APPENDIX

A-1: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS [SAMBROOK & RUSSELL, 2001B]

A. SDS-PAGE reagents:

a) Running Buffer: 25mM Tris, 250mM glycine, 0.1% (w/v) SDS. [For 1.0L (1X) buffer preparation, 3g Tris, 14.4g Glycine and 1g SDS are dissolved and volume was made up to 1.0L by milli-Q H2O].

b) SDBME buffer: 0.3% SDS, 0.2M DTT, 0.028M Tris-HCl and 0.022M Trizma base. [For 5ml (1X): 0.15ml 10% SDS, 1ml 1M DTT, 0.0221g Tris-HCl and 0.0133g Trizma base, volume was made up to 5ml by milli-Q H2O].

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) Acrylamide & bisacrylamide stock solution (1X): 30% acrylamide and 0.8% bisacrylamide solution was prepared by dissolving 30g acrylamide and 0.8g bisacrylamide in 100ml of milli-Q water and the solution was filtered by Whatman No. 1 filter paper.

e) Resolving gel (For 5ml): Acrylamide-bisacrylamide Solution: 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.

f) Stacking gel (For 2.5ml): 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.

g) Coomassie brilliant blue staining solution: 0.25% Coomassie brilliant blue in methanol:acetic acid solution. [For 100ml: 0.25g Coomassie brilliant blue was dissolved in the mixture of 50ml methanol, 40ml water and 10ml acetic acid, the solution was filtered by Whatman No. filter paper].

B. Principle:

Almost all analytical electrophoreoses 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. The amount of bound SDS is almost 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, ~1.4g of detergent is bound per gram of polypeptide. By using markers of known molecular weight, it is possible to estimate the molecular weight of any unknown polypeptide chain.

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 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, there 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. The higher pH of the resolving gel favors the ionization of glycine, and the resulting glycine ions migrate 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.

The effective range of separation of SDS-polyacrylamide gels depends a) on the concentration of polyacrylamide used to cast the gel and b) 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, has been found to be capable of resolving polypeptides that differ in size by a little as 3%. The linear range of separation of proteins is 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 Milli-Q water and then wiped out with methanol 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 tightly.

iii) The total set was kept in hot oven to increase the temperature of the glass plates because it was helpful to seal the inner side of the assembly.

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

v) Resolving gel solution was prepared of desired concentration (5-15%) and poured into the cassette and allowed 1h to polymerize. Water saturated iso-butanol was layered onto the top of the resolving gel to maintain the upper layer straight.

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

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

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

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

x) Both upper and lower Buffer tank was filled with running buffer and then comb was removed from the gel carefully.
xi) Sample was loaded in 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 moved into the resolving gel, the voltage was increased to 15V/cm and the run was continued until the bromophenol blue reaches the bottom of the resolving gel.

xii) After completion of the run, the glass plates were removed from the electrophoresis apparatus.

xiii) A spatula was used to carefully pry apart the plates. A small cut was made at the right lower corner of the gel for marking the orientation of the gel.

xiv) For coomassie staining, the gel was directly transferred to the staining solution. For silver staining, the protocol has been described in the next section.
A-2: ESTIMATION OF PROTEIN BY BRADFORD 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 to 25ml by milli-Q water and filtered through Whatman No.1 filter paper. The reagent was stored at 4°C in dark place.

B. Principle:
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 amino acids). 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 complexed with the protein.

C. Method:
To draw a standard curve 200µl BSA solution (of different concentrations) was mixed with 800µl of Bradford reagent and absorbance was measured at 595nm within 30 minutes of addition.
A-3: ASSAY OF LACTATE DEHYDROGENASE [CHATTOPADHYAY ET AL, 1994]

A. Reagent:

100 mM Tris/HCl of pH 7.5, 5 mM sodium pyruvate, and 250 μM NADH, LDH

B. Principle: The dehydrogenase activity of native LDH assays by measuring the rate of conversion from NADH to NAD+. The (absorbance)340nm of the assay mixture is measured at different intervals of time. The decrease in (absorbance)340nm with time signified enzyme activity through the disappearance of NADH, because NADH but not NAD+ had absorption maxima at 340 nm.

![NAD+ and NADH Spectrum](image)

C. Chemical Reaction: