ELECTROPHORESIS

Electrophoresis

Electro refers to electron flow or current.

Phoresis refers to movement.

Thus Electrophoresis is movement under electric current.

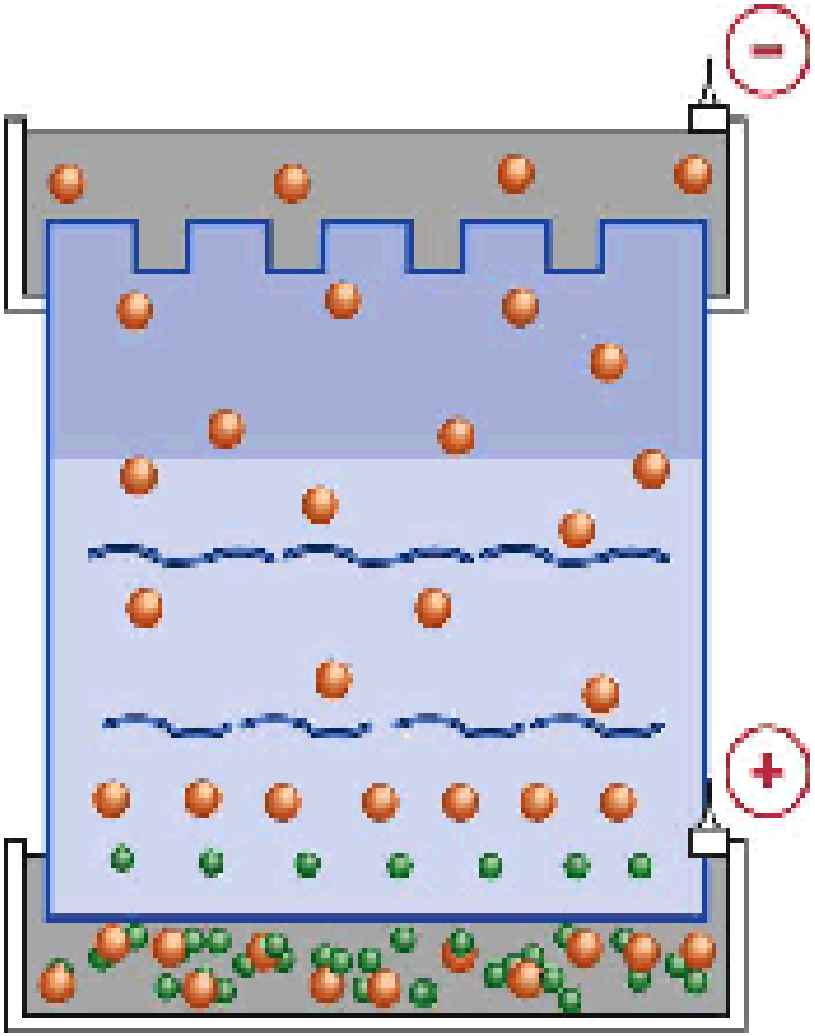
This technique therefore can separate molecules which can move in an electric field i.e charged molecules

Movement in an Electric Field

Charged molecules in an electric field behave in a predictable manner.

Positively charged molecules will move towards the negative pole while negatively charged molecules move towards the positive pole.

The Fundamentals of Electrophoresis.



Some media for Electrophoresis

Medium	Conditions	Principal Uses
Paper	**Filter paper moistened with buffer, placed between electrodes**	**Small molecules Amino acid, nucleotides**
Polyacrylamide gel	**Cast in tubes or slabs; cross linked**	**Proteins and nucleic acids**
Agarose gel	**As polyacrylamide, but no cross linking**	**Very large proteins, Nucleic acids and nucleoproteins, etc**

Types of Gel Electrophoresis

Types of Gel Electrophoresis

There are two types of gel Electrophoresis

→ One dimension
→ Two dimensions

One dimension

1. SDS-PAGE,
2. Native –PAGE
3. IEF

Several forms of PAGE exist and can provide different types of information about the protein(s).

SDS-PAGE, the most widely used electrophoresis technique, separates proteins primarily by mass.

Non denaturing PAGE, also called native PAGE, separates proteins according to their mass:charge ratio.

Two-dimensional PAGE (2D-PAGE) separates proteins by isoelectric point in the first dimension and by mass in the second dimension.

Procedure

The samples are treated with SDS (sodium dodecyl sulfate), an anionic detergent which denatures the protein by breaking the disulfide bonds and gives negative charge to each protein in proportion to its mass.

Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. SDS linearizes the proteins so that they may be separated strictly by molecular weight.

The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g giving an approximately uniform mass:charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein.

Proteins may be further treated with reducing agent, such as dithiothreitol (DTT) or TRP(Tributyl phosphine) to break any reformed disulfide bonds and then alkalated with iodoacetamide to prevent reformation of disulfide bonds.

A tracking dye like bromophenol blue may be added to the protein solution to track the progress of the protein solution through the gel during the electrophoretic run.

GEL

Gel

The gel used for SDS-PAGE is made out of acrylamide which form cross-linked polymers of polyacrylamide.

Standard gels are typically composed of two layers, one top-most layer called the stacking gel and a lower layer called separating or resolving gel.

The stacking layer contains a low percentage of acylamide and has low pH , while the acrylamide concentration of the separating gel varies according to the samples to be run and has higher pH.

The difference in pH and acrylamide concentration at the stacking and separating gel provides better resolution and sharper bands in the separating gel.

Stacking gel

The stacking gel is a large pore PAG (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2.0 pH units lower than that of electrophoresis buffer (Tris/Glycine).

This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and the volume of the sample to be applied.

Resolving gel

The resolving gel is a small pore polyacrylamide gel (3 - 30% acrylamide monomer) typically made using a pH 8.8 Tris /HCl buffer.

In the resolving gel, macromolecules separate according to their size. Resolving gels have an optimal range of separation that is based on the percent of monomer present in the polymerization reaction.

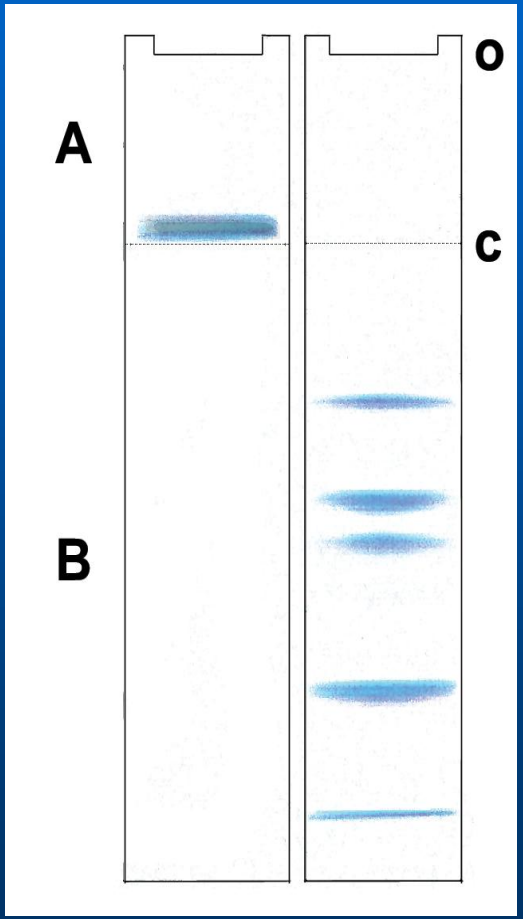
Postulated migration of proteins in a Laemmli gel system

A: Stacking gel

B: Resolving gel

o: sample application

c: discontinuities in the buffer and electrophoretic matrix



SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE).

SDS-PAGE is a method of gel electrophoresis to separate proteins based on the their mass.

Sodium dodecyl sulfate (SDS) is a detergent that breaks up the interactions between proteins.

Disrupts secondary and tertiary protein structures by breaking hydrogen bonds and unfolding protein

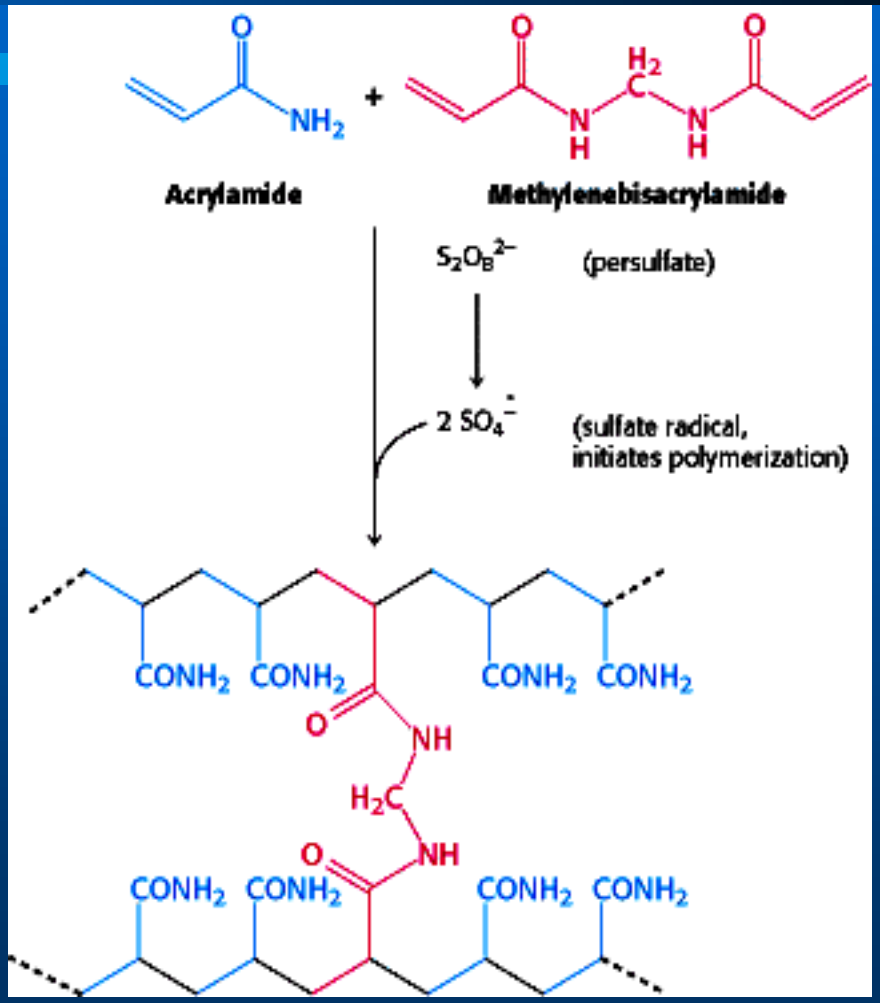
Polyacrylamide Gel Electrophoresis (PAGE)

Most commonly used for proteins.

Can offer smaller pore sizes.

Chemically inert

Rapidly formed.



How Phenomena works

The proteins are dissolved in SDS and then electrophorised.

Binds to protein in a ratio of one SDS molecule/two amino acids.

Masks' charge on protein so that all proteins are uniformly negatively charged.

The proteins denatured by SDS are applied to one end of a layer of polyacrylamide gel submerged in a buffer.

Buffer provide uniform pH and ions for conducting electric potential.

When an electric current is applied across the gel, the negatively-charged proteins migrate across the gel to the positive pole.

Short proteins will more easily fit through the pores in the gel and move fast, while larger ones will have more difficulty.

Due to differential migration based on their size, smaller proteins move farther down the gel, while larger ones stay closer to the point of origin.

After a given period of time, proteins might have separated roughly according to their sizes.

Proteins of known molecular weight (marker proteins)can be run in a separate lane in the gel to calibrate the gel.

Acrylamide polymerization

- AA concentration determines the size of the pores.
- Cross-link is usually added in 1:30 proportion to AA.

% acrylamide Average	pore diameter, nm
5.0	3.6
7.5	3.0
10.0	2.6
20.0	1.8

7.5% gel -standard for proteins 10-300 Kda

Typical size of a small protein (Myoglobin, 17 Kda) is 2.5 x 3.5 x 4.5 nm

Steps in SDS-PAGE

Extract Protein

Solubilize and Denature Protein

Separate Proteins on a gel

Stain proteins (visualization)

Analyze and interpret results

Proteins Extraction

Rupture plasma membrane to prepare tissue/ cell homogenates:

High speed blender **Sonication**

Tissue homogenize **Osmotic shock**

Solublization in a buffer

Centrifugation to remove debris

Denaturation

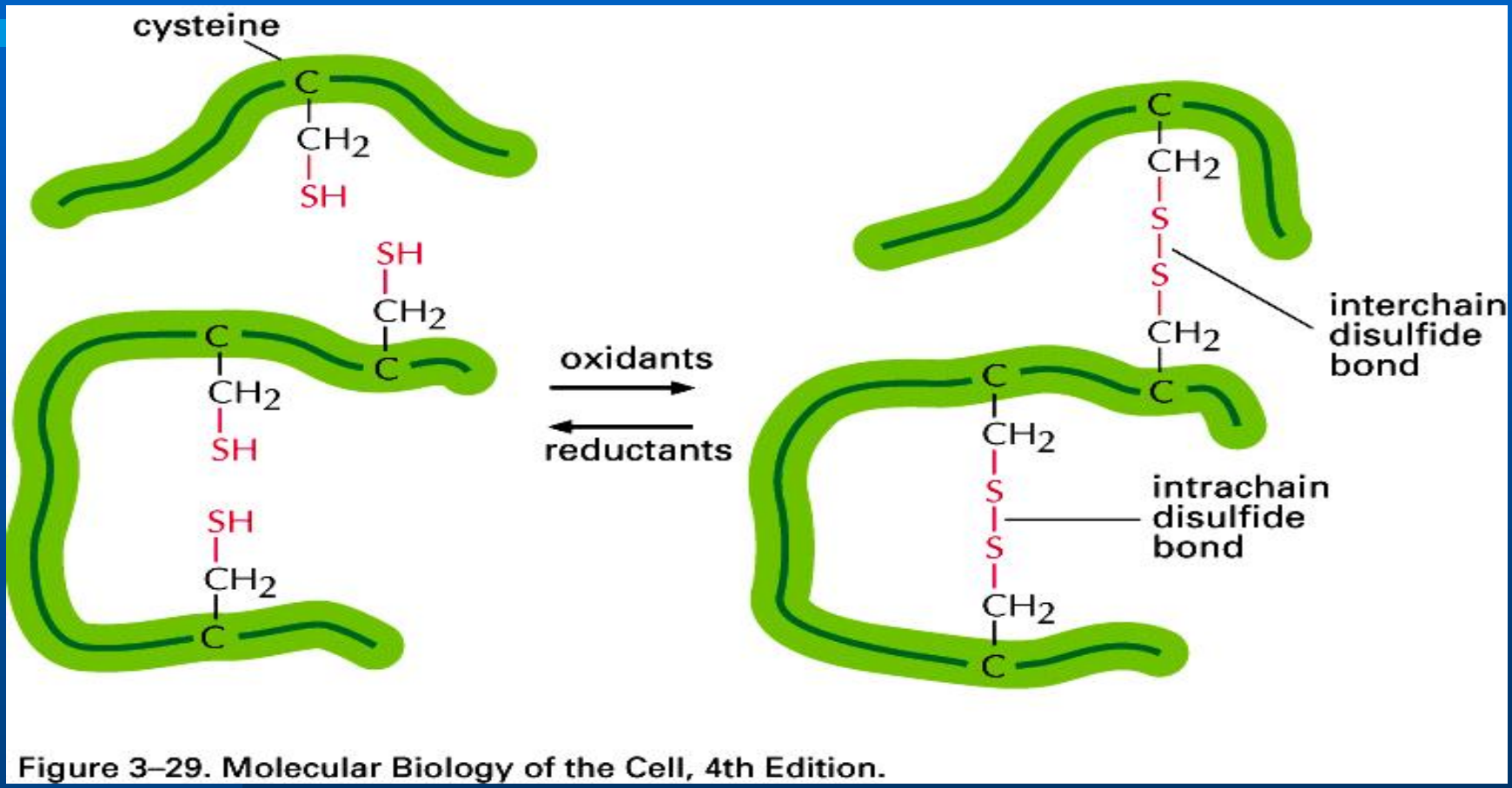
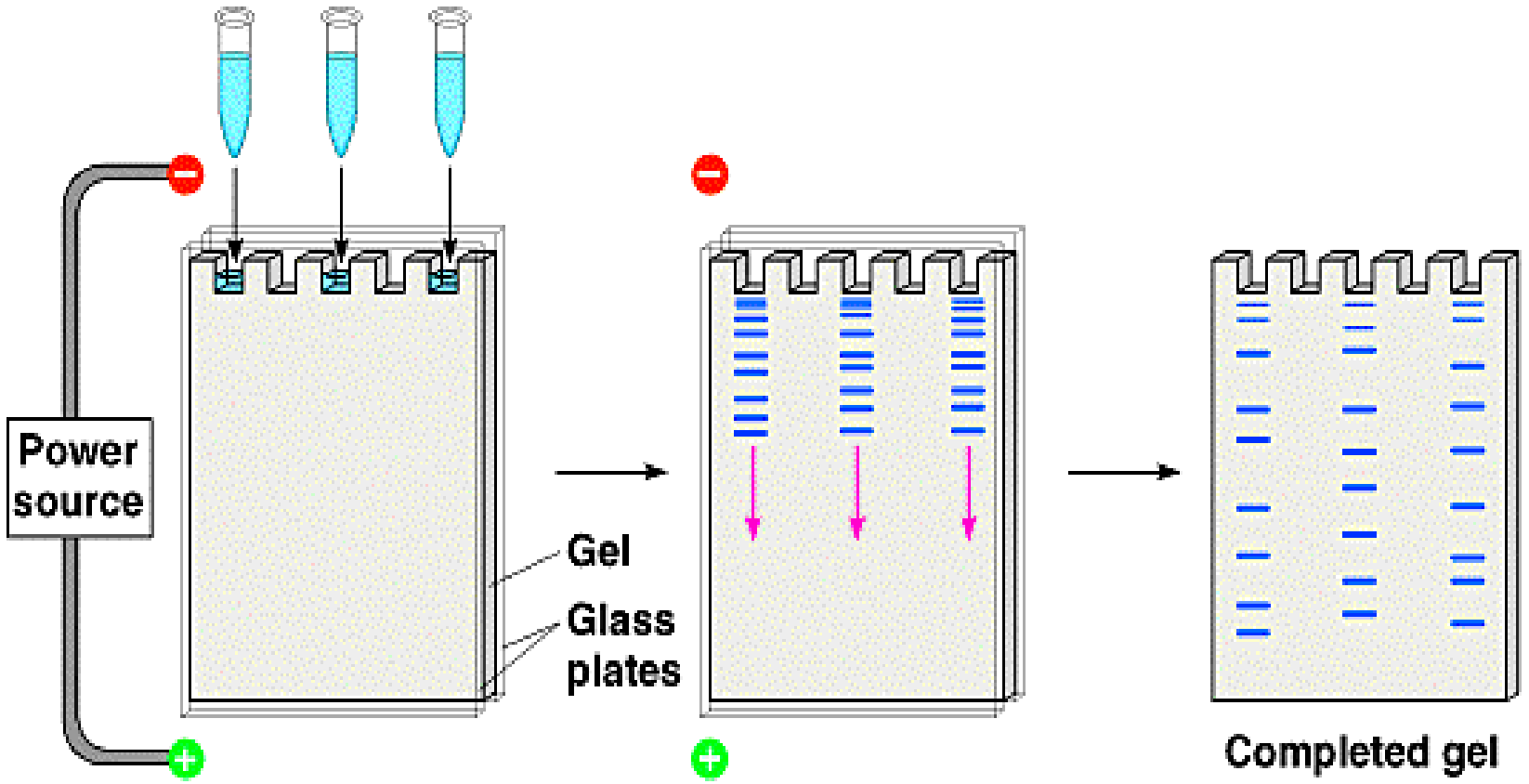
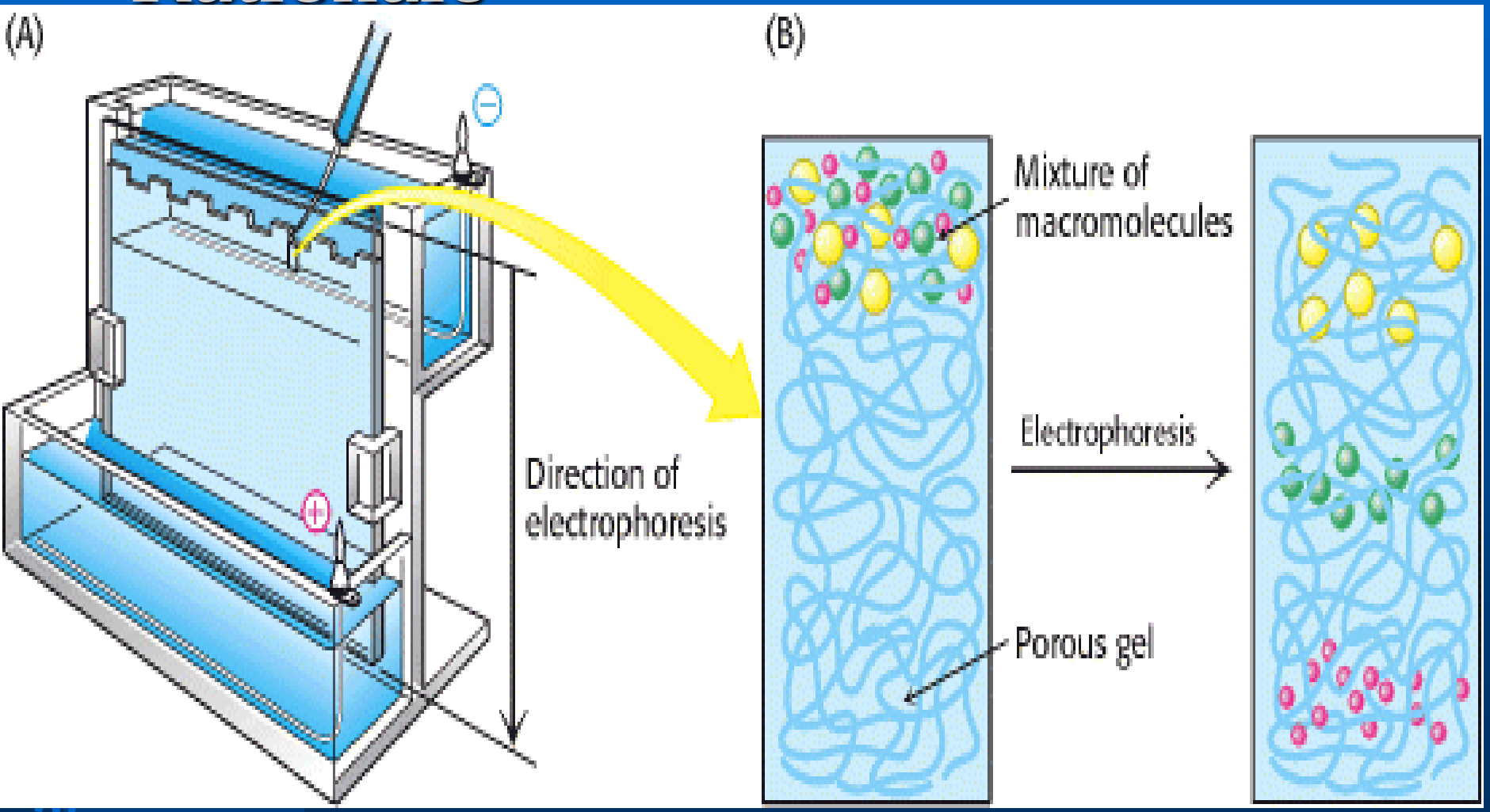


Figure 3–29. Molecular Biology of the Cell, 4th Edition.

Gel Preparation, Loading & Running



SDS-PAGE Separation Rationale



Staining and analysis

Following electrophoresis, the gel may be stained with Coomassie Brilliant Blue or silver stain to visualize the separated proteins.

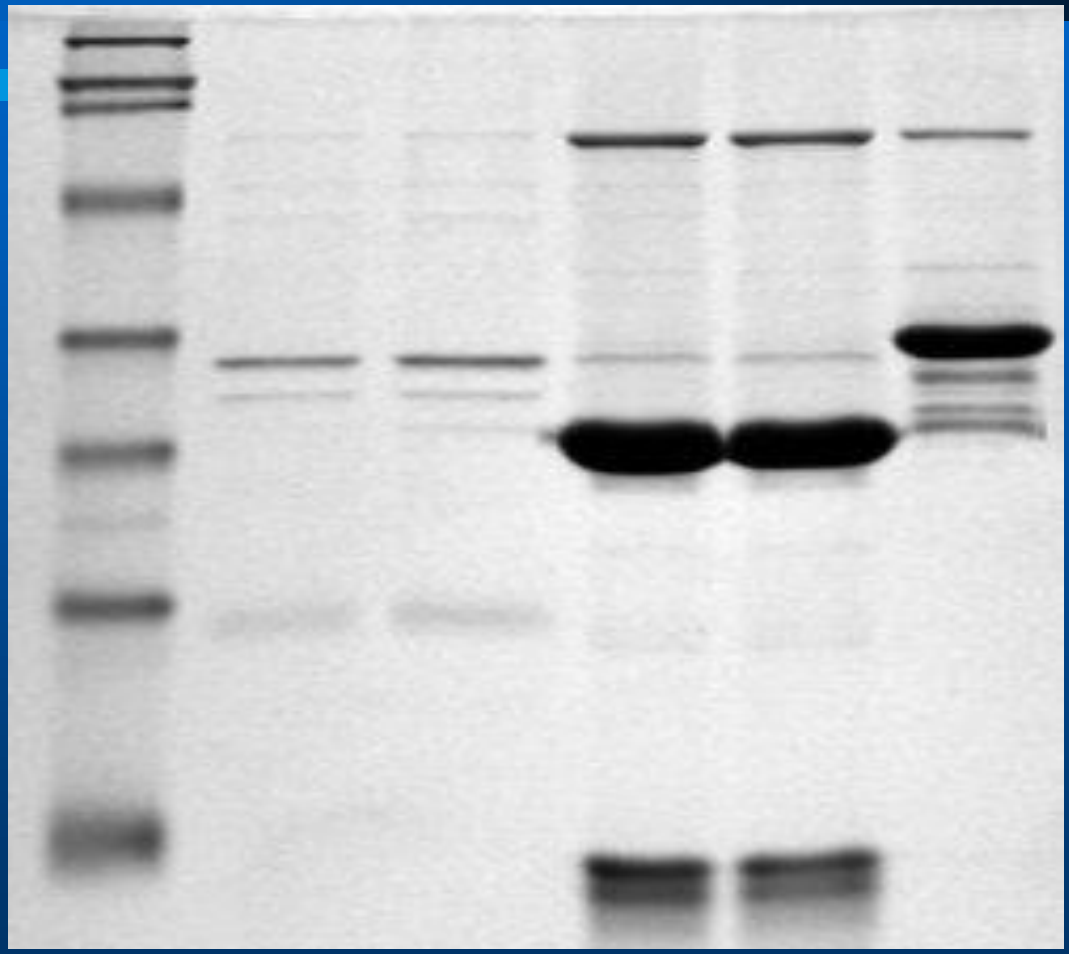
After staining, different proteins will appear as distinct bands within the gel according to their sizes(and therefore by molecular weights).

The molecular weight of a protein in the band can be estimated by comparing it with the marker proteins of known molecular weights.

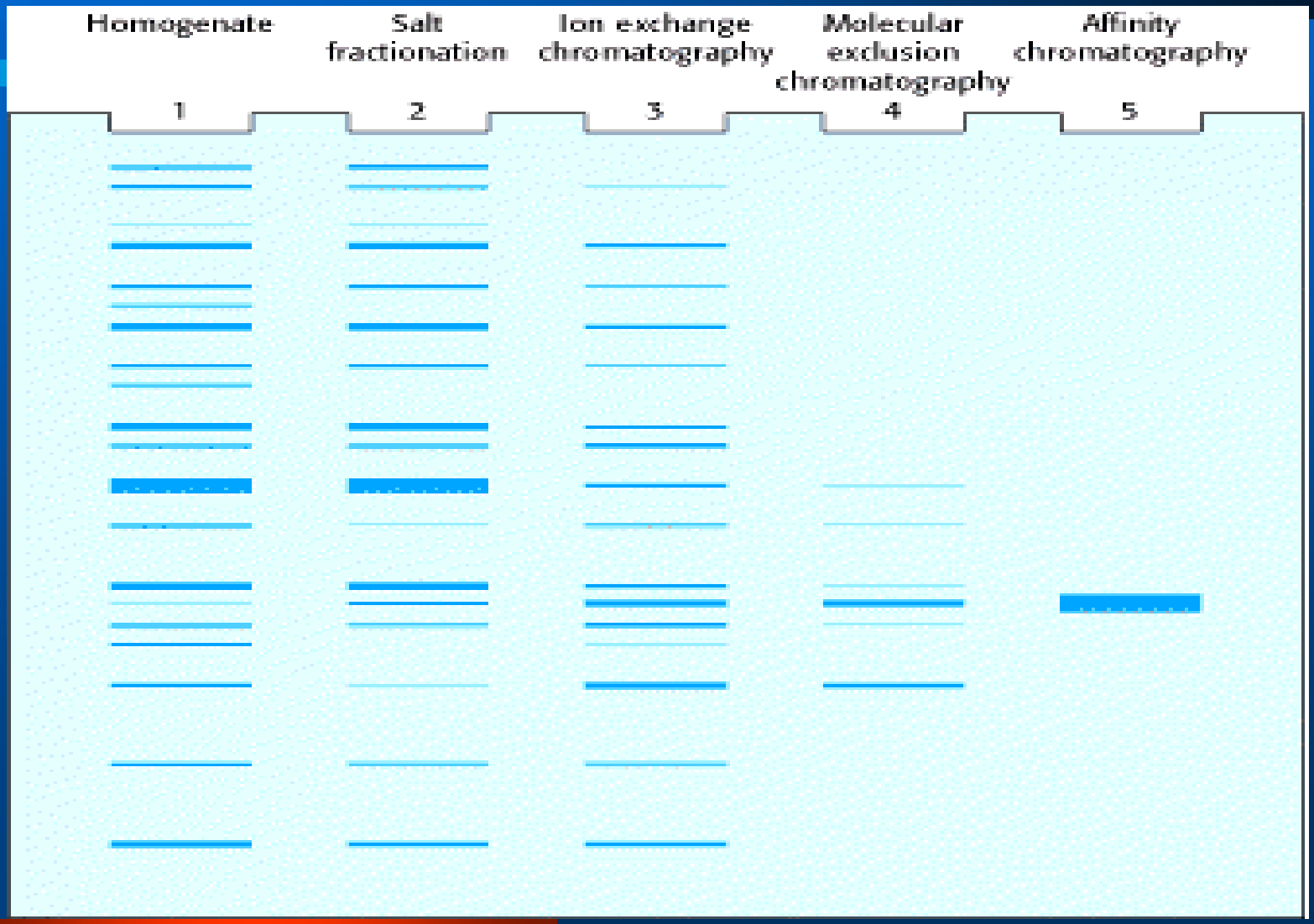
The separatesd proteins can be cut from the gel and further analyzed by other proteomics techniques.

Picture of an SDS-PAGE

The molecular marker is in the left lane

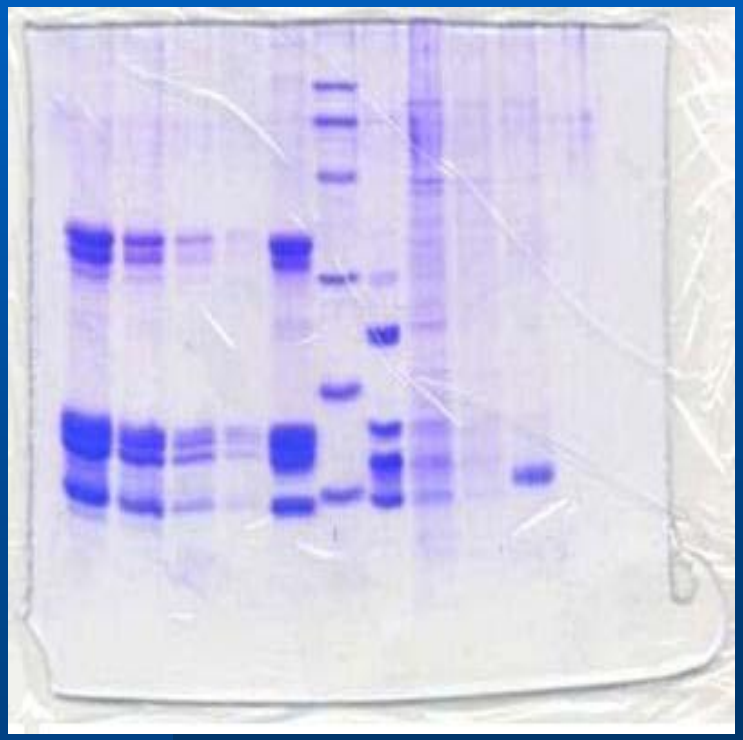


SDS-PAGE separation

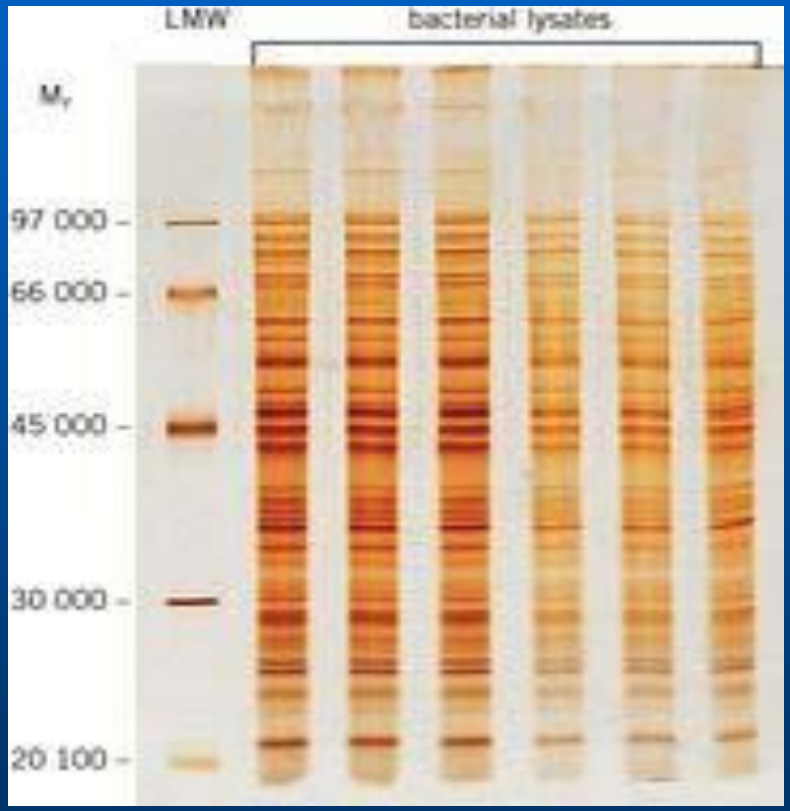


SDS-PAGE, staining

Coomassie staining



Silver staining



Native-PAGE

Native PAGE is used to separate proteins in their native states according to difference in their charge density.

Native state of protein means proteins are in properly folded state, not denatured or unfolded state.

There are no denaturants present in the gel and buffer in the gel maintains the protein in its native state.

Many proteins are shown to be enzymatically active after separation by native PAGE. Thus, it is used for preparation of purified and active proteins.

In native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. The charge depend on the amino acid composition of the protein as well as post-translational modifications.

The hydrodynamic size and mobility of native protein on the gel will vary with the nature of the conformation.

Proteins with compact conformations have higher mobility and larger structures like oligomers have lower mobility.

Native PAGE can be carried out near neutral pH to avoid acid or alkaline denaturation to study conformation as well as self-association or aggregation, and the binding of other proteins or compounds.

The apparatus is kept cool to minimize denaturation of proteins and proteolysis.

Isoelectric Focusing(IEF)

Isoelectric focusing (IEF), also known as electro focusing, is a technique for separating different molecules by their electric charge differences.

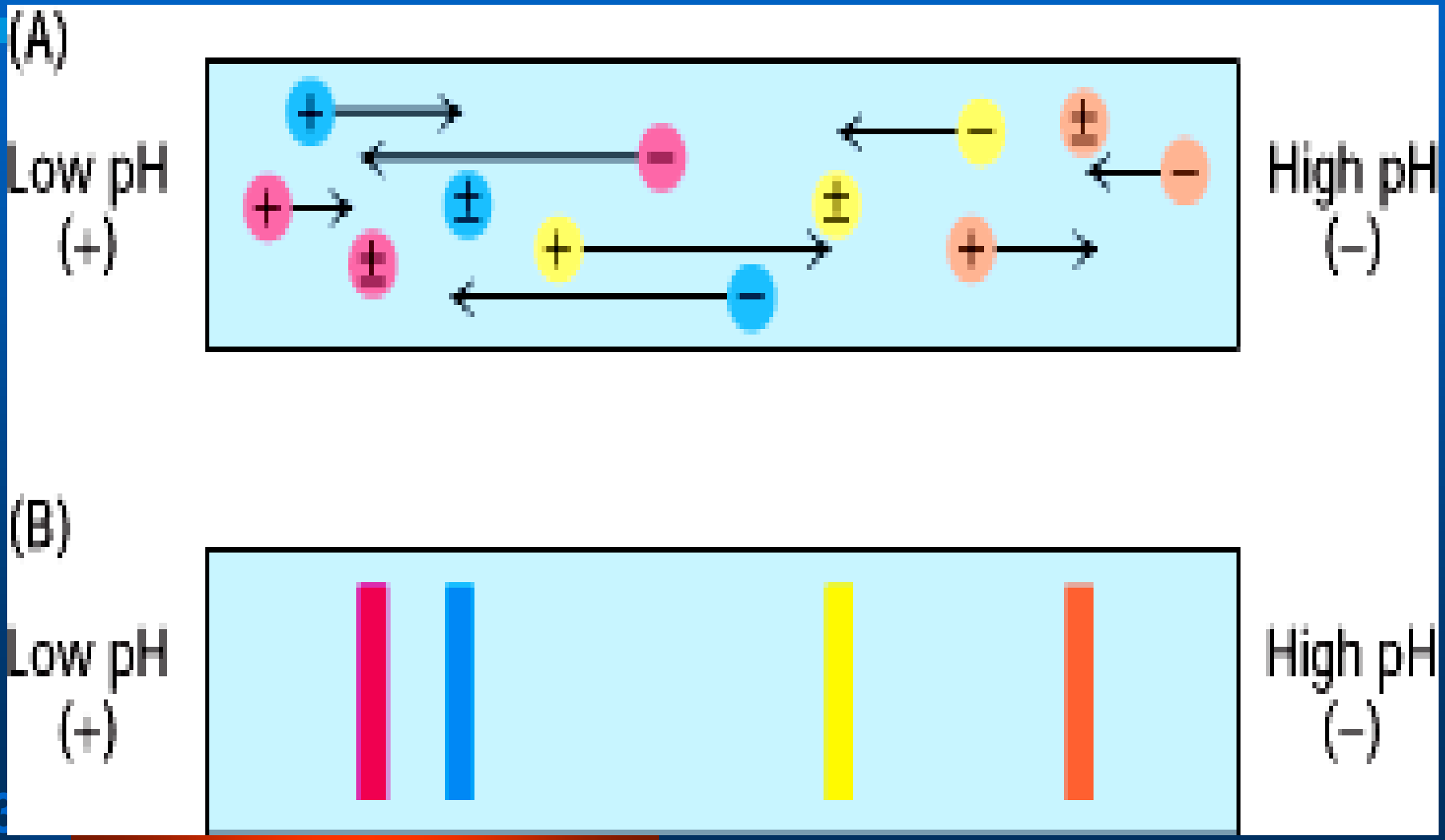
In Isoelectric focusing, proteins are separated by electrophoresis in a pH gradient based on their isoelectric point(pl).

A pH gradient is generated in the gel and an electric potential is applied across the gel.

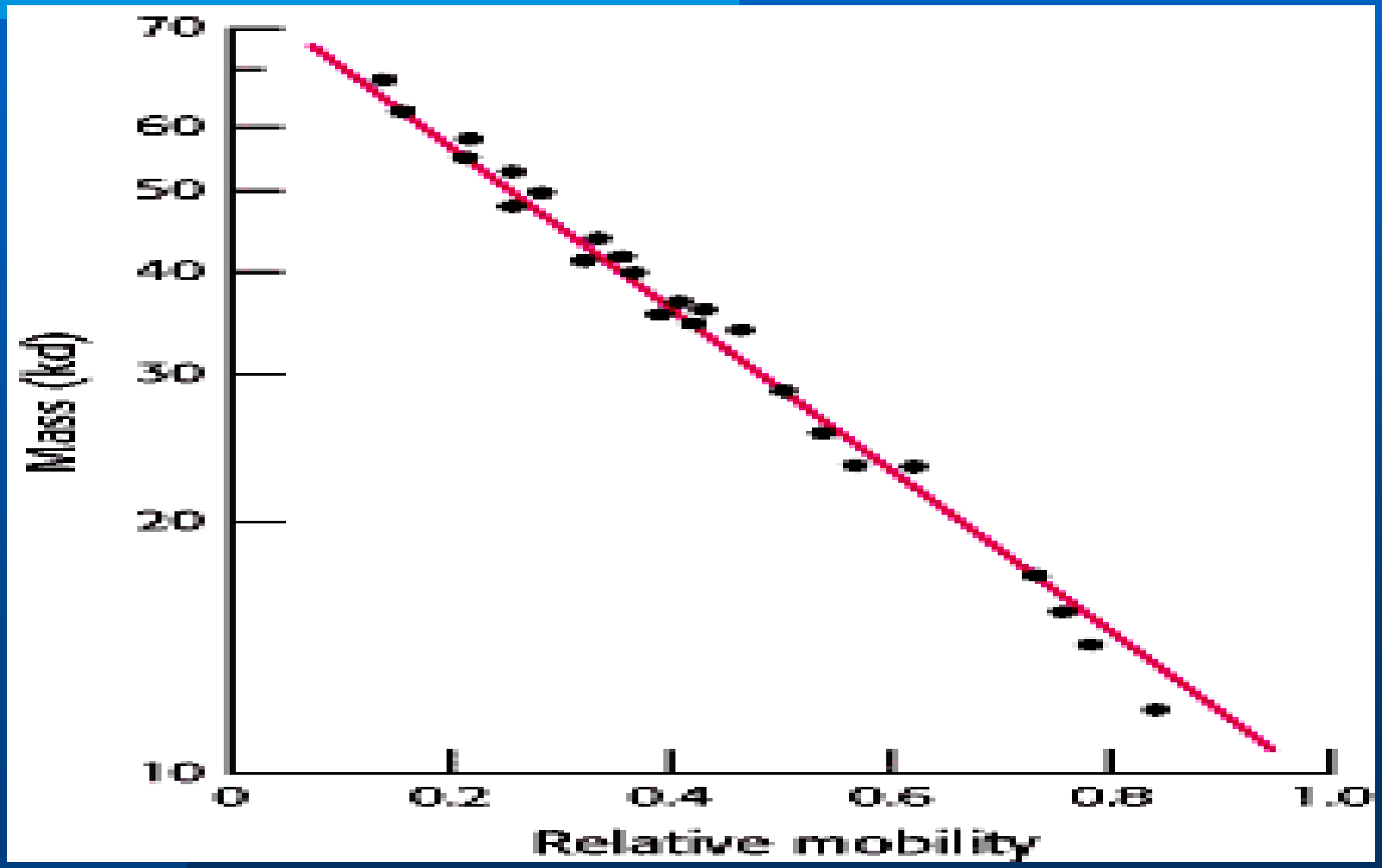
At all pHs other than their isoelectric point, proteins will be charged. If they are positively charged, they will move towards the more negative end of the gel and if they are negatively charged they will move towards the more positive end of the gel.

At its isoelectric point, since the protein molecule carry no net charge it accumulates or focuses into a sharp band.

Isoelectric focusing



Molecular weight determination



kDa	Protein, 0.1-0.2mg/ml	Source
116.0	β-galactosidase	*E.coli* (2)
66.2	Bovine serum albumin	bovine plasma (3)
45.0	Ovalbumin	chicken egg white (4)
35.0	Lactate dehydrogenase	porcine muscle (5)
25.0	REase Bsp98I	*E.coli* (6)
18.4	β-lactoglobulin	bovine milk (7)
14.4	Lysozyme	chicken egg white (8)

12% Tris-glycine SDS-PAGE
Staining with PageBlue™ Protein Staining Solution (#R0571)

Uses of SDS-PAGE

Determine Mw of a peptide/protein

Identify protein

Determine sample purity

Identify existence of disulfide bonds

Quantify amounts of protein

Second dimension in 2-D electrophoresis

Detection/Estimation of Genetic Diversity

Proteins induced in response to environmental changes

Problems with SDS-PAGE or IEF

A limited number (20~30) proteins may be separated by SDS-PAGE/IEF.

Some proteins may have either same *p*I or same molecular weight.

Cellular proteome consists of about 2-3 thousand proteins.

Solutions

Combination of SDS-PAGE/IEF

The technique is called 2-D electrophoresis

Two-Dimensional Electrophoresis

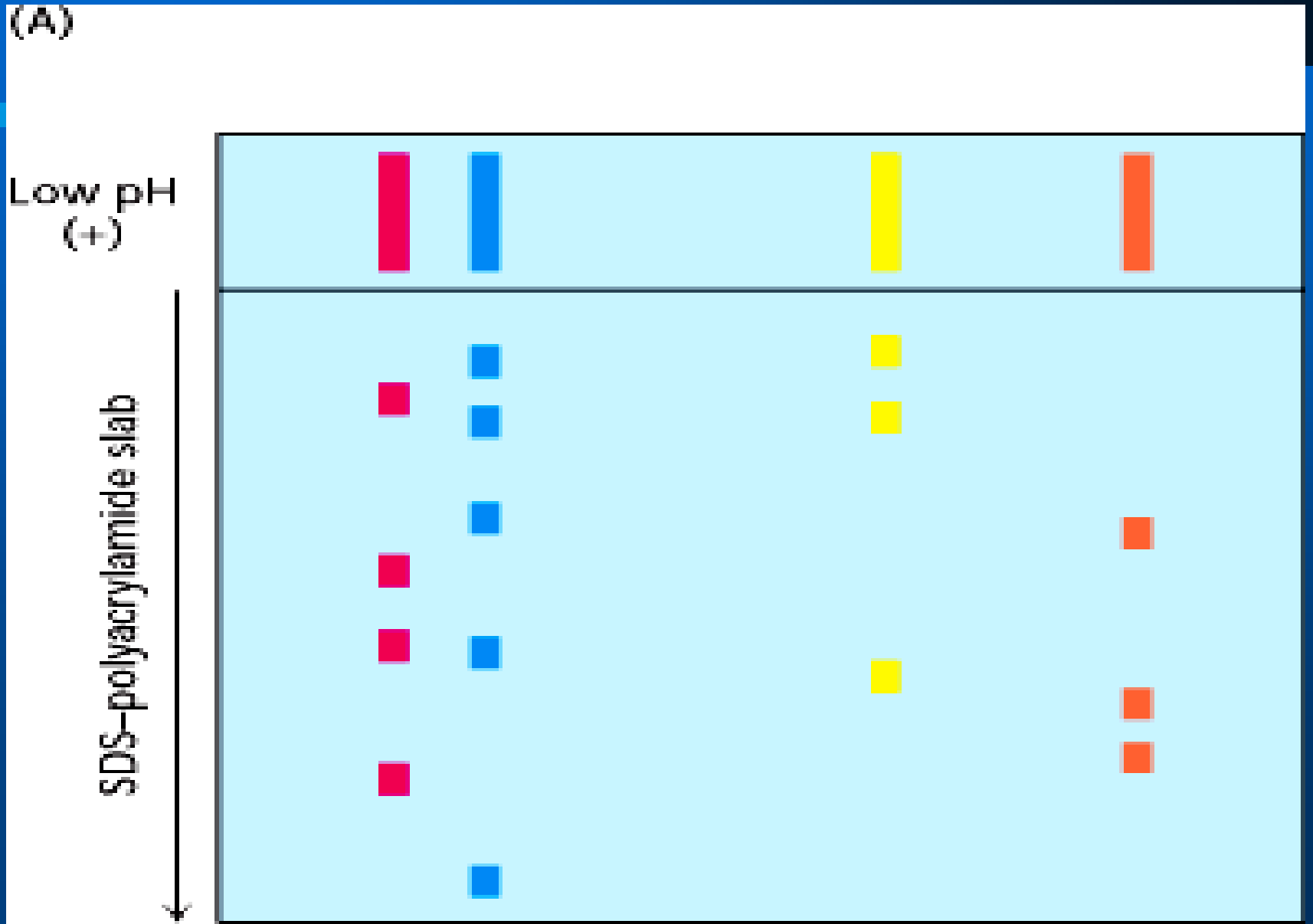
First dimension is generally IEF.

Second dimension is generally SDS-PAGE.

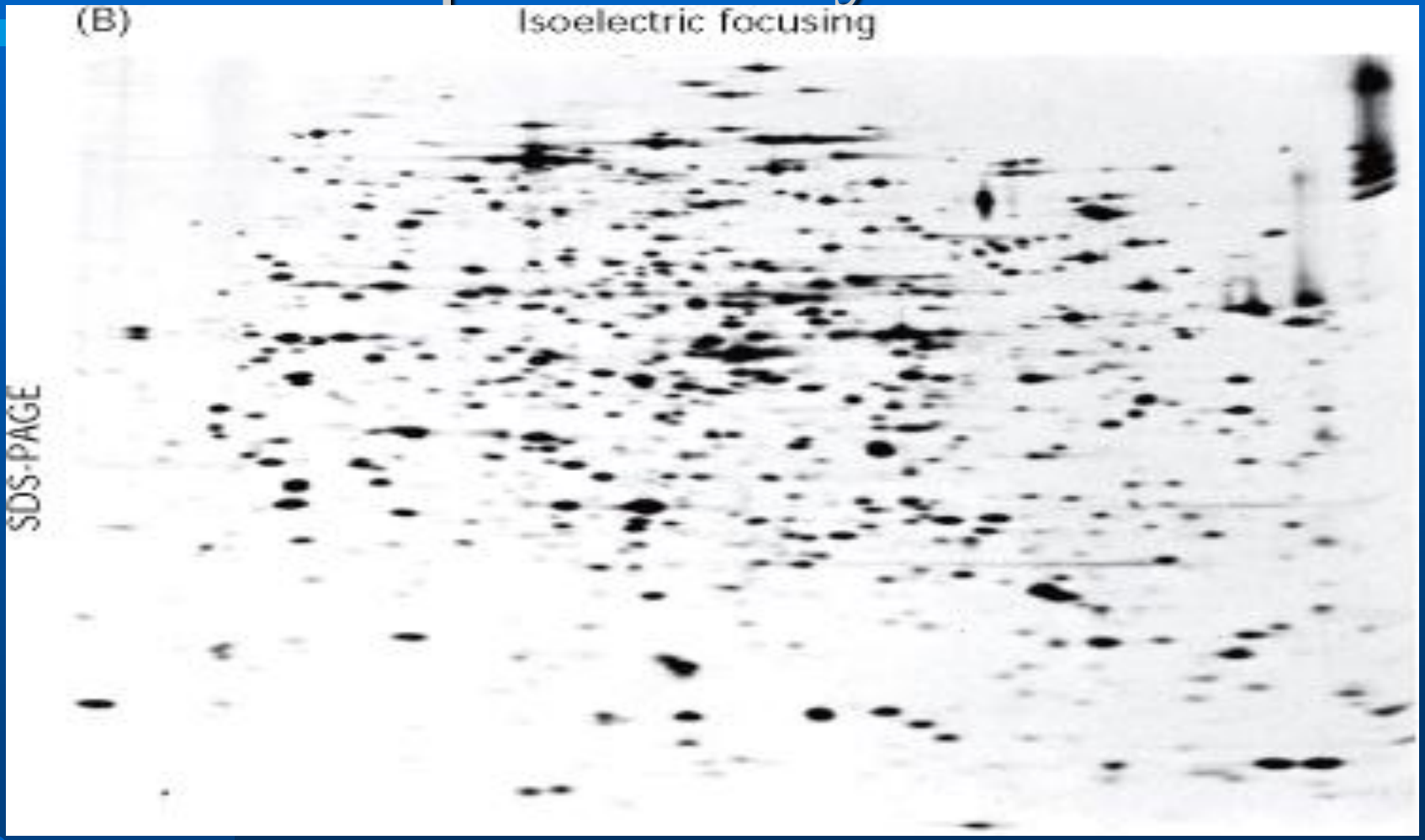
Few thousand proteins may be resolved in a single run.

Proteins have same molecular weigh or same *p*I are also resolved

2-D PAGE



Actual Separation by 2-D PAGE



Uses of 2-D PAGE

Analysis of complex mixture of proteins

Comparison of different physiological states of the same tissue

Stress exposed/control tissues

Different tissues behavior

Partial characterization of proteins

Molecular weight

Isoelectric point

Nature

Purification of proteins for further analysis e.g. sequencing/MALDI-TOF

Zymography

Zymography is an electrophoretic technique based on SDS-PAGE, that includes a substrate copolymerized with the polyacrylamide gel, for the detection of enzyme activity.

Samples are prepared in the standard SDS-PAGE treatment buffer but without boiling, and reducing agent.

After electrophoresis, SDS was removed by incubating the gel in Triton-X100.

The zymogram is subsequently stained (commonly with Amido Black or Coomassie Brilliant Blue), and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

Identification of Isoforms of α-amylase from seeds of halophytic grass, *Panicum turgidum*

Extraction Procedure

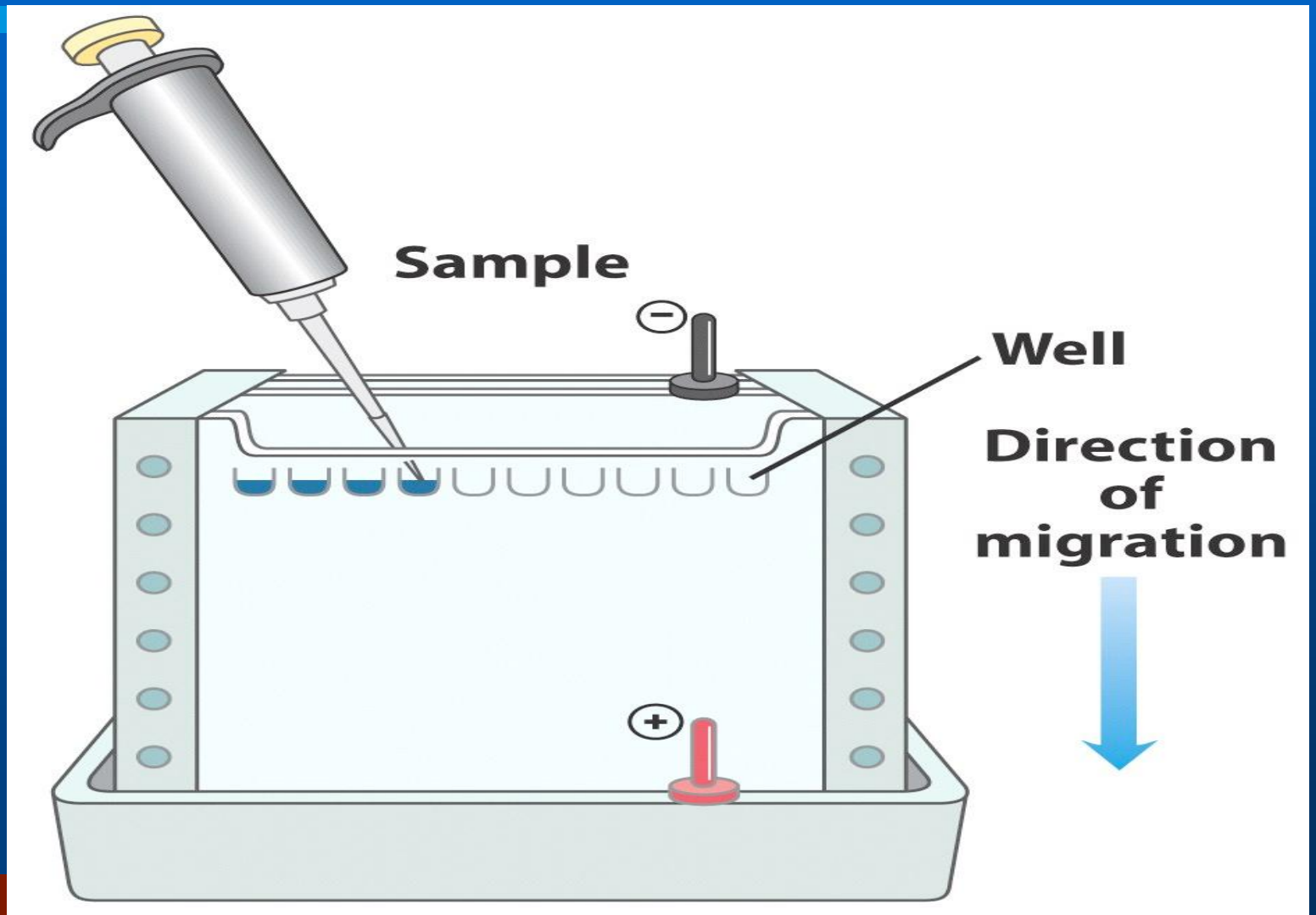
Seeds were crushed and homogenized in Phosphate buffer(50mM, pH 7.0) and centrifuged at 14000 r.p.m (4^{o}C) for 20 minutes to remove debris.

Enzyme assay

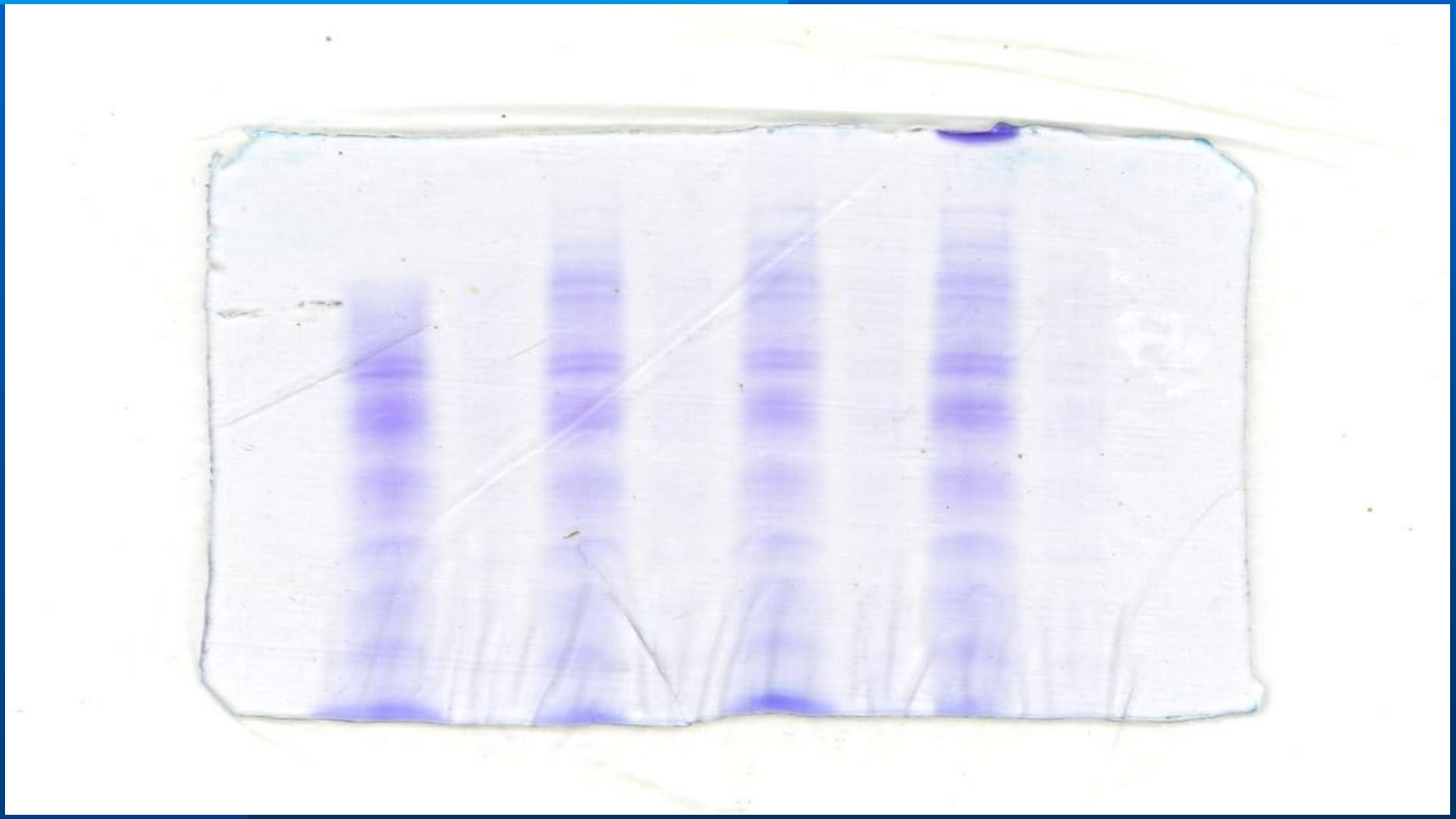
The activity of α-amylase in the extract was assayed by incubating 0.1 ml extract with 1 ml soluble starch (2 % w/v, prepared in 50mM Tris-HCl buffer of pH 7.0) at 60°C for 10 minutes.

(Ul Qader et al. 2009, Cordeiro et al. 2002)

Polyacrylamide gel electrophoresis (PAGE)



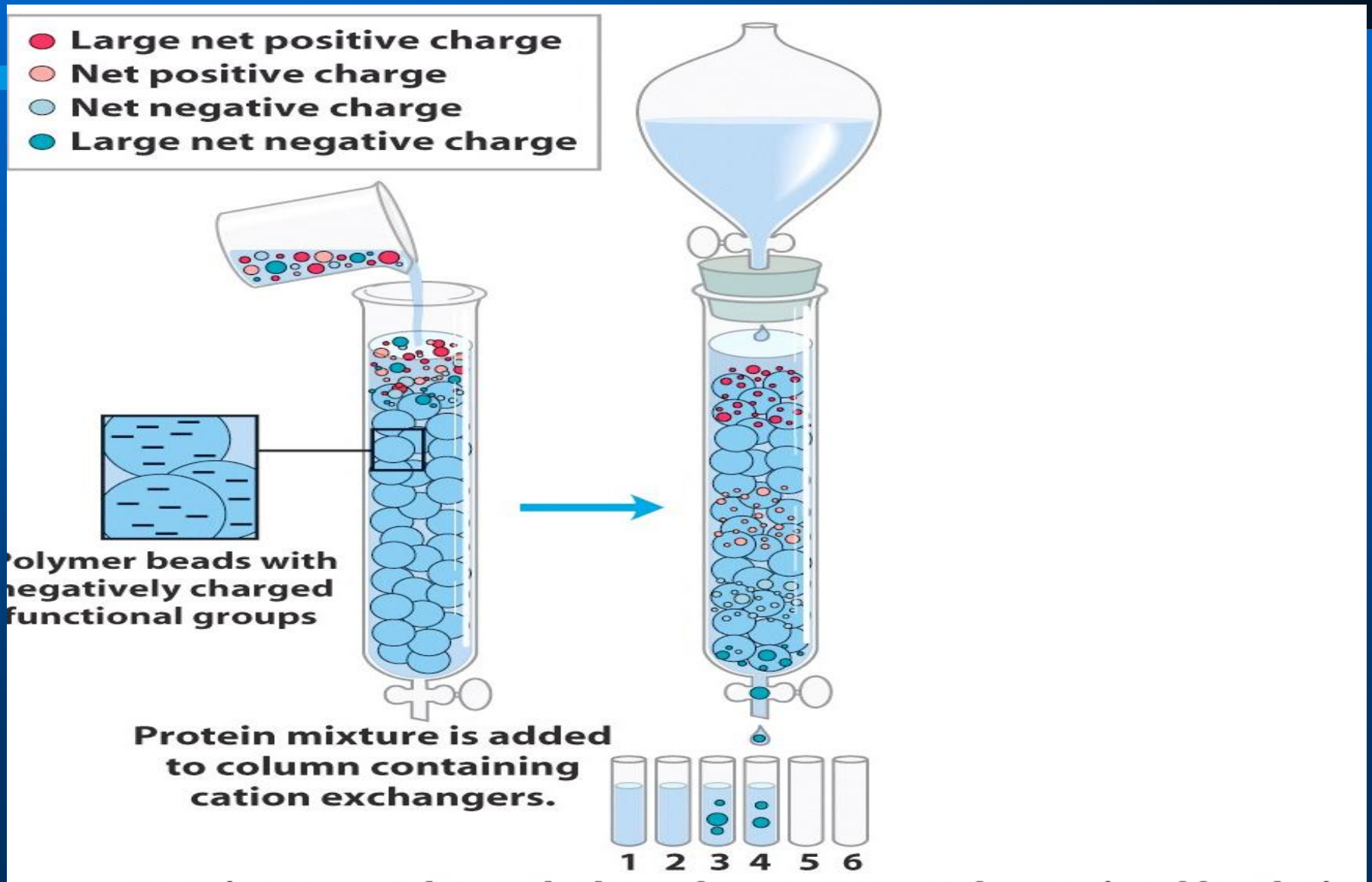
SDS-PAGE Profile of crude extract from seeds of *Panicum turgidum*



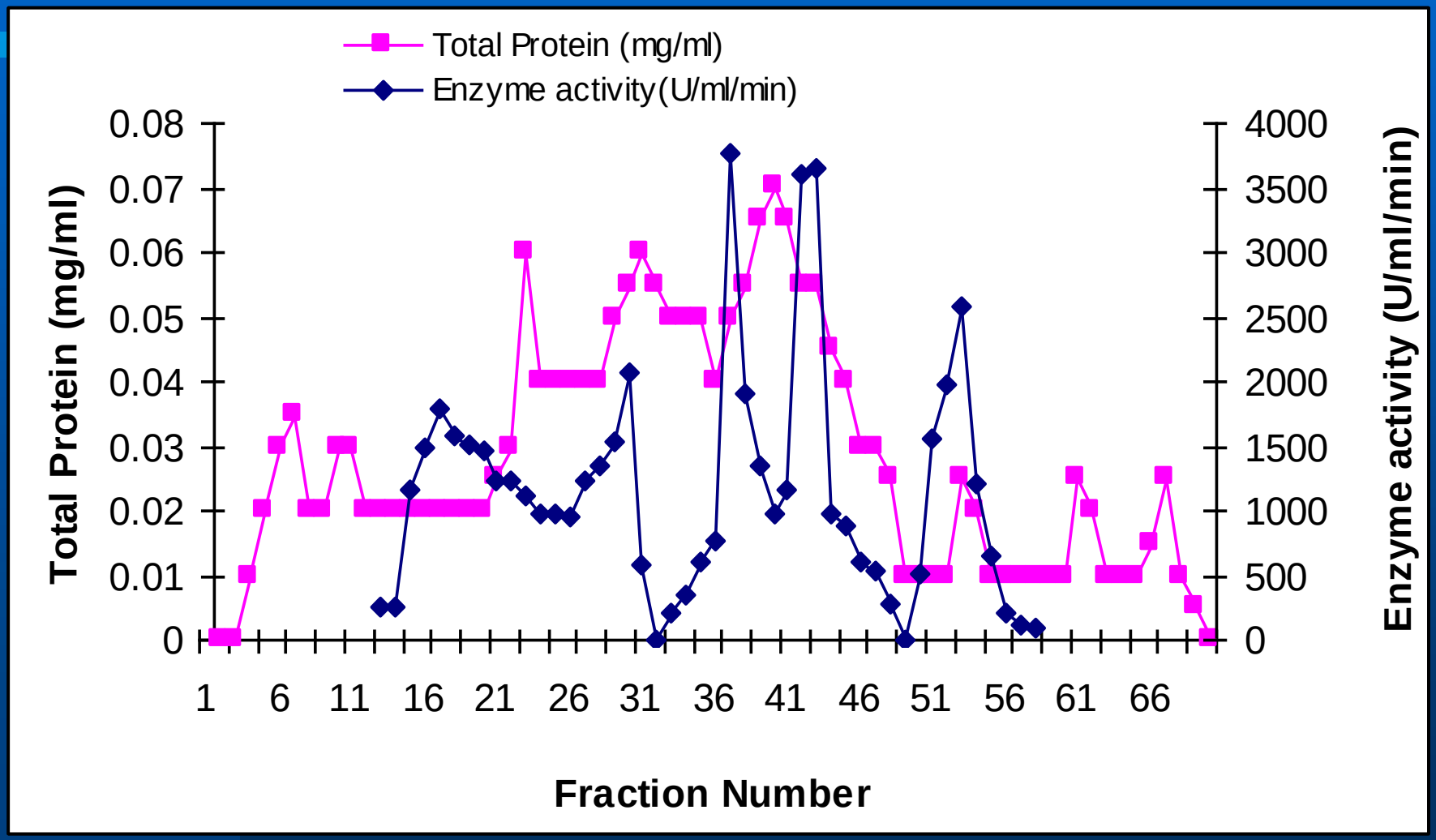
Purification of alpha amylase by gradient precipitation technique using Ammonium sulphate as a source

Percent Saturation	Total volume (ml)	Enzyme Activity (Units)	Total Protein (mg)	Specific Activity (U/mg)
Crude	8.0	3206	14.50	221
30%	0.5	80	1.29	62
40%	0.5	769	2.40	320
50%	0.5	593	2.68	221

Ion-exchange chromatography



Purification of amylase using Superdex 200



Zymography of the amylase

I →
II →
III →

Zymography of the alpha amylase from seeds of *Panicum turgidum* showing three Isoforms.

Future Plan

Complete purification

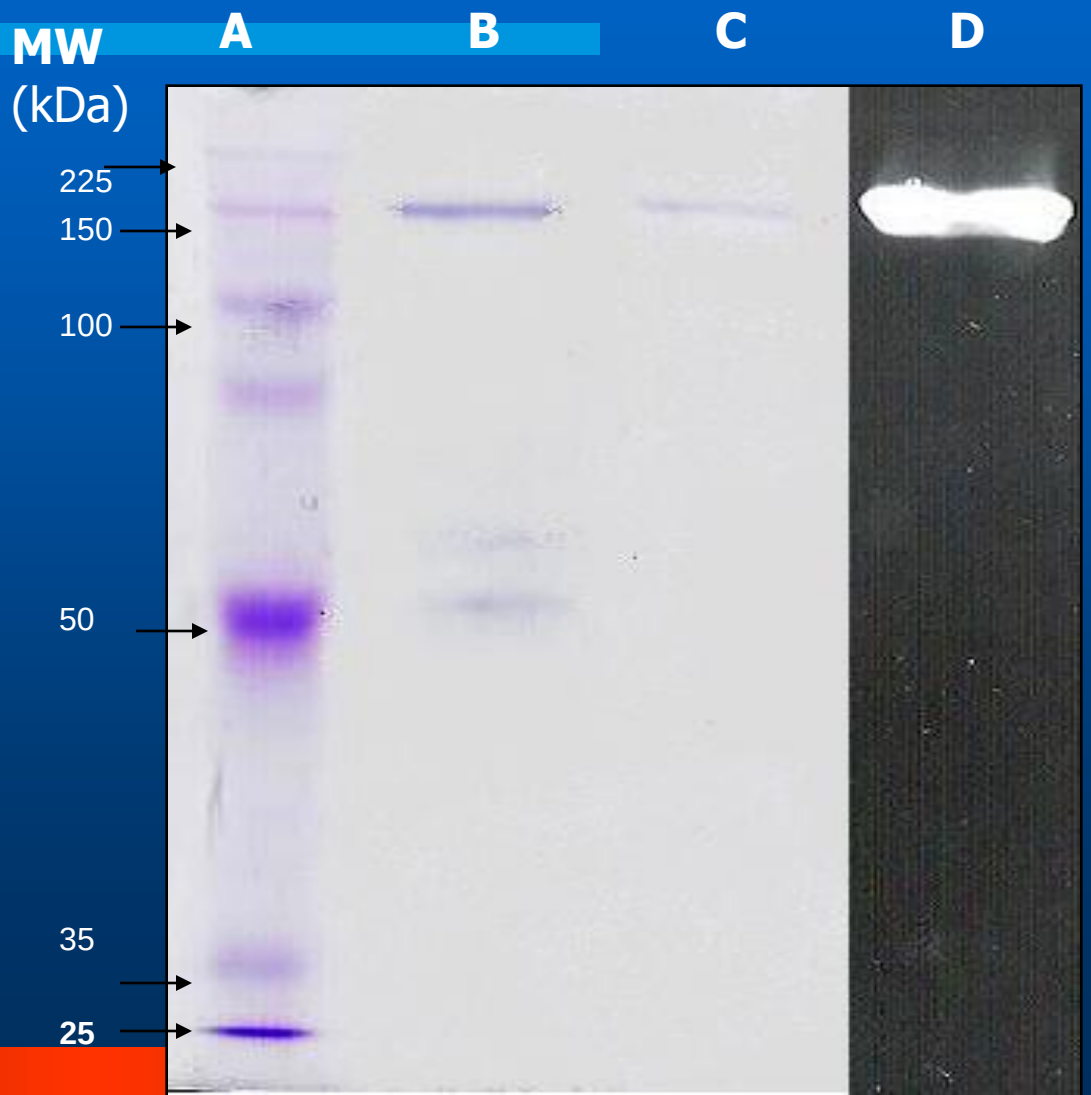
Purity to Homogeneity

Amino acid analysis

Protein sequencing

Isoforms of Alpha amylase

SDS-PAGE Profile of dextransucrase: Lane A, high molecular weight standards; Lane B, partially purified dextransucrase; Lane C purified dextransucrase (Coomassie blue staining); Lane D, assay for soluble Dextran-synthesis corresponding to lane C.



Role of Electrophoresis in Diagnosis of Different Diseases

The serum protein electrophoresis (SPEP) test measures specific proteins in the blood to help identify some diseases.

Blood serum contains two major protein groups:

Albumin and Globulin.

Using protein electrophoresis, these two groups can be separated into five smaller groups (fractions).

- **Albumin**
- **Alpha-1 globulin**
- **Alpha-2 globulin**
- **Beta globulin**
- **Gamma globulin**

Each of these five protein groups moves at a different rate in an electrical field and together form a specific pattern. This pattern helps identify some diseases.

Why It Is Done?

Serum protein electrophoresis is most often done to:

- **Screen for a disease such as multiple myeloma, macroglobulinemia or amyloidosis.**
- **Find the cause of hypogammaglobulinemia (HGG), a condition characterized by low levels of gamma globulin antibodies. HGG can make a person more susceptible to infection .**

Urinary Protein Electrophoresis

Electrophoresis on protein in urine may also be done, especially if the results of the serum protein electrophoresis test are abnormal. Normally very little protein is found in urine, but certain diseases (such as multiple myeloma) cause large amounts of protein to leak into the urine.

Although abnormal protein levels may be found in many conditions (such as kidney disease, chronic liver disease, systemic lupus erythematosus, rheumatoid arthritis, or leprosy), serum protein electrophoresis is usually not done to diagnose these conditions.

THANKS