Chapter 3

Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis
Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis

3.1. Introduction

Complete sequencing of the genome of M. tuberculosis H37Rv (Cole et al., 1998) has been a major achievement which provides a great landmark in TB research. Another major achievement has been in the definition of the composition and organization of the cell envelope, because it plays an important role in physiology and pathogenesis of M. tuberculosis, (Minnikin DE, 1982; Brennan PJ and Nikaido H, 1995; Daffe M and Draper P, 1998; Dmitriev BA et. al, 2000). The lipoproteins are the major constituents of the cell envelope. In addition, because of the secretory nature these lipoproteins act as strong immunogens.

All premature lipoproteins have signal peptides which direct their export and post-translational lipid modification. The signal peptides of these proteins have a consensus sequence consisting of four amino acids called lipobox ([LV] [ASTVI] [GAS] C) with a universally conserved cysteine at position +1; the lipobox directs processing of the pro-lipoprotein to form the mature lipoprotein (Sankaran and Wu, 1995). The M. tuberculosis H37Rv genome encodes approximately 104 lipoproteins (Sutcliffe IC and Harrington DJ, 2004), of which only a few have been functionally characterized. These lipoproteins mature by cleavage of signal peptide with the help of signal peptidase II which is encoded by lspA (Rv1539). lspA is a single gene known to be present in the genome of M. tuberculosis H37Rv which is responsible for maturation of the mycobacterial lipoproteins.

To study the potential role of lspA gene in the biology and pathogenesis of mycobacterial infections, we used an overexpression strategy. Polyclonal anti-LspA antibody was required to demonstrate the overexpression of LspA protein inside the cell, therefore the aim of this chapter was “production of polyclonal antibody against recombinant LspA protein of M. tuberculosis. To achieve our goal, we used full length of Rvl539 which has been annotated as lipoprotein-specific signal peptidase (IspA) (Cole ST et. al, 1998) in the genome of M. tuberculosis H37Rv for cloning in an expression vector.
The clone was confirmed by colony PCR, double restriction digestion and finally by sequencing. Positive confirmed clone was transformed into BL21:DE3 *E. coli* strain and LspA protein was purified using Ni-NTA affinity based column chromatography. Presence of purified protein was confirmed by using anti-His monoclonal antibody. This purified recombinant LspA protein was injected intramuscularly in rabbit to raise antibody. We confirmed the specificity of antibody by western blot analysis with purified LspA protein. This antibody was later used (Chapter 4) to confirm overexpression of LspA protein.
3.2. Materials and Methods

3.2.1. Bacterial Strains, Cells and Culture Conditions

*E. coli* strain DH5α was used in all cloning experiments for generation of constructs and for plasmid amplification. *E. coli* strain BL-21 (DE3) was used for the expression of cloned genes. Both the strains were obtained from Novagen (USA). *E. coli* DH5α and BL-21 (DE3) cells were grown in Luria-Bertani (LB) broth with shaking at 120 rpm or on LB agar plates in presence of kanamycin (25µg/ml) at 37°C.

*M. tuberculosis* H37Rv was obtained from the National JALMA Institute for Leprosy and other Mycobacterial Disease, Agra, India and served as a source of genomic DNA for the amplification of *lspA* gene by PCR.

*M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Difco) supplemented with OADC (oleic acid, albumin [bovine fraction (V), dextrose, catalase] (Difco) and 2 % glycerol at 37°C with shaking at 200 rpm.

3.2.2. Restriction Enzymes, Ladders and Plasmid

Restriction enzymes (*Bam*HI *and* XhoI) and T4 DNA ligase were procured from New England Biolabs (Beverely, MA, USA). Kits for the plasmid isolation and elution of DNA from the agarose gel were obtained from Real Biotech Corporation (RBC), Taiwan. 100 bp DNA ladder, unstained and pre-stained protein ladder were purchased from MBI, Fermentas.

Vector used for cloning and expression *i.e.*, pET-22b+ (Fig 3.1) was obtained from Novagen (Medison Wincosin, USA).

3.2.3. Cloning of *lspA* Gene of *M. tuberculosis* in pET-22b+ Expression Vector

3.2.3.1. Genomic DNA Isolation from *M. tuberculosis* H37Rv

Genomic DNA from *M. tuberculosis* H37Rv was extracted according to the CTAB (cetyl trimethyl ammonium bromide) method (Bose M et. al., 1993). *M. tuberculosis* H37Rv was grown on LJ slants at 37°C for 20-30 days. A loopful of colony was scraped from LJ slant and added in 1 ml of GTE solution (50 mM Glucose, 10 mM Tris-Cl, pH 8.0 and 1
mM EDTA). The mixture was vortexed and cells were centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and another 450 µl of GTE solution was added to the pellet with 50 µl of freshly prepared 10 mg/ml of lysozyme solution (Sigma, USA) and was incubated at 37°C overnight. Next day, 100 µl of 10% SDS was added along with 50 µl of 10 mg/ml of proteinase K (Sigma, USA) and mixed gently. The reaction mixture was kept at 55°C for 1 hr. After 1 hr, 200 µl of 5 M NaCl was added with 160 µl of preheated CTAB (cetyl trimethyl ammonium bromide) and placed at 65°C for 10 minutes. Reaction mixture was cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The cocktail was slowly mixed and centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was collected separately without disturbing the interface and the above step was repeated. To the collected aqueous phase, 0.7 volume of isopropyl alcohol was added, mixed gently and kept at -20°C for 1 hr. The mixture was centrifuged at 10,000 rpm, at 4°C for 15 minutes. The pellet was washed with 1 ml 70% ethanol and re-centrifuged at 10,000 rpm for 10 minutes. The pellet was air dried at room temperature and dissolved in 50 µl of distilled water. The DNA was checked on 0.8% agarose gel.

3.2.3.2. PCR Amplification of \textit{lspA} Gene

Primers were designed by using Gene Runner online software with \textit{BamHI} and \textit{XhoI} restriction enzymes sites incorporated in the forward and reverse primers respectively.

The sequence of primers is given below:

\textbf{Table 3.1:} List of primers for cloning of \textit{lspA} gene

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'-3')</th>
<th>Length of Primer (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lspApET FP</td>
<td>GCGGATCCTGTGCTGACGAACA</td>
<td>24</td>
</tr>
<tr>
<td>lspApET RP</td>
<td>CTCGAGCGGGGTAGCCATCGGGCTTTG</td>
<td>25</td>
</tr>
</tbody>
</table>

Full length \textit{lspA} gene (\textit{Rv1539}, 650bp) was amplified from \textit{M. tuberculosis} H37Rv genomic DNA by polymerase chain reaction (PCR) in 50 µl volume containing 200 μM dNTPs (MBI Fermentas, Canada), 1X Taq Polymerase buffer, 10 pM/µl of primers and 1 unit of Taq Polymerase enzyme (Bangalore Genei, India).
The PCR conditions were as follows:

- Initial denaturation at 94°C for 5 minutes
- Denaturation at 94°C for 1 minute
- Annealing at 65°C for 30 sec
- Extension at 72°C for 30 sec

3.2.3.3. Gel Elution of PCR Product using RBC Gel Extraction Kit

PCR amplicon was purified using HiYield Gel/PCR DNA Extraction Kit (Real Biotech Corporation (RBC), Taiwan). PCR amplified product was mixed with 6x gel loading dye and run on 1.5% agarose gel at 80V. After proper resolution the DNA band was cut and chopped in small pieces, 135mg of chopped gel was taken in 2 ml eppendorf and 500 µl of DF buffer was added. It was incubated at 55°C till gel pieces dissolved and loaded on DF column and centrifuged for 1 minute at 10,000 rpm. The flow through was discarded and 500 µl of wash buffer was added to column, spun at 10,000 rpm for 1 minute. Discarded the flow through and centrifuged the empty DF column for 5 minutes. Left the DF column opened for 2-3 minutes till complete evaporation of ethanol. In DF column 20 µl of distilled water was added to the center, after 2 minutes spun at 10,000 rpm for 2 minutes and collected the eluted product (E1). Elution was repeated with distilled water to collect E2 and E3.

3.2.3.4. Digestion of PCR Product of lspA Gene and pET-22b+ with BamHI and XhoI Restriction Enzyme

The plasmids (6µg) were digested with 80 units of BamHI and XhoI enzyme and incubated at 37°C for 4 hrs. After 4 hrs, the reaction mixture was heat inactivated at 65 °C for 20 minutes.

3.2.3.5. Purification of Digested DNA

The vector DNA was eluted and purified from the gel using RBC gel extraction kit whereas, the insert was purified with twice the volume of chilled ethanol and one tenth volume of 3 M sodium acetate, pH 5.2 and kept at -70°C for 1 hr. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and pellet was
Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis

washed with 70% ethanol. The pellet was air dried and dissolved in 10 µl autoclaved water.

3.2.3.6. Ligation of Purified Insert with Vector Backbone using Quick Ligation Kit (New England Biolabs, USA)

The concentration of the insert (PCR product) was calculated according to the size & ratio used for ligation using formula:

\[
\text{ng of insert} = \text{ng of vector} \times \frac{\text{Size of insert}}{\text{Size of vector}} \times \frac{\text{Ratio of insert}}{\text{vector}}
\]

Size of vector = 5.394 kb
Size of insert = 609 bp
ng of vector = 50 ng
Ligation was carried out at 1:3 ratio (vector: insert)
Therefore, ng of insert used was 32.11 ng

Reaction mixture was

- 2X Ligation Buffer: 10 µl
- Vector: 1 µl
- Insert: 2 µl
- Ligase Enzyme: 1 µl
- Water: 7 µl

21 µl

Reaction was incubated at 25 ºC for 15 minutes and then chilled on ice.

A control was also set where no insert was added in the ligation mixture in order to check the self-ligation of the vector.

3.2.3.7. Preparation of Competent Cells of E. coli DH5α

The competent cells of E. coli DH5α cells were prepared as per protocol (Cohen SN et al., 1972). 1% of E. coli DH5α cell inoculum from its prepared frozen glycerol stocks at -80°C was inoculated in 5 ml of LB broth and incubated at 37°C for overnight. 500 µl of overnight grown culture was inoculated in 50 ml LB broth for 2-3 hrs till O.D.
reaches 0.4. After the incubation, 50 ml overnight grown culture was kept on ice for 30-45 minutes and centrifuged at 10,000 rpm at 4°C. The pellet was suspended in ice cold 100 mM CaCl$_2$ and kept on ice for 1 hr. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and pellet of the prepared competent cells was suspended in 2 ml of 100 mM CaCl$_2$ with 15% glycerol and stored at -80°C.

3.2.3.8. Transformation of *E. coli* DH5α Cells with Ligation Mixture

The *E. coli* cells were transformed with the ligation mixture along with the control ligation mixture. A positive control was also added i.e. undigested pET-22b$^+$ in order to check the efficiency of competent cells along with negative control which only had competent cells plated on LB agar media with 25 µg/ml of kanamycin.

3.2.3.9. Mini Preparation of Plasmid DNA

The plasmid DNA was isolated by alkaline lysis method (Sambrook *et al.* 1989). The transformed colonies were inoculated in 5 ml of LB broth containing 100 µg/ml ampicillin and grown overnight at 37°C with shaking at 200 rpm. Cells were pelleted at 10,000 rpm in a microcentrifuge for 5 minutes and the supernatant was aspirated. The pellet was suspended in 200 µl of Solution I supplemented with 50 µg/ml RNase and kept at room temperature for 2 minutes. 200 µl of Solution II was added to the tube, mixed gently and incubated at room temperature for 5 minutes, 200 µl of chilled Solution III was then added and the contents were mixed gently by inverting the tube and kept in ice for 10 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and 0.7 volume of isopropanol was added and centrifuged at 10,000 rpm for 20 minutes and then supernatant was aspirated out. The pellet was washed with 70% ethanol by centrifugation at 10,000 rpm for 10 minutes. The supernatant was immediately discarded and the pellet was air dried. The dried pellet was suspended in autoclaved distilled water.

3.2.3.10. Screening of Clones

a. Screening of Clones by PCR

lspApET FP and lspApET RP (gene specific primers of *lspA* gene) primers were used by taking recombinant plasmid DNA as the template to look for the amplicon of the gene.
b. Double Digestion with *BamHI* and *XhoI* Restriction Enzymes

The plasmids which had given positive PCR amplicons were double digested with *BamHI* and *XhoI* restriction enzymes to look for the drop out of 650 bp.

c. Sequencing

The sequencing of plasmids was done commercially with T7 promoter primers in order to check for the presence of *lspA* gene and the correct reading frame of the *lspA* gene in the plasmid pET-22b⁺.

3.2.4. Expression of Recombinant LspA Protein

3.2.4.1. Induction of LspA Protein

*E. coli* Bl-21 (DE3) cells were transformed with recombinant plasmid pET-22b⁺ with *lspA* (pET-22b: *lspA*). 500 ml of transformed *E. coli* BL-21 cells were grown at 37°C for 3-4 hrs till O.D. reaches 0.4-0.6. Culture was collected in separate 2ml tube as uninduced *E. coli* culture. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.5mM and cultures were grown for an additional 4 hours at 37°C. The cells were harvested by centrifugation at 8000 rpm for 10 minutes. Both the pellet uninduced and induced *E. coli* cultures were dissolved in 1X SDS gel loading buffer and run on 12% SDS-PAGE.

3.2.4.2. Sonication of Induced Cell Pellet

The pellet of 1000 ml induced culture was dissolved in 20 ml of sonication buffer containing 300mM NaCl, 100 mM Tris-Cl pH 8.0, 10% glycerol and 1 mM PMSF and given 12 cycles of sonication. The suspension was centrifuged at 10,000rpm for 30 minutes. The pellet and supernatant were separated. An aliquot of both were mixed with 1 X SDS gel loading buffer and run on 10% SDS-PAGE.

3.2.5. Purification of LspA Protein

3.2.5.1. Solubilisation of Pellet Containing LspA Protein with 8 M Urea

The pellet was dissolved in 30 ml urea solubilisation buffer containing 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl pH 8.0 and 1 mM PMSF and kept at room temperature
on rocker for 12-16 hrs, followed by centrifugation at 10,000 rpm for 30 minutes. The pellet and supernatant were separated.

### 3.2.5.2. Column Elution of LspA Protein

The supernatant containing the required protein was mixed with 2 ml of 50% Ni NTA resin and kept on shaking for around 16-18 hrs. Supernatant mix with Ni-NTA resin was loaded on column. The LspA protein was eluted using various wash and elution buffers of different pH (Wash Buffer of pH 6.5 and 5.5: 8 M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris-Cl and 1mM PMSF Elution Buffer of pH 4.0: 8M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris-Cl and 1mM PMSF).

### 3.2.5.3. Refolding of Eluted Purified LspA Protein through Step-wise Dialysis Method

The denatured protein containing 8 M urea were refolded by gradual removal of urea by means of step-wise dialysis from 7 M to 0 M strength through 6, 5, 4, 3, 2, 1 and 0.5 molar solutions. Buffer used for dialysis had 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM PMSF, and 1mM GSH and 0.1 mM GSSG till urea concentration reached 5M. Once the urea concentration reached 5M, L-arginine was added into the buffer at the concentration of 150 mM. As the urea concentration reached 2 M, the concentration of L-arginine was increased to 300 mM. At 0 M urea concentration, the proteins were dialysed against PBS buffer (without L-arginine, urea, GSH and GSSG). The amount of lipopolysaccharide (LPS) in the protein fraction was measured quantitatively with the Limulus Amebocyte Lysate assay (Sigma, USA) as according to the manufacturer’s instructions. An additional purification step was performed to remove endotoxin contaminations (lipopolysaccharide) by transferring purified and refolded LspA protein to a column containing immobilized polymyxin B (Pierce, USA) to remove endotoxin.

### 3.2.5.4. Western Blot of Eluted LspA Protein

The western blot was carried out in order to ensure that the eluted band would be of LspA protein. After SDS-PAGE electrophoresis protein gel was transferred to nitrocellulose membrane (Bio Rad Laboratories, Hercules, USA) using transfer buffer.
Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis

(20% Methanol, 25 mM Tris-Cl & 192 mM Glycine). Transfer was run at 70 volts, 200 mA for 2 hrs. Membrane was immersed in 3% BSA in PBS to block nonspecific sites & kept at 4°C for overnight. Membrane was washed with PBS followed by three washes with PBST (0.05% Tween 20) and again one wash with PBS for 5 minutes each. Primary antibody (anti His-Tag Antibody, Abcam, USA) at a dilution of 1:3000 was added & incubated for 2 hrs on shaking. Membrane was again washed with PBS and PBST as mentioned earlier. Secondary antibody (peroxidise conjugated, Abcam, USA) was added at a dilution of 1:5000 and incubated for 2 hrs and given washings with PBS and PBST accordingly. After the washings membrane was developed with 0.5 mg/ml of 3,3- diaminobenzidine tetrahydrochloride (DAB) dissolved in PBS and 100 µl of 30% H$_2$O$_2$. Bands were visible in 15 minutes.

3.2.6. Production of Polyclonal Antibodies against Recombinant LspA Protein

The antibodies against the LspA protein was raised in 5-month-old female New Zealand white rabbit. For each immunization, 300 µg of recombinant LspA protein in 0.5 ml PBS was mixed with 0.6 ml of incomplete Freund's adjuvant (Sigma) and administered subcutaneously at five sites in rabbit, followed by four booster injections of 300 µg each 2, 3, 4 and 6 weeks after the first injection. Serum was prepared from blood collected 2 weeks after the last booster dose. The animal handling was according to the guidelines of the Institutional Ethical Committee and Indian Council of Medical Research, India.
3.3. Results

3.3.1. Cloning of *lspA* Gene (Rv 1539) of *M. tb* H37Rv in Expression Vector pET-22b+ 

The entire length of *lspA* gene (Rv1539) was amplified from genomic DNA of *M. tuberculosis* H37Rv by PCR using gene specific primers lspApET FP and lspApET RP containing *Bam*HI and *Xho*I restriction sites in the forward and reverse primer respectively. The amplified product was eluted from the gel using RBC gel extraction kit and run on 1% agarose gel (Fig 3.2A) along with 100 bp DNA ladder (MBI Fermentas, Canada). The purified PCR amplicon of *lspA* gene (650bp) was cloned into prokaryotic expression vector pET-22b+ (Fig 3.1). The recombinant clones were checked by restriction double digestion of recombinant plasmid using *Bam*HI and *Xho*I enzymes yielding a pop out of ~650 bp (Fig 3.1B). The reading frame of recombinant pET-22b+: *lspA* clone was confirmed by DNA sequencing.

![Basic Circular Map of pET-22b+ Vector](image-url)

**Fig 3.1**: Basic Circular Map of pET-22b+ Vector
Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis

3.3.2. Expression of LspA Protein

The expression of fusion protein was studied in *E. coli* BL-21 (DE3) cells. After the induction with 0.5mM IPTG, whole cell proteins were resolved by 12% SDS-PAGE. The induced LspA protein expressed at high level as compared to uninduced whole-cell lysate (Fig 3.3).

3.3.3. Purification of LspA Protein

The expressed C-terminal His-tagged recombinant protein of size 25 kDa was purified under denaturing condition using nitrilotriacetic acid (Ni-NTA) affinity based agarose column chromatography (Qiagen) according to the manufacturer's instructions. The denatured LspA protein was refolded by step-wise dialysis method (Fig 3.4A). Anti-His-Tag monoclonal antibody (Abcam, USA) was used to identify the purified refolded LspA protein (Fig 3.4B).

---

**Fig 3.2:** (A) PCR amplification of *lspA* gene. Lane1: 100 bp ladder (Fermentas). Lane 2: amplicon of *lspA* gene. (B) Screening for recombinant clones by restriction double digestion using *Bam*HI and *Xho*I enzymes. Lane 2: positive recombinant clones. Lanes 1 and 3: negative colonies. Lane 4: 100 bp ladder (Fermentas)
**Fig 3.3:** Induction of LspA protein. Lane 1: Unstained protein Marker (Fermentas). Lane 2: lysate from uninduced BL-21 cells. Lane 3: lysate from induced BL-21 cells over expressing the LspA protein following induction with 0.5mM IPTG for 4 hrs as indicated by an arrow.

**Fig 3.4:** (A) 12% SDS-PAGE for purified recombinant LspA protein. Lane 1: purified recombinant LspA protein. Lane 2: unstain protein marker (Fermentas). (B) Western blot analysis of LspA protein by using Anti-His-antibody. Lane1: Recombinant LspA protein. Lane 2: Pre-stained molecular mass marker (Fermentas).
3.3.4. Polyclonal Antibody Production against Recombinant LspA Protein

This purified recombinant LspA protein was injected intramuscularly in rabbit for raising antibody. We confirmed the specificity of antibody by western blot analysis with purified LspA protein (Fig 3.5). This antibody was used in overexpression study of LspA protein.

**Fig 3.5:** Western blot analysis of LspA protein by using anti-LspA antibody to check the specificity of antibody. Lane 1: Recombinant LspA protein. Lane 2: Pre-stained molecular mass marker (Fermentas).
3.4. Discussion

lspA is a single gene present in the genome of *M. tuberculosis* which is responsible for maturation of a large number of lipoproteins (~104) within the bacterium, which belong to class of most virulent proteins of *M. tuberculosis*. Therefore it is important to understand the physiological and biological function of the gene. To achieve this goal we prepared a large amount of this protein.

To obtain LspA protein in large amount, cloning and expression technique was used. The vector used for cloning was pET-22b which is an expression vector that utilizes the T7 RNA polymerase promoter to direct high level expression of cloned gene in *E. coli*.

*lspA* gene was successfully cloned in pET-22b vector. Positive clones were confirmed by colony PCR, restriction doubles digestion and finally by sequencing. Recombinant plasmids pET-22b: *lspA* was used to transform BL-21(DE3) for expression of the protein. His-tagged LspA protein was expressed at high level on induction with IPTG as compared to uninduced whole-cell lysate (without IPTG).

Overexpression of foreign proteins from cloned genes in heterologous hosts, such as *E. coli* often leads to the formation of insoluble intercellular aggregates of the expressed protein (Williams DC *et. al.*, 1982; Schoner RG *et. al.*, 1985). These so called inclusion bodies (IBs) are readily isolated by low speed centrifugation and usually consist of almost pure accretions of denatured forms of the foreign protein. IBs must then be solubilized and refolded into an active conformation. There are two important issues in recovering active proteins from IBs i.e. solubilization and refolding. Solubilization must result in monomolecular dispersion and minimum nonnative intra- or inter-chain interactions. Choice of solubilizing agents like urea, guanidine- HCl, or strong ionic detergents such as N-lauroyl sarcosine that are frequently used play a key role in solubilization efficiency, in the retention of structure of the protein in the denatured state, and in subsequent refolding. In most cases, 6-8 M urea and 6-7 M guanidine HCl are required to achieve extensive binding sufficient to unfold and solublize the proteins from IBs. IBs solubilized in denaturants may be a clear, nonturbid solution, but may be aggregating as soluble oligomers. Refolding is a change
Production of polyclonal antibody against purified recombinant LspA protein of M. tuberculosis

in protein conformation from unfolded to folded state. Step-wise dialysis is one of the commonly used methods to remove the denaturant and refolding of protein. In this process concentration of the denaturant is decreased gradually by the dialysis of unfolded protein against decreasing concentration of denaturant. Usually small molecules like L-arginine, sucrose etc, also called co-solutes are added to enhance the process of refolding.

The recombinant purified LspA protein formed inclusion bodies (IBs) when overexpressed in E. coli BL-21 (DE3). We therefore first solubilized LspA protein from IBs using 8 M urea and purified the protein further by using Ni-NTA affinity chromatography in denaturing conditions. This was followed by refolding of the protein by gradual removal of urea by step-wise dialysis and the purified proteins were detected using anti-His-tag monoclonal antibody.

The emulsion of 1µg of purified and refolded LspA protein with incomplete adjuvant was prepared and injected in rabbit at 5 sites to raising anti LspA antibody. The specificity of the anti LspA antibody was checked by western blot using purified recombinant LspA protein. This polyclonal anti-LspA antibody will be used in next chapter to demonstrate the overexpression and localization of LspA protein.