Isoelectric focusing

Isoelectric focusing (IEF) is one of the most commonly used techniques for the separation of proteins. IEF separations are based on the pH dependence of the electrophoretic mobilities of the protein molecules. Isoelectric focusing makes use of electrical charge properties of molecules to focus them in defined zones in a separation medium. It is the focusing mechanism that distinguishes IEF from other separation processes and makes it unique among the separation methods. In sharp contrast, the basic separation mechanism of IEF imposes forces on the molecules that directly counteract the dispersive effects of diffusion. During the separation process, the molecules in the sample accumulate in specific and predictable locations in the medium, regardless of their initial distribution. This focusing mechanism also distinguishes IEF from various modes of electrophoresis. It is important to note that IEF is a high-resolution method. It is well suited for both analytical and preparative applications.

I. SEPARATIONS BY IEF

IEF has simple operation and high-resolution output. In actual practice, IEF is easy to understand and to perform; however, a complete understanding of IEF requires a strong grasp of a number of physical chemistry principles, including acid-base titrations. This technique is applicable mainly to the fractionation of amphoteric molecules such as proteins and peptides that can act as both acids and bases. IEF is used mainly to separate proteins for analysis or purification. It measures the isoelectric points (pI) of proteins and uses the unique pI values of proteins to purify them. The pI of any particular protein is defined as the specific pH at which it carries no net electrical charge. Both analytical and preparative versions of IEF have been developed over the years. The basis for electrofocusing lies in the pH dependence of the charges on the constituent amino acid side chains, non-proteinaceous adducts, and prosthetic groups of proteins. By subjecting proteins to electrophoresis in pH gradients, they become focused on well-defined, sharp zones at pH values corresponding to their individual pI. Very subtle differences in the pI values of proteins can be detected with IEE Proteins differing in pI by less than 0.01 pH unit are routinely resolved by IEF, and separation of proteins with pI as low as 0.001 pH unit apart has been achieved. IEF works the principle on the property of individual protein molecule to stop movement at a certain pI under an electric field. But once the electric field is removed the molecules start to diffuse. IEF finds its application in proteomics. The basic of proteomics is a multi-dimensional separation of protein molecules. IEF becomes the first dimension of separation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis provides the second dimension of separation of proteins. Coupling IEF and SDS-PAGE in 2-DE affords biochemists and cell biologists a means for visualizing both the charge and size isomers of proteins in a single-gel entity. This is particularly relevant to charge isomers of proteins through differences in their pI values. It is also important for the study of post-translational modifications and their roles in protein function. Thus, IEF is and will remain an integral part of proteomic research and of protein biochemistry.

THEORY & SIMULATION OF IEF

IEF theory can be explained by a set of differential equations that could be solved analytically. Numerical simulations of IEF incorporate the dissociation rates of the components into the transport equations, generalize the discussion to multicomponent systems, and are not restricted to the steady state. The model successfully predicts pH, concentration and conductivity profiles as a function of time. The numerical models describe transient states in the formation of the steady-state distributions of ampholytes and proteins and allow mechanisms of instability to be studied. Slab Gel IEF is the most commonly used technique. Capillary IEF and chromatofocussing are variants of this method.

GENERATION OF pH GRADIENTS

IEF is a very useful practical technique where simple methods for establishing and maintaining pH gradients are available. The pH gradient is essential for this technique, and the nature of the pH gradient largely determines the quality and usefulness of the separation. The two most widely adopted methods for generating pH gradients make use of different types of synthetic buffering molecules (a) Carrier ampholytes are amphoteric electrolytes that carry both current and buffering capacity. They possess both acidic and basic functional groups and form pH gradients under the influence of electric fields. (b) Synthetic buffer compounds containing reactive double bonds, called acrylamide buffers, can be incorporated into polyacrylamide gel matrices. When used in the correct proportions, acryl- amido buffers generate immobilized pH gradients under the influence of electric fields.

PRACTICES AND PITFALLS OF SAMPLE PREPARATION

The key to successful performance of IEF and successful 2-DE lies in sample preparation. Proteins vary largely in properties.There is no universally applicable procedure for proteins. Classical sample preparation for IEF relies on non-ionic or zwitterionic reagents to disrupt protein complexes and denature proteins to ensure that the subsequent electrophoretic separations are carried out on polypeptide monomers. Since IEF separates proteins based on isoelectric point, SDS is not normally used. As SDS bonds strongly to proteins and often causes anomalous focusing and horizontal streaking in lEF. To approximate the denaturing power of boiling SDS under reducing conditions, IEF practitioners have relied on various cocktails of chaotropes, surfactants, and reducing agents. Chaotropes (such as urea) disrupt the hydrogen bonding at the protein surface and cause partial unfolding. When hydrogen in water bonds to the chaotropes instead of protein, the folded protein is more likely to open up and expose the (hydrophobic) interior. After the hydrophobic interior of the protein is exposed, the solubility is often compromised in aqueous solution. Therefore, it is desirable to have at least one surfactant present in the IEF cocktail to help solubilize the hydrophobic residues that are exposed as a result of denaturation in chaotropes.

PROTEIN DETECTION AND IMAGING

Gel-based IEF has a strong worldwide user base that supports a commercial pipeline of instrumentation as well as consumable products and will thus certainly remain a relatively low-cost, routine laboratory technique in the coming years. These techniques involve the use of many of the same dyes and stains that were developed for polyacrylamide electrophoresis gels but have been adapted for use with IEF gels.

EXPERIMENTAL PROTOCOLS: POLYACRYLAMIDES LAB GEL IEF

Carrier Ampholyte Polyacrylamide Gel IEF  
The standard protocol for slab gel IEF is presented here.  
Equipment  
Multiphor, Casting Cassette, GelBond PAG, Power Supply capable of delivering 3000V at >5 mA, Recirculating Chiller, Staining Tray, etc.

Gel Casting

(a) Stock Solutions

Carrier Ampholytes: Carrier ampholytes are usually supplied as 40% (w/v) solutions, although some narrow range carrier ampholytes are supplied at 20% (w/v).  
Acrylamide, Bis solution (T=40%, C= 3%): Dissolve 38.8g of acrylamide and 1.2 g of N,N’-methylene-bis-acrylamide, in 60-70mL of distilled, deionized water. Stir until all grains have dissolved, then filter the solution through a paper. When stored in a dark place at 4 C (refrigerator), the solution can be kept for 1 week.  
Ammonium Persulfate Solution 40% (w/v): Dissolve 400mg of ammonium persulfate in 1 ml of distilled water. This solution is stable for 1 week when stored in the refrigerator  
4~ TEMED (N,N,N’,N’-tetramethylethylenediamine) is not necessarily needed for polymerization. However, since there is some chemical difference of carrier ampholytes provided by different suppliers. TEMED is used to aid polymerization. The TEMED should be neat and not older than 1 year.

(b) Preparation of the Cassette

Pour a few milliliters of distilled water on the blank glass plate and place the support film (GelBond PAG Film) with the hydrophobic side on the water puddle. Move the film until the short edges are flush with the short edges of the glass plate and one of the long edges protrudes over a long edge of the glass plate by 1 mm. Press the film down on the glass plate with a roller. Place a U-shaped gasket cut from a 0.5-mm-thick silicone rubber on the film and put another glass plate on top of it. The upper glass plate should be treated once with RepelSilane TM to allow easy removal of the plate from the soft gel surface after polymerization. When the sandwich is clamped, it forms a cassette. Tilt the cassette vertically for filling.

(c) Polymerization

Prepare fresh monomer solution according to the recipe (Acrylamide Bis solution (40%T, 3%C) -1.9 mL, Monoethylenglycol (100%) -1.5 mL, Carrier ampholytes pH 3-10 (40% w/v) 750 mL, TEMED (100 % ) 8 mL, Distilled water 10.8 mL). Thoroughly mix the solution and deaerate it with a vacuum pump for 5 min to remove oxygen. Add 15 mL of ammonium persulfate solution and mix the monomer solution thoroughly, but carefully, without creating bubbles. Immediately pipette the monomer solution into the cassette. Overlay the upper edge of the gel with a few hundred microliters of distilled water to prevent oxygen diffusion into the upper gel. The solution should be allowed to polymerize overnight before the gel is used. The gel can be wrapped in a plastic foil and stored in a refrigerator for several weeks.

Sample Preparation

For Coomassie Brilliant Blue staining, adjust the protein concentration to around 1-3 mg/mL with distilled water. The salt concentration should not exceed 50mM; apply 10-20 mL to the gel. Also, apply 10mL of pI marker proteins (pH 3-10) to at least two lanes.

Isoelectric Focusing

Set the temperature of the thermostatic circulator to 10 Pipette 3 ml of kerosene on the cooling plate. Remove the gel from the cassette and place it on the cooling plate with the gel facing upward. The kerosene should distribute uniformly under the gel’s support foil.

(a) Electrode Strips

Soak one electrode strip with 0.5 M phosphoric acid and place it on the anodal edge of the gel layer. Soak a second electrode strip with 0.5 M sodium hydroxide and place it on the cathodal edge. Blot excess liquid from the electrode strips with filter paper before applying them to the gel.

(b) Focusing

The power supply settings are separated into four zones of operation. Prefocussing has these following settings :runtime (20 minutes), maximum voltage (700 V), maximal current (20 mA), maximal power (10 W), sample entrance is done in the following settings: runtime (30 minutes), maximum voltage (500 V), maximal current (20 mA), maximal power (10 W). The next step is separation and its running conditions: runtime (90 minutes), maximum voltage (2000 V), maximal current (20 mA), maximal power (10 W). As the final step band focusing is done: runtime (10 minutes), maximum voltage (2500 V), maximal current (5 mA), maximal power (15 W).

Protein Detection by Colloidal Coomassie Brilliant Blue Staining

The following colloidal Coomassie Brilliant Blue staining procedure is the most useful for IEF slab gels, as explained above: Dissolve 2g of Coomassie Brilliant Blue G-250 in 1 L of distilled water Add 1 L of 1 M sulfuric acid (1 M; 55.5 mL of concentrated H2SO 4 per liter) while stirring. After further stirring for 3 h, filter the solution through a paper, and then add 220 mL of 10 M sodium hydroxide (10 M; 88 g NaOH in 220 mL) to the brown filtrate. Finally, add 310mL of 100% (w/v) trichloroacetic acid and mix well. The solution will turn green. Colloidal sols of Coomassie Brilliant Blue G-250 are commercially available for protein staining. Fixing and staining are performed in one step: 3 h at 50 deg. Celsius or overnight at room temperature in the colloidal sol. Later the acid is washed out by soaking the gel in water for 1-2h. The green bands become blue and more intense as the water drives the dye molecules into the proteins.

Immobilized pH Gradient IEF

Equipment

Multiphor, Casting Cassette, Gradient Maker, GelBond PAG, Power Supply capable of delivering >3000 V with the minimum current safety switch turned off, Recirculating Chiller, Staining Tray.

Gel Casting

Preparation of immobilized pH gradient gels is much more complicated and prone to errors than making laboratory-cast carrier-ampholyte IEF gels. Only a small selection of ready-made IPG gel slabs is commercially available. Therefore, the entire procedure is described here.

(a) Stock Solutions

Immobiline – 0.2 molar stock solutions:  
Acids: pK-3.6, 4.6.  
Bases: pK-6.2, 7.0, 8.5, and 9.3.  
The solutions are stabilized against autopolymerization and hydrolysis and have a shelf life of at least 12 months when stored in the refrigerator (4 to 8 deg. Celsius).  
Acrylamide, Bis solution (T=40%, C=3%): Dissolve 38.8g of acrylamide and 1.2 g of N,N’-methylene-bis-acrylamide, in 60-70 mL of distilled, deionized water. Stir until all grains have dissolved, then filter the solution through a paper. When stored in a dark place at 4 deg. Celsius (refrigerator) the solution can be kept for 1 week. This solution is commercially available from several suppliers. Caution! Acrylamide and N,N’-methylene-bis-acrylamide are toxic in the monomer form. Avoid skin contact and do not pipette by mouth.  
Ammonium persulfate solution 40% (w/v): Dissolve 400mg of ammonium persulfate in I mL of distilled water. This solution is stable for one week when stored in the refrigerator (4 deg. Celsius).  
TEMED (N,N,N’,N’-tetramethylethylenediamine) (100%): TEMED should not be older than 1 year and should be kept in the refrigerator.  
4 M HCl: Dissolve 33.0 mL of concentrated HC1 in 67.0mL of distilled water.

(b) Preparation of the Cassette

Pour a few milliliters of distilled water on the blank glass plate and place the support film (GelBond PAG Film) with the hydrophobic side on the water puddle. Move the film until the short edges are flush with the short edges of the glass plate and one of the long edges protrudes over a long edge of the glass plate by l mm. Press the film down on the glass plate with a roller. Place a U-shaped gasket cut from a 0.5-mm-thick silicone rubber on the film and put another glass plate on top of it. The upper glass plate should be treated once with RepelSilane TM to allow easy removal of the plate from the soft gel surface after polymerization. When the sandwich is clamped, it forms a cassette. Chill the cassette in a refrigerator in order to delay the start of polymerization. This measure is taken to ensure that the density gradient has settled before polymerization begins. Note that settling of a gradient in a 0.5-mm-thin cassette is much slower than for an l mm cassette.

(c) Casting a pH Gradient Gel and Polymerization

Immobilized pH gradients are cast in a similar way as porosity or additive gradient gels. Two monomer solution is prepared. They are dense and light solution. Dense solution is prepared in the following composition: Immobiline pK 3.6 – 551 mL, Immobiline pK 6.2 – 227 mL, Immobiline pK 7.0 – 45 mL, Immobiline pK 8.5 – 167 mL, Acrylamide Bis solution (40% T, 3% C) -750 mL, Glycerol (87 %) – 2.2 mL, TEMED (100 %) – 4 mL. The above solution is filled with distilled water to make a solution of 7.5 mL. Ammonium Persulfate 8 mL is added before adding into the cascade. Light solution is prepared in the following composition: Immobiline pK 4.6 – 57 mL, Immobiline pK 6.2 – 25 mL, Immobiline pK 7.0 – 244 mL, Immobiline pK 8.5 – 79 mL, Immobiline pK 9.3 – 179 mL, Acrylamide Bis solution (40% T, 3% C) -750 mL, Glycerol (87 %) – 400 mL, TEMED (100 %) – 4 mL. The above solution is filled with distilled water to make a solution of 7.5 mL. Titrate the solution to pH 7.0 using 4.0 mol HCl solution and TEMED solution. Ammonium Persulfate 8 mL is added before adding into the cascade. The pH gradient is stabilized by a glycerol density gradient. The gradient maker consists of two communicating chambers. First the light, basic, solution is pipetted into the rear cylinder, the channel between the cylinders is opened very briefly and immediately closed again to fill up the channel with light solution, thus avoiding an air bubble barrier between the two solutions. The dense, acidic solution and a stirrer bar are placed in the front cylinder, the mixing chamber. A compensation bar is placed into the rear cylinder, the reservoir, to balance the volume of the magnetic stirrer and the difference in density: 25% glycerol is added to the dense solution and 5% to the light one so that it is easier to overlay the gel solution in the cassette with water before polymerization. The casting cassette is removed from the refrigerator and placed close to the gradient maker, with the glass plate holding the film facing the operator. This allows a better control of the tip of the tubing, which is inserted into the center of the cassette between the upper edges. At this time, the ammonium persulfate is added and mixed first with the dense solution by briefly turning on the magnetic stirring motor, and second with the light solution using the compensation bar. The magnetic stirrer motor is turned on and adjusted to a speed producing a small vortex is obtained. Fast rotation must be avoided in order to prevent the development of air bubbles. The channel between the chambers is opened and the clamp at the front tubing is released. The gel solution will flow into the cassette through the tubing under the influence of gravity. Overlay the upper edge of the gel with a few hundred microliters of distilled water to prevent oxygen diffusion into the upper gel. Do not use alcohol-containing overlay solutions. The gel is allowed to polymerize for 2 h at room temperature. At first, the gradient will not be straight. It takes about 10min for the gradient to settle completely.

(d) Gel Washing, Drying, and Rehydratation

Remove the gel from the cassette and wash it four times for 15 min, each in 0.5 L of distilled water, on a shaker. In order to avoid curling of the drying gel, incubate it for another 15 min in 1.5% (v/v) glycerol in distilled water. Dry the gel at room temperature in a dust-free cabinet. When the gel is dry, immediately cover it with an inert plastic film and store it in a plastic bag in a freezer. Rehydratation can either be performed in a vertical cassette or in a reswelling tray. The vertical rehydration cassette also allows rehydration with a urea gradient perpendicular to the pH gradient. The gel casting cassette can also be used for rehydration. When the gel is just rehydrated in distilled water, the matrix is completely reconstituted.

Sample Preparation

For Coomassie Brilliant Blue staining, adjust the protein concentration to around 1-3 mg/mL with distilled water. The salt concentration should not exceed 50 mM. Apply 10-20 mL to the gel. Apply 10 mL of pI marker proteins (pH 3-10) to at least two lanes.

Isoelectric Focusing

Set the temperature of the thermostatic circulator to 10 deg. Celsius. Pipette 3 mL of kerosene on the cooling plate. Place the gel on the cooling plate with the gel facing upward, and with the acidic side at the anode. The kerosene should distribute uniformly under the gel’s support foil. Usually, no electrode strips are needed.  
(a) Sample Application: For focusing in IPG gels, the sample must be applied without prefocusing in order to use the initial current to transport the sample proteins into the gel. All sample application modes shown in Figure 10 can be used. Additionally, in IPG gel IEF, holes can be punched into the gel because the gradient is fixed. Also, in IPG gels, the position of the optimal sample application point is dependent on the kind of sample and should be selected with the help of a reference in the literature or laboratory manual, or must be determined with a step trial test.  
(b) Focusing: For IPG gels, 10 deg. Celsius is the optimal temperature. When 8M urea has been added to the rehydration solution, 20 deg. Celsius is chosen. Because the pH gradient is already established in the gel, the power supply settings are very simple: one phase with the maximum set to 3500 V, 1.0 mA, and 5.0 W. The minimum separation time is dependent on the pH gradient. For non-denaturing IEF it is suggested to use 5 h; even in narrow-interval pH gradients, all proteins will have focused by this time. Because the gradient cannot drift, the pattern remains stable