. 1998). Moreover, OMPs have been shown to play roles in pathogenic processes, including regulating, proteases, adherence, toxins, motility and colonization in host cells (Bina et al. 2006). In this context, *Salmonella* OMPs have been proposed to confer protection against typhoid (Hamid and Jain 2008).

"Surface presentation of antigen" (SpaO) protein of *Salmonella* play their role in adhesion and invasion processes (Hueck 1998) and allows the pathogen to enter and colonize host cells (Collazo and Galan 1996). The SpaO gene is expressed through variety of species including *Salmonella paratyphi, typhi and typhimurium* (Parkhill et al. 2001). It was suggested that for the treatment of typhoid caused by *Salmonella paratyphi* SpaO might be used for generation of vaccine (Ruan et al. 2008). Recombinant SpaO could therefore be a target antigen, as it has been reported to have good immunogenicity and confer a degree of immunoprotection.

Moreover, OMPs of Gram-negative bacteria can act as a barrier against host protection, while through β-barrel proteins allowing the uptake of nutrients (Nikaido 2003). These outer membrane β-barrel proteins play vital roles in virulence and adhesion, as well as acting as enzymes. These β-barrel proteins are predicted to make up only 2%-3% of the proteome (Gromiha and Suwa 2007). A class of "porins" that include β-barrel proteins usually act as channels of solute diffusion through variable selectivity (Basle et al. 2006). One of well-known porin protein name LamB that is important for growth through reducing maltose concentration (Luckey and Nikaido 1980). Recombinant LamB proteins could therefore be a target for vaccine development.

During the high quality recombinant proteins generation inclusion bodies formation considered a vital bottleneck that needs the suitable strategies for the proper refolding and solubilization (Singh et al. 2015). In *Escherichia coli* (*E. coli*) expression systems the production of high levels of recombinant proteins often result in inclusion bodies formation (Chrunyk et al. 1993), which can be a great barrier to the high quality recombinant protein purification and production (Burgess 2009). The high yield expression of desired protein achieved by several factors such as high inducer concentration, strong promoter and high temperature. However, quality control of bacterial protein systems is often difficult, with partially folded and misfolded proteins aggregating to form inclusion bodies (Carrio and VillaverdeI 2005).

Inclusion bodies that have significant biological activity are recognized as non-classical inclusion bodies (Garcia-Fruitos et al. 2012) and are characterized through a loose molecular arrangement of protein molecules. These inclusion bodies solubilized in denaturants such as urea (Upadhyay et al. 2007) and can be purified under suitable conditions to yield the proteins of interest (Singh et al. 2017. The aim of this study was to develop an optimized method for the expression, purification and renaturation of outer membrane SpaO and LamB from *S. typhi*.

2. Material and Methods

Bacterial strains and vectors

Escherichia coli strains DH5α and BL21 (DE3) used in this study were obtained from Invitrogen. Cultured the bacteria in Luria-Bertani (LB) broth at 37°C. Selection of clone were done with the use of ampicillin (100 µg/mL) and Kanamycin (50 µg/mL), ligase enzymes, and restriction endonucleases were obtained from New England BioLabs. The pET28a vector and Taq polymerase (Pyrobest and Ex.taq) were from laboratory stocks.

Amplification of SpaO and LamB genes

A previously Isolated and characterized strain of *Salmonella enterica serovar typhi* was used. Total genomic DNA was isolated from *Salmonella serovar typhi* according to Sambrook et al. (1989). Reported primers (Ruan et al. 2008) were used to amplify SpaO gene, while primers for LamB gene were designed by adding the restriction sites *Nde1* and *Xho1* at the 5' and 3' ends (Table 1). Each PCR amplification reaction mixture (50 µL) contained 100 pmol primer, 2.5 mM dNTPs, 25 mM $MgCl_2$, and 5U Taq polymerase (Thermo

Fisher Scientific). The amplification conditions for SpaO gene were 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 59 s; and 72°C for 10 min. The PCR amplification conditions for LamB were 94°C for 5 min; 30 cycles of 94°C for 0.45 s, 55°C for 0.45 s, and 72°C for 0.45 s; and 72°C for 10 min. The size of the amplicon was measured by comparing it with standard markers. The PCR products were purified by GeneJET PCR purification kit (Thermo Fisher Scientific) and cloned into a pTZ57R/T vector (Thermo Fisher Scientific). Recombinant clones were confirmed by digestion with *NdeI* and *XhoI,* respectively.

Table 1. Name, sequences, length and melting temperatures of SpaO and LamB gene Primers.

Primers	Sequences	Lenght	Tm (C°)	GC
SpaO-F	5'CGCCATATGTCATTGCGTGTGAGACAG-'3	27	48	50
SpaO-R	5' CTCGAGTTCCCCATTACCAGACTC- '3	24	47	50
LamB-F	5' CATATGATGATTACTCTGCGCAA- '3	23	42	41
LamB-R	5' CTCGAGTTACCACCAGATTTCCA- '3	23	43	41

SpaO: Surface presentation of antigen; LamB: Maltose outer membrane porin; F: Forward; R: Reverse.

Amplification of SpaO and LamB from *S. typhi*

The amplified SpaO and LamB segments were linked with linearized pET28a using T4 DNA ligase and transformed to *E. coli* BL21 (DE3) cells (Novagen). Plasmids were isolated using a plasmid isolation kit (Qiagen). Double digestion was performed by *NdeI* and *Xho*I to confirm insertion. For the expression analysis, single bacterial colonies containing the desired SpaO or LamB gene were grow with kanamycin in 5 mL LB broth (50 µg/mL) and incubation was carried out in orbital shaker for 5 hr at 37°C. 0.5 mM IPTG was used for induction of culture on reaching an OD_{600} of 0.6 overnight to allow generation of the soluble proteins. Bacterial lysis was carried out by resuspending the recombinant proteins in protein binding buffer (20 mM Tris-Cl at pH 8.0, 20 mM imidazole, 250 mM NaCl). The pellet was sonicated for 3 min (5 s on pulse and 8 s off pulse) and samples were analyzed by 12% SDS-PAGE.

Optimization of different buffers for induction, solubilization, and refolding of recombinant proteins

The cultures containing SpaO and LamB were induced with 200 and 400 µL of 0.5 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG), respectively. The cells after incubation for 5-4 h harvested and centrifuged at 4,000 rpm for 20 min at 4°C. The pellet of cells was resuspend in 70 mL binding buffer at pH 8.0 (20 mM Tris-Cl, 20 mM imidazole, 250 mM NaCl). Cells were lysed by sonication for 30 min (5 s on pulse and 8 s off pulse). The cell pellet containing inclusion bodies was washed at 4°C overnight with IB buffer at pH 6.0 (10 mM Tris, 5 mM EDTA, 200 mM NaCl, 1 M urea, 1% Triton X-100). The cell pellet was then resuspended in 30 mL of solubilization buffer at pH 8.0 (10 mM Tris-HCl, 100 mM NaH2PO4, 100 mM NaCl) containing 8 M urea for SpaO or 6 M urea for LamB overnight at 37°C with stirring. On the next day, samples were centrifuged for 30 min at 4°C at 15,000 rpm. Collected the supernatant and proteins refolded by dialysis in 3 M urea overnight.

Optimization of elution buffers for the purification of recombinant proteins

The rSpaO and rLamB supernatants (30 mL) were purified with Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography using a chelating Sepharose fast flow column (Hochuli 1990). The column was equilibrated for 2 to 3 h using 20 mL IB solubilization buffer at pH 8.0. Allowed the supernatant to bind with the column followed by washing with wash buffer (20 mM Tris-HCl, 250 mM NaCl, 50 mM imidazole). Recombinant proteins that were bound eluted by using elution buffer (20 mM Tris-HCl, 250 mM NaCl) containing 250 mM imidazole for rSpaO and 150 mM imidazole for rLamB. The collected 1-mL fractions were evaluated by 12% SDS-PAGE.

Western Blotting

The rSpaO and rLamB proteins induced by 0.5 mM IPTG along with control as uninduced culture pellet were examined by 12% SDS-PAGE. Both proteins onto nitrocellulose membrane were electroblotted (Towbin

et al. 1979). The nitrocellulose membrane for 2 h was blocked in 5% non-fat milk in 1 x TBST (Tris-buffered saline with tween). Incubation of membrane was carried out with primary mouse, anti-His–tag monoclonal antibody at a dilution of 1:5,000 in 5% non-fat milk for overnight at 4^{0}C on shaker. Next day wash the membrane three times in 1 × TBST, incubated the membrane with secondary anti-rabbit polyclonal antibody on shaker for 1 hr at a dilution of 1:8,000 at room temperature. After washing the membrane with 1 × TBST, the proteins were visualized by a UV detector.

3. Results

The specific primers for the amplification of SpaO and LamB by PCR are shown in Table 1. As expected, the sizes of SpaO and LamB products were approximately 912 bp and 1340 bp, respectively, as shown in Figure 1. The amplified products were individually cloned in the pTZ57R/T vector. The recombinant plasmids were screened by colony PCR using specific primers and by restriction digestion of the minipreps (Figure 2). Both recombinant plasmids were successfully sub-cloned in pET28a vector. The integrity of recombinant pET28a-SpaO and pET28a-LamB were confirmed by double digestion and colony PCR. The 912 bp and 1340 bp fragments were observed to be generated by the pET28a vectors (5369 bp), respectively (Figure 3).

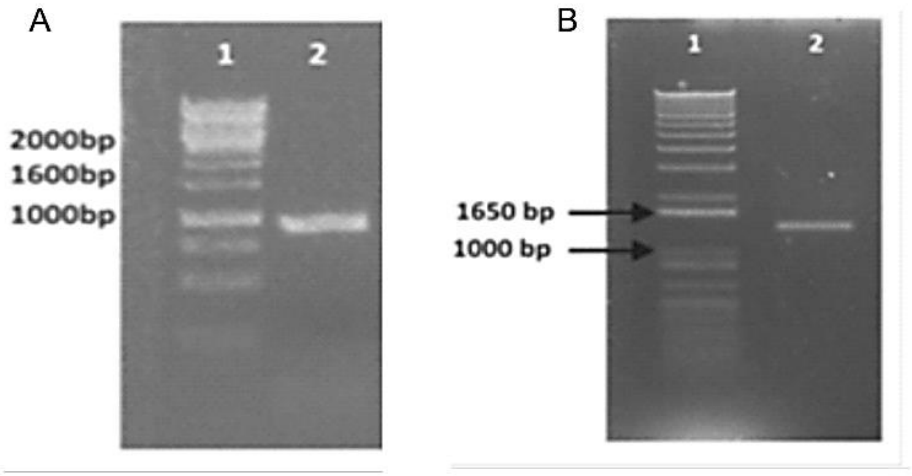

Figure 1. A - Optimization results of outer membrane protein of *S.typhi* SpaO, 1 DNA Ladder 1 Kb; 2 was SpaO amplification product approximately 912 base pair; B - Amplification LamB gene, 1 DNA Ladder; 2 PCR product 1340 base pair fragment.

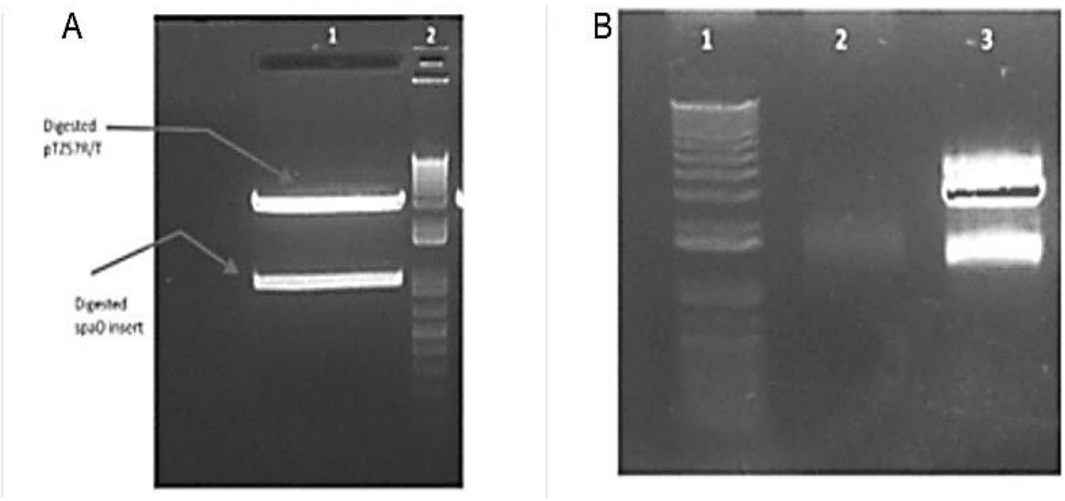

Figure 2. A - pTZ57R/TSpaO gene digested with enzymes *Nde*1 and *Xho*1: A1 - SpaO/pTZ plasmid double digestion; A2 - DNA Ladder 1kb. B - Restriction digestion pTZ57R/T plasmid having LamB gene with *Nde*1 and *Xho*1 enzymes: 1kb DNA ladder; 2 double digestion of LamB/pTZ57R/T Plasmid.

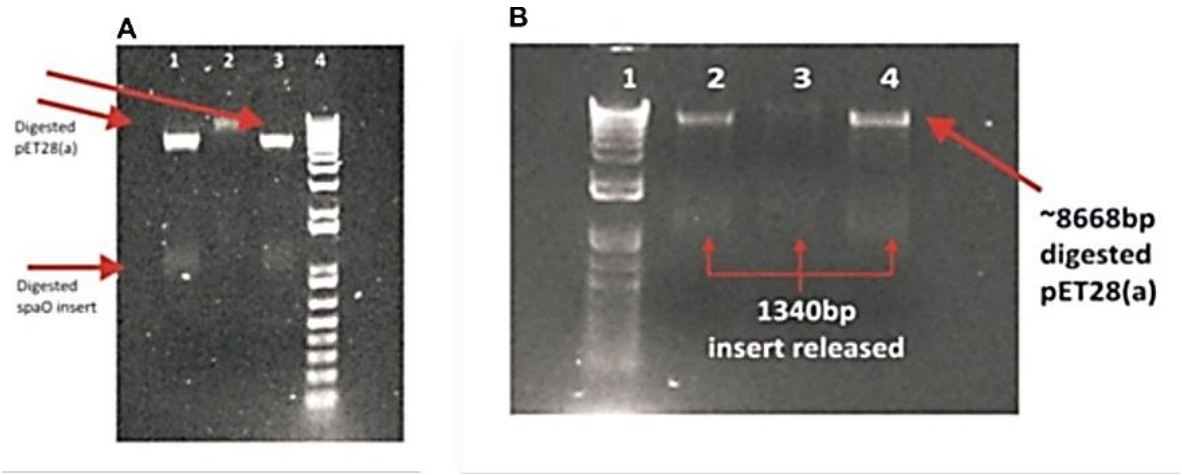

Figure 3. A - pET28(a) plasmid having SpaO gene digestion with *Nde*1 and *Xho*1: A1 and A3 - SpaO/PTZ Plasmids double digested with *Nde*1 and *Xho*1; A2 - vector with plasmid, A4 - DNA ladder. B - pET28a-LamB 1% agarose gel electrophoresis: B1 – ladder 1kb DNA; B2, B3 and B4 - pET28a-LamB restricted with *Nde*1 and *Xho*1.

Overexpression of recombinant proteins

The two constructed prokaryotic expression systems pET28a-SpaO-*E. coli* BL21DE3 and pET28a-LamB-*E. coli* BL21DE3 were shown to express rSpaO and rLamB, respectively. Induction with 200 µl and 400 µl of 0.5 mM ITPG at 37°C for 4-5 h was found to be optimal to achieve high expression levels of SpaO and LamB, respectively. After IPTG induction, whole-bacterial lysates were analyzed by 12 % SDS-PAGE, which yielded bands at approximately 33 and 49 kDa corresponding to rSpaO and rLamB proteins (Figure 4). Both the supernatant and cell pellets were analyzed for the presence of recombinant proteins. The level of expressed proteins from the inclusion bodies was also determined (Figure 5).

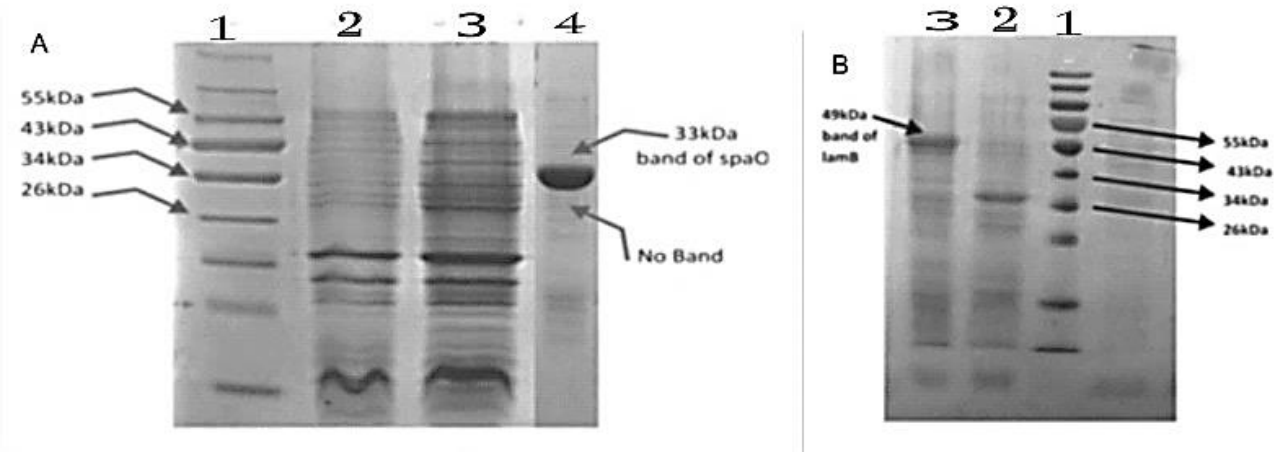

Figure 4. Expression analysis of rSpaO and rLamB: A - SDS-PAGE; A1 - protein marker; A2 - negative control uninduced; A3 – in supernatant there is no band of rSpaO; A4 – upon IPTG induction rSpaO (33kDa) expressed in pellet. B1 - expressed rLamB in pellet; B2 - negative control showing no band; B3 - protein marker; B4 - No band is shown in supernatant.

Purification of recombinant protein

Under native conditions lysis revealed both rSpaO and rLamB proteins in the pellet fraction were insoluble. While no discernible recombinant, proteins were found in the supernatant. Lysis under denaturing conditions was carried out to extract the recombinant SpaO and LamB proteins, which were solubilized in 8 and 6 M urea, respectively, followed by refolding in 3 M urea. Both proteins SpaO were purified by Ni-NTA affinity chromatography using elution buffer containing 250 mM imidazole and elution buffer comprising 150 mM imidazole for rLamB. The SDS-PAGE analysis confirmed the purified recombinant proteins (Figure 6).

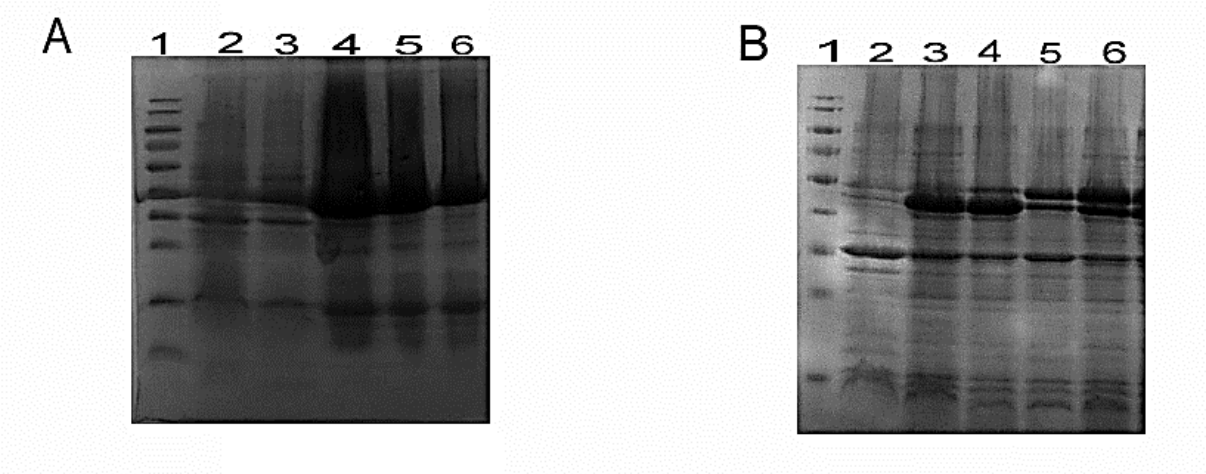

Figure 5. rSpaO solubilization protein; A1 - protein marker; A2 – rSpaO showing solubilization in IB solubilization buffer (8M Urea). A3- showing supernatant after sonication. B - Solubilization of rLamB. B1 - protein marker; B2 - expressed rLamB in pellet; B3 - showing supernatant after sonication; B4 – there was not any band seen in IB wash buffer; B5 – rLamB solubilization upon 8M urea.

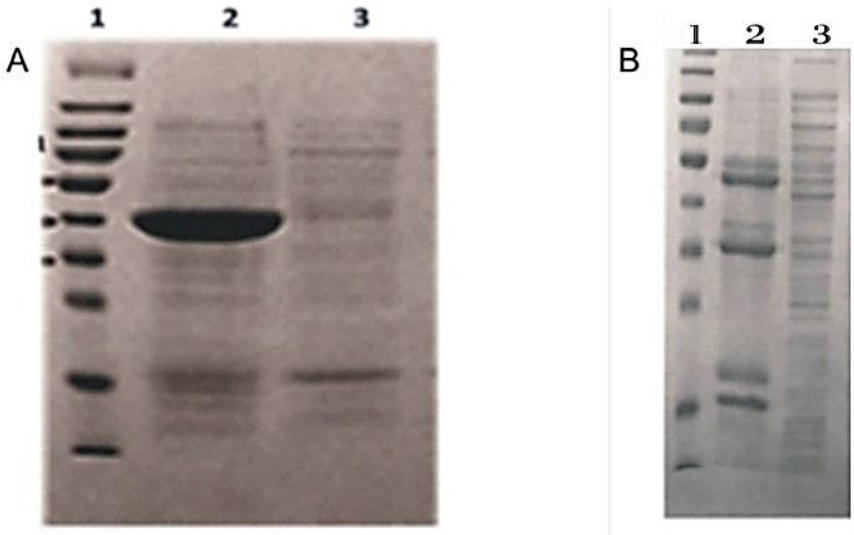

Figure 6. Purified SpaO A1 - protein molecular marker (kDa); A2 – purified rSpaO. B - SDS-PAGE (12%) analysis of purified rLamB: B1 - protein molecular marker (kDa); B2 – purified rLamB.

Western Blot analysis

Both expressed proteins antigenicity was done by western blot analysis. The SDS-PAGE showed 33 and 49 kDa protein bands corresponding to rSpaO and rLamB proteins (Figure 7 and 8).

4. Discussion

Gram-negative bacteria OMPs have been demonstrated to be potential targets for protective immunity against infections (O'Toole et al. 1995). In the present study, recombinant DNA technology was used to obtain two outer membrane proteins SpaO and LamB from *S. typhi*. Among the available protein expression systems, *E. coli* is the furthermost commonly used approach due to its well-investigated genetics, rapid growth, and relatively low cost. Moreover, there are a gradually huge number of mutant host strains and cloning vectors, which are available (Sorensen and Mortensen 2005). In this study, we successfully optimized an approach to express and purify SpaO and LamB from *S. typhi*.

For the OMP SpaO gene from *S. typhi*, we first carried out PCR amplification by means of specific primers. An amplicon of 912bp for SpaO produced as reported by RUAN et al. (2008). The SpaO sequence was cloned in a pET28a expression vector (5.3 kb) and transformed to cells of BL21 (DE3) *E. coli.* The results showed that induction by 200 µL 0.5 mM IPTG formed inclusion bodies that expressed SpaO at high level.

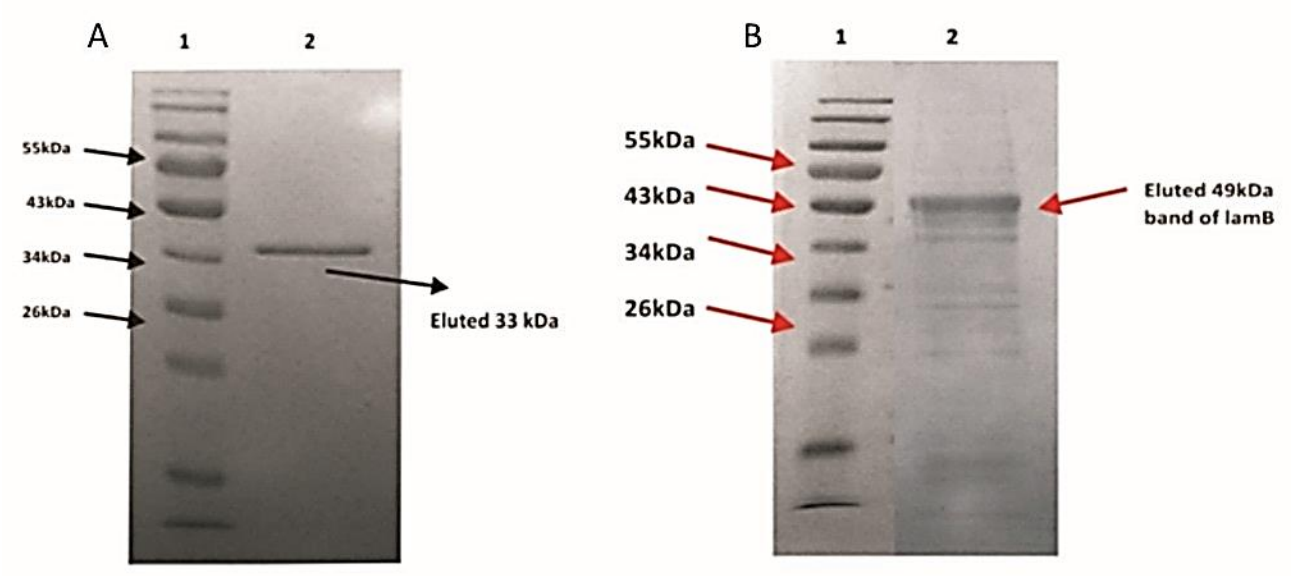

Figure 7. A - Western blot with anti His tag monoclonal antibody: 1 rSpaO inducted by 0.5M IPTG; 2 protein marker (kDa). B - Western Blot of rLamB: B1 – a 49 kDa band shown; B2 - protein marker.

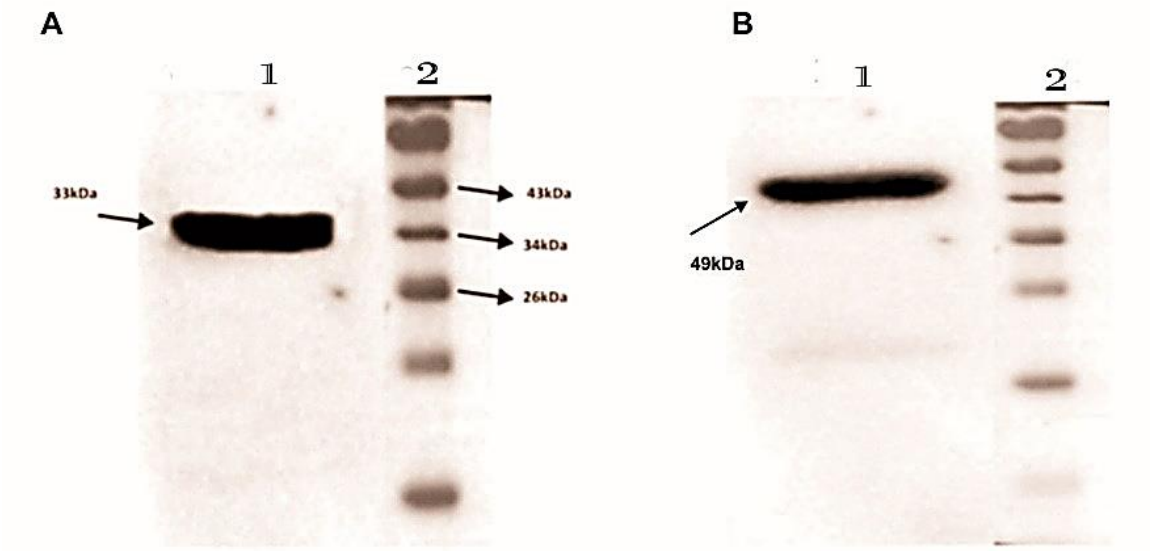

Figure 8. A - Western blot analysis of recombinant SpaO with anti His tag monoclonal antibody: 1 recombinant SpaO induced with 0.5mM IPTG; 2 protein molecular marker (kDa); B - Western Blot analysis of LamB: 1 showing a strong band of 49 kDa. 2; protein marker.

Earlier studies revealed that in *E. coli* high expression levels of recombinant proteins frequently leads to the aggregation of the expressed protein molecules in inclusion bodies (Chrunyk et al. 1993). Lack of eukaryotic chaperons (Carrio and Villaverde 2001), High concentration of inducers, a strong promoter system and the post-translational machinery are the factors that can affect the inclusion bodies formation (Singh et al. 2015). Intact proteins can be recovered from inclusion bodies by solubilization, refolding, and purification (Vallejo and Rians 2004). However, inclusion bodies formation hampers the recovery of recombinant protein by the solubilization method (Singh et al. 2015). Solubilization is the critical step in obtaining the desired protein in solution without inducing any chemical and deleterious alterations. Several detergents can be used to solubilize inclusion bodies including strong denaturants such as urea (Schmid et al. 1996), milder detergents for example sodium dodecyl (Stockel et al. 1997), sarkosyl, n-cetyltrimethylammonium bromide (CTAB) and guanidinium salts (Cardamone et al. 1995). A study conducted by Fischer and Rudolph revealed that high concentration of urea (6-8 M) can be used for solubilization of inclusion bodies or guanidine hydrochloride (Rudolph et al. 1997). After obtaining inclusion bodies expressing SpaO proteins, we carried out solubilization in 8 M urea, that is in line with earlier studies on OmpF (Wang et al. 2017), OmpC (Kumar and Krishnaswamy 2005), and 49 kDa OMP (Kumar et al. 2009)

results in inclusion bodies formation. Refolding of denatured proteins can be achieved through the controlled removal of excess denaturants, which allows the protein to refold spontaneously (Singh and Panda 2005). There are numerous refolding approaches including dialysis, slow dilution, reverse dialysis and rapid dilution (Kumar and Krishnaswamy 2005). In our study, we refolded the rSpaO proteins by dialysis in different concentrations of urea, which revealed 3 M urea was optimal for proper refolding. In order to achieve functionally active protein, with a use of 250mM imidazole in elution buffer SpaO was purified by Ni-NTA affinity chromatography. We achieved 75% purity, giving an 33kDa protein, Moreover, which was confirmed with Western blot analysis. These results were in accordance with a purity of 75% for rSpaO by Ni-NTA affinity chromatography reported by Mao *et al.* (2006).

Similarly, for the OMP LamB gene from of *S. typhi*, PCR amplification was performed with specific primers. DNA sequence analysis revealed a full-length gene of approximately 1340 bp, which revealed high-level homology through a previously reported LamB gene from different bacteria (Upadhyaya et al. 2007). Subsequently, purified and eluted 1340 bp sequence was cloned in pET28a expression vector (5.3 kb). After transformation with the recombinant plasmid, *E. coli* BL21 (DE3) cells were induced by ITPG under different conditions. We found that induction with 400 µL of 0.5 mM IPTG formed inclusion bodies that highly expressed LamB (49-kDa band in Western blot analysis). Kaur and Jain (2013) expressed successfully a protein of 49 kDa in a pET28 (a) expression vector. We next solubilized the rLamB proteins in 6 M urea, which is in line with the approach used by Hamid and Jain (2008). The rLamB proteins was then refolded by dialysis in 3 M urea as above. To achieve functionally active protein, rLamB was purified by Ni-NTA affinity chromatography using an elution buffer containing 150 mM imidazole. A single band of purified rLamB at approximately 49 kDa revealed by Western blot analysis. These findings are in line with Kaur and Jain (2013), who purified successfully a protein of 49 KDa upon 150 mM imidazole. Moreover, Hamid and Jain (2010) used pQE60 plasmid to cloned OMP from *S. typhimurium* of 49kDa and purified by Ni-NTA affinity chromatography upon different pH conditions.

5. Conclusions

Our results indicated that rSpaO and rLamB proteins in the *E. coli* expression system could be formed into inclusion bodies upon 200 and 400 µL IPTG induction, followed by solubilization in 8 and 6 M urea, respectively, and refolding in 3 M urea. Purification of rSpaO and rLamB was then achieved by Ni-NTA affinity chromatography with buffer containing 250 and 150 mM imidazole, respectively. These optimized methods can be used to generate recombinant proteins for the development of vaccines in the future.

Authors' Contributions: LATIF, K.: acquisition of data, analysis and interpretation of data and drafting the article; NAZ, S.: acquisition of data, analysis and interpretation of data; ALTAF, I.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article and critical review of important intellectual content; HUANG, J.D.: acquisition of data and analysis and interpretation of data; BASHIR, R.: acquisition of data and analysis and interpretation of data; ASLAM, F.: acquisition of data and analysis and interpretation of data; XING, S.: acquisition of data and analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

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