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METHODS IN BIOLOGY

CONTENT

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1. Molecular Biology and Recombinant DNA Methods 1

2. Histochemical and Immunotechniques 100

3. Biophysical Method 128

4. Statistical Methods 154

5. Radiolabeling techniques 197

6. Microscopic techniques 209

7. Electrophysiological methods 237

8. Methods in field biology 256

9. Computational Methods 281

Molecular Biology and Recombinant DNA Methods

Syllabus

• Isolation and Purification of Nucleic Acid (RNA and DNA)

• Analysis of RNA, DNA and Proteins, Gel Electrophoresis, Isoelectric Focusing.

• Molecular Cloning.

• Preparing CDNA Libraries, Plasmid Vectors, Phagemids, Cosmid and YACs.

• Expression of Recombinant Proteins, Isolation of Specific Nucleic Acid Sequences, Detection of Post-translation Modification of Proteins, DNA Sequencing, Site-Directed in vitro Mutagenesis, Gene Knock Out, Microarray.

• Isolation, Separation and Analysis of carbohydrates and lipids.

DNA technology is the chemical manipulation of the genotypes and resulting phenotypes of organisms such that living organisms are modified; alternatively, no-longer living organisms or their no-longer-living parts may be analyzed chemically at the level of genotype. The use of DNA technology has revolutionized how scientists study the genetics, biochemistry, even the ecology and evolutionary biology of organisms, plus has allowed the development of novel biological products, indeed whole industries are now devoted to DNA-technology based on production and analysis of biological materials. Together the manipulation and analysis associated with DNA technology, genetic engineering and biotechnology are based on a number of technologies generally referred to as molecular techniques. Molecular techniques include (though are not limited to):

(i) Gene cloning (which is associated with various molecular techniques including in vitro restriction enzyme digests and DNA ligation plus additional, less-artificial manipulations including transformation and transduction).

(ii) Creation of cDNA.

(iii) Polymerase chain reaction.

(iv) Gel electrophoresis.

(v) Various blotting techniques (Southern blotting, Northern blotting, Western blotting, etc.).

(vi) RFLP analysis.

(vii) DNA sequencing.

Isolation and Purification of Nucleic Acids (DNA and RNA)

Three major types of techniques or combinations of them, are employed in the isolation of nucleic acids: differential solubility, absorption methods or density gradient centrifugation. The choice of method will depend on the type OF DNA being isolated and the application. A major goal of nucleic acid isolation is the removal of proteins. The separation of nucleic acids from proteins is generally easily accomplished due to their different chemical properties. In particular, the highly charged phosphate backbone makes the nucleic acids rather hydrophilic as compared to proteins which are more hydrophobic. Separating the different types of nucleic acids can be more problematic in that they all have similar chemistries. On the other hand, though, this similar chemistry results in a few basic procedures which are common to many nucleic acid isolation protocols. Most nucleic acid isolation protocols involve a cell lysis step, enzymatic treatments, differential solubility (e.g., phenol extraction or absorption to a solid support) and precipitation.

Cell Lysis

Nucleic acids must be solubilized from cells or other biological material. This solubilization is usually carried out under denaturing conditions such as: SDS, alkali, boiling or chaotropic agents. These denaturing conditions efficiently solubilize the nucleic acids and generally do not adversely affect them. In addition, the denaturing conditions promote the removal of proteins during the subsequent steps and inhibit the activity of nucleases which will degrade the nucleic acids.

Enzymatic Treatment

Another approach in the isolation of nucleic acids is to degrade unwanted components. For example, inclusion of proteases (e.g., proteinase K) in the lysate will promote the removal of proteins. Proteinase K is still active at 55°C in the presence of 0.5% SDS. The elevated temperature and SDS improve solubility and inhibit any DNAse activity that may be present in the lysate. Nucleases can also be used to remove unwanted nucleic acids. For example, many DNA extraction protocols include a RNAse treatment step and vise-versa. It is important that the RNAse be free of DNAse activity. DNAse-free RNAse is easily prepared by boiling commercial RNAse for 10 minutes. The stability of

RNAse makes the preparation of RNAse-free DNAse more difficult. RNAse-free DNAse should be purchased from a reliable vendor or tested before it use.

Phenol Extraction

Phenol is an organic solvent that is used to separate proteins from nucleic acids. Proteins are hydrophobic and partition in the organic phase. Nucleic acids are highly charged and partition in the aqueous phase. The advantages are of phenol extraction are that it is easy to carry out and can be adapted to many applications. It is also easily applied over a wide range of volumes (40 uL to several mL). In particular, phenol extraction is widely used for the isolation of high molecular weight genomic DNA. Phenol extraction is accomplished by mixing the sample with an equal volume of phenol which has been previously saturated with a Tris buffer at pH 8 containing EDTA and NaCl. The phenol should be molecular biology grade phenol should and store at -20°C until preparing the saturated solution. The saturated solution is stored at 4°C. Phenol is easily oxidized, as evidenced by yellowing and the oxidation products can break DNA. Oxidized phenol should be discarded. Depending on the application, the two phases are completely mixed by vortexing or gently mixed (e.g., high molecular weight DNA). The phases are separated by centrifugation and the upper aqueous phase, which contains the nucleic acids, is retained. Proteins will often be visible as flocculent material at the top of the phenol phase. The two phases need to be carefully separated in that the nucleic acids and proteins tend to be at the interface. Leaving too much of the aqueous layer behind will lead to undue loss of material and aspirating too close to the interface can include protein. The aqueous phase can be re-extracted with phenol to remove more protein. Phenol is a hazardous waste material that needs to be disposed of properly.

A common variation of phenol extraction is a mixture of phenol:chloroform: isoamyl alcohol (25:24:1). The more organic chloroform removes lipids, denatures more protein and mixes less with the aqueous phase leading to more efficient extraction.

Ethanol Precipitation

Nucleic acids can be precipitated from dilute solutions with ethanol. This precipitation can be a concentration step or a means to change buffers, especially after phenol extraction. Typically either sodium acetate or potassium acetate, pH 5.0-5.5, is added to a final concentration of approximately 0.3 M. The sodium and acidic pH will neutralize the highly charged phosphate backbone and to promote hydrophobic interactions. Two-to-two and a half volumes of ethanol are added and the sample is incubated as -20°. If the nucleic acids are small in size and/or in low concentrations an extended incubation (several hours

to overnight) is needed. The precipitated DNA is collected by centrifugation. The pellet is rinsed with 70% ethanol to remove any excess salt, dried and dissolved in the appropriate buffer. A variation is to substitute ammonium acetate if the 'hard' salts are a problem. Another modification is to use an equal volume of isopropanol (instead of 2-242 volumes of ethanol) which minimizes the increase in sample volume.

Isolation of High Molecular Weight Genomic DNA

High molecular weight chromosomal DNA is usually isolated by multiple rounds of phenol extraction and enzyme treatments as discussed above. Shear forces, which can break long DNA molecules, need to be avoided during all steps and samples should never be vortexed. Therefore, the phenol extraction is carried with gentle rocking for several hours. These precautions against shear forces are not necessary in the isolation of loss molecular weight DNA. Another common modification at the ethanol precipitation step is 'spool out' the high molecular weight genomic DNA on the end of a sealed Pasteur pipet. The precipitated DNA is wrapped around the end of the pipet is then allowed to partially dry and then dissolved in the appropriate buffer. This minimizes the contamination with RNA and low molecular weight DNA fragments.

Plasmid Minipreps and Adsorption Methods

Historically, phenol extractions were used for the isolation of most forms of nucleic acids. It is now more common to use techniques based upon adsorption chromatography for the isolation of smaller DNA molecules, such as plasmids.

Various kits are available for the rapid isolation of small quantities of plasmid DNA. The procedure consists of Solubilizing the bacteria in an alkali solution followed by neutralization with sodium acetate. The neutralization results in the precipitation of some of the protein and the genomic DNA which is removed by centrifugation. The Soluble material is then mixed with a resin in the presence of chaotropic agents (usually guanidine hydrochloride). The resins are usually either based on silica or diatomaceous Earth. Under these conditions DNA binds to the matrix, but proteins and RNA do not. The DNA is eluted in a low salt buffer. These methods are rapid and yield a highly purified plasmid DNA which can generally be used directly in most applications without further processing.

Another common application for an adsorption method is the isolation of DNA fragments following gel electrophoresis. In this case, the agarose gel piece containing the DNA is dissolved in NaI, a chaotropic salt and the DNA adsorbed to silica. The DNA is then eluted with a low salt buffer and sometimes gentle heating.

Toppersnotes Unleash the topper in you

Isolation of RNA

Most RNA isolation protocols also involve phenol extractions and are similar to DNA isolations. However, there are some differences and special considerations. In particular, precautions against RNAse activity must be taken. RNAse is an extremely stable and active enzyme. Gloves should be worn at all times and sterile plasticware should be used whenever possible to avoid introducing exogenous RNAse to the sample. Glassware needs to treated with DEPC-water and autoclaved to inactivate any RNAse. Buffers should be prepared from DEPC-water or RNAse inhibitors included. The cell lysis and solubilization of RNA will typically be carried out in guanidine salts (especially guanidine thiocyanate). Guanidine is a strong chaotropic agent and will inhibit RNAses. The strong denaturing effect of this salt will also promote better phenol extraction. The phenol extraction is the same as the DNA isolation except that the phenol is usually saturated with a buffer of pH 5-6. The lower pH will result in some DNA partitioning in the organic phase. If DNA contamination is a problem, it is possible to purchase RNAse-free DNAse. Longterm storage is best as a precipitate in 70% ethanol at -20°C.

Precipitation of RNA with LiCl

Lill has been used to selectively precipitate RNA. Large RNAse (rRNA, mRNA) are insoluble at high ionic strength, whereas small RNAse (tRNA and 5 S rRNA) and DNA generally remain soluble. Following either phenol or guanidine extraction, an equal volume of 8 M LiCl is added. The sample is mixed vigorously and incubated at -20°. The precipitate is collected by centrifugation and reprecipitated if necessary.

Affinity Chromatography

Most eukaryotic mRNA contains a stretch of A residues at its 3' end which added post-transcriptionally. It is possible to isolate mRNA by affinity chromatography on oligod T columns. The RNA solution is passed over an oligo-dT column under conditions which promote base pairing. Only RNA with a poly A tail binds. The poly A RNA is eluted under conditions (usually low salt and high temperature) which break the base pairing.

Density Gradient Centrifugation

Density gradient centrifugation can also be used in the analysis and isolation of nucleic acids. Double-stranded DNA, single-stranded DNA, RNA and proteins all have different densities and therefore, can be separated by isopycnic (i.e., equilibrium) centrifugation. CsCl is the standard medium for the density gradient centrifugation of nucleic acids and

are especially useful for the purification of large amount of highly purified DNA. The gradients are carried out in the presence of ethidium bromide which fluoresces when bound to DNA. The DNA bands are detected by illumination with ultraviolet light and easily recovered with a syringe and needle by puncturing the wall of the disposable tube and aspirating the DNA. The CsCl can be removed by dialysis or by precipitating the DNA. The %G:C content affects the density of DNA (Figure). This can result in multiple bands on CsCl gradients if DNA composed different G:C compositions are centrifuged. For example, minor bands, called satellite DNA, are often observed when total DNA from an organism is analyzed by CsCl gradients. These satellite bands are usually due to highly repetitive DNA or organellar DNA. Mitochondrial DNA is noted for its high A:T content. These satellite DNA bands can be purified from the genomic DNA by density gradient centrifugation.

Nucleic acids can also be separated according to size by rate zonal centrifugation on sucrose gradients. However, this method is not widely used since gel electrophoresis is generally a more convenient method for the size fractionation of nucleic acids.

Analysis and Quantification

The quality and quantity of isolated nucleic acids can be determined spectrophotometrically. Nucleic acids have an A_{max} of 260 nm and proteins have A_{max} of 280 nm. The A260/A280 ratio is therefore, indicative of the degree of purity of the nucleic acid. A260/A280 ratios of 1.6-1.8 or 1.8-2.0 are usually acceptable for DNA and RNA, respectively. The standard extinction coefficient used for ssDNA, dsDNA and RNA are 0.03 mL/ug, 0.02 mL/ug and 0.025 mL/ug, respectively. Formulas which take into account protein and other contaminants are also available. Indirect spectrophometric assays for DNA quantification are also available, but rarely used. In some instances fluorometry using fluorescent dyes that bind DNA and/or RNA is used to determine nucleic acid concentrations.

Protein Isolation and Purification

(i) In order to purify a protein you need a source.

(ii) It might be blood or some other biological fluid, but most often it is a cell, usually a specific type-liver, muscle, yeast, bacteria, etc.

(iii) The cells must be broken open-homogenized—to release the protein in a soluble form.

(iv) Homogenization conditions must be worked out that release the protein from the cell without damaging the protein.

(v) Membrane-bound proteins can also be purified, but different approaches are required.

Fractional Precipitation

(a) In concentrated salt solutions, usually ammonium sulphate is used; some proteins are more soluble than others. By varying the concentration of ammonium sulphate, one can achieve some limited purification of proteins. This technique is often used in the first step of protein purification.

(b) Small proteins are more soluble than large proteins.

(c) The larger the number of charged side chains, the more soluble the protein.

Column Chromatography

(a) The invention of column chromatography was a critical event in biochemistry, because it was the basis for the development of procedures for obtaining pure proteins.

(b) Studies on pure proteins are essential for understanding the structural and functional properties of proteins.

(c) In column chromatography, an adsorbent is placed in a glass tube.

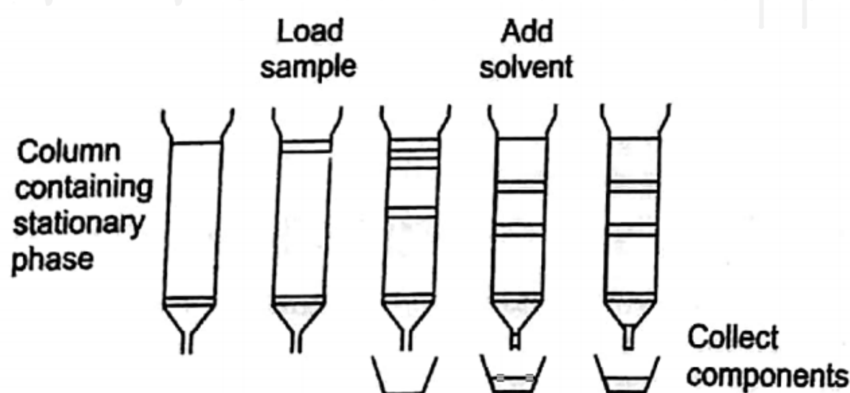

Fig. 37.1 Process of column chromatography

(d) A protein mixture is passed into the column and binds to the adsorbent.

(e) By proper choice of the eluting buffer, specific proteins can be eluted from the adsorbent and separated from other proteins in the mixture.

(f) By repeating this procedure with several different adsorbents, pure protein can be obtained.

(g) Because proteins are not very stable, low temperature (4°C) and neutral pH must often be employed.

(h) The properties of some adsorbents are described below.

Ion Exchange Chromatography

(a) Ion exchange resins have fixed charges-either positive or negative.

(b) Proteins bind to the resin via electrostatic interactions

(c) The strength of these interactions depends on the net charge on the protein, which is a function of pH and the nature of the weak acid amino acid side chains and the salt concentration of the buffer-high salt concentrations reduce the interaction.

Affinity Chromatography

(a) This is a more specific interaction in which a ligand specifically recognized by the protein of interest is attached to the column material.

(b) When a mixture of proteins is passed through the column, only those few that bind strongly to the ligand will stick, while the others will pass through the column.

(c) By changing the buffer one weakens the interaction between the protein and the ligand, which causes the protein to be eluted from the column.

(d) A variation is immuno-affinity chromatography; in which an antibody specific for a protein is immobilized on the column and used to affinity purify the specific protein.

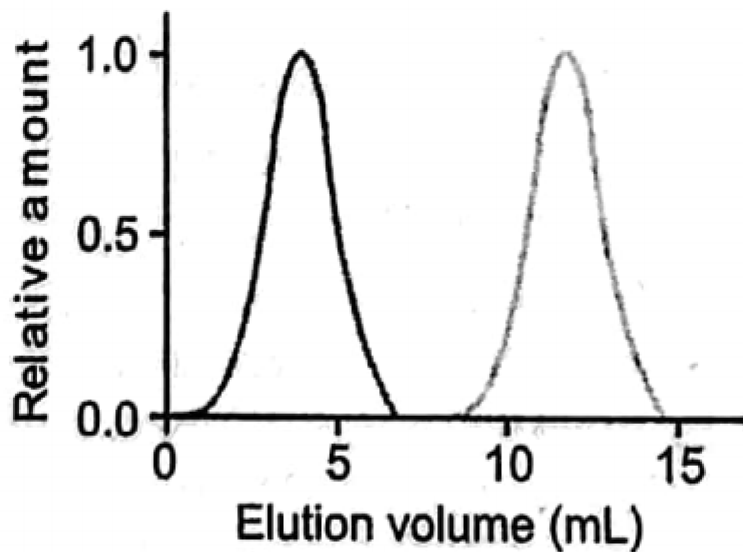

Fig. 37.2 A typical curve of protein during chromatography

Gel Filtration Chromatography (Molecular Sieve or Size Exclusion Chromatography)

(a) The column consists of material that separates proteins based on their size and shape.

(b) A wide range of molecular exclusion limits is available for separating proteins of all sizes.

(c) For any particular column dimensions and material, the volume of buffer required to elute a specific protein depends on the molecular weight of the protein. Thus, one can separate proteins by size.

(d) If one calibrates the column by determining the elution volume of proteins with known molecular weights, then a calibration curve relating elution volume and molecular weight can be constructed.

(e) Such a calibration curve can then be used to estimate the molecular weight of an unknown protein.

Dialysis/Ultrafiltration

- Semipermeable membranes are available, which allow passage of small molecules but exclude the passage of proteins. Sacs made of such material allow the salt and buffer components of a protein solution to be changed to another buffer.

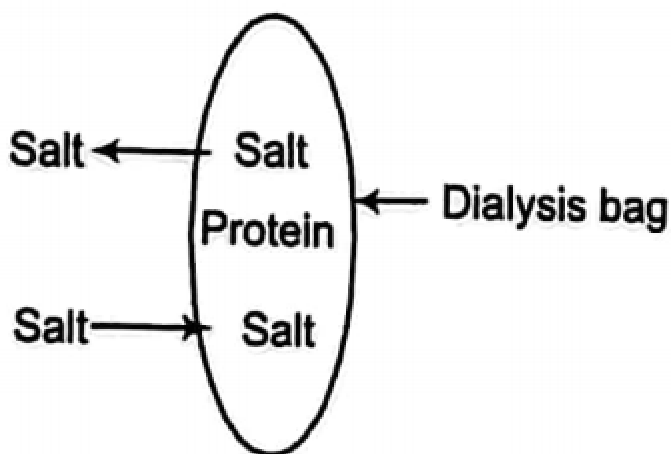

Fig. 37.3 Process of dialysis

Analysis of DNA, RNA and Protein

DNA and RNA form the nucleic acid, while protein is the structural macromolecule of the cell. These are analysed by electrophoresis centrifugation, etc.

Gel Electrophoresis

Electrophoresis of macromolecules can be carried out in solution. However, the ability to separate molecules is compromised by their diffusion. Greater resolution is achieved if electrophoresis is carried out on semi-solid supports such as polyacrylamide or agarose gels. Gels are formed by cross-linking polymers in aqueous medium. This will form a 3-dimensional meshwork which the molecules must pass through. Polyacrylamide is a common gel for protein electrophoresis, whereas agarose is more commonly used for nucleic acids. Agarose gels have a larger pore size than acrylamide gels and are better suited for larger macromolecules. However, either type of gel can be applied to either nucleic acids or proteins depending on the application. Gels are formed from long polymers in a cross-linked lattice. The spaces between the polymers are the pores. Higher concentrations of the polymer will result in smaller average pore sizes. Polyacrylamide gels are formed by covalently cross-linking acrylamide monomers with bis-acrylamide with a free radical like per sulphate (SO_4). The cross-linking of the acrylamide polymers results in 'pores' of a defined size. The total acrylamide concentration and the ratio of bis-acrylamide to acryl amide will determine the average pore size. The polyacrylamide solution is poured into a mold and polymerized. This mold can be a cylindrical tube, but 18 usually a 'slab' poured between two glass plates.

Since, the gel is solid with respect to the mold, all molecules are forced through the gel. Smaller molecules will be able to pass through this lattice more easily resulting in larger molecules having a lower mobility than smaller molecules. In other words, the gel acts like a molecular sieve and retains the larger molecules while letting the smaller ones pass through (this is opposite of gel filtration, where the larger molecules have a higher mobility because they to not enter the gel). Therefore, the frictional coefficient is related to how easily a protein passes through the pores of the gel and size will be the major determinant of the mobility of molecules in a gel matrix. Protein shape and other factors will still affect mobility, but to a lesser extent.

Substituting size for the frictional coefficient results in:

Mobility ≅ (voltage) (charge)/ (size)

In other words, the mobility of a protein during gel electrophoresis is primarily a function of its charge/mass ratio.

Equipment

Equipment to conduct gel electrophoresis is relatively simple. They consist of a mold to form the gels, an apparatus to hold the gel and contain buffers and a power supply capable of delivering the required voltage or current. There are many types of apparatus for carrying out electrophoresis depending on the application. Gels can be either in a vertical or horizontal configuration. Polyacrylamide gels are run in a vertical fashion and agarose gels tend to be run in a horizontal position. Gels can either be formed as cylinders by using glass tubing as a mold (often called tube gels) or formed as rectangular slabs. These slab gels are formed by polymerizing the acrylamide solution between glass plates separated by spacers. Typically the gel is 0.75-1.5 mm thick. At the top a 'comb' is used to form sample wells. Slab gels allow multiple samples to be compared on the same gel, thus eliminating gel-to-gel variations. The formed gel is placed into the apparatus to that the top and bottom of the gel are in contact with chambers containing buffer. These chambers contain electrodes which are connected to a power supply. Thus, an electric field is generated across the gel when a voltage is applied. The buffer in the chambers is generally different that the buffer making up the gel for protein electrophoresis and in some applications the buffers in the lower and upper chambers may be different. In most applications, the buffers are such that the protein has a negative charge and therefore, the anode (positve pole) will be in the lower chamber and the cathode (negative pole) will be in the upper chamber. However, there are applications in which the proteins of interest may be positively charged and therefore, the electrodes will be reversed.

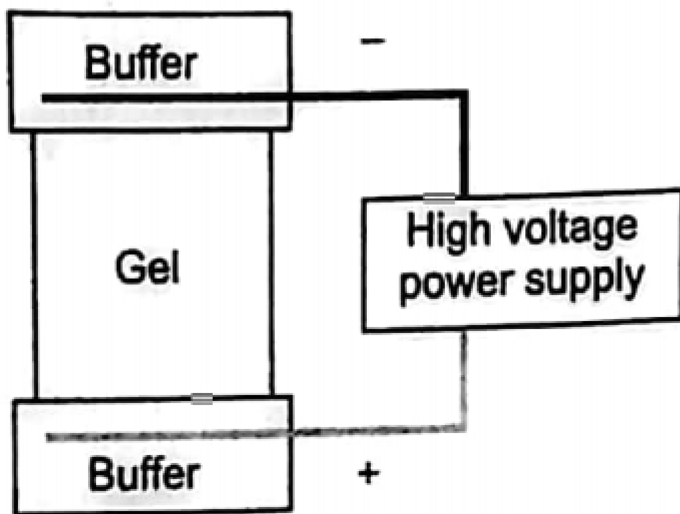

Fig. 37.4 Electrophoresis

Protein Characterization

(a) SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is a variant of electrophoresis in which the buffers contain SDS, a detergent that binds to proteins.

(b) Most proteins bind SDS at a constant ratio, about 1 SDS for every 2 amino acids.

(c) The large negative charge resulting from the bound SDS masks the native charge on the protein, so that all proteins have essentially the same charge to mass ratio.

(d) This means that the rate of movement in the electric field depends only on the polypeptide chain molecular weight. Mercaptoethanol is used to reduce disulphides.

(e) In addition, the SDS causes all proteins to adopt a random-coil structure, which means that shape does not affect movement through the gel.

(f) Thus, SDS-PAGE is very useful method for determining the molecular weight of the individual polypeptide chains of a protein.

(g) Western blotting is a technique for detecting a specific protein in a mixture.

(h) Gel electrophoresis and SDS-PAGE electrophoresis are primarily useful as analytical techniques, although they can be used for purification.

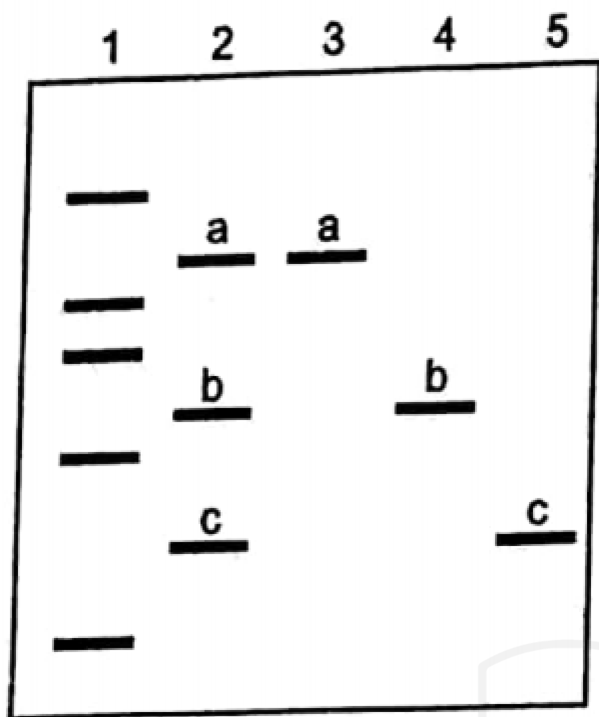

Fig. 37.5 A typical SDS-PAGE gel

(i) In this figure, lane 1 would contain standards of known molecular weight, lane 2 a mixture of three unknown proteins and lanes 3-5 the three unknown proteins.

(j) SDS-PAGE can be used to determine the molecular weight of a protein.

(k) The molecular weights of the three unknown proteins can be determined from a calibration curve constructed by plotting the log of the molecular weight of the standard proteins vs. the distance traveled in the gel.

(l) The distance traveled depends on the porosity of the gel. The lower the porosity the faster (further) the proteins move.

(m) Depending on the molecular of the unknown proteins, different porosity gels will need to be used.

Isoelectric Focusing

Isoelectric focusing (IEF) separates proteins based on their isoelectric points. The isoelectric point is defined as the pH at which a protein has no net charge (i.e., the number of negative and positive charges are equal) and is a measure of the protein's net charge. Separating proteins according to their net charge is accomplished by generating a pH gradient in an electric field. The effect of protein size on mobility is minimized by carrying out the electrophoresis gels with large pore sizes such as low acrylamide concentrations

(e.g., 3.5%) or agarose. This large pore size minimizes the molecular sieving. A pH gradient is generated with carrier ampholytes. These ampholytes are a mixture of aliphatic amines and either carboxylic or sulphonic acid. They have a high buffering capacity, low molecular weight (300-600 Da) and a range of PK_a values. Initially the pH of an ampholyte solution will be the average of the pK_a values of the mixture. Application of an electric current will cause the ampholytes to migrate toward the electrodes according to their charges. Ampholytes that have pK_a values above the pH will be positively charged and those with pK_a values below the pH will be negatively charged. As the ampholytes migrate, this will result in changes in the local pH due to the buffering action of the ampholytes. This change in the local pH will affect the charge on the ampholytes depending upon the pK_a The ampholytes will continue to migrate until they reach a position in which the local pH equals their pK_a (i.e., no net charge). The end result is a pH gradient in which the most basic ampholytes are found at the cathode, a dilute alkali solution (e.g., NaOH) and the most acidic ampholytes are at the anode, a dilute acid solution (e.g., H,PO.). Carrier ampholytes with defined pH ranges can be purchased or prepared by isoelectric focusing. Proteins are also ampholytes and will migrate within the pH gradient until they reach a pH equal to their isoelectric point. The carrier ampholytes are needed since the protein concentration is generally not high enough to establish a stable pH gradient and the isoelectric points of the proteins may not be uniformly distributed along a pH gradient. Sample preparation is important for IEF in that many reagents can adversely affect isoelectric focusing. In particular, the ionic strength should be as low as possible. Precipitation of proteins with acetone is a method for removal of excess salts, as well as concentrating the protein. Separations can be performed under either native or denaturing conditions. Urea is the preferred denaturing agent since it is uncharged. Similarly, non-ionic detergents, such as Triton X-100 or NP-40, are less likely to interfere with the formation of pH gradients. Protein precipitation is sometimes a problem in that proteins tend to be less soluble at their isoelectric points and their local concentrations can be quite high. In addition, the high voltages and high resistance associated with IEF generates substantial heat which increases protein precipitation. Many apparatuses for IEF will have a cooling mechanism to disperse the excess heat. Inclusion of urea and NP-40 in the gels will also minimize protein precipitation. IEF is an equilibrium phenomenon since the components of the system migrate until they have no net charge. As the system approaches equilibrium the resistance approaches infinity since there are no ions to conduct the current. However, the pH gradient will start to breakdown before true equilibrium is reached and the ampholytes will migrate into the anode and cathode buffers. This gradient breakdown is accompanied by a lowering of the resistance.

Therefore, the progress of IEF can be followed by performing the electrophoresis under constant voltage and monitoring the current. Initially the current will rapidly drop in concordance with the rapid migration of the ampholytes. As the ampholytes lose their net charge, the resistance increases and the current decreases (E=IR). The rate at which the current decreases levels off as the system approaches equilibrium. The current will start to rise again when the pH gradient starts to breakdown. IEF needs to be discontinued before this point. The pH gradient can be determined with marker proteins with known isoelectric points or by measuring the pH along the gel. This is accomplished by slicing the gel into pieces, eluting the ampholytes into distilled water and measuring the pH.

Two-Dimensional Gel Electrophoresis

Conventional electrophoresis separates proteins according to their charge/mass ratios. SDS-PAGE separates proteins according to subunit or polypeptide, mass. IEF separates proteins according to isoelectric point (or charge). It is possible to sequentially combine the different types of electrophoresis and run two-dimensional (2-D) gels. A common form of 2-D gel electrophoresis is to first separate proteins by IEF in 'tube' gels. These gels are then equilibrated in SDS gel electrophoresis sample buffer and subjected to SDS-PAGE in a 'slab' gel.

(It is necessary to carry out the IEF first since, SDS interfere with the isoelectric focusing.) The 2-D separation results in higher resolution since proteins are being separated according to two distinct properties (i.e., charge and size).

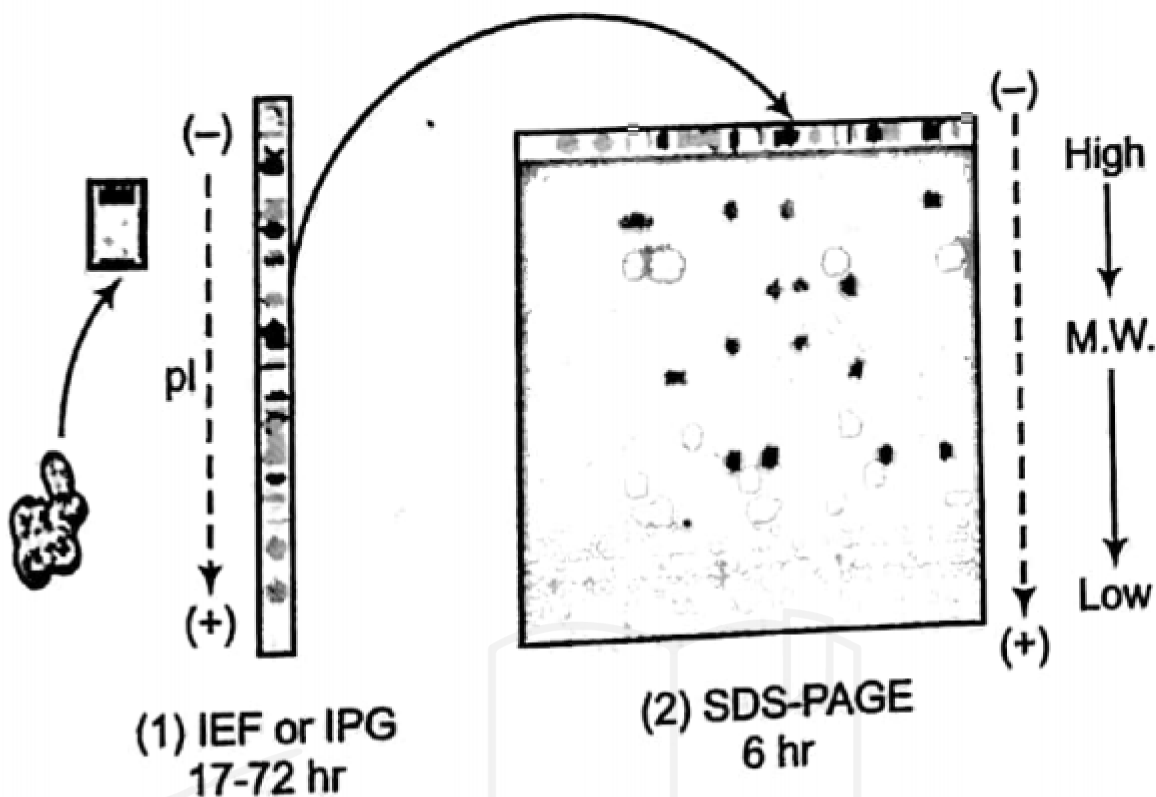

Fig. 37.6 Two-dimensional electrophoresis

Molecular weight and shape are fundamental physical properties of a protein. Estimates of molecular weight can be obtained using SDS-PAGE or gel filtration, as described above. One very useful technique for measuring molecular weight and shape is centrifugation.

Centrifugation

(i) A particle that is subjected to a centrifugal field by being spun in a centrifuge is subjected to a force, $f = m(1 - v\rho)\omega^2 r$, where m is the mass of the mass of the particle, r is the distance of the particle from the center of rotation and w is the angular velocity. (1 - nr) is the buoyancy factor which accounts for the fact that particle is buoyed up by the surrounding solvent of density r (g/mL).

(ii) v is the specific volume of the particle (mL/g) (= 1/density of the particle).

(iii) If y = p then the particle will not move.

(iv) The movement of the particle through the solvent is resisted by a frictional coefficient, f, that depends on the shape of the particle.