Electrophoresis of Serum Proteins

Properties of Proteins

Practical Lesson on Medical Chemistry and Biochemistry

General Medicine

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2010/2011
1 Electrophoresis of serum proteins in 0.5% agarose

Principle:
Native electrophoresis of serum proteins in agarose gel is still one of the basic examinations in clinical chemistry, and in our practical lesson serves as a general example of electrophoretic separation of proteins. In this arrangement of electrophoresis the proteins are native, i.e., not denatured. In an alkaline buffer (pH 8.5-9) they gain negative charges and migrate from the negative electrode to the positive one. The support consists of an agarose gel, which, unlike acrylamide, has pores too large to substantially limit the protein movement. As a result, the proteins are separated according to their surface charge densities. If resolved in this way, the human serum yields several classical protein fractions: albumin moves the farthest, followed by several globulin bands, denoted consecutively as α1, α2, β (usually separated to β1 and β2), and finally γ globulins.

![Typical results of an authentic electrophoretic separation of human serum proteins, including densitometric evaluation (assembled from pictures available at www.sebia.com).](image)

The whole experiment consists of several steps:
- Casting the agarose gel: agarose is a polysaccharide galactan obtained from seaweed. In order to get a liquid agarose solution, the mixture of buffer and solid agarose must be heated to boiling, then during cooling the agarose fibers in the solution non-covalently associate and form a gel. Work with agarose is facilitated by an interesting phenomenon of hysteresis – the temperature at which the solution solidifies to the gel is much lower than the temperature needed for gel dissolution.
- Serum that should be analyzed is diluted 10-times. Glycerol is added to increase the sample density, which facilitates sample application. It is also spiked with Bromophenol Blue, an anionic dye that migrates ahead of proteins during electrophoresis; in this way the course of electrophoresis is visualized.
- Application of serum samples into the wells in agarose gel under the surface of electrophoretic buffer. The actual electrophoretic separation of proteins follows.
- Fixation in the mixture of methanol and acetic acid denatures protein molecules and prevents diffusion of resolved protein fractions after the electrophoresis.
- Staining: protein bands in the gel are visualized by means of a suitable organic dye that non-covalently binds to proteins. The dyes employed most frequently for this purpose are Coomassie Brilliant Blue or (like in our experiment) Amido Black.
• Destaining: the excess of the dye is removed from the gel in order to provide a clear background on which the protein bands are visible.

• Evaluation of the resulting electrophoreogram can be either visual (qualitative) or densitometric (quantitative). Our results will be evaluated only visually. However, if this electrophoresis is performed in clinical chemistry, intensity of the stained protein bands is also measured with a densitometer. In principle, it is based on photometry: the instrument continuously reads the absorbance of the sample lane. The areas under the peaks of the resulting absorbance curve are proportional to the amount of protein in the bands (see figure above).

Reagents and tools:
1. Agarose for electrophoresis
2. Electrophoretic buffer: sodium barbital 5.5 g/l citric acid 0.25 g/l, pH 8.7-9.0.
3. Human serum diluted 10x with 0.5 % Bromophenol Blue in 30 % glycerol
4. Fixing/destaining solution: acetic acid-methanol 1:9
5. Staining solution: 0.5% Amido Black 10B in acetic acid-methanol 1:9
6. Power supply for electrophoresis
7. Horizontal electrophoretic chamber
8. Containers for fixation, staining and destaining

ATTENTION: The voltage and electric current regularly used in electrophoresis is more than sufficient to cause a serious injury!!!
Take all necessary care, especially avoid liquid spills around the electrophoretic chamber under voltage, before any manipulation with the chamber first switch off the power supply!

Procedure (demonstration):

a. Pour 50 ml of the electrophoretic buffer over 0.25 g of agarose in an Erlenmeyer flask, insert a stirring bar, close with alumina foil, and heat until boiling with continuous stirring. This amount of agarose is for two gels.

b. As soon as the solution starts boiling, switch off heating, and allow the dissolved agarose to cool down to less than 60 °C (possible to hold the flask with bare hand).

c. Assemble the electrophoretic equipment for pouring: insert the silicone sealings to grooves on both sides of the gel tray, and position the tray into the chamber so that the sealings face the chamber side walls.

d. Check that the chamber is in horizontal position and pour 25 ml of dissolved agarose. Insert (only) one comb to one side of the tray.

e. Allow the gel to solidify for at least one hour. Do not move the chamber during this period.

f. Both the electrophoretic chamber with gel and the electrophoretic buffer should be pre-cooled in a refrigerator before the experiment.

g. Re-set the chamber for electrophoresis: Take the tray out from the chamber and remove the silicone sealings. Rotate the tray 90° and reinsert to the chamber so that the sample wells are placed above the color strip on the chamber floor.

h. Pour about 250 ml of the ice-cold electrophoretic buffer over the gel, very carefully remove the comb (the wells must not break!) and allow the gel to equilibrate for about 5 minutes.
i. Application of serum samples to the wells: under the buffer surface, 10 µl per well, using an automatic pipette with a yellow tip.

j. Close the chamber and connect it to the power supply. Set the voltage to 100 V and let the electrophoresis proceed until the Bromophenol Blue reaches about 0.5 cm from the gel edge (lasts about 30 minutes).

k. Switch off the power supply, disconnect the cables and remove the tray.

l. Push the gel off the tray to the first portion of fixing solution. Fixation 3 x 5 minutes, always in fresh batch of fixing solution. The used solution should be removed by aspiration rather than simple pouring out as the gel is soft and prone to mechanical damage.

m. Stain the dry gel in the Amido Black solution for about 30 minutes.

n. Destain the gel background in several portions of destaining solutions, about 1-2 hours (check the progress of destaining). Store the gel in distilled water at 4 °C after destaining.

o. Evaluate the resulting electrophoreogram: make a drawing into your laboratory notebook and attempt to identify the visible protein bands.

2 Reactions of proteins: Biuret reaction

Principle:

Proteins give a characteristic violet color when mixed with copper(II) ion in alkaline solution. The color change results from complexation of copper(II) by nitrogen atoms of adjacent peptide bonds:

The reaction depends on the presence of peptidic bonds, not on the properties of amino acid side chains, hence, all proteins indiscriminately react. In general, any substance that has at least two adjacent groups -CO-NH₂ (amide) or at least two peptide bonds -CO-NH-, will give the reaction. Thus, the simplest reacting compounds are oxamide H₂N-CO-CO-NH₂, or biuret (bis-urea, dimer of urea) that gave the reaction its name:
The biuret reaction is still commonly employed as a quantitative assay for protein in a biological sample, for instance a patient’s serum.

N.B.: The reaction is called after biuret because this compound also reacts. However, when the biuret reaction is used to measure proteins, which is the commonest case, the compound biuret as such is actually not used at all, since it is neither in the sample nor in the reagent.

**Reagents:**

1. Sodium hydroxide, 2 mol/l (from the basic set)
2. Copper sulfate 70 g/l (from the basic set)
3. Solution of egg white protein (2 egg whites separated from yolks and shaken in 1 l saline)

**Procedure:**

a. Pre-dilute the copper(II) sulfate solution from your set of chemicals: to about 2 ml of distilled water add CuSO₄ drop wise to a weak blue color.

b. Combine about 1 ml of egg white solution with about 1 ml of sodium hydroxide solution. Then add several drops of the diluted copper(II) sulfate. Notice the color change (best seen against a white background).

## 3 Dialysis

**Principle:**

Molecules of proteins in solution can be separated from low-molecular-mass substances by natural or artificial membranes that allow diffusion of small molecules but not proteins. This process of differential diffusion through membranes of a limited permeability is called **dialysis**. The membranes suitable for this purpose are called **semi-permeable**, the cellophane used most widely in a laboratory for this purpose allows penetration of molecules up to MW 10,000 (so called cut-off value). In the body, in fact, all the cellular and subcellular membranes are semi-permeable in complex ways, i.e. they allow flow of water and small molecules to various degrees, but not flow of proteins. In research laboratory, dialysis is a simple method of choice if, for instance, the task is to remove excess of salt from a protein solution, or to transfer proteins into a different buffer. In clinical practice, a widely used method of kidney function replacement is called **haemodialysis**, since it is based on the same principle: blood of a patient flows along special semi-permeable membranes that allow equilibration with the solution at the other side of the membrane (and removal of the waste metabolic products) while plasma proteins and blood cells are retained.

This quick and simple experiment demonstrates the phenomenon of limited membrane permeability: a cellophane tubing is filled with an albumin solution combined with a small amount of copper(II) sulfate. The closed cellophane bag is then immersed into sodium hydroxide solution. Concentrations of small ions on both sides of the membrane gradually equilibrate, while albumin cannot pass the semi-permeable cellophane and (together with the copper ions likely bound to albumin) stays inside the bag. A visible color change is caused by hydroxide ions diffusing into the bag where they meet the albumin and the biuret reaction occurs (complex of Cu(II) with peptide bonds in alkaline medium). On the other hand, sulfate ions leave the bag and enter the dialyzing solution outside where they can be (after sufficient time period) proven with the sulfate ion reagent – barium(II) ions.
Reagents and tools:
1. Albumin solution for dialysis containing:
   - Bovine serum albumin 20 g/l
   - KI 90 mmol/l
   - Sodium-potassium tartrate 95 mmol/l
   - Na$_2$SO$_4$ 250 mmol/l
2. Copper sulfate 70 g/l (from the basic set)
3. Sodium hydroxide 1 mol/l (Do not confuse it with the NaOH solution from the basic set!!)
4. Barium nitrate 60 g/l (from the basic set)
5. Cut cellophane tubing
6. Dish with distilled water for wetting the cellophane
7. Needle for opening the cellophane tubing

Procedure:

a. Fill a beaker with sodium hydroxide solution 1 mol/l (do not use the NaOH from your set of chemicals!). Put a small portion of this solution aside into a test tube for future analysis.

b. Dip a piece of cellophane tubing into a dish with distilled water to soften it, open it at one end with a needle and make a tight knot on the tubing to close it at this end.

c. Open the tubing at the other end. Into the tubing measure 8 ml of the albumin solution, and add 4 drops of copper(II) sulfate from your set of chemicals.

d. Close the cellophane bag by making another tight knot at the other end.

e. Wash the bag briefly with distilled water (to clean the end of the tubing from eventual contamination with albumin), and place it into the prepared beaker with 1 M NaOH.

f. Keep observing the bag: As OH$^-$ ions cross the membrane, the appearance of the bag is going to change already within the first three minutes. Carefully record all the observed changes and explain them.

g. The outside solution in the beaker remains colorless but its composition changes as well as the sulfate anions diffuse out from the bag. They can be demonstrated with barium(II) ions giving an insoluble white precipitate of barium(II) sulfate. Attempt this proof after at least 15 minutes of dialysis, or at the very end of the lesson. The precipitate of BaSO$_4$ must be differentiated from Ba(OH)$_2$, which is also white and insoluble, but unlike barium sulfate it dissolves after addition of HCl or HNO$_3$.

Take a small aliquot of the beaker content to a test tube, and also a sample of the original NaOH solution to another tube as a negative control. Add the same amount of barium(II) nitrate solution from your set of chemicals to both tubes and compare the appearance of solutions. If you find both precipitates the same, try adding HCl drop wise to both tubes. Record all observed changes and try to explain them.
4  Reversible precipitation of proteins

Solubility of a protein in water is basically determined by the presence of polar amino acid residues in its primary structure. Some proteins dissolve easily in water (e.g. albumin), others not at all (e.g. collagen). Speaking only about proteins that are basically water soluble, stability of aqueous solution of such a protein depends on the intensity of its surface charge. Among other factors, the surface protein charge is controlled by pH. If pH equals the isoelectric point of the protein, its net charge disappears and solubility of the protein is at its lowest (see the separate chapter on electrophoresis for detailed explanation on protein charge and the isoelectric point).

Higher concentrations of inorganic salts (especially ammonium, alkaline metals and alkaline earth metals) lead to precipitation of proteins from the solution. It is because the inorganic ions neutralize the surface protein charges, and also compete with the proteins for the solvent molecules, which stripes proteins from their hydration coats, necessary to keep the proteins dissolved. Likewise, proteins are precipitated with ethanol in the presence of low amount of salts. Ethanol dehydrates the proteins and also decreases the dielectric constant of the medium (dipoles more attract each other). However, the ethanol can also cause protein denaturation (see below); to minimize this effect, temperature needs to be lowered below 0°C.

Since proteins differ in their susceptibility to precipitation with salts, pH change, and/or ethanol, with suitable protocol a mixture of proteins can be fractionated. A classical example is fractionation of serum with ammonium sulfate: globulins precipitate in half-saturated while albumin in fully saturated (NH₄)₂SO₄. Cold fractionation with ethanol according to Cohn separates proteins of human plasma up to 5 fractions. In all these cases the precipitation is reversible, meaning that if the precipitating factor is removed, proteins dissolve again and their biological activity is preserved.

Reagents:
1. Solution of egg white protein (the same as for the task 2)
2. Crystalline sodium chloride, with measuring cup
3. Ethanol
4. Acetic acid diluted 12 g/l (from the basic set)
5. Sodium hydroxide 2 mol/l (from the basic set)
6. Parafilm

4.1 Precipitation of proteins with alcohol

Procedure:
Take 1-2 ml of the protein solution into a test tube, add few crystals of sodium chloride, and shake. Then add about 0.5 ml of ethanol. Within several minutes a protein precipitate appears.

4.2 Precipitation of egg-white protein with sodium chloride and its reversal

Procedure:
Take about 2 ml of the protein solution into a test tube, add three measures of sodium chloride, about five drops of diluted acetic acid (from your set of chemicals), close with parafilm, and shake. A white protein precipitate appears.

Next, try to demonstrate that the protein precipitation is reversible: add about 2 ml of deionised water (from squeeze bottle) and a few drops of sodium hydroxide (from your set of chemicals). Close with parafilm, shake well and wait until the foam/bubbles settle down. Is the egg-white protein solution clear again?
5 Precipitation of proteins by denaturation

Various chemicals as well as physical conditions (high temperature) can destroy the conformation of proteins. The side chain interactions holding together the secondary, tertiary, and (if present) the quaternary structure of a protein are disrupted, whereas much stronger peptidic bonds (and therefore the primary protein structure) are preserved. This process is called denaturation and in majority of cases is irreversible. Biological activity of a protein is dependent on its native conformation and after denaturation disappears. Denaturation is usually (but not always) accompanied by changes in solubility of proteins, i.e. precipitation also occurs.

Reagents:
1. Solution of egg white protein (the same as for the task 2)
2. Lead(II) nitrate 5 g/l
3. Copper sulfate 70 g/l (from the basic set)
4. Nitric acid concentrated (from the basic set)
5. Trichloroacetic acid, CCl\(_3\)COOH 30 g/l
6. Sulfosalicylic acid (2-hydroxy-5-sulfobenzoic acid) HO\(_3\)SC\(_6\)H\(_7\)-2-(OH)COOH, 200 g/l
7. Acetic acid 12 g/l (from the basic set)
8. Acetic acid 100 g/l

5.1 Precipitation of proteins with heavy metal salts

Principle:
Heavy metal ions (lead, copper, silver, mercury) react with proteins to complex salts and even in small amount cause their denaturation and precipitation. The heavy metal ions in excess can donate charges to the protein and then the precipitate can dissolve again, but the protein remains denatured. It is for such reactions that proteins can act as an antidote in heavy metal poisoning, e.g. milk is given in cases of poisoning by sublimate (mercury(II) chloride).

Procedure:
Put about 1 ml of the protein solution into two test tubes. Add one drop of lead(II) acetate solution into the first test tube, and one drop of copper(II) sulphate solution into the other tube. Observe whether proteins precipitate.
Try to dissolve the precipitates again by addition of the heavy metal salts in excess.

5.2 Precipitation of proteins with mineral acids

Principle:
Concentrated mineral (inorganic) acids precipitate and denature protein molecules by means of dehydration and formation of insoluble salts. The precipitation of proteins by nitric acid was used in the past as a test for protein in patient’s urine (the Heller’s test).
Procedure:
1 ml of the concentrated nitric acid in a test tube is carefully (using glass pipette, on the tube wall) overlaid with about 1 ml of the protein solution so that bulk mixing of the two solutions is avoided. Protein precipitation can be seen as a white ring at the interface between both solutions.

5.3 Precipitation of proteins with organic acids

Principle:
The effects of organic acids on proteins are analogous to the effects of mineral ones. In clinical chemistry, trichloroacetate has been used for deproteination of serum prior to further analysis in which proteins would interfere with. Sulfosalicylic acid is a classical reagent for protein detection in urine.

Procedure:
Put 1-2 ml of the protein solution into two test tubes. Add several drops of trichloroacetic acid solution into the first test tube and several drops of sulfosalicylic acid into the second one. Observe whether proteins precipitate.

5.4 Precipitation of proteins with high temperature (boiling)

Principle:
Although there are fascinating extremophilic bacteria thriving in deep sea vents at temperatures above 100 °C, most ordinary proteins are easily denatured by heat. Differences exist: some proteins lose their tertiary structure and precipitate at 50-60 °C, while others require shorter or longer boiling. Thermal denaturation is not always followed by precipitation – consider for instance the results when boiling eggs and milk. Whether a heat denatured protein would precipitate or not, depends also on other factors, such as presence of salts and pH of a solution. In general, the closer the pH to the isoelectric point, the more easily the protein precipitates.

Procedure:

a. Measure 2 ml of the protein solution into a test tube and heat to boiling in water bath. Proteins precipitate.

b. Measure 2 ml of the protein solution into another test tube, add one drop of acetic acid 12 g/l (from your set of chemicals), and heat to boiling. Compare the course of precipitation with the previous tube: the weakly acidic pH is close to the isoelectric point and the precipitation should occur faster now.

c. Measure 2 ml of the protein solution into yet another test tube, in this case add about 0.5 ml of acetic acid 100 g/l, and heat to boiling. Now the medium is strongly acidic and it can be expected that the protein in solution, albeit denatured, remains ionized and will not precipitate.

Literature:
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The dialysis experiment setup was designed by MUDr. Martin Vejražka, PhD.