Chapter II

Materials and Method
### Chapter II

#### 2.1. Bacterial Strain

#### 2.2. Culture Media

- **A.** Composition Of Materials Used In MOPS Medium
- **B.** Luria Broth (LB)

#### 2.3. Protein estimation by Bradford Assay method

#### 2.4. Assay of inducible periplasmic enzyme Alkaline phosphatase in *E.coli*

#### 2.5. Immunization

- 2.5.1. Collection of antibody against alkaline phosphatase
- 2.5.2. Detection of presence of antibody in the serum by Ocketlony double diffusion technique

#### 2.6. SDS-Polyacrylamide gel-electrophoresis of proteins

#### 2.7. Silver Staining of Protein bands in SDS-PAGE

#### 2.8. Autoradiography

| 2.1. | Bacterial Strain | 37 |
| 2.2. | Culture Media | |
| **A.** Composition Of Materials Used In MOPS Medium | 37 |
| **B.** Luria Broth (LB) | 39 |
| 2.3. | Protein estimation by Bradford Assay method | 39 |
| 2.4. | Assay of inducible periplasmic enzyme Alkaline phosphatase in *E.coli* | 40 |
| 2.5. | Immunization | |
| 2.5.1. | Collection of antibody against alkaline phosphatase | 41 |
| 2.5.2. | Detection of presence of antibody in the serum by Ocketlony double diffusion technique | 42 |
| 2.6. | SDS-Polyacrylamide gel-electrophoresis of proteins | 42 |
| 2.7. | Silver Staining of Protein bands in SDS-PAGE | 46 |
| 2.8. | Autoradiography | 47 |
Chapter II: Materials & Methods

2.9. Determination of subcellular localization of periplasmic protein Alkaline phosphatase

2.10. Detection of target protein by Immunoprecipitation Technique

2.11. Immunodetection of proteins by Western Blotting

2.12. Isolation of aggregated protein

2.13. Important Chemicals
2.1. Bacterial Strain

AP signal sequence mutant strain of E.coli (MPh1061) and its corresponding wild type strain (MPh42) [Benson et al., 1985] were obtained from Dr. Jonathan Beckwith, Department of Microbiology & Molecular Genetics, Harvard Medical School, Boston, USA.

Genotype of the strains are as follows:
MPh 42: F^- araD139 Δ(ara-leu)7697 Δlac74 galE galK rpsL phoR
MPh 1061: F^- araD139 Δ(ara-leu)7697 Δlac74 galE galK rpsL phoR phoA61

2.2. Culture Media

A. Composition Of Materials Used In MOPS Medium : [VanBogelen and Neidhardt, 1999]

a. MOPS buffer [for 200ml stock (10X)]:
MOPS - 16.744gm, Tricine - 1.432gm, FeSO_4 - 0.0056gm, NH_4Cl - 1.016gm, K_2SO_4 - 0.096gm, CaCl_2 - 0.00011gm, MgCl_2 - 0.208gm, NaCl - 5.844gm, K_2HPO_4 - 0.6022gm [for PO_4-less medium (10X) MOPS was prepared without adding K_2HPO_4], Micronutrient - 2ml [from (100X) stock].

pH was adjusted to 7.5 using 10M KOH before making the volume up to 200ml with MilliQ water. Sterilized through 0.22µm membrane filter and stored at -20°C.

Composition of micronutrient [for 200ml stock (100X)]:
(NH_4)_6(MO_7)_{24} - 0.0008gm, H_3BO_3 - 0.0050gm, CoCl_2 - 0.0014gm, CuSO_4 - 0.0006gm, MnCl_2 - 0.0032gm, ZnSO_4 - 0.0006gm. Sterilized through 0.22µm membrane filter and stored at room temperature.

b. Supplements of amino acids [for 200ml stock FSA-cys (10X)]:
Chapter II: Materials & Methods

Alanine - 0.1426gm, Arginine - 0.1686gm, Asparagine - 0.1200gm, Aspartic acid - 0.1064gm, Glutamic acid - 0.1766gm, Glutamine - 0.1754gm, Glycine - 0.1200gm, Histidine - 0.0766gm, Iso-leucine - 0.1050gm, Leucine - 0.2099gm, Lycine - 0.146gm, Phenyl alanine - 0.1322gm, Proline - 0.0920gm, Serine - 0.1019gm, Threonine - 0.0952gm, Tryptophan - 0.0408gm, Tyrosine - 0.0724gm, Valine - 0.1405gm, Methionine - 0.0596gm [for radiolabeling experiments FSA was made using 1/10th concentration of normal methionine i.e. 0.00596gm], Cystein - 0.0032gm/2ml (as the Cystein is unstable in FSA, it was prepared as separate stock). Total volume was made up with 200ml Milli-Q water. Sterilized through 0.22μ membrane filter and stored at 4 - 8°C.

c. Bases [for 200ml stock (10X)]:
Adenine - 0.054gm, Cytosine - 0.0444gm, Uracil - 0.0448gm, Guanine - 0.0604gm. Made up to the volume using 0.015M KOH. Sterilized through 0.22μ membrane filter and stored at 4 - 8°C.

d. Vitamins -
Vit - A [for 10ml stock (100X)]:
B1 (Thiamine-HCl) - 0.0134gm, Ca-pantothanate - 0.019gm, p-Amino benzoic acid - 0.0054gm, p-Hydroxy benzoic acid - 0.0056gm, 2,3-Dihydroxy benzoic acid - 0.0062gm in 10ml 0.02M KOH. Sterilized through 0.22μ membrane filter and stored at 4°C.

Vit - B [for 20ml (50X)]:
Riboflavin - 0.00376gm, Nicotinic acid - 0.0123gm, Pyridoxine-HCl - 0.02gm, D-Biotin - 0.0025gm. Solubilised in 20ml Milli-Q water, sterilized by filtration and stored at 4°C.

e. Carbon source:
35.7% Glucose stock solution. [50ml of Milli-Q water was added to 35.7gm glucose and dissolved by heating in boiling water bath, volume was made up to 100ml, cotton plugged and kept for 10min into the boiling water bath for sterilization.] Stored at 4°C.

Recipes for 100ml (1X) MOPS culture medium:
Chapter II: Materials & Methods

MOPS buffer [10X]: 10ml
Supplements of amino acids [10X]: 10ml
Bases [10X]: 10ml
Vit - A [100X]: 1ml
Vit - B [for 20ml (50X)]: 2ml
Carbon source: 1ml

Milli-Q water was added to make up the volume. Sterilized by filtration through 0.22μ filter membrane.

B. Luria Broth (LB):
10.0gm Bactotryptone, 10.0gm NaCl, 5.0gm Yeast extract in 1000ml distilled water; pH adjusted to 7.5 with 5N NaOH solution. Sterilized by autoclaving.

LB-agar plates were prepared using 1.5% Bacto-agar supplements before sterilization by autoclaving.

2.3. Protein estimation by Bradford Assay method [Bradford, 1976]

A. Bradford Reagent:
(For 25ml) : Coomassie Brilliant Blue - 2.5mg, 85% Orthophosphoric acid - 2.5ml, 95% EtOH - 1.25ml, 1(N) NaOH - 1.25ml, Volume was made up by mille-Q, Filtered through Whatman No.1 filter paper.

B. Principle:
Chapter II: Materials & Methods

The Bradford assay works by the action of Coomassie brilliant blue G-250 dye (CBBG). This dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. It should be noted that the assay primarily responds to arginine residues (eight times as much as the other listed residues). CBBG binds to these residues in the anionic form, which has an absorbance maximum at 595 nm (blue). The free dye in solution is in the cationic form, which has an absorbance maximum at 470 nm (red). The assay is monitored at 595 nm in a spectrophotometer, and thus measures the CBBG complex with the protein.

C. Method:

To draw a standard curve 750μl sample were taken with equal volume of Bradford reagent and incubated for 30 minutes at room temperature. The absorbance was measured at 595nm immediately after incubation.

2.4. Assay of inducible periplasmic enzyme Alkaline phosphatase in E.coli

A. Principle:

The activity of this enzyme is assayed spectrophotometrically by following the colour change of the color-less substrate paranitrophenyl phosphate (pNPP) that results from its dephosphorylation by the enzyme alkaline phosphatase to yield paranitrophenol (Garen and Levinthal, 1960). The yellow colored paranitrophenol has the absorption maxima at 410nm (Fig 2.2.). When the substrate concentration is in excess of 0.1mg/ml in Tris buffer (pH 8.2), the amount of enzyme is proportional to that of the liberated paranitrophenol per minute (Garen and Levinthal, 1960). One unit was defined as the amount of enzyme, which led to a change of
absorbance of p-nitrophenol by 0.1 per 6 min of enzyme-substrate reaction (Basu and Poddar, 1997).

B. Method:
i). 1ml of aliquots were withdrawn over 0.2ml toluene for whole cell enzyme assay at different time intervals and toluenized by vigorous vortexing.

ii). 2ml of pNPP (0.2% in 1M Tris.Cl, pH 8.2) was added to each of the sample.

iii). Incubated at 37°C up to the development of yellow color of p-nitrophenol.

iv). Reaction was stopped with 0.5ml of 13% potassium dihydrogen phosphate.

v). The absorption maxima was obtained at 410nm and absorbance of the samples was measured at that wavelength (410nm) against a reference blank.

vi). Enzyme unit was calculated according to its definition.

2.5. Immunization

2.5.1. Collection of antibody against alkaline phosphatase

i). Rabbits were injected interdermally in four sites with antigen (100µg per rabbit) emulsified with complete & incomplete Freunds adjuvant (Sigma) for the primary injection.

ii). Subsequent injections of antigen (50µg per rabbit) emulsified with incomplete Freunds adjuvant (Sigma) were at 10days intervals.

iii). Blood was drawn 3days after booster dose (100µg per rabbit emulsified with incomplete Freunds adjuvant) from the marginal vein of the ear.
iv). Collected blood was then incubated for 30 min at 37°C to separate serum from the whole blood cells.

v). Centrifuged at 10,000rpm for 10 min at 4°C to isolate the clear serum.

vi). The antiserum titer was checked by the method of immunodiffusion.

2.5.2. Detection of presence of antibody in the serum by Octerlony double diffusion technique:

i). 1% molten agarose in Milli-Q water was poured on a clean glass plate to make a thin layer.

ii). Grooves were made by gel puncher and filled with antiserum and antigen (pure AP diluted in PBS buffer 25times) 20μl each.

iii). Kept at 4°C overnight to visualize the precipitin line indicating the presence of AP antibody in the blood serum.

Fig 2.1. Immunodiffusion study of the isolated serum. A: 25 times diluted Alkaline phosphatase; B: Serum collected after immunization; C: Pre-immune serum.

2.6. SDS-Polyacrylamide gel-electrophoresis of proteins
[Sambrook and Russell, 2001b]

A. SDS-PAGE Reagents:
Chapter II: Materials & Methods

a. Running Buffer:
25mM Tris, 250mM glycine, 0.1% (w/v) SDS. [(For 1lttr) Tris - 3gm, Glycine - 14.4gm, SDS -1gm.]

b. SDBME buffer:
0.3% SDS, 0.2M DTT, 0.028M Tris-HCl and 0.022M Trizma base.
[For 5ml: 0.15ml 10% SDS, 1ml 1M DTT, 0.0221gm Tris-HCl and 0.0133gm Trizma base.]
c. Composition of gel loading buffer for SDS-PAGE (1X):
50mM Tris-Cl (pH-6.8), 100mM DTT, 2% SDS, 0.1% Bromophenol blue, 10% Glycerol.
[For 5ml: 0.25ml 1M Tris-Cl (pH-6.8), 0.5ml 1M DTT, 0.1ml 10% SDS, 0.5ml 1% Bromophenol Blue, 0.5ml glycerol, 3.15ml milli-Q H2O].
d. Resolving gel:
Acrylamide-bisacrylamide: 10% or 12% (from 30% acryl-bisacryl stock solution), Tris-Cl (pH-8.8): 375mM (from 1.5M stock solution), Ammonium per sulfate: 0.07% (from 1% stock solution), SDS: 0.1% (from 10% stock solution), TEMED: 2.5µl (for 5ml).
e. Stacking gel:
Acrylamide-bisacrylamide: 3% (from 30% acryl-bisacryl stock solution), Tris-Cl (pH-6.8): 125mM (from 0.5M stock solution), Ammonium per sulfate: 0.07% (from 1% stock solution), SDS: 0.1% (from 10% stock solution), TEMED: 3µl (for 2.5ml).
f. Coomassie brilliant blue staining solution:
0.25% in Coomassie brilliant blue methanol:acetic acid solution. [for 100ml: 0.25gm Coomassie brilliant blue, 100ml methanol: water: acetic acid (50:40:10) solution].

B. Principle:
Almost all analytical electrophoreses of proteins are carried out in polyacrylamide gels under condition that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination
with a reducing agent and heat to dissociate the proteins before they are loaded onto the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. At saturation, ~1.4 g of detergent is bound per gram of polypeptide. By using markers of known molecular weight, it is possible to estimate the molecular weight of the polypeptide chain(s).

In most cases, SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a pH and ionic strength different from that of the buffer used to cast the gel. The SDS-polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The ability of discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS-polyacrylamide gels.

The sample and stacking gel contain Tris-Cl (pH 6.8), the upper and lower buffer reservoirs contain Tris-Glycine (pH 8.3), and the resolving gel contains Tris-Cl (pH 8.8). All component of the system contain 0.1% SDS [Laemmli, 1970]. The chloride ions in the sample and stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between the leading and trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient, which sweeps the polypeptide from the sample and deposits them on the surface of the resolving gel. There the higher pH of the resolving gel favors the ionization of glycine, and the resulting glycine ions migrates through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size of sieving.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by a bifunctional agent such as N,N'-methylene-bis-acrylamide.

The effective range of separation of SDS-polyacrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking. Polymerization of acrylamide in the absence of cross-linking agents generates viscous solutions that are of no practical use. Cross-links formed from bisacrylamide add rigidity and tensile strength to the gel.
and form pores through which SDS-polypeptide complexes must pass. The size of these pores decreases as the bisacrylamide:acrylamide ratio increases, reaching a minimum when the ratio is ~1:20. Most SDS-polyacrylamide gels cast with a molar ratio of bisacrylamide:acrylamide of 1:29, which has been shown empirically to be capable of resolving polypeptides that differ in size by a little as 3%.

The sieving properties of the gel are determined by the size of the pores, which is a function of the absolute concentration of acrylamide and bisacrylamide used to cast the gel. The linear range of separation of proteins obtained with gels cast with concentrations of acrylamide that range from 5% to 15%.

C. Method

i). The glass plates, comb and spacer were washed by double distilled water and then wiped out with alcohol soaked lint free paper towel to remove any greasy adhered substances.

ii). Spacers were placed in between dry glass plates and tightly wrapped with tape by the sides of the plates and clamped by gel holding clip to hold glass plate-spacers-glass plate assembly firmly.

iii). 1% molten agar was used to seal the inner sides of the gel cassette.

iv). Resolving gel solution was prepared of desired concentration and poured in to the cassette and allowed 1 hour to polymerize. Water saturated iso-butanol was layered onto top of the acrylamide solution.

v). After polymerization of the resolving gel, the gel top was washed with milli-Q water gently and the excess water was removed using absorbent paper without touching the resolving gel surface carefully.

vi). Stacking gel solution was prepared and poured onto the resolving gel. Then comb was placed and allowed another 1 hour to polymerize.

vii). After completion of gel casting tape was removed and cassette was freed from clamp.

viii). The gel cassette was placed tightly in the electrophoresis equipment.

ix). Both upper and lower Buffer tank was filled with running buffer and then comb was removed from the gel carefully.

x). Sample was loaded in to the groove. Then electrophoresis apparatus was attached to an electric power supply. A voltage of 8V/cm was applied to the gel. After the dye front has
Chapter II: Materials & Methods

moved into the resolving gel, the voltage was increased to 15V/cm and was ran the gel until the bromophenol blue reaches the bottom of the resolving gel.

xii). After the run the glass plates was removed from the electrophoresis apparatus.

xiii). A spatula was used to carefully pry apart the plates. Cutting right corner from the bottom of the gel marked the orientation of the gel.

Then the gel was fixed with fixing solution to pretreat for further staining of the protein bands.

2.7. Silver Staining of Protein bands in SDS-PAGE

A. Reagents:

a) Fixer (100ml):
   MeOH: 50ml, Acetic acid: 12ml, 37% HCHO: 50μl, DD H2O: 38ml.

b) Pretreat (100ml):
   20mg Na2S2O5. 5H2O.

c) Impregnation (100ml):
   AgNO3: 200mg, 37% HCHO: 75μl, DD H2O: 100ml.

d) Developer (250ml):
   Na2CO3: 15gm, 37% HCHO: 125μl, Na2S2O3. 5H2O: 1mg.

B. Principle:

   Silver staining, although more difficult to perform compared to that of Coomassie Brilliant Blue staining of proteins, is significantly more sensitive. The use of silver staining allows detection of proteins resolved by gel electrophoresis at concentration nearly 100-fold lower than those detected by Coomassie Brilliant Blue staining. The identification of proteins by silver staining is based on the differential reduction of silver ions, in a reaction similar to that used in photographic processes. A number of methods have been developed to stain polypeptide with silver salts after separation by SDS-polyacrylamide gel electrophoresis. In every case, the process relies on differential reduction of silver ions that are bound to the side chains of amino acids (Merril et al., 1984).
Chapter II: Materials & Methods

C. Method:

i). Gel was first fixed in fixing solution for more than 1 hour.

ii). After fixing the gel was washed three times 20 minutes each in 50% EtOH.

iii). Before impregnation the gel was pretreated in Pretreat solution for 1 minute.

iv). Rinsed in double distilled water thrice, approx 20 second each.

v). The gel was then kept in Impregnation solution for 20 minutes to saturate enough.

vi). Rinsed with double distilled water twice.

vii). Developed using Developer solution. The black bands were appeared nearly 10 minutes depending on the concentration of proteins in the bands.

viii). Then the gel was washed in double distilled water for two minutes twice.

ix). The reaction was Stopped using 50% MeOH, 12% AcOH solution approximately 10 minutes.

x). Finally washed in 50% MeOH for more than 20 minutes.

2.8. Autoradiography [Link, 1999]

A. Reagents & apparatus:

a) Fix solution
10% acetic acid, 20% methanol solution.

b) Solution for Fluorography
1M Sodium salicylate.

a) 3MM Whatmann chromatographic paper

b) Cling wrap

c) Gel drier

d) Autoradiographic cassette with intensifier screen.

e) KODAK X-OMAT X-Ray film

f) Fixing solution (KODAK)

g) Developing solution (KODAK)

B. Method:
After gel electrophoresis the gel was carefully kept in fixing solution. After fixing gel was silver stained, when required.

For fluorography, gel was then soaked in 1M sodium salicylate solution for 30min.

Gel was then placed on top of a piece of 3MM paper taken to the size of the gel.

Wrapped with cling wrap sheet.

Dried the gel in a gel drier.

The dried gel was then placed in a cassette with a X-ray film and kept the whole assembly in -70 refrigerator.

After exposure the gel was removed from the cassette in a dark room and developed the film emerging in developing solution and fixing solution consecutively.

The film was then air dried and photograph was taken using white-light transilluminator.

2.9. Determination of subcellular localization of periplasmic protein

Alkaline phosphatase [Sambrook and Russell, 2001a]

A. Reagents:

b) Buffer A:

1mg/ml lysozyme, 20% (w/v) Sucrose, 30mM Tris-Cl (pH 8.0) and 1mM EDTA (pH 8.0).

i) Buffer B:

0.1 M Tris-Cl (pH 8.0)

B. Principle:

The method relies on the ability of lysozyme to form spheroplasts simultaneously releasing soluble periplasmic fraction and corresponding freezing and thawing to release soluble cytoplasmic fraction. The insoluble part contains the membrane fraction.

C. Method:

i). 1ml of the induced culture was transferred to a microfuge tube and centrifuged at 10,000 rpm for 1 min at 4°C.
Chapter II: Materials & Methods

ii). The cell pellet was suspended in 100µl of freshly prepared solution of buffer A and stored on ice for 20 minutes.

iii). The cells were recovered by centrifugation at 10,000 rpm for 1 min at 4°C and the supernatant, the periplasmic fraction, was stored on ice.

iv). The cell pellets were resuspended in 100µl on buffer B and lysed the cell by freezing and thawing (i.e., placed the cells in -70°C or dry ice to freeze and then thawed at 37°C). The process was repeated at least thrice.

v). Centrifuged the suspension at 10,000 rpm for 5 min at 4°C. The supernatant contained the proteins of cytoplasmic fraction. The pellet, which contain mainly the membrane fraction, were then suspended in 100µl of buffer B.

vi). Fractions were then loaded in SDS-polyacrylamide gel.

2.10. Detection of target protein by Immunoprecipitation Technique[Oliver and Beckwith, 1982]

A. Buffers & Reagents:

a) First washing buffer:
10mM Tris pH-8. [for 1ml: 990µl milli-Q, 10µl 1M Tris, pH-8].

b) Cell lysis buffer [1ml]:
1% SDS, 10mM Tris pH-8, 1mM EDTA.[100µl 10% SDS, 10µl 1M Tris pH-8, 2µl 0.5M EDTA pH-8, 888µl milli-Q].

c) Triton Buffer [1ml]:
2% Triton X- 100, 50mM Tris pH-8, 150mM NaCl, 1mM EDTA. [20µl Triton X-100, 50µl 1M Tris pH-8, 150µl1M NaCl, 2µl 0.5M EDTA pH - 8, 778µl milli-Q].

d) Triton washing buffer [10ml]:
1% Triton, 50mM Tris pH-7.5, 1M NaCl. [0.1ml Triton, 0.5ml 1M Tris pH - 7.5, 9.4ml 1M NaCl].

e) Tris washing buffer [10ml]:
10mM Tris pH – 8. [100µl 1M Tris pH – 8, 9.9ml Milli –Q].

f) Protein A-CL agarose suspension (Bangalore Genei Pvt. Ltd.).

g) Antibodies.
Chapter II: Materials & Methods

B. Principle:

Immunoprecipitation is used to detect and quantitate target antigens in mixtures of proteins. The power of the technique lies in its selectivity: The specificity of the immunoglobulin for its ligand is so high that the resulting antibody-antigen complexes can be purified from contaminating proteins. Furthermore, immunoprecipitation is extremely sensitive and is capable of detecting as little as 100pg of radiolabeled protein. When coupled with SDS-polyacrylamide gel electrophoresis, the technique is ideal for analysis of the synthesis and processing of foreign antigens expressed in prokaryotic and eukaryotic hosts or in In vitro systems.

The target protein is usually immunoprecipitated from extracts of cells that have been radiolabeled. Protein A, a 42kDa cell wall component of staphylococcus aureus, binds to sites in the second and third constant regions of the Fc portion of the immunoglobulin (IgG) of many mammalian species efficiently. Protein A coated Sepharose beads are used to precipitate down the antibody-antigen complex from the labeled cell extracts. Antibodies bind to protein A chiefly by hydrophobic interactions that can be disrupted at low pH. Most antibodies can withstand transient exposure to low pH, and this treatment is now the standard method to release them in an active form from protein A-Sepharose beads.

C. Method:

i). 0.5ml cell culture OD – 0.3 at 600 nm was taken in phosphate less MOPS medium.
ii). Pulse labeled for 90 seconds and chased by 80μl of cold 0.2M methionine.
iii). Aliquot were taken at different time interval at chasing period.
iv). 0.5ml labeled culture centrifuged for 5min at 4°C.
v). Wash by washing buffer and resediment.
vii). 20μl of this mixture was added to 200μl of triton buffer.
viii). Centrifuge for 5min at 10,000rpm.
ix). Collect the supernatant; add 1-5μl of antiserum (according to the stock concentration).
x). Incubated overnight at 0°C.
Chapter II: Materials & Methods

xii). Incubate at 0°C for 30 min.

xiii). Immunocomplex were sedimented at 10000 rpm for 2 min.

xiv). Wash three times the immunocomplex by Triton washing buffer.

xv). Again wash the complex by Tris washing buffer.

xvi). Resuspend the complex in 50 μl sample buffer (DTT was not added to the sample buffer for non-reducing gel).

2.11. Immunodetection of proteins by Western Blotting

[Magi et al., 1999]

A. Reagents:

a) Transfer Buffer for western blot:

192 mM Glycine, 25 mM Tris, 20% Methanol (v/v), [for 1 litre: 14.413 gm Glycine, 3.0285 gm Tris, 200 ml Methanol, dissolved in Milli-Q water]

b) TBS Buffer

10 mM Tris, 0.9% NaCl. [for 500 ml: 0.6057 gm Tris, 4.5 gm NaCl, pH 7.4 by HCl].

c) Ponceau S staining solution:

0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid. [for 100 ml: 0.2 gm, 3 gm TCA, 80 ml Milli-Q water; dissolved and then the volume is made up].

d) Blocking solution:

3% BSA in TBS, 0.1% (w/v) Triton X-100; [1.5 gm BSA, 50 μl Triton X-100 in 50 ml TBS].

e) Washing solution:

5% (w/v) Triton X-100 in TBS.

f) Western blot developing solution:

5 ml 4-chloro 1-naphthol 0.3% in methanol, 7 μl H₂O₂, 20 ml 0.05 M Tris-HCl pH 6.8.

B. Principle:

Immunoblotting is used to identify and measure the size of macromolecular antigens (usually proteins) that react with a specific antibody. The proteins are first separated by electrophoresis through SDS-polyacrylamide gels and then transferred electrophoretically from the gel to a solid support, such as a nitrocellulose membrane. After the unreacted binding sites of the membrane are blocked to suppress nonspecific adsorption of antibodies, the
immobilized proteins are reacted with a specific monoclonal or polyclonal antibody. Antigen-antibody complexes are finally located by radiographic, chromogenic, or chemiluminescent reactions.

C. Method:

i). Following separation of proteins by gel electrophoresis, the gel was placed in acetic acid-methanol equilibration buffer, and gently agitated for 30 minutes at room temperature.

ii). The anode side of the blotting cassette was placed in a dish of cooled blotting buffer.

iii). A piece of buffer wetted scorch bite was placed on the top of the anodic side of the cassette.

iv). Before placing on top of the scorch bite on the anodic side of the cassette, a sponge pad was submerged carefully to displace any trapped air in blotting buffer.

v). A piece of 3MM Whatman paper was placed on to the sponge pad, and rolled with a glass tube to ensure air bubbles are removed.

vi). Then the prewetted transfer membrane [BIORAD Supported NC membrane] was placed on top of the 3MM paper carefully and rolled with a glass tube.

vii). The equilibrated gel was then placed on top of the blotting membrane and ensured that no bubbles are trapped in between them.

viii). Another piece of 3MM paper, sponge pad and scorch bright was then placed correspondingly with caution not to trap any air bubble.

ix). The cathode side was then placed and clamped to the anode side of the cassette.

x). The assembled cassette was then quickly transferred into the blotting tank filled with cooled transfer buffer.

xi). Connected to the power supply and carried on transfer for 1 hour at 100V at 4°C.

xii). After the transfer the nitrocellulose (NC) membrane was removed from the cassette and placed in a glass tray filled with Ponceau S staining solution. The band of protein was monitored (washing excess stain by distilled water after 3 minutes of staining) for assurance of the transfer.

xiii). The stain was distained by washing with TBS several times.

xiv). The nonspecific binding sites in the membrane was blocked in blocking solution three times, each for 10 minutes.

xv). Membrane was then incubated overnight with primary antibody in blocking solution.

xvi). Washed for 3 X 10 minutes in blocking solution.
Chapter II: Materials & Methods

xvii). Incubated 2 hours in the secondary antibody solution (antibody diluted in blocking solution).
xviii). Washed again for 3 X 10 minutes in blocking solution.
xix). Then washed in Triton in TBS washing solution for 30 minutes.
xx). Washed in 0.05M Tris-HCl, pH-6.8, two times, each for 30 minutes.
xxi). After completion of washing step the membrane was soaked in developing solution until the color appears.
xxii). Stopped the reaction with washes in distilled water.
xxiii). The membrane was then air-dried and photographed immediately.

2.12. Isolation of aggregated protein

[Rosen et al., 2002]

A. Reagents:
a) Buffer A:
10mM potassium phosphate buffer pH-6.5, 1mM EDTA, 20%(w/v) sucrose, 1mg/ml lysozyme. [For 5ml: 0.05ml 1M potassium phosphate buffer pH-6.5, 0.01ml 0.5M EDTA, 1gm sucrose, 5mg lysozyme.]
b) Buffer B:
10mM potassium phosphate buffer pH-6.5, 1mM EDTA. [For 5ml: 0.05ml 1M potassium phosphate buffer pH-6.5, 0.01ml 0.5M EDTA].
c) 10% (v/v) NP40:
[For 5ml: 0.5ml NP40 in 4.5ml Milli-Q H2O].

B. Method
i). Aliquots of bacterial culture was rapidly cooled to 0°C in an ice-water bath & centrifuged for 10 minutes at 5000g at 4°C.
ii). Pellets were resuspended in buffer A (40µl buffer A for 8ml culture).
iii). Incubate for 30 minutes on ice.
iv). Then 360µl buffer B was added (corresponding to the 40µl buffer A).
v). Mixed and sonicate (level 2, 50% duty, 8 cycles) on ice.
vi). Centrifuge at 2000g for 15 minutes at 4°C.
vii). Supernatant was again centrifuged at 15000g for 20 minutes at 4°C for isolation of (aggregated protein and membrane) insoluble cell fraction.

viii). Pellet fraction were frozen and resuspended in 400μl of buffer B by brief sonication.

ix). Centrifuged at 15000g for 20 minutes at 4°C.

x). Washed pellet fractions were resuspended in 320μl of buffer B by brief sonication.

xi). Then add 80μl of 10% NP40.

xii). Centrifuged at 15000g for 30 minutes at 4°C.

xiii). Step 10 and 12 were repeated for complete removal of contaminated membrane protein several time.

xiv). Pellet obtained was resuspended in 400μl buffer B by sonication and centrifuged at 15000g for 30 minutes at 4°C.

xv). Finally resuspended in 200μl of buffer B by sonication and subjected to gel electrophoresis.

2.13. Important Chemicals

All the electrophoresis reagents and growth medium components were purchased from Pharmacia Amersham. E.coli alkaline Phosphatase, CCCP, complete & incomplete Freunds adjuvant was obtained from Sigma. ProteinA-CL agarose was purchased from Bangalore Genei and ethanol was from Bengal Chemical and Pharmaceuticals Ltd., Calcutta. 35S-methionine (15mCi/ml) was purchased from Board of Radiation and Isotope Technology, India.