Basic organic reactions
Properties of Proteins
Electrophoresis of Serum Proteins

Practical Lesson on Medical Chemistry and Biochemistry

General Medicine

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Contents

Task 1 - Electrophoresis of serum proteins in 0.5% agarose

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. Samples of serum proteins suitably diluted and with 0.5 % Bromophenol Blue and 25 % 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 and heat to boiling in the microwave (take care not to overflow). 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 about 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 (about half of the volume) 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 run 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.

The following staining/destaing procedure is lengthy and will be performed by technicians aside the practical lesson. The gel is to be carefully transferred to a 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. Next, the gel is stained in the Amido Black solution for about 30 minutes. Then the gel background is clarified in several portions of destaining solutions, about 1-2 hours (good to check the progress of destaining). The fixed and properly stained/destained gel can then be stored in distilled water at 4 °C.

l. Look at the stained gel from a previous day and evaluate the result: make a drawing into your laboratory notebook and attempt to identify the visible protein bands.

Task 2 – Basic organic reactions

2.1 Oxidation of alcohols

Reagents:

1. Potassium permanganate (saturated solution) 
2. Samples (encoded as A, B, C) containing:

   Methanol  , 2-Propanol  , *tert*-Butanol 

Procedure:

Pour about 1 ml of sample A, B, C or distilled water into 4 appropriately labelled test tubes. Add approx. 4 drops of potassium permanganate solution to each tube, and mix gently. Reaction should be visible within 5 minutes. Observe the color changes of the samples.

2.2 Coupling reactions of diazonium salts

Reagents:

1. Sulfanilic acid (Diazo reagent I)  5 g/100 ml of 0.6 mol/l HCl
2. Sodium nitrite (Diazo reagent II)  5 g/100 ml of distilled water
3. β-naphthol 2 g/100 ml of ethanol 

Procedure:

Mix 1 ml of Diazo reagent I with 5 drops of Diazo reagent II. Add solution of β-naphthol dropwise and examine the resulting color.

2.3 Reactions of carbonyl group

Reagents:

1. Sodium hydroxide (2 mol/l) 
2. Sodium nitroprusside 
3. Silver nitrate (20g/l) 
4. Ammonia (aqueous solution) 
5. Fehling solution I (cupric sulfate 70g/l) 
6. Fehling solution II (sodium hydroxide 250 g/l, potassium/sodium tartrate 350 g/l) 
7. Schiff reagent (fuchsine decolorized by sulfur dioxide) 
8. Acetone 
9. Formaldehyde 
10. Acetic acid 
11. Formic acid 

Procedure:

- **Legal's test**

Dissolve several crystals of sodium nitroprusside in water. Put about 0.5 ml of acetone to one test tube, and 0.5 ml of acetic acid to the other. Add 2-3 drops of this solution into each sample and slowly alkalize by adding NaOH dropwise. Examine the final color.

- **Fehling's test**

Prepare Fehling's reagent by mixing equal volumes (about 2 ml each) of Fehling's solution I and II.
Dispense the reagent to two long tubes. Add several drops of formaldehyde to about 2 ml of Fehling's reagent to one tube, and several drops of acetic acid to the reagent in the other one. Heat both tubes gently in a boiling water bath and check the color change.

- **Tollens' test**

Prepare Tollens' reagent:
In a beaker, mix equal volumes of silver nitrate and sodium hydroxide. Silver oxide will precipitate. Add aqueous ammonia dropwise until silver oxide nearly dissolves. Avoid excess of ammonia – the silver oxide does not have to dissolve completely.
Then dispense the reagent to three long test tubes, about 1 ml each. Add several drops of formaldehyde to the first tube, formic acid to the second, and purified water to the third. Heat in a water bath. Examine the color changes.

- **Schiff's reaction**

Put about 1 ml of formaldehyde to first test tube, a similar amount of aqueous acetic acid to the second one, and purified water to the third. Add about two drops of Schiff's reagent to each sample, shake gently and record the color changes.

2.4 Esterification

Reagents:

Salicylic acid 

Methanol 

Ethanol 

Sulfuric acid (concentrated) 

Benzoic acid 

Procedure:

Choose appropriate reagents to prepare either ethyl benzoate (benzoic acid ethyl ester) or methyl salicylate (salicylic acid methyl ester).

Mix approx. 0.5 g of benzoic (salicylic) acid with 1.5 ml of ethanol (methanol). Add 10 drops of concentrated sulfuric acid (**carefully!!!**) and incubate for a few minutes in a hot (not boiling) water bath. Successful esterification can be easily monitored, since both esters have a very intensive scent, which resembles that of mint. Especially the smell of methyl salicylate, the predominant compound of mouthwashes, is characteristic.

In addition, because the esters are less soluble than the parent compounds, they likely appear in the tube as a white precipitate. If you do not see the precipitate, pour the whole reaction mixture into a beaker with a small amount of cold water. The precipitated ester will appear as white crystals.

Task 3 - Reversible precipitation of proteins

Reagents:

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

3.1 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 deionized water (from a 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?

Task 4 - 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 disappears with denaturation. 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 3)
2. Lead(II) acetate 5 g/l 
3. Nitric acid concentrated 
4. Sulfosalicylic acid (2-hydroxy-5-sulfobenzoic acid) $HO_3SC_6H_3$-2-(OH)COOH, 200 g/l 
5. Acetic acid 12 g/l (from the basic set)
6. Acetic acid 100 g/l 

Procedure:

4.1 Precipitation of proteins with heavy metal salts

Put about 1 ml of the protein solution into a glass test tube. Add one drop of lead(II) acetate solution into the tube. Observe whether the protein precipitate.

Try to dissolve the precipitate again by the addition of the lead(II) acetate solution in excess.

4.2 Precipitation of proteins with mineral acids

Put about 1 ml of the protein solution into a glass test tube. Then add carefully 1 ml of the concentrated nitric acid so that bulk mixing of the two solutions is avoided (use a plastic dropper, hold the tube tilted and carefully dispense the acid on the wall; the acid is heavier than water and will flow under the protein solution). Protein precipitation can be seen as a white ring at the interface between both solutions. The ring can also be yellow due to nitration of aromatic amino acid residues (xanthoproteic reaction).

4.3 Precipitation of proteins with organic acids

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

4.4 Precipitation of proteins with high temperature (boiling)

a. Measure 2 ml of the protein solution into three long test tubes. The first tube is a control. To the second tube add also one drop of acetic acid 12 g/l (from your set of chemicals), and to the third one add about 0.5 ml of acetic acid 100 g/l.

b. Place all three tubes to water bath and heat to boiling.

c. Keep observing the solutions - proteins should precipitate by heat but not necessarily with the same rate. Record any such differences.

d. It can be expected that in the second tube the weakly acidic pH brings protein close to its isoelectric point and the precipitation should occur faster than in the control tube. In contrast, in the third tube the medium is strongly acidic and it can be expected that the protein in solution, albeit denatured, remains ionized and will not precipitate. However, your results can be different depending on the exact amount of acetic acid added. Record your actual observations!