BCHEM 365
Lecture 6
October 10, 2017

SDS-PAGE

- Typically, the resolving gel used is a 15 % polyacrylamide gel. This gives a gel of pore size in which proteins of relative molecular mass (M_r) 10,000 move through the gel relatively unhindered, whereas proteins of M_r 100,000 can only just enter the pores of this gel
- Gels of 15 % polyacrylamide are therefore useful for separating proteins in the range M_r 10,000 to 100,000
- However, a protein of M_r 150,000, for example, would be unable to enter a 15 % gel and would require a larger pore gel (e.g., a 10% or even 7.5% gel)

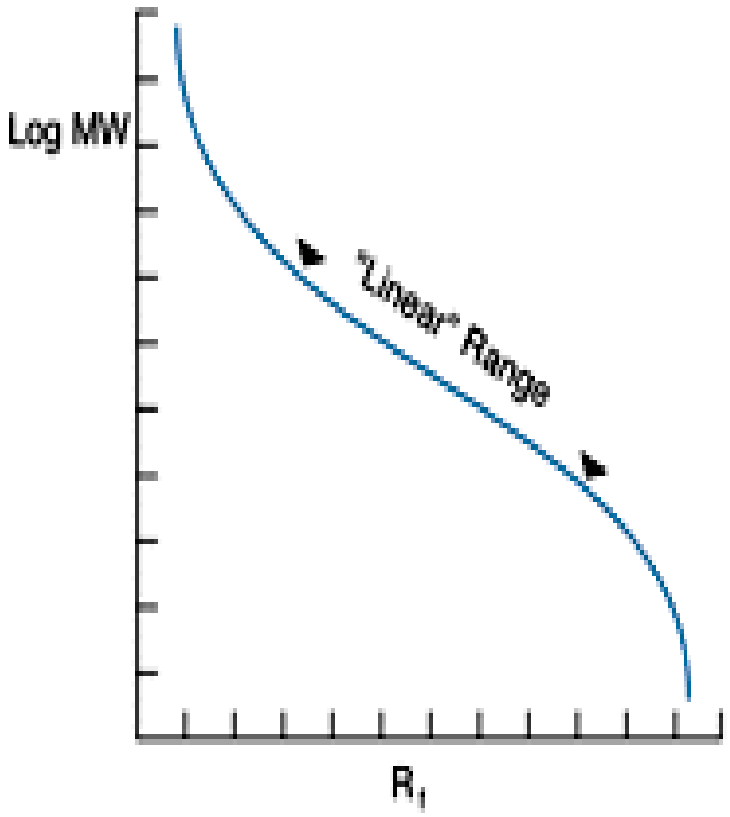
Applications of SDS-gel electrophoresis

- 1. Molecular weight determination
- 2. Determination of number of subunits in a protein
- 3. Purity of a protein

Protein molecular weight determination

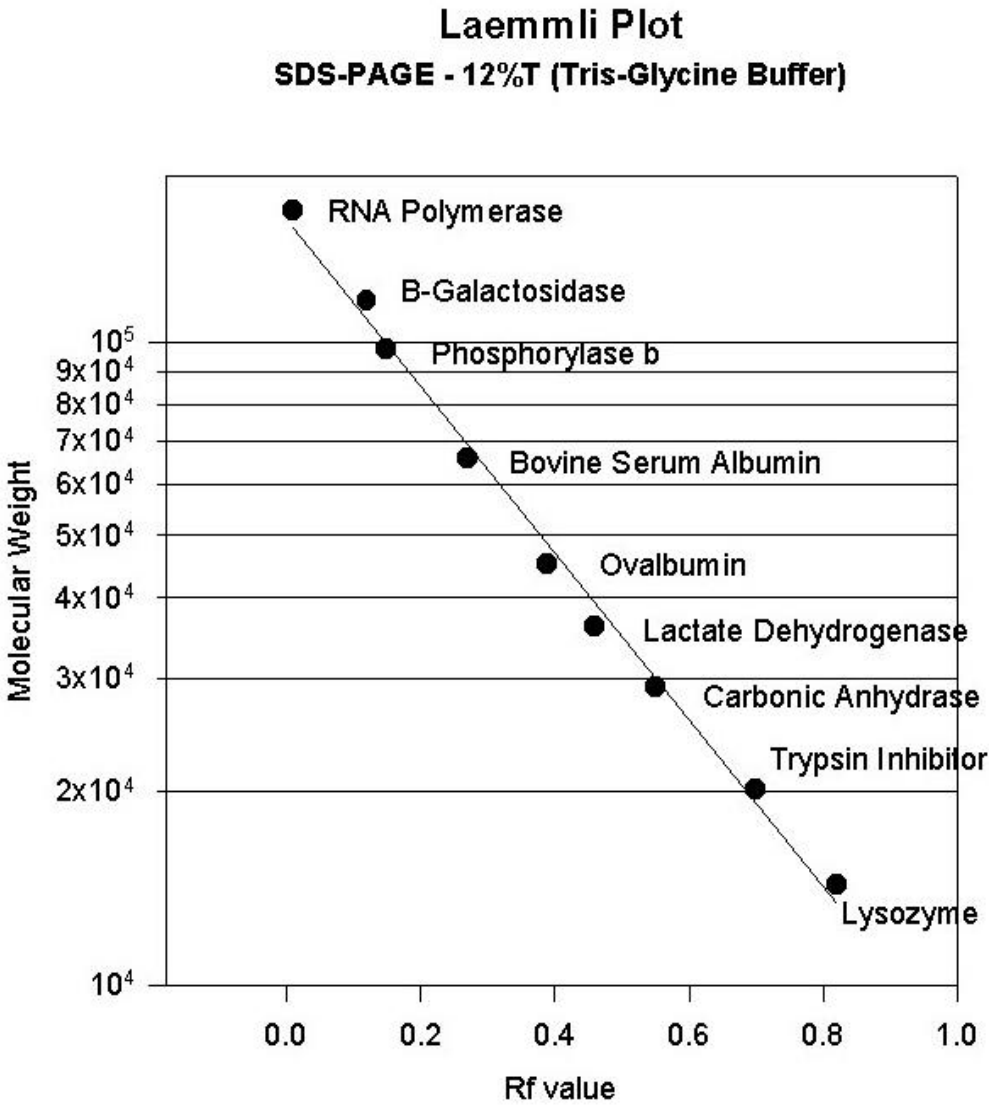
- The relative molecular weight (M_r) of a protein can be determined by comparing its mobility with those of standard proteins run on the same gel
- The M_r of the unknown protein is obtained from a graph of calibration curve of distance moved (R_f) against log M_r for each of the standard proteins (MW ladder)
- The actual plot of log(MW) vs. R_f is sigmoidal because at high MW the sieving effect of the gel hinders penetration of most proteins, while at low MW the sieving effect is negligible and proteins migrate almost at their free mobility, which in SDS is independent of MW

A graph of log(MW) of proteins vs. R_f is sigmoidal but is nearly linear for a range of molecular weights depending on concentration of acrylamide. The linear or proportionality range , in which log of MW is directly proportional to th distance travelled on gel requires that proteins are fully denatured and appropriate gel concentration is selected



Protein molecular weight determination

An empirical plot of log(MW) against R_f for several protein standards used to determine the MWs of proteins. It is required that the protein be fully denatured



Worked example

- Question: Table 1 shows the distance moved in SDS-PAGE by a series of marker proteins of known relative molecular mass (M_r: the ratio of the mass of that molecule to 1/12 of the mass of carbon-12 and is a dimensionless number)
- A purified bacterial protein (X) run on the same gel showed a two bands, A and B that had moved a distance of 45 mm and 64 mm, respectively. What is the nature of this protein? Find the M_r of protein X? Assign reasons for separation into two bands.
- Note: Beta-Lactoglobulin is a dimer with a relative molecular mass of 36,800. Under the reducing conditions the disulfide bridges linking the subunits are reduced so that the two monomer polypeptide chains are seen on the gel

Table 1. Data for worked example

Protein	M_r	Distance (mm)
Transferrin	78,000	6.0
Bovine serum albumin	66,000	12.5
Ovalbumin (egg albumin)	45,000	32.0
Glyceraldehyde-3-phosphate dehydrogenase	36,000	38.0
Carbonic anhydrase	29,000	50.0
Trypsinogen	24,000	54.0
Soybean trypsin inhibitor	20,100	61.0
Beta-Lactoglobulin	18,400	69.0
Myoglobin	17,800	69.0
Lysozyme	14,300	79.0
Cytochrome c	12,400	86.5

Solution

- Construct a calibration graph by plotting log M_r versus distance moved for each of the marker proteins. Determine the relative molecular mass of protein X from the graph. M_r is approximately 31,000.
- Note that this method is accurate with an error rate of 5 to 10 %, so the answer is 31,000 ±3100 for band A
- You are to calculate the M_r for band B and discuss the result

Monitoring purity of proteins

- SDS-gel electrophoresis is often used to assess the purity of a sample.
- A pure protein should give a single band on SDS-PAGE, unless the molecule is made up of two unequal subunits, where two bands, corresponding to the two subunits, will appear
- Observation of two bands or more shows impure proteins

Isoelectric focusing

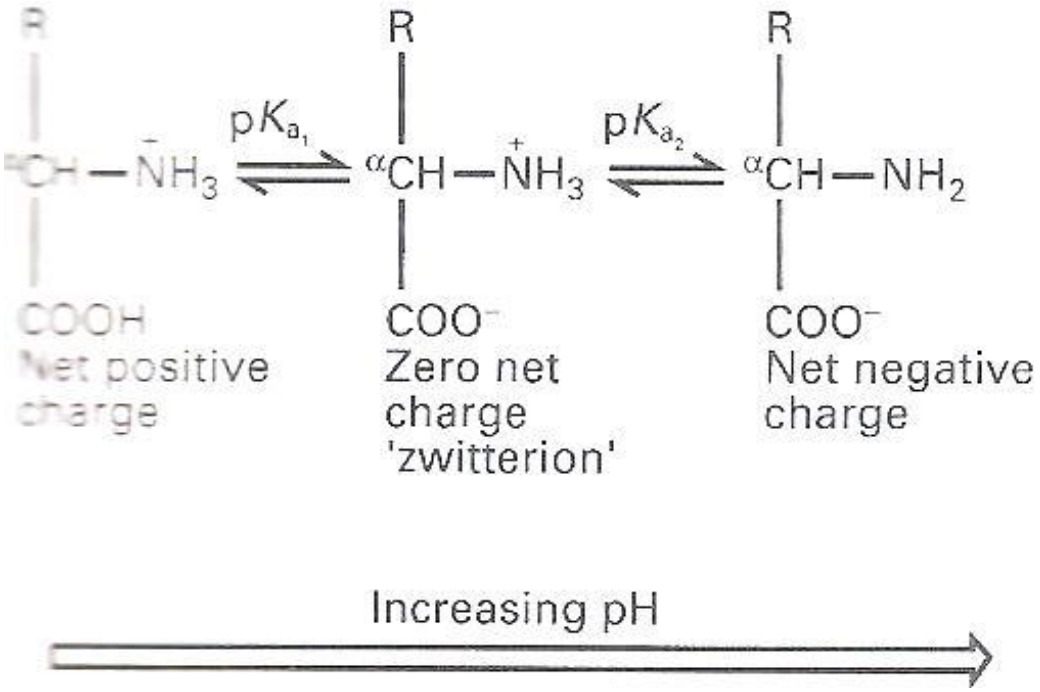
- Proteins are amphoteric molecules, that is, they can carry positive, negative, or zero net charge depending on the pH of their environment
- The charges on proteins arise from functional groups present on the amino acids which make up the protein. Twenty amino acids varying in shape, size, charge, and chemical reactivity are found in proteins
- Each of the functional groups has a pK_a value
- $HA \rightarrow A^- + H^+$ with an acid dissociation constant, K_a
- pK_a is the pH at which half of the members of that functional group are protonated
- At pH values above the pK_a that group can be considered fully protonated, and below the pK_a, is fully deprotonated
- Thus, as the pH of the environment changes, the net charge carried by a protein will change
- At high pH, most proteins will have many deprotonated surface groups and will carry net negative charge

Isoelectric focusing

- At low pH, H^+ are in excess and most proteins have a net positive charge
- At some intermediate pH, different for every protein, the net charge on the protein will be zero, that is, the protein carries equal numbers of positive and negative charges.
- The pH at which a protein has a net charge of zero is its **isoelectric point (pI**). At this pH, the protein has no net electrophoretic mobility.
- In solution, a protein is positively charged at pH values below its pI and negatively charged when the pH is above its pI

Isoelectric focusing

- Separation of proteins on the basis of differences in isoelectric points is called **Isoelectric focusing**
- The method has high resolution, and can separate proteins that differ in their isoelectric points by as little as 0.01 pH unit
- The numerical value of isoelectric point is related to the pKa values, and is given by the equation:



$$pI = \frac{pK_{a1} + pK_{a2}}{2}$$

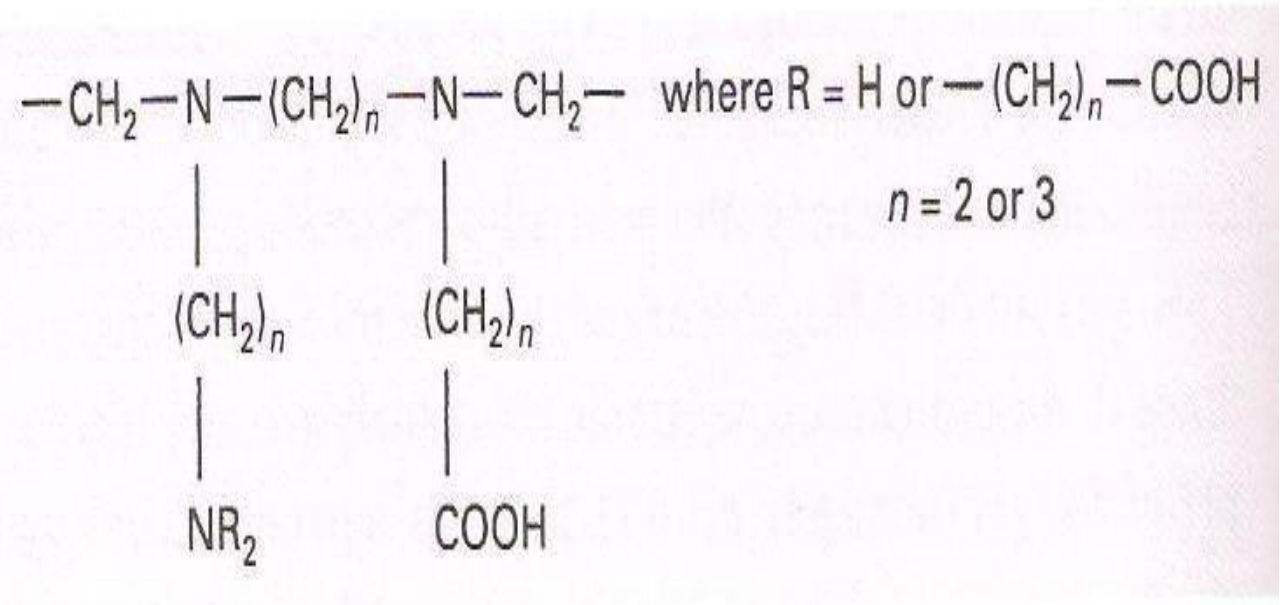
Isoelectric focusing (IEF)

- In isoelectric focusing (IEF) a **pH gradient** is established along the length of a gel. Proteins migrate through this gradient until they reach their pI, where they stop moving

Making of pH gradient for IEF gels

- pH gradient for IEF is prepared using a mixture of **ampholytes**, each having a different pI
- The most commonly used ampholytes are complex mixtures of synthetic polyamino-polycarboxylic acids (Fig. 6.1).
- Concentration of ampholytes used must be high enough to cause a change in the pH of their surroundings
- Like protein molecules, the ampholytes migrate through the gel until they reach a region where the pH is equal to their pI

Fig. 6.1. General formula of ampholytes



Isoelectric focusing

- The gel is set up with a uniform mixture of ampholytes throughout, and its anode and cathode ends are immersed in dilute acid and base, respectively
- A well designed ampholyte system will produce a smooth and gradual gradient of pH with no abrupt charges or "steps’
- Ampholytes near the ends of the gels will be positively charged if near the positive electrode, and negatively charged if near the negative electrode.
- Ampholytes are sold in **broad range** covering pH of 2 - 12 or **narrow range** of 2 pH units across the gel, say pH 5 – 7
- Commercial ampholytes include Bio-Lyte and Pharmalyte

Materials used as ampholytes for IEF gels

Many reagents can behave as ampholytes and may be used. Below, we find the merits and demerits of each

- Materials which behave as ampholytes: amino acids, proteins, and polyacidic and polybasic synthetic molecules
- Amino acids have poor conductivity and poor buffering capacity in their zwitterionic state, making them poor ampholytes
- Proteins are good ampholytes, but they interfere with analysis of the sample, by introducing new proteins into the mixture
- Polyamino-polycarboxylic acids have good buffering capacity and conductivity across a broad pH range, and usually manufactured in a relative molecular weight range of 300 - 500, which is small enough to avoid interference with most subsequent processing
- Polyamino-polycarboxylic acids have a disadvantage of binding tightly to the proteins being separated, due to ionic interactions, and can be very difficult to remove

Ampholytes

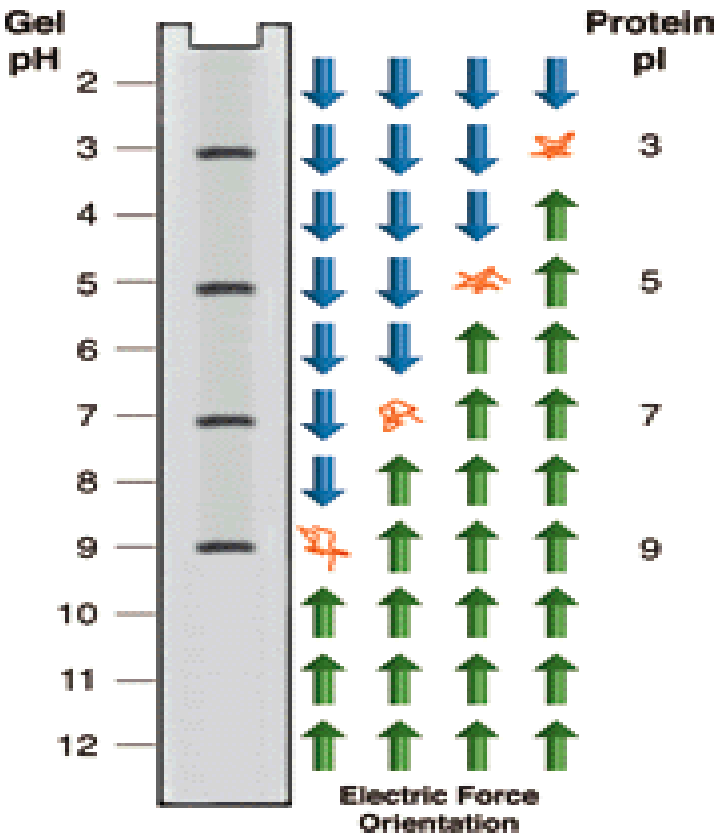
- Optimum concentration of ampholytes for IEF gels is 2 % (w/v)
- Ampholyte concentrations below 1% (w/v) often result in unstable pH gradients
- Concentrations above 3% (w/v) are difficult to remove from gels and can interfere with protein staining

Isoelectric focusing

- On the pH gradient gel, if a protein is in a pH region above its pI, it has a negative charge and will move into lower pH regions in attempt to look for its pI
- If it is in a pH region below its pI, it has a positive charge and will move into higher pH regions to look for its pI
- Hence, proteins are continually swept back or forward until they settle on the appropriate pI (Fig. 6.2)
- This sweeping back process is called "focusing“. IEF is thus an equilibrium electrophoresis system, run until protein movement ceases

Isoelectric focusing (IEF)

Fig. 6.2. A gel is prepared having pH gradient extending the length of the gel. Protein samples soaked on paper discs are layered on the gel. Protein diffuses from paper into gel and is swept back or forward by the electric field depending on its pI and wherever it was placed on the gel. A protein stops migrating when it enters a zone in which the surrounding pH equals its isoelectric point. At any other position on the gel, the protein acquires a charge which causes it to migrate toward its pI (green and blue arrows). On the gel are 4 protein molecules having pI 3, 5, 7, and 9 (red)



Isoelectric focusing

- When the current is switched on, proteins begin to migrate into the gel, with the most charged, i.e., those furthest from their pI moving fastest
- Gradually, proteins separate into zones of defined pH
- IEF is most frequently carried out as the **first step in 2-dimensional electrophoresis.** The apparatus best suited for this use is a **tube gel** system. The gels are cast and run in glass tubes with an internal diameter matched to the thickness of the second dimension gel. 1.5 mm gels are commonly used

Isoelectric focusing

- After running the IEF gel, it is extruded from the tube and laid across the top of the second dimension gel.
- IEF gels can also be run as **slabs**, which allows an increased sample throughput. Slab IEF gels can be cut into strips for loading onto second dimension gels
- Since the method requires that proteins move freely according to their charge under the electric field, IEF is carried out at **low percentage gels** to avoid any sieving effect within the gel
- Polyacrylamide gels (4%) are commonly used, but agarose may also be used, especially for the study of high M_r proteins that may undergo some sieving even in a low percentage acrylamide gel

Isoelectric focusing (IEF)

- To prepare a thin-layer IEF gel slab, carrier ampholytes, covering a suitable pH range, and riboflavin are mixed with the 4 % acrylamide solution
- The mixture is poured over a glass plate (typically 25 cm × 10 cm), which contains the spacer. The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerized by photoplymerization by placing the gel in front of bright light.
- Photodecomposition of riboflavin generates a free radical, which initiates polymerization. This takes 2-3 hours. Once gel has set, the glass plates are pushed apart gently to reveal the gel stuck to one of the glass plates

Isoelectric focusing (IEF)

- Two electrode wicks, made of thick (3 mm) strips of filter paper, one soaked in phosphoric acid (as anode) and the other soaked in sodium hydroxide (as cathode) are laid along the long length of each side of the gel and the power is turned on
- Ampholytes form a pH gradient between the anode and cathode. The power is then turned off and protein samples soaked on small discs of filter paper are layered on the gel
- Voltage is again applied for about 30 min to allow protein samples to electrophorese off the filter paper and into the gel, at which time the paper squares can be removed from the gel
- Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their pI will be positively charged and will initially migrate toward the cathode

Isoelectric focusing (IEF)

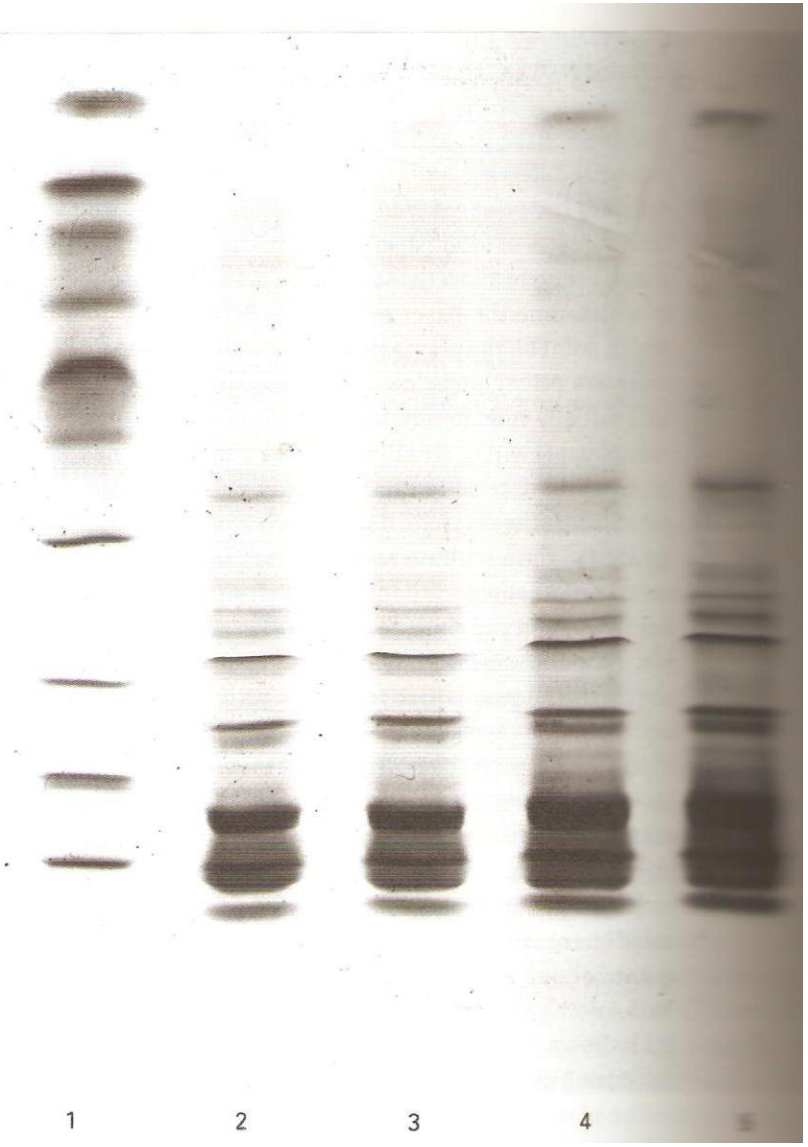
- Proteins that are initially at pH regions below their pI will be positively charged. As the proteins move, the surrounding pH will be steadily increasing, and therefore, the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point
- The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, proteins that are initially at pH regions above their pI will be negatively charged and will migrate toward the anode until they reach their isoelectric point and become stationary.
- Because samples will always move toward their pI, it is not critical where on the gel they are placed
- To achieve rapid separation (i.e., 2-3 hours of running the gel), relatively high voltage (up to 2,500 V) are used. As considerable heat is produced, gels are run on cooling plates (10^{o}C) and power packs are used to stabilize the power output and minimize thermal fluctuations

Isoelectric focusing (IEF)

- Following electrophoresis, the gel must be stained to detect the protein samples. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel
- The gel is therefore first washed with fixing solution, such as 10 % trichloroacetic acid (TCA)
- This precipitates proteins in the gel and allows the much smaller ampholytes to be washed out
- Gel is then stained with Coomassie Brilliant Blue and then destained. A typical IEF gel is shown in Fig. 6.3

Isoelectric focusing (IEF)

Fig. 6.3. A typical isoelectric focusing gel. Lane 1 contains a mixture of standard proteins of known isoelectric points. Lanes 2-5 show increasing loadings of venom from the Japanese water moccasin snake. Source: Bio-Rad Labs.



Applications of Isoelectric focusing

1. For preparative purposes
2. Determination of pI of a protein
3. Determination of heterogeneity of a protein
4. Separating isoenzymes

Preparative IEF

- For separation of mixtures of proteins, that is protein purification

Determination of pI of a protein

- The pI of a given protein may be determined by running a mixture of proteins of known isoelectric points on the same gel
- A number of mixtures of proteins with differing pI values are commercially available covering the pH range 3.5 -10. After staining, the distance of each band from one electrode is measured and a graph of distance for each protein against its pI plotted
- By means of this calibration line, the pI of an unknown protein can be determined from its position on the gel
- Read the article attached (Robertson et al., 1987)

Heterogeneity in a protein

- IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein
- For example, a protein may show a single band on an SDS gel, but may show more than one band on an IEF gel
- This may occur, for example, when a protein exists in mono-, di-, and tri-phophosphorylated forms.
- The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels. However, the small charge difference introduced on each molecule by PO_4^{3-} groups can be detected by IEF

Isoelectric focusing

- IEF is particularly useful for separating isoenzymes, which are different forms of the same enzyme often differing by only one or two amino acid residues. Different amino acids introduce different charges, hence, different pIs
- The approach has found particular use in forensic science, where traces of blood or other biological fluids from suspect, victim, or perpetrator can be analyzed and compared according to the composition of certain isoenzymes
- In this case, detection may be carried out by overlaying the corresponding substrate on the IEF gel