Chapter 2
Materials & Methods

2.1 Reagents and Chemicals

Fine chemicals were procured locally and also from Sigma Chemical Company, USA and were of analytical grade. Restriction enzymes, T4 DNA ligase, RNase were purchased from New England Biolabs MA, USA. Enhanced Chemiluminiscence Labeling kit and Detection reagent, Taq DNA polymerase and Hybond N+ Nylon membranes were purchased from Amersham International, Birmingam, UK. Novex NuPage gels for analyzing protein samples were purchased from Invitrogen, US.

Automated DNA sequencing kit was from Perkin-Elmer Applied Biosystems, NJ, and USA. PCR purification column were purchased from Qiagen, USA, Buffers, reagents and media were prepared with glass-distilled water. Bacterial culture media were purchased from Hi-media, India.

2.2 Bacterial strains used in the study

a) *E.coli* DH5α,

b) *E.coli* BL-21 (DE3) and *E.coli* BL21 (DE3) pLys S

c) *M.tuberculosis* H37Rv

d) *M.tuberculosis* (clinical isolates)

2.3 Culture media

Luria-Bertani (LB) broth was prepared using 10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride in 1000 ml of distilled water, pH adjusted to 7. 2 before sterilization. Solid LB media was prepared with 1. 5% agar.

2.4 Antibiotics

Plasmid bearing strains of *E. coli* were grown in LB media containing 100μg/ml ampicillin with or without chloramphenicol 35 μg /ml.

2.5 Growth conditions

Cultures on solid media were grown at the required temperature in appropriate incubators. Liquid cultures were grown on water bath gyratory shaker (200 rpm) at 37°C. Culture growth was monitored by measuring optical density at 600 nm in spectrophotometer (Hitachi U2000).

2.6 Storage of cultures

Bacterial host strain and recombinant strains were maintained in Petri-plates with / without respective antibiotics. For long term storage they were maintained in the form of stabs at room temperature (RT) and 30% glycerol culture stock were stored at –80°C. Host and recombinant bacteria were lyophilized and stored for indefinitely at room temperature (RT).

2.7 Adenosine deaminase Assay

Principle

ADA is easily assayed by measuring the amount of ammonia formed during the 60 minutes of incubation at 37°C. Ammonia reacts in presence of sodium nitrosyl pentacyanoferrate (III) as a catalyst, with sodium hypochlorite and phenol, in alkaline solution, producing a deep blue indophenol. The ammonia

concentration is directly proportional to the absorbance of the indophenol at 650nm.

Adenosine deaminase is an enzyme involved in hydrolytic transformation of adenosine into inosine. It is widely distributed in mammalian tissues, particulary in T-lymphocytes.

A high concentration of ADA incurr in several forms of tuberculosis, but also in other infectious diseases such as mononucleosis, typhoid fever and in the initial stage of HIV.

Through a series of biochemical modifications, the usual colorimetric procedure to assay ADA activity became more sensitive and stable.

Such modifications of the technique also allowed the assay of ADA activity in very small samples of biological fluids. Thus this technique proved to be a very important method for the clinical diagnosis of tuberculosis.

Materials

Vial 1: Phosphate buffer 2 ml, was freshly prepared.

Vial 2: Buffered adenosine 5 ml. Crystals in this solution was dissolved by placing the flask in warm water bath.

Vial 3: Buffered standard solution of ammonium sulfate 1 ml. Solution B was diluted 1:1000 with distilled water (0.1 ml of ammonium sulfate solution with 100 ml of distilled water). This diluted solution is stable for one week.

Vial 4: Phenol-catalyst solution 10 ml. 10 ml of phenol catalyst solution diluted with 40 ml of distilled water (1:4). Stable for six months.

Vial 5: Sodium hypochlorite in alkaline solution 10 ml. 10 ml of sodium hypochlorite solution diluted with 40 ml of distilled water). Stability of this diluted solution is at least 6 months.

Precautions and Storage

All reagents of the kit was stored at 4°C.

Procedure

1. Following solutions were added:

Solution	Sample	Blank of Sample	Standard	Blank of reagents
	A1	A2	A3	A4
Phosphate buffer	-	-	10 µl	110 µl
Adenosine	100µl	100µl	-	-
Standard Solution	-	-	100 µl	-
Sample	10µl	-	-	-

2. Tubes were incubated for 60 minutes at 37°C.

Phenol catalyst	300µl	300µl	300µl	300µl
Sample	-	10µl	-	-
Alkaline Hypochlorite	300µl	300µl	300µl	300µl

3. All the tubes were incubated at room temperature for 30 minutes.

4. Reading was carried out photometrically with the aid of a tube reader at a wavelength of 630 nm. Distilled water served as the blank.

Following formula was used to interpret the results

$$\text{ADA activity in U/L} = \frac{A1 - A2}{A3 - A4} \times 50$$

One U/L of ADA activity corresponds to 3 nmoles of ammonia released into the reaction mixture per hour at 37°C.

If the O.D. of the sample > 1, the test was repeated after a dilution of the sample between 5 and 10 times with the phosphate buffer (vial 1) and the result was multiplied with the dilution factor.

2.8 Processing of clinical samples

Lymph nodes were chopped finely using a sterile scalpel and homogenized in lysis buffer (Tris-HCl 25mM; EDTA 10mM; Glucose 50mM;) until the solution becomes turbid. This was centrifuged at 10,000g for 20 minutes. The supernatant was discarded and pellet was processed for further studies.

Cerebrospinal fluid and **Plueral effusion** were centrifuged at 2,500 rpm (Eppendorf) for 15 minutes, supernatant was discarded and pellet was used to extract DNA.

2.9 Auramine-rhodamine staining

Materials

Auramine O

Rhodamine B

Phenol crystals

Hydrochloric acid, concentrated

Ethanol, 70%, 95%

Potassium permanganate

Water, distilled

Preparations

Auramine O-Rhodamine B

1.5 g of Auramine O, 0.75 g Rhodamine B, 75 ml glycerol (glycerine) and 10 ml heated phenol crystals dissolved in 50 ml of distilled water. This solution was filtered through glass wool.

Acid alcohol

Concentrated hydrochloric acid 0.5 ml was added to 100 ml of 70% ethanol.

Potassium Permanganate

0.5 g of potassium permanganate ($KMnO_4$) was dissolved in 100 ml of distilled water.

Procedure

1. Sample was smeared and air dried.
2. Smear was fixed on electric slide warmer at 65-75 BC for at least 2 hours, or Bunsen burner was used (care was taken to avoid overheating).
3. Smear was flooded with Auramine O-Rhodamine B solution (Appendix) and allowed to stain for 15 minutes, making certain that the staining solution remains on the smear.

4. Smear was rinsed with chlorine free water and drained. (Chlorine may interfere with fluorescence).
5. Smears was flooded with acid alcohol and allowed to de-stain for 2 minutes.
6. Step was repeated again.
7. Smear was flooded with counter stain potassium permanganate for 2 minutes. Counter staining time was strictly followed to avoid quenching of flourescence of acid fast bacilli

2.10 Growth of *M.tuberculosis* from clinical specimen

Lowenstein-Jensen Medium

The concentrated sediment obtained after processing the clinical specimen was spread on the L-J slant and incubated at 37°C up to 8 weeks. The slants were examined every week for the growth of *M.tuberculosis*. The primary cultures were subcultured when confluent.

1) Mineral salt solution was prepared as follows and sterilized by autoclaving:

KH2PO4	4.0g
MgSO4	0.4g
Magnesium citrate	1.0g
L-Asparagine	6.0g
Glycerol	20.0ml
Distilled water was added to make	1 litre

2) Eggs were cleaned and air-dried. Egg fluid was collected as sterile as possible in a large glass flask and homogenized by shaking with sterile glass pieces.

3) 1% malachite green solution.

The final medium was made by mixing 600ml of solution (1) ,1000ml of (2) and 40 ml of (3).

About 20 ml aliquots were poured into screw-capped tubes and inspissated at 80°C for 45 minutes.

Selective Kirchners medium

Formulation g/litre

Sodium dihydrogen phosphate	7.54
Potassium dihydrogen phosphate	2.00
Magnesium sulphate	0.34
Trisodium citrate	2.5
L-Asparagine	5
Phenol red	0.012

All the above mentioned chemicals were dispersed in 1 litre deionised water. 20ml Glycerol was added to it and mixed well. After complete dispersing aliquoted in McCartney bottles and sterilised at 121°C for 10 minutes. 1ml. sterile heat inactivated horse serum was added along with selective agents mentioned below:

Polymixin B	200 units/ ml
Carbenicellin	100 µg/ml
Trimethoprin	10 µg/ml
Amphotericin	10µg/ml

2.11 Isolation and purification of nucleic acid

Plasmid DNA preparation

Plasmid DNA isolation from *E.coli* was based on the modified method of Birmboim and Doly (1979) (All centrifugation steps in this procedure were performed in an eppendorf centrifuge at 12,000 g).

a) 1.5 ml of O/N grown culture of *E. coli* containing plasmid was centrifuged for 1 minute. The supernatant was removed. The residual medium was aspirated out after a brief centrifugation.

b) The bacterial pellet was suspended in 100 µl of ice-cold TEG buffer (25 mM Tris-HCl, pH.8.0, 10mM EDTA pH 8, 50 mM Glucose) by vortexing.

c) Freshly prepared 200 µl of alkaline – SDS solution (1% SDS in 0.2 N NaOH) was added to the cells, mixed by inverting tubes gently 3 to 4 times and incubated on ice for 5 minutes.

d) 150 µl of 3M potassium acetate solution (5M Potassium acetate 60 ml, Glacial acetic acid 11.5 ml, H_2O 28.5 ml, pH 4.8) was added, immediately

mixed by gentle inversion. Placed on ice for 15 minute and then centrifuged for 15 minutes at 4°C.

e) The supernatant was removed carefully into a fresh tube. To this, RNase at a final concentration of 20 µg/ml was added and incubated at 37°C for 30 minutes.

f) The sample was extracted once with Tris buffered phenol, chloroform and isoamyl alcohol (25:24:1) and twice with choloroform, isoamyl alcohol (24:1).

g) DNA was precipitated by 0.6 volume of ice cold isopropanol and placed in -20°C for 10 minutes. DNA was pelleted by centrifuging at 12,000 rpm for 15 minutes at 4°C.

h) Supernatant was discarded, DNA pellet was washed with 70% ethanol and centrifuged again at RT for 5 minutes at 12000 rpm. The DNA pellet was dried using speed vacuum (Virtis, Sentry, USA). The DNA was dissolved in appropriate volume of either double distilled water or TE (10mM Tris. Cl and 1mM EDTA, pH.8) and stored at –20°C.

2.12 Agarose gel electrophoresis

Horizontal submerged gels were used to separate the DNA. TAE buffer of pH 8.3 (98 mM Tris, 89 mM Acetic acid and 2 mM EDTA, pH 8.3) was used and the electrophoresis was performed at 5.8 v/cm at room temperature. The gel loading buffer contained 20% glycerol in TE with 0.01% Orange-G or 0.001% Bromophenol Blue.

Depending on the size of DNA fragments, 0.7 to 1. 2% agarose gel were employed in this study. Gels were stained with approximately 1 µg/ ml of ethidium bromide, viewed under UV transilluminator. Photographs were taken either with Tracktel video-densitometer. Either 100 bp or 1 kb ladder (Gibco, BRL, USA) and *Hind* III digest of lambda phage DNA (New England Biolabs, MA, USA) were used as DNA molecular weight markers.

To prevent cross contamination stock solutions (including solutions required for processing of samples for DNA extraction) were prepared with Millipore-filtered and doubly autoclaved. These were prepared in separate room, aliquoted, stored and used once only. Daily chemical decontamination of surfaces and weekly decontamination of equipment were done with 0.5% sodium hypochloride. Prevention of DNA contamination was further accomplished by physically separating different steps in the PCR procedure, and using different sets of pipettes and tips.

2.13 Polymerase chain reaction (PCR)

Table 2.1: Primers used in the amplification of *M.tuberculosis*

Primers Used	Name	Purpose	Size	Annealing Temperature
5'gacaacgacgtgcgcctact 3'	Primer1	To amplify TRC4	123 bp	58°C
5'-gaccgaattagcgtagctcc-3'	Primer2			
5' cctgcgagcgtaggcgtcgg 3'	IS1	To amplify IS6110	173 bp	58°C
5' ctcgtccagcgccgctccgg 3'	IS2			
5'cgcgatccgatgccgctcagctctcgta 3'	VF1	To amplify *OxyR*	936 bp	58°C
5'cgaattcctacgtgctgcgggaagtga 3'	VR1			

Polymerase chain reaction was performed under the following conditions.

i) 200 µM of each of dNTP

ii) 1X PCR buffer (50mM KCl, 10mM TRIS-Cl, pH 8.3)

iii) 2.5 mM $MgCl_2$

iv) 0.5µM of each primer (Table: 4.1) and

v) 1 units of Taq DNA polymerase

PCR was performed either on Perkin-Elmer 9600 or MJ Thermal Cycler. The cycling parameters varied on the primer sets used.

Steps	Conditions	Temperature
i.	Initial denaturatiuon	95°C for 5 minutes
ii.	Denaturation	95°C for 30 seconds
iii.	Annealing	58°C for 30 seconds
iv.	Extension	72°C for 90 seconds
v.	Final extension	72°C for 5 minutes

2.14 Purification of PCR product for cloning

Purification of PCR product was done by QIAquick PCR purification kit (Qiagen GmbH, Germany).

a) One volume of PCR sample (100 µl) with or without mineral oil was mixed with 5 volumes (500µl) of buffer PB.

b) QIAquick spin column was placed on a 2 ml collection tube.

c) PCR mixture was applied onto the QIAquick spin column and spun for 60 seconds at 10,000 x g.

d) Flow through was discarded and the column was placed back into the same collection tube.

e) To the column 750µl of PE buffer was added and spun for 60 seconds.

f) Flow through was collected and discarded, again spun for 60 seconds to remove solution completely from column.

g) Column was placed into a clean 1.5 ml eppendorf tube, 50 µl of water or 10mM Tris-HCl, pH.8 was added onto the column to elute PCR product and spun for 60 seconds. PCR products were safely stored at –20°C.

2.15 Automated DNA sequencing

DNA sequencing was performed by the di-deoxy chain termination method (Sanger *et al.*, 1977) using automated DNA sequencing by Dye terminator cycle sequencing kit from Perkin-Elmer Applied Biosystems, NJ, USA on AB1 377.

Automated DNA sequencing was performed on ABI 377 facility using ABI prism Dye-terminator cycle sequencing ready reaction kit from Perkin-Elmer applied biosystems, NJ, USA. pRSET forward and reverse primers were used in the pRSET B vector. Full length sequencing of pSNOXYR was performed with insert specific primers (Table: 2.1).

A) Following protocol was used as per the recommendations of the manufacturer of the kit.

a) Template DNA (50ng) 5 µl

Amersham Shrimp alkaline Phosphatase 1 µl

Exonuclease 1 µl

This reaction mixture was incubated at 37°C for 15 min and 80°C for 15 min to inactivate enzymes. In the same tube, the following sequencing reaction mix was added.

b) Perkin Elmer ready reaction mix 8 µl

Primer (5 pM) 5 µl

c) Reaction tubes were placed in the thermocycler Perkin Elmer 1800 to generate nested sequences using the following parameters:

Step 1: 95°C 30 sec

Step 2: 55°C 30 sec

Step 3: 72°C 60 sec and

Linked to Step 4: to maintain temperature at 4°C

Steps 1, 2 and 3 repeated 25 cycles.

B) Nested DNA sample was mixed with the tube containing 90 µl of 95% ethanol and 3 µl of sodium acetate. The tube was incubated on ice for 15 minutes.

C) Tubes were centrifuged at 15, 000g for 15 min at 4°C and the supernatant was carefully decanted.

D) DNA pellet was washed in 250 µl of 70% ethanol, spun at RT for 5 min at 12 000g. Samples were dried in a speed vacuum centrifuge for about 10 minutes.

E) Sample was re-suspended in loading buffer 4µl (deionized formamide: 50 mM EDTA, 5:1) and boiled for 5 min and snap chilled before loading on a standard 6% polyacrylamide DNA sequencing gel.

2.16 Plasmid vectors employed for the present study

The T7 expression vector (Invitrogen, USA), contains very specific T7 viral promoter and N- terminal poly-histidine tag for purification. The presence of the both ColE1 and F1 origins of replication has offered flexibility to handle this vector for a wide spectrum of methodologies in molecular biology. The availability of this vector in three possible reading frames i.e., pRSET A, B and C (Appendix 1) and give the researcher the choice to select a vector that maintains the right reading frame of their insert. These plasmids are relaxed plasmids their copy number is high and the expression of the protein is also high. It however needs specialized *E. coli* host, BL 21 (DE3) or BL21 (DE3) pLysS, Which has the T7 RNA polymerase operon driven by a *lac* UV5 promoter incorporated in its chromosome as a lysogen DE3 (Rosenberg *et al.*, 1987). Furthermore, being a lon protease deficient strain, *E. coli* BL21 (DE3) protects the heterologous protein from proteolytic degradation. Leaky expression of T7 RNA polymerase from the above operon is taken care of by $lacI^q$ repression. These vectors also contain a nucleotide sequence that encodes a metal binding domain, a series of six

consecutive histidine amino acids expressed as N-terminal fusion to the protein of interest. This metal binding domain (six histidine tagged moieties) on the fusion peptide has high affinity for the divalent ions (like nickel, copper and cobalt) and facilitates one step purification of the protein using immobilized metal affinity columns (IMAC) (Crowe *et al.*, 1995).

2.17 Restriction digestion and ligation of DNA

The restriction digestions were performed using enzymes from New England Biolabs, (USA) in the recommended buffers. Approximately 1 to 2 units of enzymes were used per μg of DNA. When the double digestion were performed, the most appropriate buffer was used and simultaneously the efficiency of each enzyme was verified separately in the selected buffer using control DNA. For ligation, T4 DNA ligase was used and ligations were performed at 16°C for 16 h. Different molar ratios of insert DNA to vector plasmid DNA were used.

2.18 Transformation of *E.coli* with plasmid DNA

Transformation of *E. coli* with plasmid DNA was based on the method of Hanahan, (1983) using $CaCl_2$ for the preparation of competent cells.

a) A single colony of freshly revived *E. coli* culture was inoculated in 2 ml LB and allowed to grow overnight at 37°C.

b) 100 μl of O/N culture was inoculated in 50 ml LB in 100 ml conical flask and allowed to grow in shaker at 37°C till OD_{600} = 0.6.

c) Culture was chilled on ice by gentle swirling for 15 minutes and centrifuged at 3500 g for 5 minutes at 4°C.

d) The cell pellet was resuspended in 10 ml of ice cold 100mM $MgCl_2$ and incubated on ice for 20 minutes.

e) Cells were pelleted as in step c and the pellet was resuspended in 2 ml of 100 mM $CaCl_2$ and incubated on ice for 1 hour.

f) Heat shock was given at 42°C for 90 seconds and then, immediately chilled on ice.

g) To this tube, 900 µl of LB was added, allowed it to grow for 1 hour at 37°C incubator and 100 µl of above transformed culture was spread plated onto LB agar plate containing 50µg/ml of ampicillin. These plates were incubated at 37°C for 16 hours.

Bacterial transformants were screened by picking up a small portion of the colonies using a sterile toothpick and suspended in 50 µl of 0.1 X TE. Bacterial suspensions was lysed by boiling for 10 min and immediately snap chilled. It was then centrifuged at 10,000 x g for 10 min at 4°C and 1 µl of the supernatant was used as template for PCR.

2.19 Expression of the recombinant proteins in *E. coli*

In down stream processing, the most widely used bacteria is *E. coli* that they are genetically modified to express foreign proteins safely. Genetically engineered bacteria is well characterized than any other bacteria. For expression

of proteins, *E. coli* BL21 (DE3) chromosomal DNA was integrated with T7 RNA polymerase under the control of *lacUV5* promoter and expression of T7 polymerase can be induced using 1mM IPTG. This strain is lon- so the foreign proteins possibly expressed without degradation. Following procedure was adopted to express recombinant proteins pSNOXYR.

a) Recombinant *E. coli* BL21 (DE3) transformed with pSNOXYR plasmid, was cultured in LB media supplemented with 100µg of ampicillin and grown O/N at 37°C in water bath shaker (200 rpm).

b) 2% of pre-inoculum was transferred into required amount of LB with 100µg of ampicillin and grown at 37°C in shaking water bath till it reaches the OD_{600} 0.6.

c) Culture was induced with 1mM IPTG and allowed to grow for further 3 h at 37°C.

d) The culture was harvested by centrifuging at 10 000 for 5 min. The pellet was preserved at –20°C for further use.

2.20 Purification of recombinant protein using immobilized metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography in which an immobilized metal ions such as copper, zinc or a transition metal ion such as cobalt or nickel is used to bind protein selectively by reaction with **imidazole** group of **histidine** residues (Porath *et al.*, 1975). The metal atoms are immobilized by attachment to an iminodiacetate or Tris (carboxymethyl) ethylendiamine substituted agarose.

Further immobilization of the protein involves the formation of the coordinated bond with the metal that must be sufficiently stable to protein attachment and retention during washing to remove weakly associated contaminating protein from matrix. The elution of interested proteins was achieved either by lowering pH 4.5 thereby destabilizing the protein–metal complex or by using competitive ligands like imidazole 250mM or by using 50 mM EDTA.

Thus IMAC is an excellent method for purifying recombinant proteins as poly- histidine fusion. One such expression vector is pRSET which has 6 x His residues moiety at the N-terminal. Usually efficiency of protein purification depends on the solubility and nature of the recombinant protein formed in prokaryotic expression system. In general the highly expressed recombinant proteins in *E. coli* will usually accumulate in the form of insoluble inclusion bodies. Since pRSET is T7 based expression vector, expression of proteins by IPTG induction leads to inclusion bodies formation of pSNOXYR. The protein was solubilized in with a choatropic agent like urea and the protein purified under denaturing conditions.

Immobilization of the nickel to the matrix

The chelating sepharose fast flow matrix (Pharmacia) was used in the present study. It was the iminodiacetic acid as the chelating ligand, which couple to the agarose (Sepharose Fast Flow) by epoxy coupling method. The column was prepared as described by the manufacture.

Charging the column

The choice of metal in our study was nickel. Charging the column is achieved by passing a solution of nickel chloride (0.1M) prepared in distilled water at the neutral pH 7.0, through the column. After charging, the gel is washed thoroughly with starting buffer. This is followed by the equilibration of the gel with the starting column buffer (0.1M phosphate buffer pH 8.0, 0.01M Tris HCl pH 8.0 and 8 M Urea).

Binding and elution of protein from nickel immobilized column

The protein was solubilized in lysis buffer (0.1M phosphate buffer pH 8. 0, 0. 01M Tris pH 8. 0 and 8M Urea, 5mM Imidazole) for 1 hour at room temperature and sample was centrifuged at 26,000 g for 15 min to remove the debris. The supernatant containing the solubilized recombinant protein was mixed with column matrix and allowed to bind for 4 hours on shaking. The column was packed without trapping bubbles and unbound protein was removed by washing the column with 10-20 column volumes of start buffer. (0. 1M Phosphate buffer pH 8. 0, 20 mM Imidazole) until the fractions showed zero at A_{280}. This was followed by the elution of the desired protein using an agent that competes for either the ligand or the target molecules. Normally an increased concentration of imidazole (0. 1- 0. 3M) with affinity for the chelated metals used. The other method of elution is by decreasing the pH of the buffer or by EDTA (0. 5M), which are strong chelators and strip the metal and firmly bound metal from the column. The eluted fractions were collected and the protein concentration quantified by measuring the absorbancy at 280nm. The eluted fractions containing the protein were pooled and urea was removed by stepwise dialysis.

SDS-PAGE analysis was carried out with the wash and the eluted fractions to test the purity of the eluted protein.

2.21 SDS- PAGE gel electrophoresis

Proteins present in cell extracts were separated by SDS-PAGE according to the method of Laemmli (1970) with some modifications. The compositions of the various reagents are described below.

Buffers, reagents and gel compositions for SDS-PAGE:

a) Monomer solution: 20% acrylamide and 0.8% N', N'-Methylene bis-acrylamide. The solution was filtered through Whatman 1mM paper and stored at 4°C.

b) Stacking gel buffer: 0.5 M Tris HCl, pH 8.0

c) Separating gel buffer: 1.5 M Tris HCl, pH 8.8

d) 10% SDS

e) Ammonium per sulfate, 140 mg/ml prepared fresh.

f) Electrophoresis buffer: 0.025 M Tris HCl, 0.192 M Glycine, 0.1% SDS; pH 8.3

g) Sample solubilizing buffer: 10% (v/v) 2-mercaptoethanol, 50% sucrose, 0.025 Bromophenol blue in 1/4X stacking gel buffer. For a 10% (w/v) separating gel, 15 ml monomer solution, 4.5 ml separating gel buffer,

10.5ml distilled water, 300 µl SDS, 150 µl ammonium per sulphate 15 µl of TEMED (N, N, N', N'-tetra methylethylene diamine) were used.

h) For 12% separating gel, 18 ml of monomer solution, 4.5 ml of separating gel buffer, 7.5 ml of distilled water were used and rest of them were same as that for 10% gels.

i) For a 4% (w/v) stacking gel, 2 ml of monomer solution, 2/4 ml stacking gel buffer, 150µl of SDS, 5.6 ml of distilled water and 5 µl of TEMED were used.

j) Protein estimation was carried out as per the protocol of Lowry *et al.*, (1953).

Electrophoresis was performed at room temperature at a constant current of 30 milli amperes. After electrophoresis, the gel was removed and soaked in staining solution (0.25 g Coomassie brilliant blue R250 dissolved in 45ml of ethanol, 10 ml of glacial acetic acid, 45 ml of distilled water). After 2 hours, the gel was rinsed briefly to remove excess of stain by using distilled water and immersed in destaining solution which is the same as staining solution composition without Coomassie brilliant blue.

2.22 Cyanogen Bromide (CnBr) digestion of protein (Matsudaira, 1993)

1. Protein was TCA precipitated and washed with acetone and then dried.
2. Stock solution of 0.66 M CnBr was prepared as per instruction: 132 ml of 5M CnBr in acetonitrile and 868 ml of 70% formic acid.
3. 10 ml of the 70 mg/ml CnBr solution was added to 10 mg of protein.
4. Digestion of the protein was carried out for about 36 to 72 hours at room temperature.

5. The tube contents were dried in speed vacuum and additional 100 ml of water was added to the tubes and dried in the Speed vacuum.
6. The resulting peptides was separated on 12% SDS- PAGE depending on the size and amounts of the peptides.

2.23 Western blotting

After electrophoresis was complete, the gel was immersed for 10-15 minutes in transfer buffer to eliminate swelling. In the mean time, NCP cut to the desired size was incubated for 5 to 10 minutes in transfer buffer (Tris 25 mM, pH; Glycine 192 mM methanol 20%, SDS 0.1%). The nitrocellulose membrane was overlaid on the gel by avoiding air bubbles and sandwiched between the filter paper and scotch brite pad. The gel was placed cathodic to the NCP. The transfer was carried out at 300 mA for 200 minutes (Bio-Rad). After the transfer was complete the pre stained molecular weight marker lane was cut out and dried. Rest of the NCP was stained with Ponceu's reagent for 5 min to confirm the presence of protein interest. NCP was washed thoroughly with TBS and blocked for one hour at 37°C or overnight at 4°C with 3% fat free skimmed milk powder in TBS/T (25 mM Tris pH 7.5 and 150 mM NaCl 0.05%, Tween-20). The NCP was washed in wash buffer for three times of five minutes duration. The NCP was incubated for 3 hours with the desired primary antibody diluted in TBST. After intensive washing in the wash buffer, the NCP was incubated for one hour with secondary antibody conjugated with alkaline phosphatase for one hour. After extensive washing of the blot with wash buffer and incubated with substrate buffer (100mM Tris- Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min, The color development was carried out using 66 µl Nitroblue Tetrazolium (50mg/ ml in 70% diethyl formamide) and 33 µl of 5- Bromo 4- chloro- indolyl phosphate (50mg/ml in diethyl formamide) in 10 ml of detection buffer. The reaction was stopped by washing in 10mM EDTA for 10 min.