**3.4. Biochemical genetic markers: Principles and types**

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 = Eq

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**

**I. Based on 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.

**II. Based on Support media**

**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.

**Polyacrylamide gel electrophoresis (PAGE):**

**Native polyacrylamide gel electrophoresis:** Acrylamide monomer (CH2= CH CO NH2) is co-polymerised with a cross linking agent, usually N.N’- methylene bisacrylamide {CH2 (NH CO CH = CH2)2}, in the presence of a catalyst accelerator chain initiator mixture. This mixture consists of freshly prepared ammonium per sulfate 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.

**Cellulose acetate gel electrophoresis:**

In this type of electrophoresis, protein migration essentially takes place in the buffer film on the gel surface. The medium has therefore no influence on the electrophoretic 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.

**Two - dimensional (2D) electrophoresis:**

The techniques of isoelectric focusing and polyacrylamide gel electrophoresis have been combined to produce two-dimensional separation of proteins. This technique is increasingly used now a days and its great resolving power is due to the use of two independent properties of proteins. The proteins are first separated by isoelectric focusing (this is the first dimension), which separates proteins according to their charge (isoelectric point). The proteins are subsequently separated by SDS-PAG electrophoresis (this is the second dimension) at right angles, which separates proteins according to their size (molecular weight). This technique results in a series of spots distributed throughout the polyacrylamide gel.

**Source of current:**

The source of DC is a simple battery. However, for prolonged and constant supply, alternating current (AC) after rectification to DC is employed. Unlike in DC, the electron flow in AC is not unidirectional. But this is rectified by equipment called electrophoresis power supply. These equipments are made to supply constant voltage, current or power.

**Constant Voltage**: Almost all power supplies provide constant voltage. Voltage gradient of 15/cm is generally set for electrophoresis at room temperature (250C). But, when higher voltage is employed, heat generation is unavoidable. Hence for all types of electrophoresis using agarose gel, which is heat labile, a constant low voltage is given. Increased resistance during the run is reflected in the decrease of mA.

**Constant Current:** This provision is available in imported power supplies, and is generally required for Disc-PAGE to generate localized voltage gradient. Up to 5 mA/gel rod and 25 mA/gel slab is provided. Voltage and temperature rise during electrophoresis can be lowered by buffer circulation through a coolant.

**Buffers:**

It is a solution of a weak acid and one of its salts. It resists changes in H+ and OH- ion concentrations and maintains constant pH. Each buffer has its own ‘buffering capacity’ (the rate of change of pH to the number of equivalents of acid or base added). The following are the commonly employed buffers and their pH values with regard to electrophoresis.

**Buffer   pH value**

Phosphate buffer around 7.0

Tris-Borate-EDTA buffer (TBE) around 8.0

Tris-Acetate EDTA buffer (TAE) above 8.0

Tris Glycine buffer (TG) more than 8.5

Tris -Citrate-EDTA buffer (TCE) around 7.0

Tris -EDTA buffer (TE) around 8.0

Tris -Maleic acid -EDTA buffer (TME) around 7.5

Lithium Borate - buffer (LB) around 8.6

**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 –196oC in liquid nitrogen or at –80oC in ultra low freezers retains 90% of initial activity indefinitely and at –40oC most tissues will remain usable for a year or more, but at –18oC 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 f1sh stocks have not only proven valuable for estimating population divergence, but also have focused attention on the underlying evolutionary forces that promote differentiation.

**Staining Systems:** Once a gel has been run, it is necessary to identify the positions of the protein products of a particular locus. Most proteins are colourless and way to detect the separated bands is by staining them like histological specimens using the histochemical stains developed by histologists. For example, to detect all forms of protein, the technique is to first denature them and ‘fix’ them in position in the gel by fixation with an acid. They are then stained and, finally, excess stain washed from the gel, much as a tissue specimen is processed for microscopic examination. The pattern of stained bands is called ‘zymogram’. Proteins are usually stained with wool dyes, such as Ponceau red S, Amido black 10 B, Kenacid blue or Coomassie Brilliant Blue R. Other stains may also be used, as in histology, to locate a general class of proteins, *e.g.* the alcian blue stain for acid mucopolysaccharides and Oil red O for lipoproteins.

There are major shortcomings in the use of such non-specific protein stains. Normally a large number of zones are stained and, without functional information for each zone in each sample, the relationships between the zones are difficult to determine. As a consequence, the number of loci and alleles involved in the patterns observed can rarely be determined. An alternative approach is to locate areas of the gel containing specific proteins by using specific histochemical staining techniques in which some functional property of the protein is used to detect its presence. These techniques vary widely, being limited only by the ingenuity of research workers. For example transferrin, the iron-transporting protein of vertebrate serum, may be detected by adding radioactive iron to the sample before electrophoresis, and then identifying the location of the protein-bound radioactive iron after electrophoresis.

The most common staining techniques used in electrophoresis detect particular enzymes. Since each enzyme catalyses a specific reaction, any enzyme can be histochemically localized provided that either a substrate or, more commonly, a product involved in its reaction can be made visible (figure 1A and B). Coloured bands appear on the gel wherever product is formed by the action of the relevant enzyme. When the substrate is detected, the entire gel is coloured, except for areas where the substrate has been used up by the enzyme. Enzymes can also be detected by coupling the reaction so catalysed to one that forms a product, which can be detected. This is achieved by adding an appropriate *linking enzyme,* along with other necessary reagents,

to the staining mixture (figure 1C). The most frequently used technique is to identify reactions involving hydrogen ion transfers from the substrate to nicotinamide adenine dinucleotide- (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (i.e. to convert NAD to NADH or NADP to NADPH). These are called dehydrogenase reactions. Phenazine methosulphate (PMS) is then used to transfer the hydrogen ion from the NAD to one of the tetrazolium salts, usually methyl-thiazolyl blue (MTT) or nitro blue tetrazolium (NBT). The soluble, weakly yellow coloured, tetrazolium salts are reduced to insoluble blue formazan, which appears as a purplish zone on the gel wherever the reaction occurs.

Specific enzymes can be stained in this fashion. Particular dehydrogenases are detected by the addition of the correct substrates for the enzyme along with PMS and MTT (figure 1B). Enzymes that can be coupled to a dehydrogenase can also be stained by using the correct combination of substrates and linking enzymes (*e.g*. figure 1C).

Thus, electrophoresis, a ubiquitous biochemical method that allows separation as well as visualization of macromolecules. This may be considered as the core technique of all molecular based studies. In the filed of genetics, it may be utilized as the base technique in examining the genetic diversity of individuals/population, which in turn may help in establishing genetic relatedness between taxa to provide major role in conservation and management strategies.

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**Fig.1. Types of staining systems for enzymes.**

**A. The enzyme being stained for converts the substrate directly into a visible product.**

**B. The enzyme being stained for converts the substrate into a product that is not visible, but can be made visible by the addition of other histochemicals.**

**C. The enzyme being stained for converts the substrate into a product that is not visible but can be converted by a linking enzyme into a second product that can be made visible.**

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**Fig. 2. Horizontal gel electrophoresis**

