

Lecture 11: Isoelectric focusing (IEF)

We have studied in previous lectures that proteins have ionizable amino acids side chains. Ionization state of these side chains and N-terminal amino group and C-terminal carboxyl provides net charge to a protein at given pH. As pH changes ionization state of the amino acids side chains also changes (can be described by the Henderson-Hasselbalch equation). This results in change in overall charge on protein.

Proteins with different amino acid sequences have different amino acid side chains composition. Thus, at a given pH net charge may be different. In other words, the charge on protein depends on what kind of amino acids it has.

At acidic pH most proteins are positively charged while in alkaline pH negatively charged. However, in between pH net charge on protein can vary considerably. This variation is because of different protein sequence or difference in post-translational modification. pH where net charge on a given protein is zero, called isoelectric point of the protein. Isoelectric point is an important biophysical parameter.

If we do an electrophoresis in pH gradient (We shall also discuss how to make pH gradient during later part of the lecture) and protein sample (protein mixture) is loaded at pH 7.0:

All proteins with isoelectric point above pH 7.0 will have positive charge and move towards negative electrode. As they move towards negative electrode due to pH increase (due to pH gradient) net positive charge on protein would decrease. When protein reaches pH which equals isoelectric point of protein, net charge on the protein becomes zero and electrophoretic mobility becomes nil (protein remains confined near isoelectric point in the pH gradient).

All proteins with isoelectric point below 7.0 will have negative charge and move towards positive electrode. As they move towards positive electrode there is a decrease in the net negative charge on the protein due to pH decrease (due to pH gradient). When protein reaches pH which equals isoelectric point of protein, net charge on the protein becomes zero and electrophoretic mobility becomes nil (protein remains confined near isoelectric point in the pH gradient). Please refer to Fig. 1 for detail. This process of separating proteins based on isoelectric point is called isoelectric focusing.

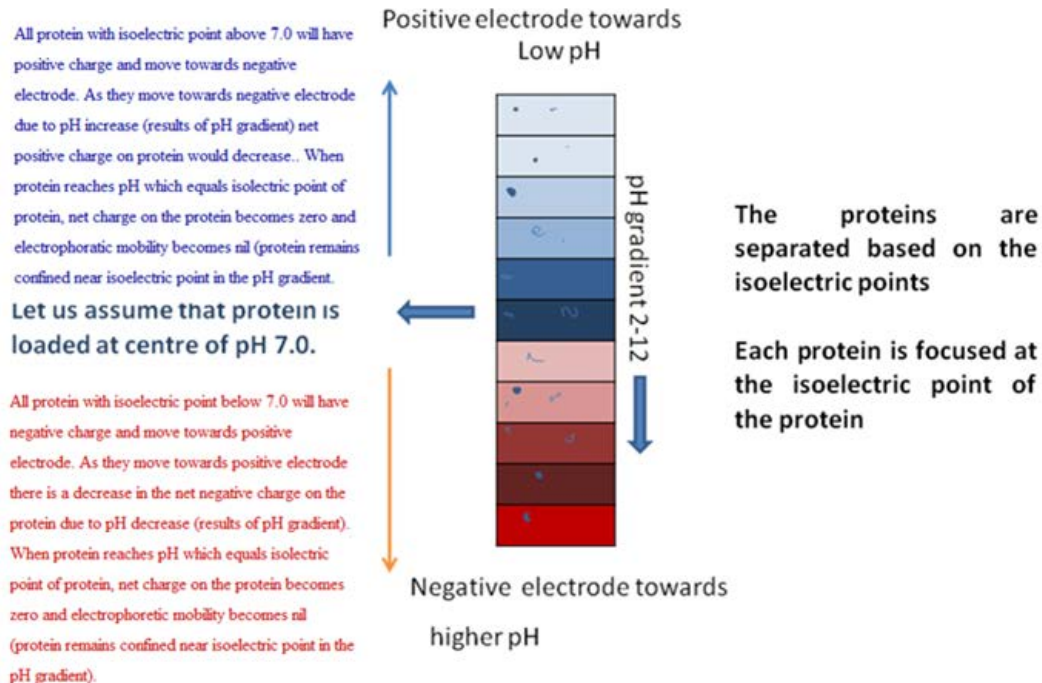
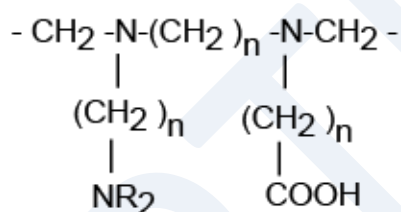


Figure 1: Process of separating proteins based on isoelectric point

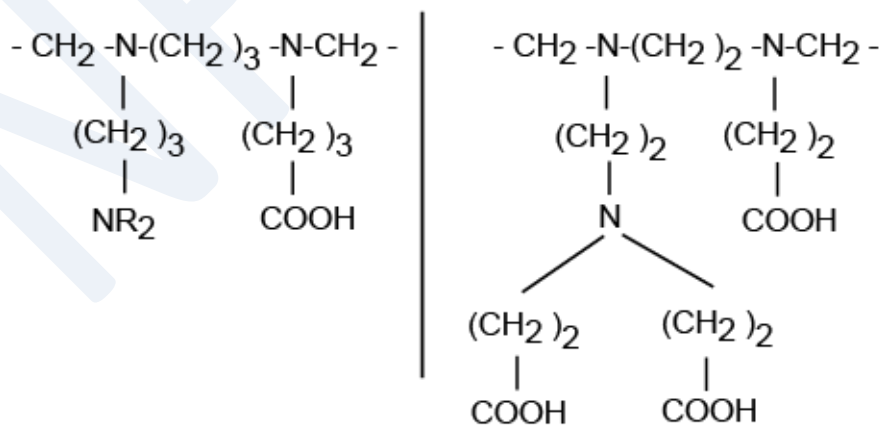
How pH gradient is generated?

It is technically difficult to generate a stable pH gradient by buffer system. A stable pH gradient is generated using Ampholytes. Ampholytes are synthetic, low molecular mass heteropolymer of oligoamino and oligocarboxylic acids (containing both acidic and basic groups). Using various combination of amino and carboxylic acid group, ampholytes with different isoelectric points are synthesized. Ampholytes have another property which makes them very useful. They have very high buffering capacity at their isoelectric point. Mixture of ampholytes with different isoelectric point ranges are commercially available (pH 7-12; pH 4.0-7.0 etc). General structure of ampholytes are shown in Fig. 2.

General structure of Ampholytes



For Example



[Various combination of amino and carboxylic group provides different isoelectric point]

Figure 2: General structure of ampholytes.

-Ampholytes of a given range (for example pH 2.0-12.0) is mixed with acrylamide/bis acrylamide while being polymerized (no SDS). If this polymerization is done at pH 8.8 and electrophoresis is performed, what happens?

Ampholyte molecules with maximum isoelectric point (above pH 8.8) will have maximum positive charge and move fastest and reach closest towards negative electrode. The ampholyte with second maximum isoelectric point will not move further because the space taken by ampholytes with higher isoelectric point. As ampholytes have high buffering capacity at isoelectric point a higher pH is maintained. As soon as it moves forward, it will experience a pH higher than its isoelectric point. Protein will get negative charge and move back (Fig. 3)

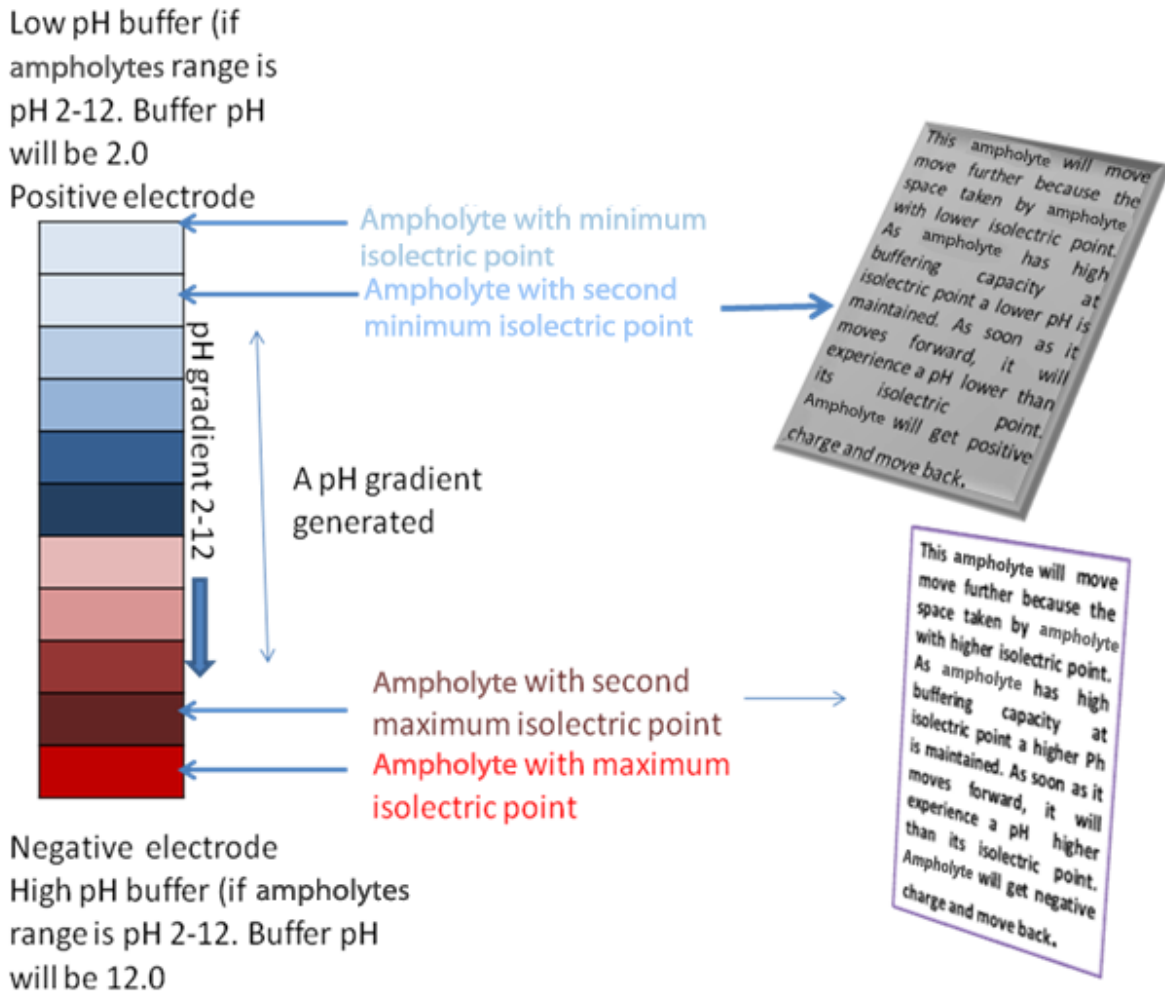


Figure 3: pH gradient using ampholytes

Similar, ampholytes molecule with minimum isoelectric point will have maximum negative charge (at pH 8.8) and move fastest and reach closest towards positive electrode. The ampholytes with second minimum isoelectric point will not move further because the space taken by ampholytes with lower isoelectric point. As ampholytes has high buffering capacity at isoelectric point a lower pH is maintained. As soon as it moves forward, it will experience a pH lower than its isoelectric point. Ampholytes will get positive charge and move back. This way a isoelectric point gradient is generated in solid support (like polyacrylamide). **As ampholytes have high buffering capacity at isoelectric point, the isoelectric point gradient effectively makes a pH gradient.** Now refer to our discussion earlier in this lecture about protein in pH gradient. What happens if we use pH gradient generated by ampholytes? Protein will get separated according to isoelectric point. As the pH gradient is in solid support, diffusion is minimized after electric field is stopped. Gel is sliced in small pieces and proteins with different isoelectric points are separated (Fig. 4)

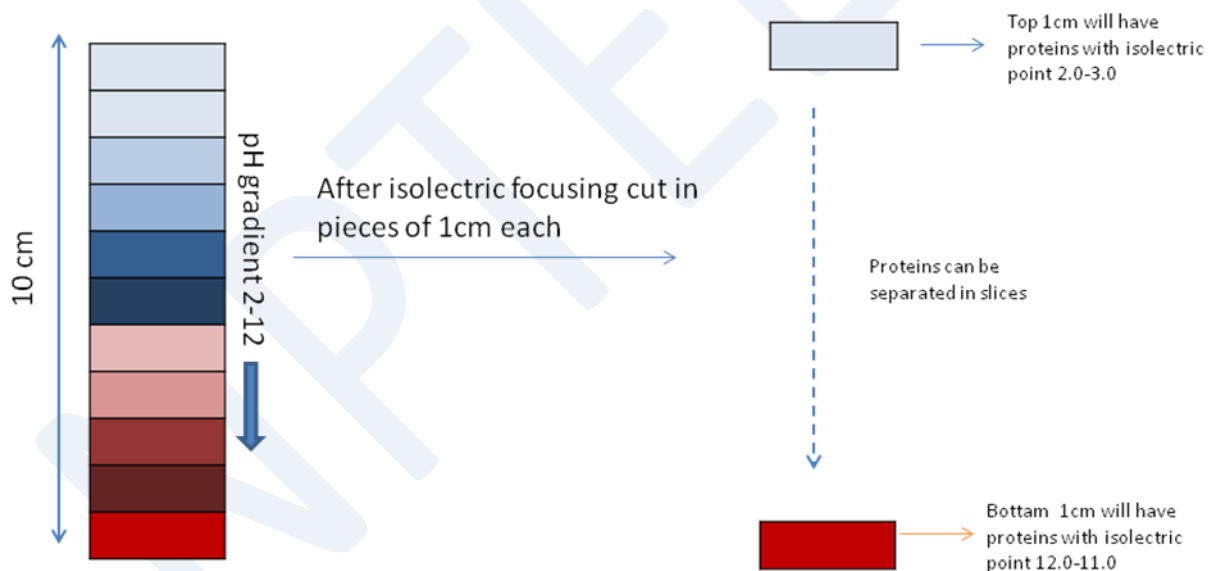


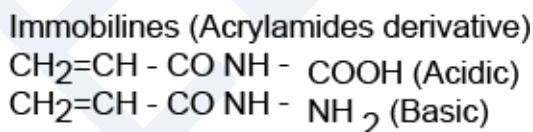
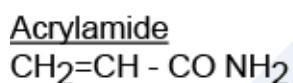
Figure 4: Separation of proteins in pH gradient

As shown in Fig. 4, each cm of gel contains proteins having isoelectric point differing in one unit. This may accommodate more protein per slice and effective purification may not be achieved in a protein mixture having many proteins.

By carefully choosing range of ampholytes better separation may be achieved. For example if we know the isoelectric point of desired protein, range of ampholytes can be narrowed. If we consider that the desired protein's isoelectric point is 5.0, we may use ampholytes of range 4-7 (3 unit pH difference) in 10 cm gel. This will result in 1cm slice accommodating proteins differing in 0.3unit (10cm/3unit pH difference) difference in isoelectric point.

Other less common method for generating pH gradient

Some times immobilines are also used to generate pH gradient. Immobilines are derivatives of acrylamide. It could be acidic or basic and polymerized (as we have seen in case of acrylamide). Solutions of acidic and basic immobilines are used to generate pH gradient as shown in Fig. 5. Once pH gradient is generated, the gel can be used for isoelectric focusing.



* Immobilines can also polymerized like acrylamide

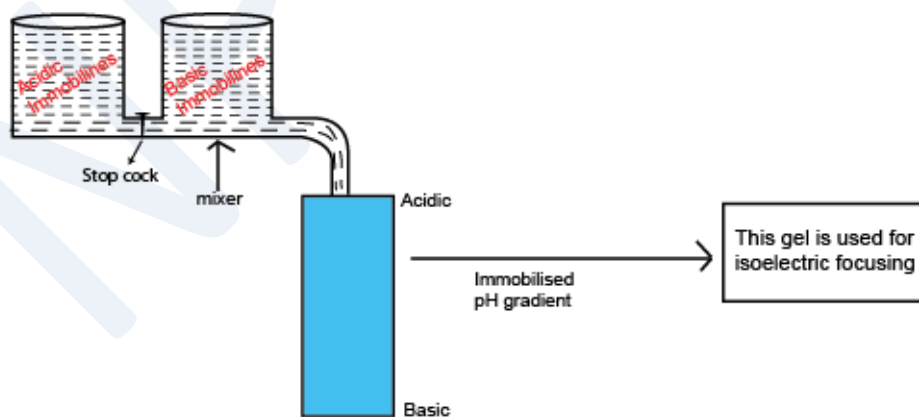


Figure 5: Preparation of pH gradient using immobilines