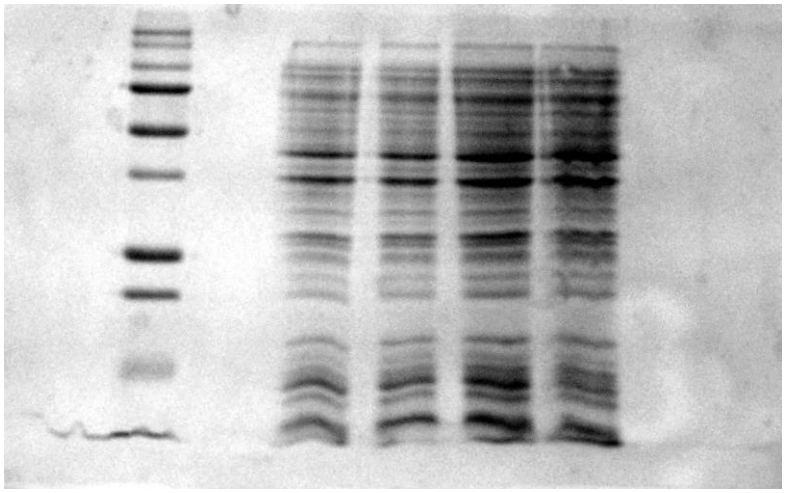
CHAPTER 1 Techniques

1.1 SDS-PAGE Electrophoresis

1.1.1 Introduction

Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis is a technique used for the analysis and separation of proteins based on their size. Samples are placed on a matrix of polyacrylamide gel and an electric field is applied. The protein molecules migrate towards the positive electrode, with the smaller molecules moving at a faster rate through the matrix. Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

Figure 1.1 An SDS-PAGE gel.



1.1.2 Ladder marker

Lane 2 in the above gel contains a Precision Plus protein standard, all blue (bio-Rad). The marker contains ten protein bands; 10 kD, 15 kD, 20 kD, 25 kD, 37 kD, 50 kD, 75 kD, 100 kD, 150 kD and 250 kD. The marker comes in loading buffer.

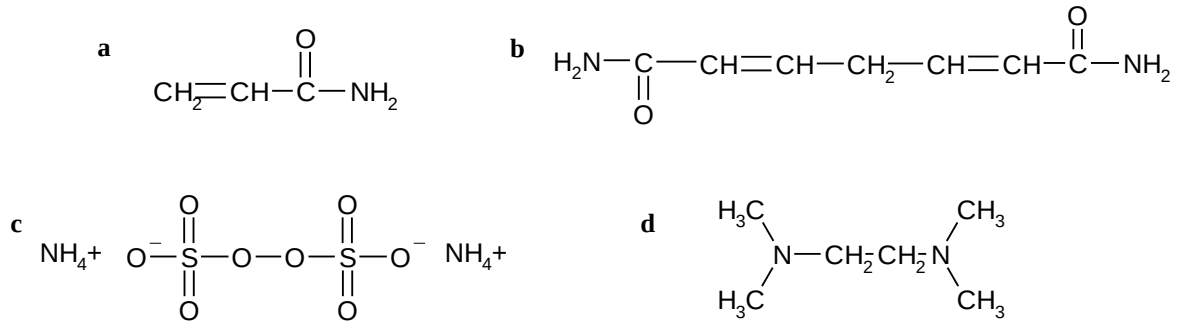
1.1.3 Recipes

15 % Seperating gel

30% acrylamide/ 0.8% bisacrylamide	3.5 ml (wear gloves!!!)
Tris-HCl pH 8.8	2.62 ml
water	0.84 ml
10% SDS	70 µl (0.1 %)
10% AMPS	46 µl
TEMED	4.6 µl
	7 ml

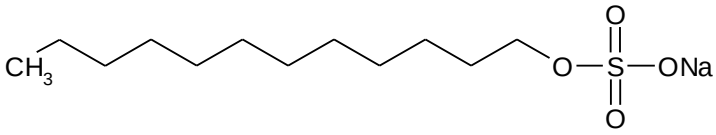
Acrylamide (Fig. 1.1a) and bis-acrylamide (Fig 1.1b) polymerise to form a sieving matrix. The polymerisation forms long chains of acrylamide which are cross linked by bis-acrylamide. The polymerisation is initiated by free radical formation. The addition of ammonium persulfate (AMPS) (Fig. 1.1c) forms free radical oxygen species in aqueous solution by a base catalysed reaction. N,N,N',N'-tetramethylethylenediamine (TEMED) (Fig. 1.1d), a tertiary amine base, serves as the catalyst.

Figure 1.2 Structures of gel components.



Sodium dodecyl sulfate (SDS) is an anionic detergent used to solubilise and denature the protein samples. Most proteins bind SDS in a ratio of 1.4 g SDS per gram of protein. The intrinsic charge of the protein is insignificant compared to the overall negative charge provided by the bound SDS. The charge to mass ratio is essentially the same for each protein molecule and therefore migration through the gel is based only on their size.

Figure 1.3 Structure of SDS.



5 % Stacking gel

30% acrylamide/ 0.8% bisacrylamide	1.11 ml (wear gloves!!!)
Tris-HCl pH 6.8	0.83 ml
water	4.64 ml
10% SDS	33 µl (0.05 %)
10% AMPS	33 µl
TEMED	6.6 µl
	7 ml

The different compositions of the separating and stacking gels are an example of a discontinuous buffer system. The main advantage of this technique is that samples are concentrated into narrow starting zones or stacks. This results in very sharp protein bands on the gel and therefore is a high resolution technique.

A discontinuous buffer system has four important features[1]: (i) there is a large pore (low acrylamide concentration) stacking gel between the sample zone and the separating gel; (ii)

the separating gel has a higher acrylamide concentration appropriate to the size range of the proteins to be separated; (iii) the loading buffer and both gels contain chloride ions, while the running buffer contains glycinate ions; and (iv) the pH of the resolving gel (pH 8.8) is higher than that of the loading buffer and stacking gel (pH6.8).

In the stacking gel at pH 6.8, glycinate is poorly dissociated and therefore has a low electrophoretic mobility. Chloride is highly dissociated at pH 6.8 and therefore has a high mobility. When an electric field is applied the chloride (leading) ions will move rapidly down the gel, moving away from the slower moving glycinate (trailing) ions and leaving a zone of lower conductivity. Conductivity is inversely proportional to the field strength and therefore a steeper voltage gradient is established in this zone. The trailing ions are accelerated so they migrate down the gel immediately behind the leading ions. The sample proteins have intermediate mobilities between the leading and trailing ions and therefore are concentrated into a thin band between them. To minimise the separating effect of the gel, the concentration of acrylamide is kept low.

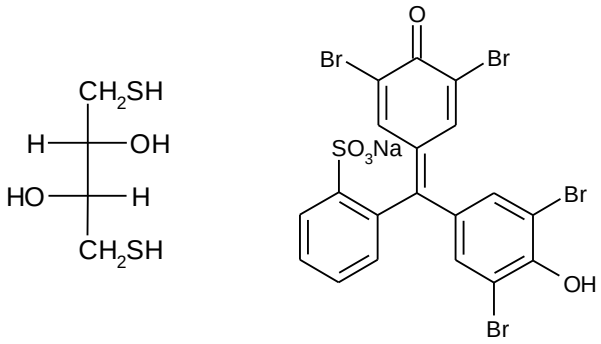
At the interface between the stacking and separating gels the pH suddenly increases and the pore size decreases. At pH 8.8, the degree of dissociation of glycinate is much higher and therefore its mobility is increased, almost to that of the chloride ions. The trailing ions therefore overtake the sample proteins as they migrate down the gel. This effect, together with the decreased pore size, causes the proteins to become unstacked. The proteins are then separated according to their size.

Loading buffer

Tris-HCl pH6.8	1 ml
DTT	200 mM
SDS	4%
Bromophenol blue	1 grain
Glycerol	2 ml
Water	10 ml

Dithiothreitol (DTT) is present to reduce disulfide bonds in the protein sample prior to electrophoresis. The bromophenol blue serves as a tracking dye. Glycerol gives the solution more viscosity and therefore it will sink into the wells when loading.

Figure 1.4 Structures of DTT and Bromophenol blue.



Running buffer

Tris	1.5 g
Glycine	7.2 g
10% SDS	5 ml
Water	up to 500 ml

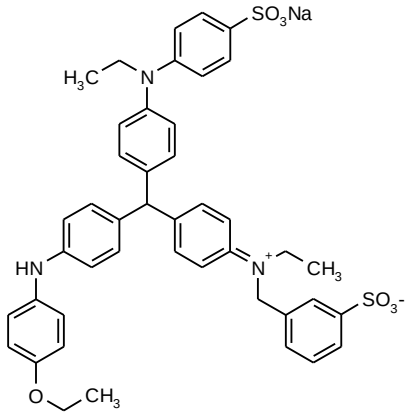
The running buffer is poured into the gel unit tank, enough to cover the bottom of the gel and therefore the positive electrode is submerged. This ensures that a current will flow.

Staining buffer

Coomassie blue	0.1%
Acetic acid	10%
Methanol	40%

Coomassie blue requires an acidic medium for the generation of electrostatic attraction between the dye molecules and the amino groups of proteins. The dye is thought to interact with the basic groups of polypeptides. 1 mg of protein will bind as much as 1.2 to 1.4 mg of dye.

Figure 1.5 Structure of coomassie blue.



De-staining buffer

Acetic acid	10%
Methanol	40%

1.1.4 Verticle electrophoresis unit

The electrophoresis unit generally comprises;

- a tank with lid, power leads and connectors,
- a gel running/casting module,
- a gel casting frame,
- a notched glass plate which fits with a plain glass plate separated by 1 mm spacers,
- a 1 mm thick sample comb.

1.1.5 CopA/Z gels

As CopA and CopZ are small proteins, they do not show up well on the usual SDS-PAGE gels. Therefore gels with higher acrylamide percentages are used.

Seperating gel

30% acrylamide/ 0.8% bisacrylamide	3.5 ml (wear gloves!!!)
Tris-HCl pH 8.8	1.5 ml
10% SDS	60 µl
water	1.49 ml
TEMED	3.3 µl
10% AMPS	23.3 µl

Stacking gel

30% acrylamide/ 0.8% bisacrylamide	0.5 ml (wear gloves!!!)
Tris-HCl pH 6.8	1.125 ml
10% SDS	25 µl
water	1.325 ml
TEMED	5 µl
10% AMPS	25 µl

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[1] Dunn, M. J. *Gel Electrophoresis: Proteins.* BIOS Scientific Publishers Ltd., **Oxford**, 1993.