

Protein Analysis and DNA Polymorphism

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Introduction

Each fish species is chemically composed of different proteins at varying levels, so techniques that separate proteins may help to identify different species. Of these techniques, electrophoresis is the most important one. Many biological molecules such as proteins are made up of amino acids with electrically charged side chains. Basic amino acids such as arginine, histidine and lysine are positively charged while the acidic amino acids such as aspartic acid and glutamic acid carry negative charges. Thus, virtually all proteins have a net charge depending on the relative proportions of amino acids, unless they are at their “iso-electric point” (pI), the definite pH at which the net charge of the protein molecule is zero. The basis of electrophoretic separation is that proteins of different net charge and different molecular size will migrate at different rates within an electric field and it is a very useful technique for the separation of cellular proteins and DNA.

The term electrophoresis comes from the Greek, and means, “transport by electricity” and has been known since the end of 19th century. In 1807, a Russian Physicist, Alexander Reuss observed a novel phenomenon - when electricity was passed through a glass tube containing water and clay, colloidal particles moved towards the positive electrode. The term electrophoresis

describes the migration of a charged particle under the influence of an electric field. Charged molecules are having either positive or negative charge. At a given pH, the biological molecules exist in solution as electrically charged particles. Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The theory of movement of a particle in electrophoresis is as follows: When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, E , which is the applied voltage (V), divided by the distance (d), between the electrodes. The force that drives a charged molecule towards an electrode is the product of potential gradient, and the charge of q coulombs on the particle. However, the frictional force that retards the movement of a charged molecule is function of hydro-dynamic size of the molecule, shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer.

The velocity (v) of charged molecule in an electric field-

$$v = \frac{Eq}{F}$$

F

where, F = frictional coefficient, which depends upon the mass and shape of the molecule; E = electric field (V/ cm); q = the net charge on molecule; and v = velocity of the molecule.

Most of the large molecules possess both anionic (basic positively charged) and cationic (acidic-negatively charged) groupings as part of their structure and hence are termed as “amphoteric molecules” or “Zwitterions”. The actual charge of protein molecule is the result of the sum of all single charges. Because dissociation of the different acidic and basic groups takes place at different hydrogen ion concentrations of the medium, pH greatly influences the total charge of the molecule. At lower pH, they migrate to the negative pole (cathode) and at higher pH to the positive pole (anode). Ionic strength also affects the migration, low ionic strength permits high rate of migration. The choice of buffer strength may be seen to be crucial, since it determines the amount of electrical power that can be applied to the system. The rate of migration will also depend upon the charge density (the ratio of charge to mass) of the proteins concerned; the higher the ratio of charge to mass, the faster the molecule will migrate.

Gel Electrophoresis

In this type of electrophoresis, gel is used as the support media instead of thin paper sheets etc. Most electrophoresis is carried out on the inert media.

Types of gel electrophoresis

I. Based on Buffer System

II. Based on Support media

Buffer System

Continuous buffer System

Continuous buffer electrophoresis is the simplest and most commonly used method. The same buffer is used to provide electrical contact between the platinum electrodes and the support medium and to soak the gel. In the gradient pore method an acrylamide gel is prepared other. Protein solutions are applied at the end of the gel where pore size is largest and allowed to migrate in an electrical field until they reach the point where pore size prevents further movement. Separation is purely on the basis of molecular size and electrical charge is used only to induce movement.

Discontinuous buffer System

In discontinuous buffer or multiphasic electrophoresis, the electrode chambers contain a different buffer to that in the gel. The front at which the two buffers meet concentrates different proteins so that they enter the main electrophoresis system as a very narrow zone. This will enhance the resolution.

Supporting Media

A variety of anti-convection media are being used in zone electrophoresis. They exhibit several properties. An ideal medium should have the following features: -

Chemical nature inert

Availability easy

Electrical conductivity high

Adsorptivity low

Sieving effect desirable

Porosity controlled

Transparency high

Electro-endosmosis (EEO) low

Rigidity moderate to high

Preservation feasible

Toxicity low

Preparation easy

The original material, filter paper, has now been replaced by a variety of gels. Cellulose acetate and agar have large pore size and are used for separation of large protein molecules and for immunodiffusion. Starch and polyacrylamide have a pore size to the molecular size of many proteins, so there is also a molecular sieving effect. The pore size of acrylamide gels is adjustable. Protein resolution depends on the pH and ionic strength of the buffer, the pore size of the gel and the current applied. Buffer pH is usually 8 to 9 ensuring that all proteins are negatively charged and migrate to anode. Heat is produced in proportion to the product of Volt x Ampere so temperature must be controlled by using cooling devices. The details of various media are discussed below.

Starch Gel Electrophoresis

In this type of electrophoresis, starch is used as the support media. The molecular sieving properties of starch make it good choice for the separation of complex mixtures of structural molecules and physiologically active proteins. Starch gel is prepared by mixing hydrolyzed potato starch with an electrolyte buffer, cooking until a gel of uniform consistency is achieved, followed by pouring the gel into a mold. The ends of the longer section of the gel are placed gently on the absorbent towels or filter paper in the lower (anodal) buffer tank. Each tank is approximately one-third filled with chilled electrode buffer.

Voltage, current and duration of the electrophoresis and other requirements vary widely among buffer systems for particular electrophoretic separations. Gels should be run in the cold chambers to produce sharp and straight lines of migration and to minimize heat production in overnight conditions usually requiring a constant 200V and variable current of not more than 30mA. An important application of starch gel electrophoresis is the analysis of allozyme pattern.

Poly Acrylamide Gel Electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis

Acrylamide monomer ($\text{CH}_2 = \text{CH} \text{CO} \text{NH}_2$) is co-polymerised with a cross linking agent, usually N,N'-methylene bisacrylamide $\{\text{CH}_2 (\text{NH} \text{CO} \text{CH} = \text{CH}_2)_2\}$, in the presence of a catalyst accelerator chain initiator mixture. This mixture consists of freshly prepared ammonium persulfate as (0.1 to 0.3% w/v) initiator together with about the same concentration of a suitable base, for example, dimethyl aminopropionitrile (DMAP) or N, N, N', N' - tetra methylenediamine (TEMED) as catalyst. Out of the two, the most used is TEMED and proportional increase in its concentration speeds up the rate of gel polymerization. Gelation occurs due to vinyl polymerization. Prior degassing of solution is required since molecular oxygen inhibits chemical polymerization. The relative proportion of acrylamide monomer to cross-linking agent determines the porosity of a gel. Gels may be defined in terms of the total percentage of acrylamide present. Gels may be prepared containing from 3% to 30% acrylamide, corresponding to pore sizes of 0.5nm and 0.2nm, diameter respectively. Polyacrylamide gels may be prepared with a high degree of reproducibility and the precise porosity. This feature makes the method particularly suitable for resolving mixtures of proteins. This feature makes the method particularly suitable for resolving mixtures of proteins. Other features of polyacrylamide

gels include their minimal absorption capacity, their lack of electro-endosmosis and their general suitability for *in situ* quantitative analysis (as they do not absorb UV) and for various types of histo-chemical analysis. For running of the polyacrylamide gels, the gel slab (earlier gel rods made in glass tubes of uniform diameter were used) is loaded with the sample and attached to the lower submarine unit, filled with the buffer and connected to the power pack for the DC supply.

Denaturing Polyacrylamide gel electrophoresis

In the electrophoretic technique previously discussed, the mobility of biological molecules is influenced by both charge and size. But, if protein samples are treated with certain chemicals so that they have a uniform charge, the electrophoretic mobility then depends primarily on size. The molecular weights of proteins may be estimated if they are subjected to electrophoresis in presence of detergent, SDS and disulfide reducing agent mercaptoethanol. When protein molecules are treated with SDS, the detergent disrupts the secondary, tertiary and quaternary structure, leaving the molecule to produce polypeptide chain in a random coil, imparting an overall negative charge and masking the individual variation in charge. The presence of mercaptoethanol assists in protein denature by reducing all disulfide bonds. In essence, polypeptide chains of constant charge/mass ratio and uniform shape are produced. The electrophoretic mobility of the SDS-protein complexes will be influenced primarily by molecular size; the

larger molecules will be retarded by the molecular sieving effect of the gel, while the smaller molecules will have greater mobility.

In practice, a protein of unknown molecule weight and structure is treated with 1% SDS and 0.1 mercaptoethanol in electrophoresis buffer. A standard mixture of proteins with known molecular weights must also be subjected to electrophoresis under the same conditions. After electrophoresis followed by staining, the molecular weight may be determined.

Agarose Electrophoresis

The electrophoretic technique used to characterize DNA and RNA is through agarose gels. The mobility of nucleic acid in agarose gels is influenced by agarose concentration and the size and shape of the nucleic acid. Agarose concentration of 0.5 to 3% is most effective for nucleic acid separations. Gels with agarose concentration less than 0.5% are rather fragile and must be used in a horizontal slab arrangement or in a refrigerated chamber. Like proteins, nucleic acids migrate at a rate that is inversely proportional to the logarithm of their molecular weights; hence molecular weight can be estimated from electrophoretic results using standard nucleic acids of known molecular weight. Passage of a molecule through a gel is influenced by the shape and size of the molecule. A small, compact molecule would be expected to have a greater mobility than rod like, linear molecules.

Most agarose gel electrophoresis experiments are carried out with horizontal slab gels. This method is chosen over vertical mode because low agarose concentration can be used for maximum mobility.

Isoelectric Focusing (IEF)

Isoelectric focusing uses a polyacrylamide gel with large pore size containing a mixture of polyamino, poly carboxylic acids with different isoelectric point (pI) s. These form a stable Ph

gradient along the gel in an electric field. Strong acid applied at the anode and strong base in the cathode contain and stabilize the gradient. Proteins migrate under the influence of their charge until they reach the point in the gel where the pH is equivalent to their isoelectric point and so their charge is neutralized. At the isoelectric point, proteins in the electrical field do not migrate to either of the poles. High resolutions are achieved permitting separation of proteins differing only by 0.01 pI.

Sources and Extraction of Proteins

For electrophoretic separation, proteins must be in solution. These can be body fluids such as plasma, serum, milt, haemolymph or aqueous extracts of tissue proteins. Extracts are often made from muscle, liver, eyes, etc in distilled water, 10% sucrose solution or specific extraction buffer. Crustaceans can be sampled non-lethally by removing a walking leg. With larvae or other small organisms, it is necessary to use the whole animal. The pH and ionic concentration of the buffer used, permits differential extraction of proteins.

After removal from the animal, proteins begin to denature rapidly and so the tissue must be used immediately or stored deep-frozen. Storage at -196°C in liquid nitrogen or at -80°C in ultra low freezers retains 90% of initial activity indefinitely and at -40°C most tissues will remain usable for a year or more, but at -18°C denaturation is relatively rapid and will produce altered electrophoretic pattern.

Sarcoplasmic proteins (water-soluble proteins)

The soluble proteins of the sarcoplasm, located within the sarcolemma are referred to as sarcoplasmic proteins. Among them, some albumins and so called myogens; to which belong most of the glycolytic enzymes are the real water-soluble proteins. (The other fractions of sarcoplasmic proteins are soluble in low salt concentrations). The genetic differences between species are more pronounced in this than in other group of proteins, as they are responsible for widely divergent enzymatic transformations in the muscle cell. Hence, the separation patterns of profiles obtained on electrophoresis or isoelectric focusing (IEF) can be used for the unequivocal identification of the species.

Myofibrillar proteins (salt-soluble proteins)

They are salt soluble proteins present in the myofibrils of the muscle fibre. Of the different myofibril proteins, myosin and tropomyosin find application in fish species identification by electrophoresis. Fish myosin, similar to myosin of other vertebrates, is a hexameric protein consisting of two identical heavy chains and four light chains, of which two of them are identical. Electrophoretic pattern of heavy chains from different species are similar whereas that of the light chains is different for different species. Hence, an electropherogram of myosin light chain isolated from fish muscle is used for species identification. Electrophoresis of most of the fish muscle tropomyosin gives a single band whose electrophoretic mobility is different for different species. Tropomyosin is a heat stable protein that can be extracted from heat-treated fish products, thus useful in identifying the species of fish of the product by studying the SDS-electrophoretic pattern of tropomyosin.

Eye-lens Proteins

The soluble proteins of the eye-lens have great value in taxonomic studies, because they are synthesized only one cell type present in the eye as a single layer. Three saline soluble eye lens proteins are distinguishable by electrophoretic and immunological techniques. There are alpha, beta and gamma crystallines in order of decreasing electrophoretic mobilities, each of which constitutes a family of similar, but no identical proteins. Protein with alpha-crystallin characteristics have been found in all vertebrate species and regarded as a classical organ-specific protein. The beta- and gamma- crystallin patterns are species-specific and can be used to resolve taxonomic disputes using ultra-thin IEF technique.

Allozymes

Isozymes are functionally similar and separable forms of enzymes encoded by one or more loci. Isozyme products of different alleles at the same locus are termed as “**allozymes**”. The most important quality of allozyme data is the codominant nature of inheritance of gene products and thus genetic interpretation (genotype) of the phenotype is facilitated because all products are normally visible and not masked by dominance of one over another. Other advantages include function of most of the proteins are known and, extensive database is available for many fish species. Allozyme electrophoresis has been used in defining genetic markers for stock identification on the basis of differences in allelic frequencies between stocks in many species. Using allozyme markers, it is possible to determine whether a population is a random mating one with equilibrium genotypes frequencies or sample comprises of an assembly of genetically distinct units. Their allele frequencies primarily respond to mutation, gene flow and drift. One of the limitations of enzyme variants as genetic markers is the low level polymorphism observed in some species and populations. The extensive allozymes studies undertaken on fish stocks have not only proven valuable for estimating population divergence, but also have focused attention on the underlying evolutionary forces that promote differentiation.