Protein Extraction and Purification Methods Electrophoresis

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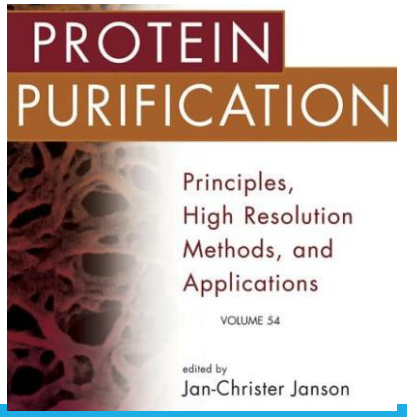


- The easiest way to carry out protein electrophoresis is in a gel, which serves as an anticonvective medium. The separation results are easily visualized by staining the gels with dyes, which specifically bind to proteins.
- Electrophoresis of proteins in gels has become a widespread separation technique, because it has a very high resolving power, is fast, easy to perform in relatively simply designed instruments, and the consumables do not cost very much. The principle of electrophoresis is also relatively easy to understand.

PRINCIPLE

- Each protein has a specific electrophoretic mobility, m, which determines its migration velocity, v, in an electric field E (measured in V/cm), and is therefore decisive for the separation:

$$v = m \times E.$$

- Electrophoretic mobility is dependent on the net charge and the size of the molecule. Proteins with different electrophoretic mobilities migrate in the electric field with different migration velocities and form discrete zones.

❖Because proteins are amphoteric molecules, they will have different net charges that depend on their pH environment.

❖Figure 15.2 presents a schematic of a net charge curve of a protein.

❖The shape of the curve and its intersection with the x-axis describe the behavior of a protein at a certain buffer pH value and in the electric field.

❖It should be mentioned that the net charge curves of proteins can be determined by a simple-to-perform electrophoretic technique called titration curve analysis (2), which is very valuable for ion-exchange chromatography (IEC, see Chapter 4) and isoelectric focusing..

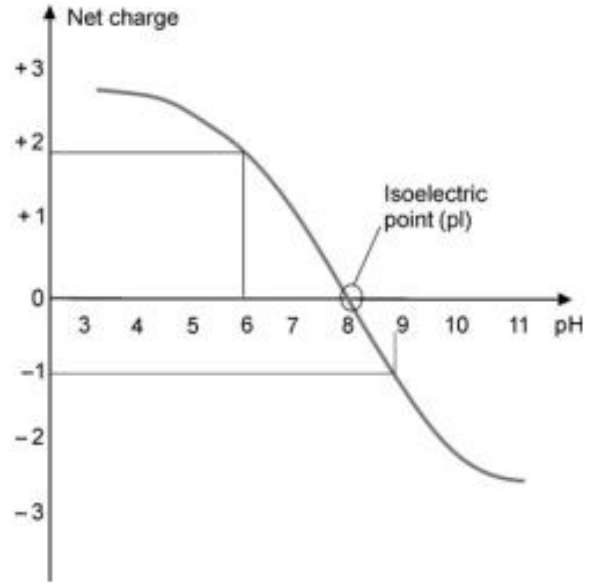


Figure 15.2 Schematic of a net charge curve of a protein. A protein with this net charge curve has an isoelectric point at pH 8, has two positive charges at pH 6, and one negative charge at pH 9.

There are several electrophoretic separation principles that can be applied on proteins: moving boundary electrophoresis, isotachophoresis, isoelectric focusing, and zone electrophoresis. This chapter deals only with zone electrophoresis methods (often called just electrophoresis). The separation principle of zone electrophoresis is shown in Figure 15.3.

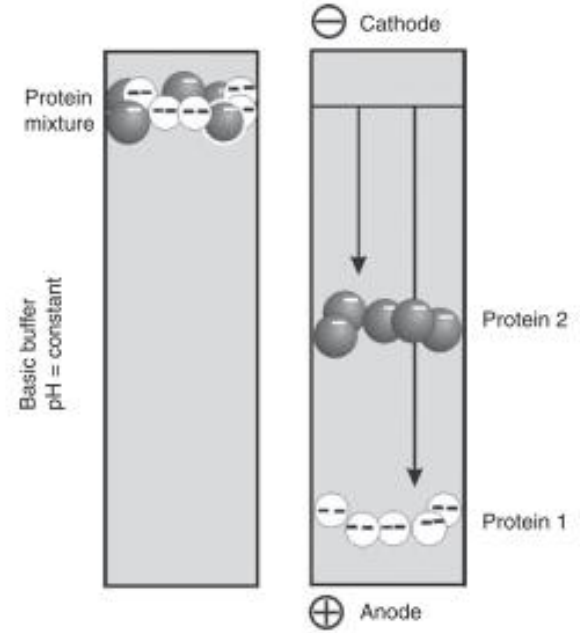


Figure 15.3 Separation principle of electrophoresis. Proteins with different net charges and sizes migrate in an electric field in the presence of buffer with different migration velocities. The different proteins form discrete zones.

- Polyacrylamide gels are predominantly used for protein electrophoresis; in some exceptional cases agarose gels are used.
- Agarose gels are used for protein electrophoresis in clinical diagnostics and when large pores for the analysis of large proteins over 800 kDa are needed. Agarose is a polysaccharide obtained from red seaweed.
- Polyacrylamide forms a much more restrictive gel, is mechanically and chemically more stable, has much lower electroendosmosis, and is clearer than agarose.

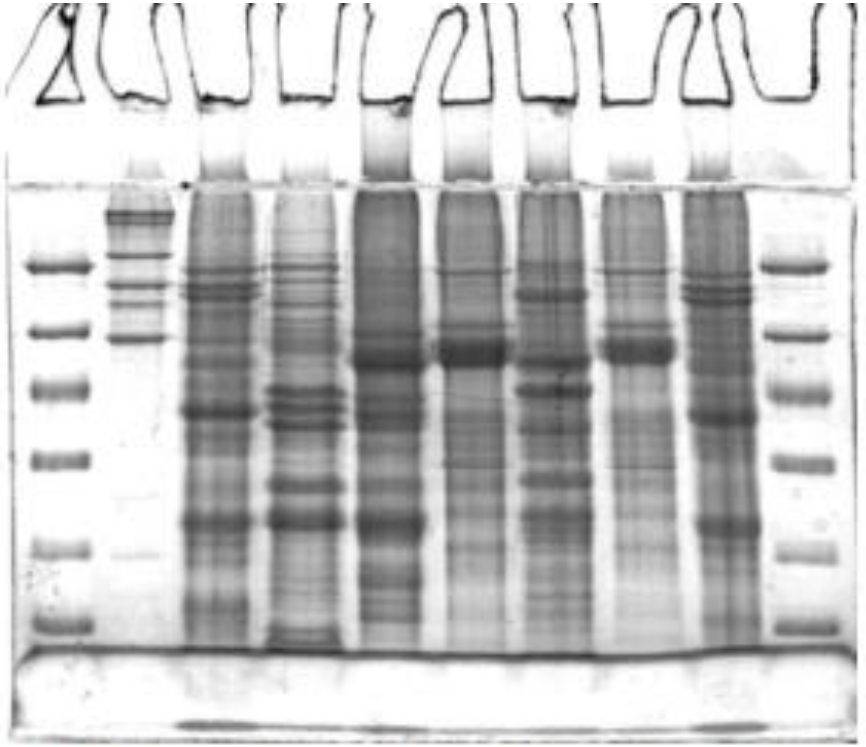


Figure 15.6 Vertical SDS polyacrylamide gel $T = 12.5\%$, $C = 3\%$. The two lateral lanes are low molecular weight markers from 14.4 to 94 kDa; the second lane from the left contains high molecular weight markers from 53 to 220 kDa. The other seven lanes contain buffer extracts of legume seeds. Hot Coomassie Brilliant blue staining.

❖Vertical slab gels are mostly polymerized between glass plates and lateral spacers, which determine the gel thickness. The gels are left in these cassettes from casting to the end of the run. The samples are loaded in slots on the top edge (Fig. 15.5B,C), which have been formed by inserting a comb during polymerization. Combs with different thicknesses and numbers of *teeth* are available.

❖Prefabricated gels are shipped in the cassette, including the inserted combs, and they are then inserted into the appropriate apparatus.

❖The upper and lower gel edges are in direct contact with the electrode buffers. To prevent mixing of the samples with the upper buffer, ≙20% glycerol or sucrose is added to the sample buffer in order to increase the density of the samples markedly.

❖In the simplest design for vertical electrophoresis (B) the gels cannot be cooled. The applied electrophoretic field has therefore to be limited accordingly.

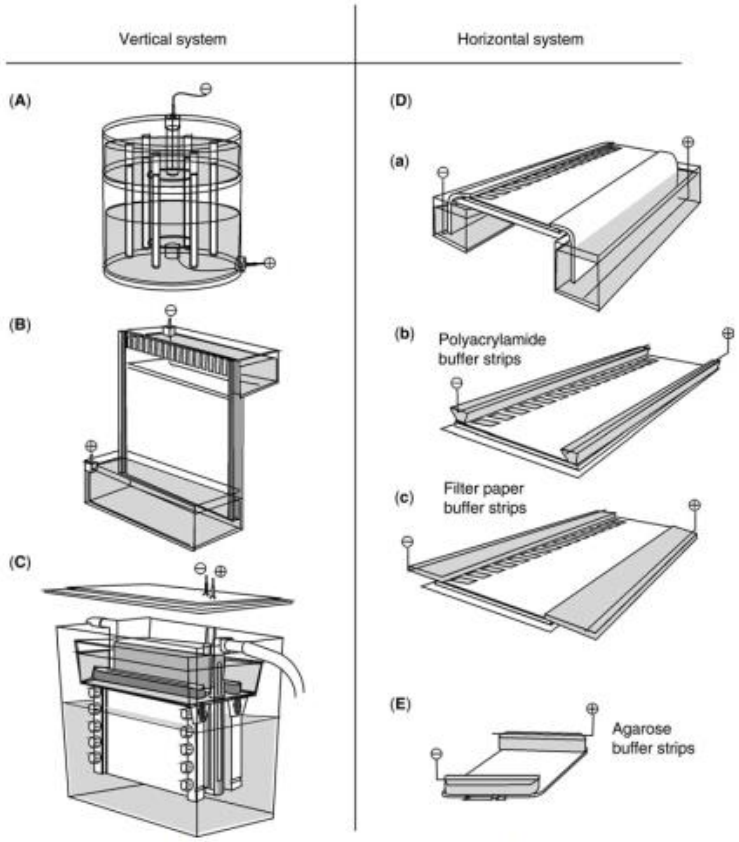


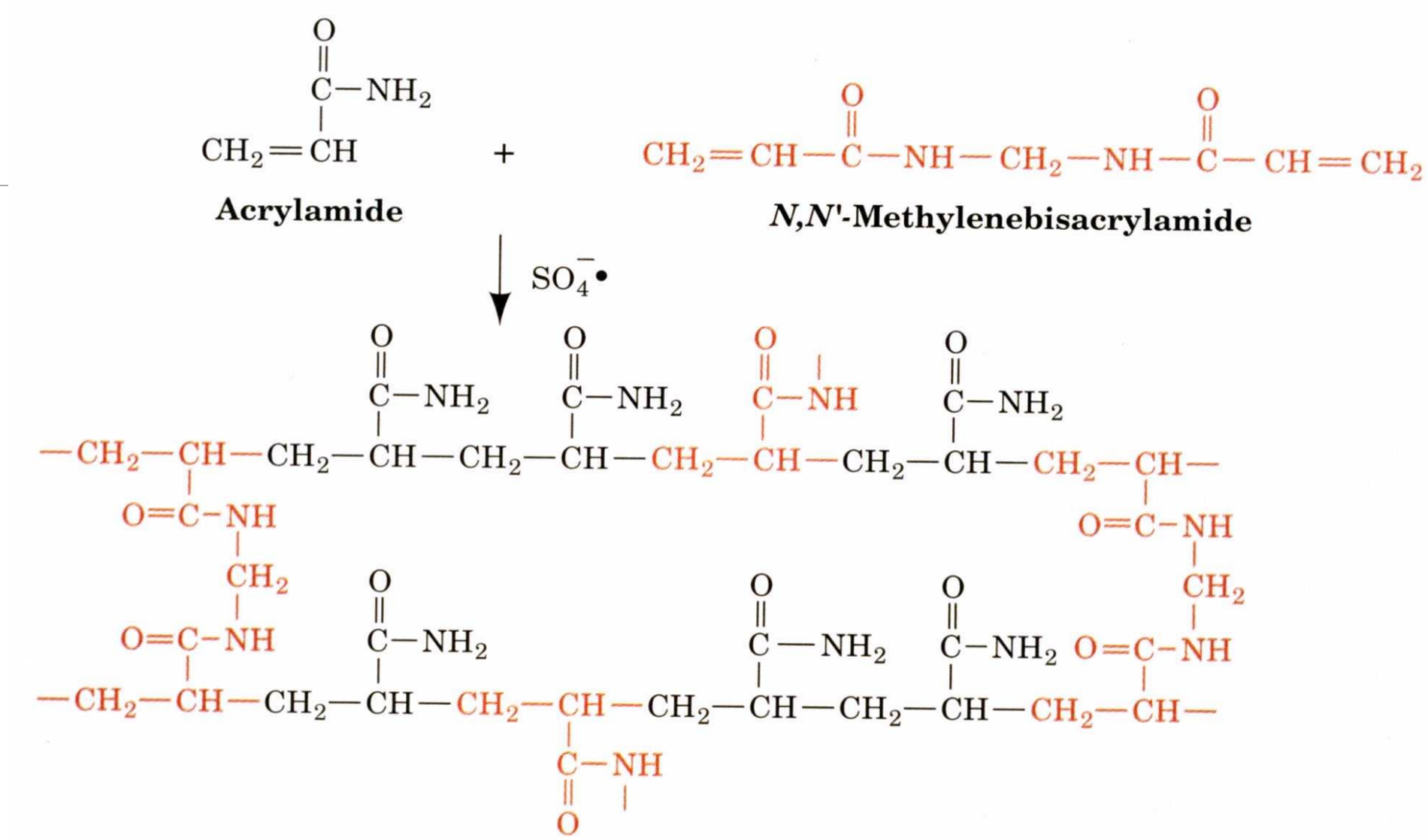
Figure 15.5 Schematic representation of different set-ups for protein electrophoresis in gels. (**A**) System for gel rods in glass tubes. (**B**) Vertical slab gel system without cooling. (**C**) Vertical system for two or four slab gels with cooling via the lower buffer. (**D**) Horizontal flatbed system with cooling plate (like the Multiphor™ II, GE Healthcare): (**a**) gel connected to buffer tanks via paper wicks; (**b**) disposable polyacrylamide buffer strips; (**c**) disposable filter paper strips soaked with buffer. (**E**) Horizontal minigels for the PhastSystem® with disposable agarose buffer strips.

Types of electrophoresis

1. Paper and cellulose acetate electrophoresis
2. Thin layer electrophoresis
3. Polyacrylamide gel electrophoresis (PAGE)
4. Agarose gel electrophoresis
5. Capillary electrophoresis

Gel Electrophoresis

- In this method, polyacrylamide is used as a support matrix. Acrylamide monomers covalently bond with crosslinker N, N'-methylene bisacrylamide to form a polymer.
- The polymerization process can be done chemically or photochemically. In chemical methods, ammonium persulfate and in photochemical methods, riboflabin is used.
- These act as free radical initiators. The formed radicals allow the acrylamide molecules to be linked together in the form of a chain.
- N, N, N',N’-tetramethylethylenediamine (TEMED) is used as catalyst in both applications. TEMED allows bisacrylamide to bind to the growing polymer chain. Thus, long chain polyacrylamide is polymerized with crosslinker bisacrylamide.


Acrylamide
N,N'-Methylenebisacrylamide
SO4−•

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide polymers obtained from acrylamide monomer are obtained. The porosity of polyacrylamide can be changed by changing the acrylamide content. PAGE method can be applied to separate native (natural) proteins. This technique is also used to determine the molecular mass by denaturing the proteins using sodium dodecyl sulfate (SDS). SDS-PAGE is a protein electrophoresis technique widely used for research in molecular biology. This technique also provides a much better separation than routine use in clinical laboratories. It also provides separation of a large number of subunits of proteins.

The gels used in gel electrophoresis according to the type of matrix can be starch, agarose or polyacrylamide. The matrix used in electrophoresis must be unloaded. If the matrix is loaded, the progression of the loaded biomolecules is prevented. As the concentration of the substance in the matrix increases, the pore diameter becomes smaller. According to pore diameters, the largest porous gel is agarose, then starch and the smallest porous gel is polyacrylamide.

Two factors are important in this process.

% T and% C

% T = Total amount of monomer

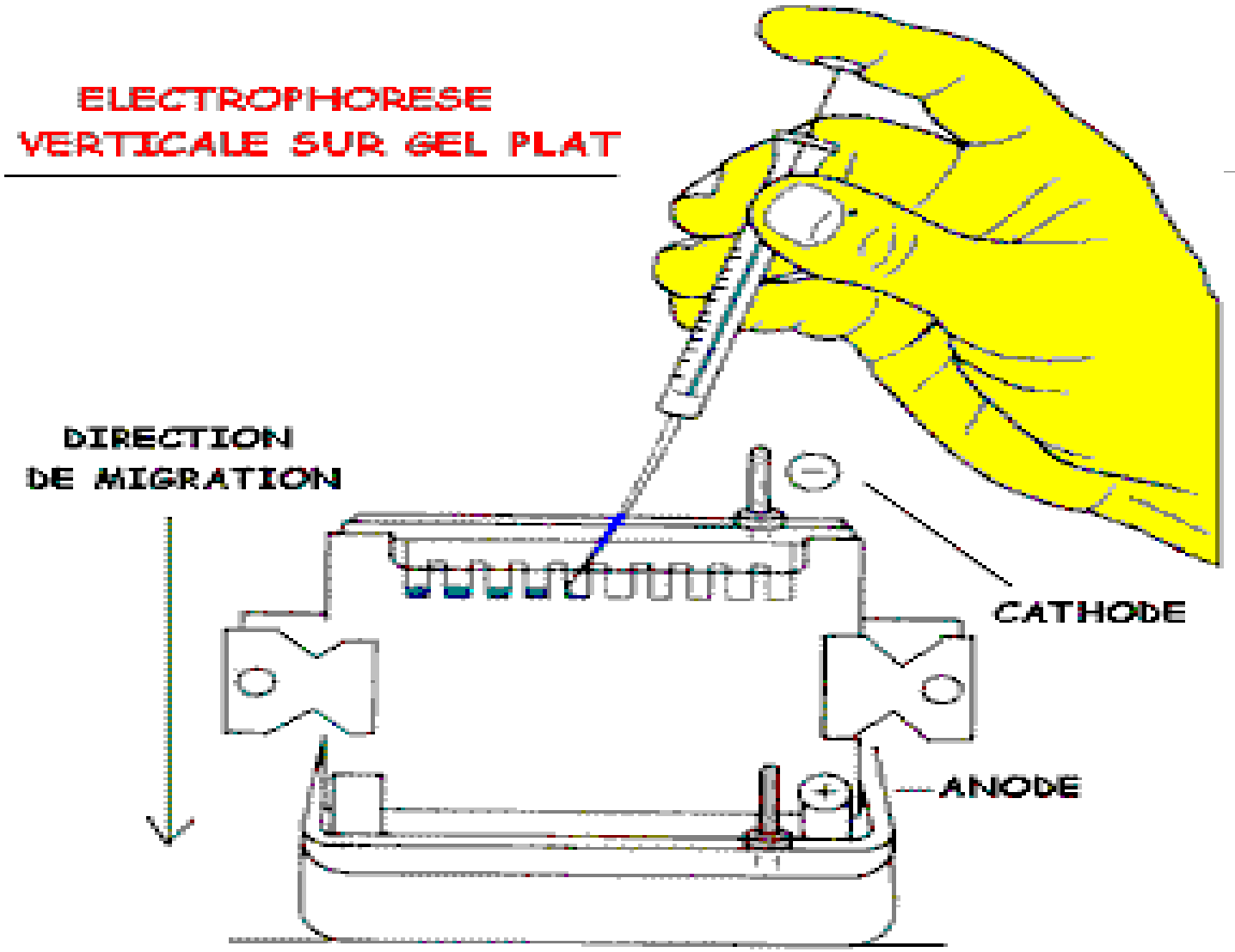
% C = Bisacrylamide amount in total monomer (Cross-link ratio)

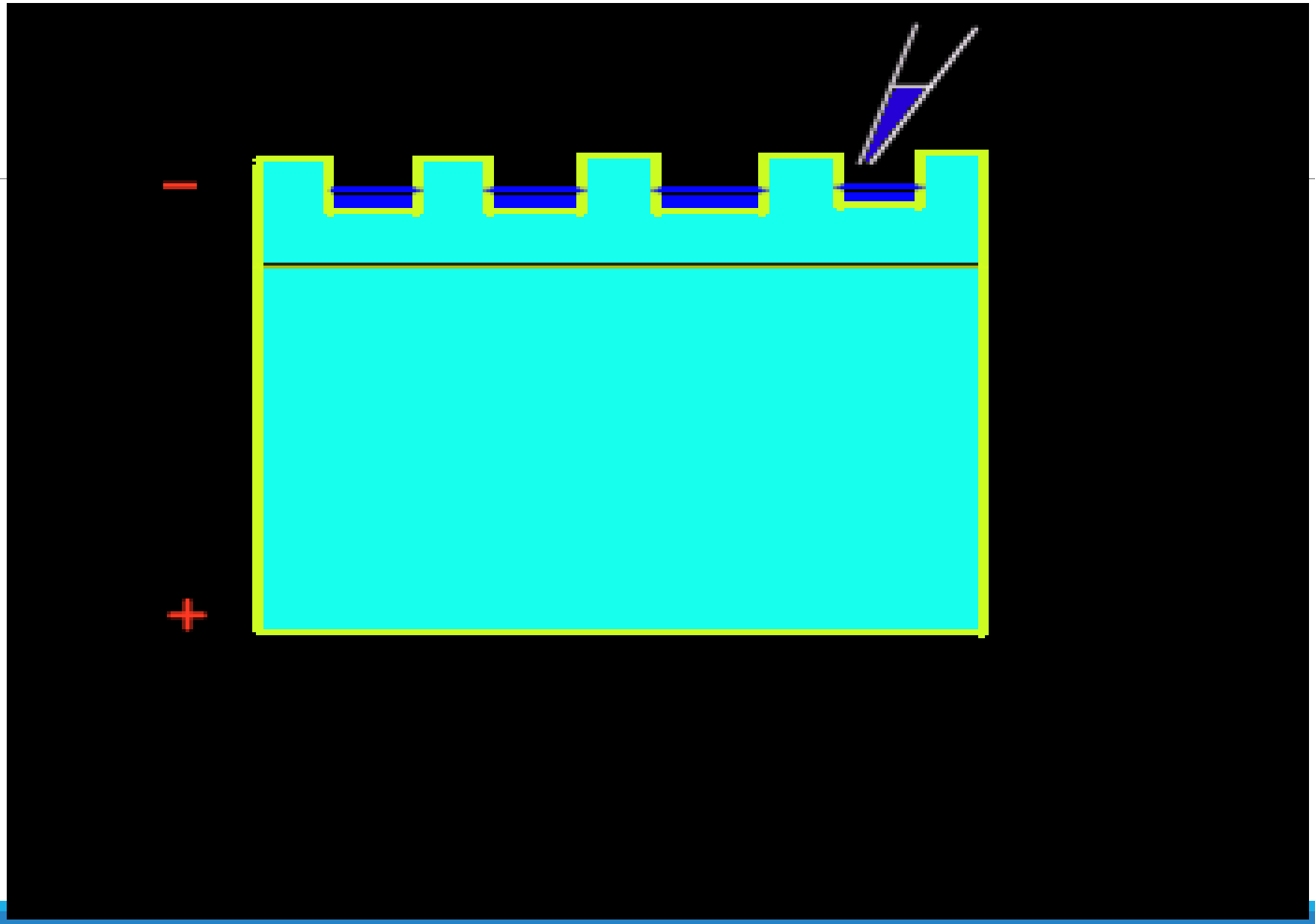
$$\% C = \frac{\text{bisacrylamide (g)}}{\text{acrylamide (g)} + \text{bisacrylamide (g)}} \times 100$$

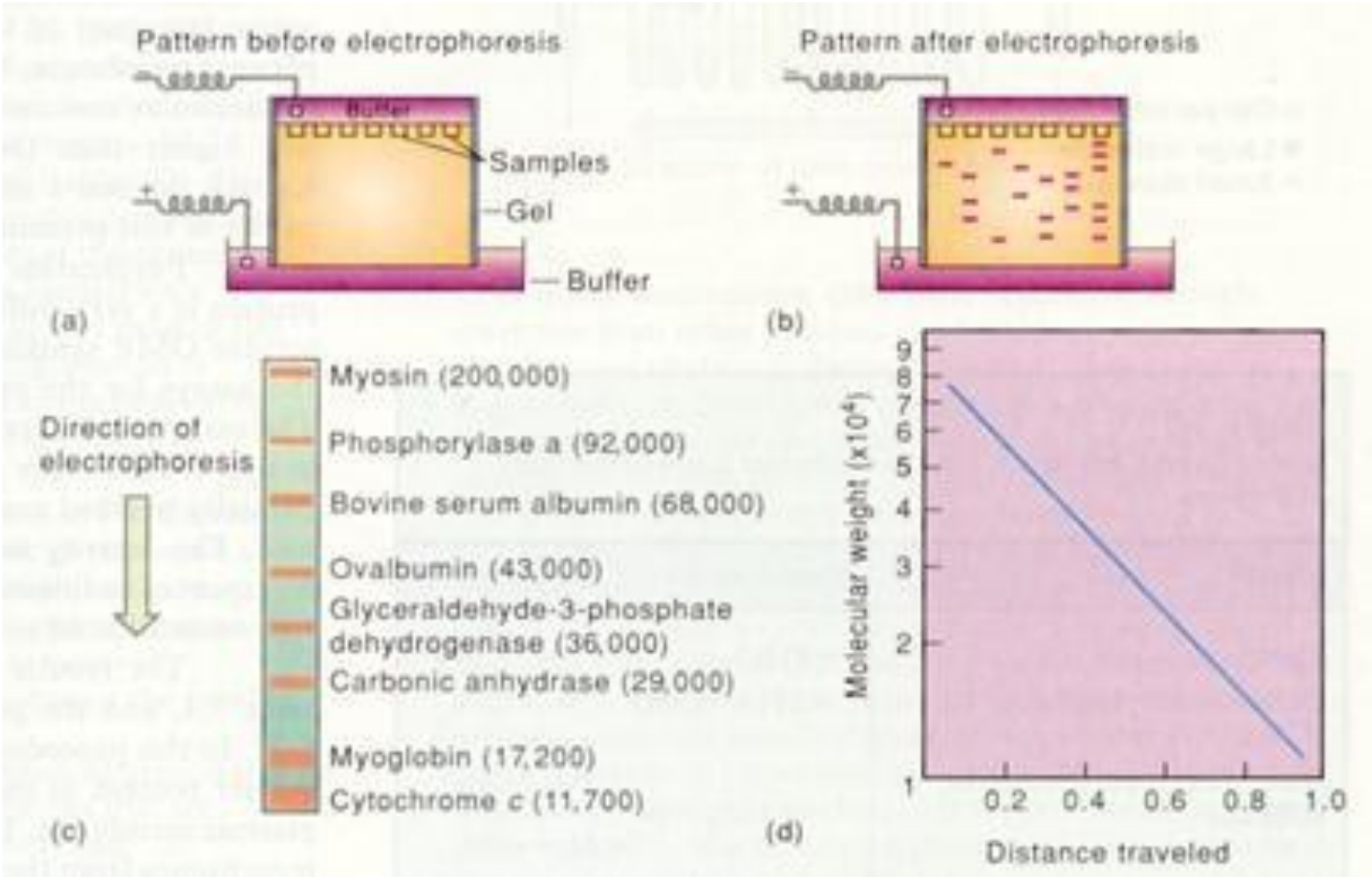
- The more crosslinked the acrylamide units, the greater the% C, the tighter the gel. The smallest pore diameter should be% C, around 5%.
- In this method, the monomer prepared according to the purpose is filled between two glasses or gel tube and left to polymerization after the necessary substances are put. When the gel mixture is poured into the tube or cassette, a curved surface is formed due to the tension on the top surface. Since this leads to distorted tape dispersion, a thin layer of n-butanol must be formed before starting polymerization.

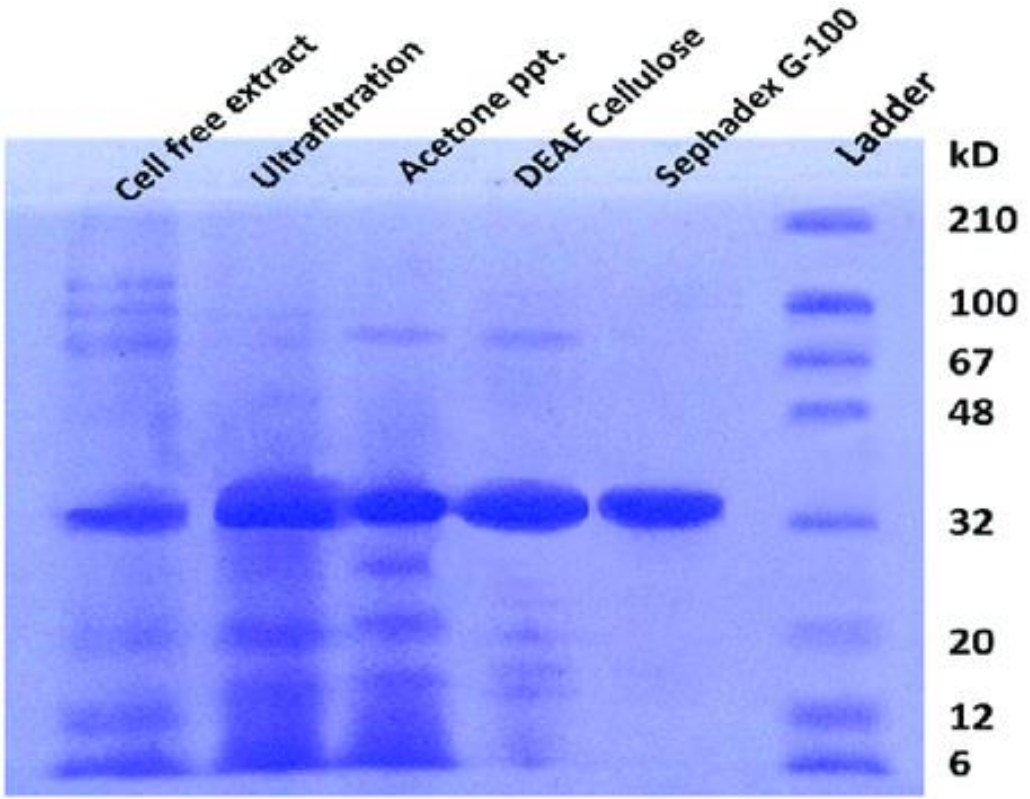
- There are two different systems in gel electrophoresis, continuous and discontinuous.
- In the batch system, there is a second gel on the bottom gel.
- After the lower gel is polymerized, the upper gel is placed and the comb is placed. After the comb is removed after completion of the polymerization, voids are formed in the upper gel. The upper gel loading gel (stacking gel) is large pores, the lower gel separating gel (seperating gel) is small pores.
- The applied sample first passes through the big porous loading gel, then passes through the sub-gel and separation is achieved.
- The two parts in the batch system are prepared at different concentration and pH. This technique is the most widely used form of PAGE. The continuous gel contains a single gel.

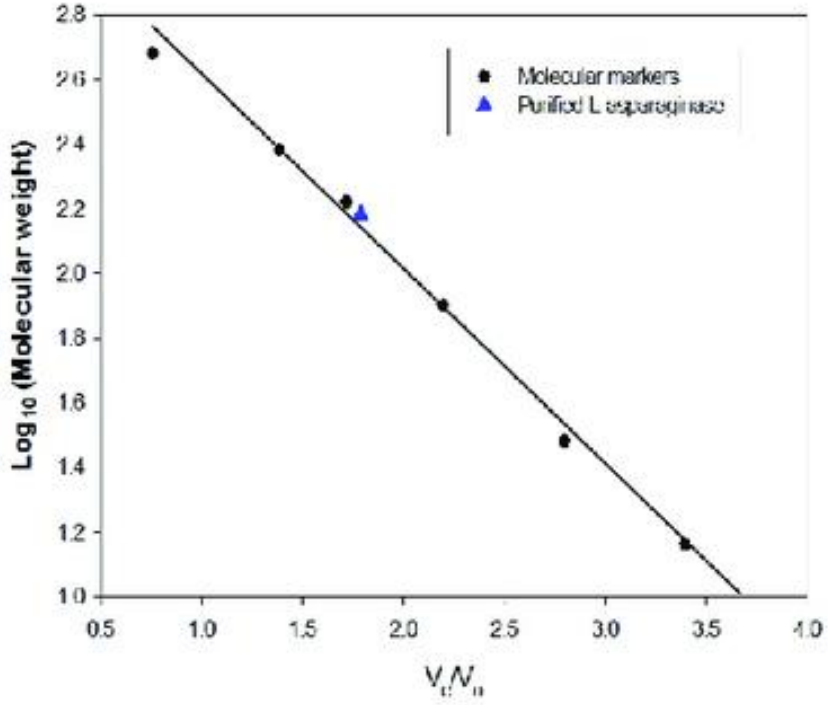
ELECTROPHORESE VERTICALE SUR GEL PLAT

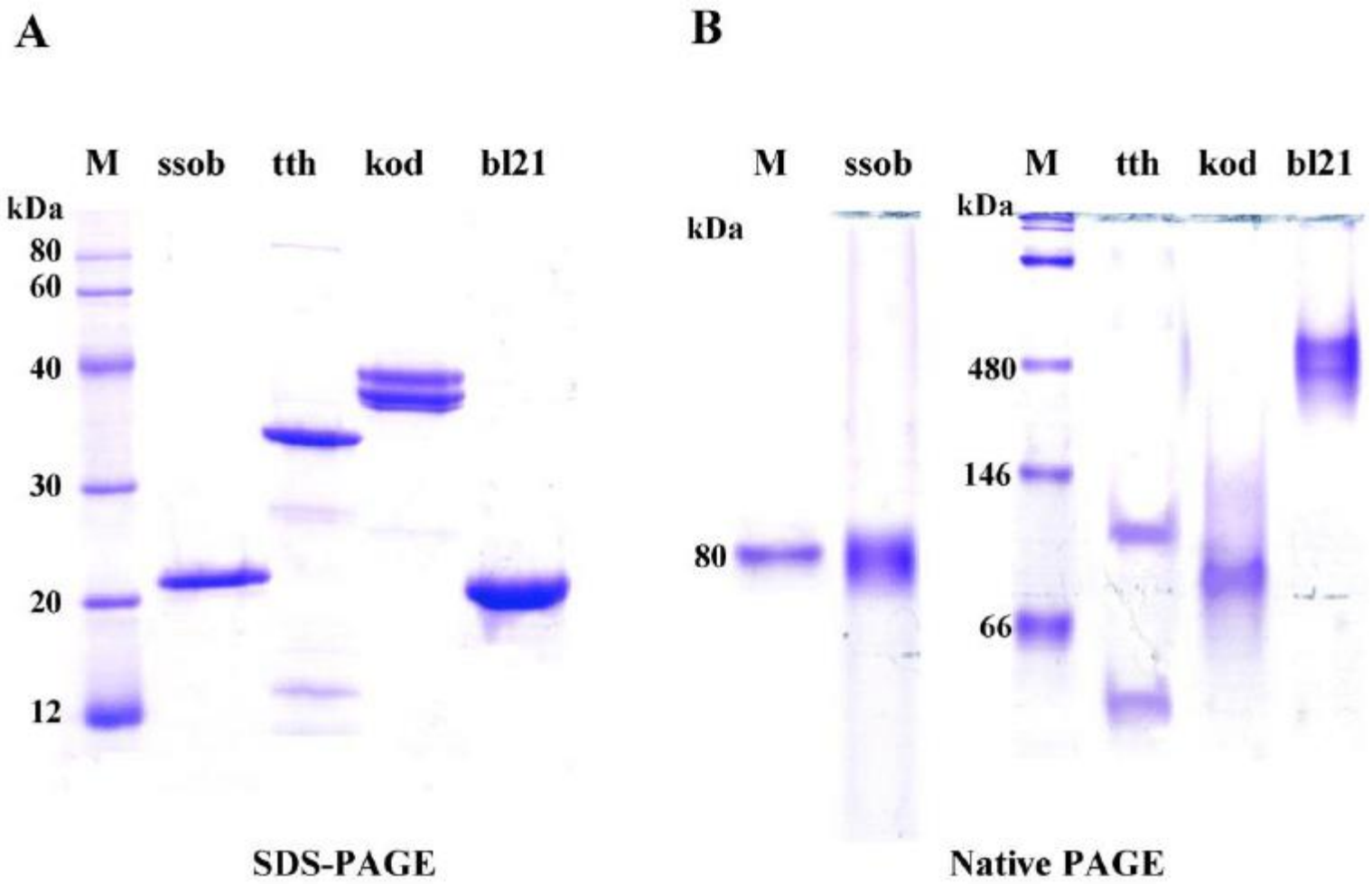





Pattern before electrophoresis
Buffer
Samples
Gel
Buffer
(a)
Pattern after electrophoresis
(b)
Direction of electrophoresis
Myosin (200,000)
Phosphorylase a (92,000)
Bovine serum albumin (68,000)
Ovalbumin (43,000)
Glyceraldehyde-3-phosphate dehydrogenase (36,000)
Carbonic anhydrase (29,000)
Myoglobin (17,200)
Cytochrome c (11,700)
(c)
Molecular weight (x10⁴)
9
8
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
Distance traveled
(d)


Cell free extract
Ultrafiltration
Acetone ppt.
DEAE Cellulose
Sephadex G-100
Ladder
kD
210
100
67
48
32
20
12
6


Molecular markers
Purified L asparaginase
Log10 (Molecular weight)
Ve/Vo
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0


A
M
ssob
tth
kod
bl21
kDa
80
60
40
30
20
12
SDS-PAGE
B
M
ssob
kDa
80
M
tth
kod
bl21
kDa
480
146
66
Native PAGE