Electrophoresis of Serum Proteins

Properties of Proteins

Practical Lesson on Medical Chemistry and Biochemistry

General Medicine

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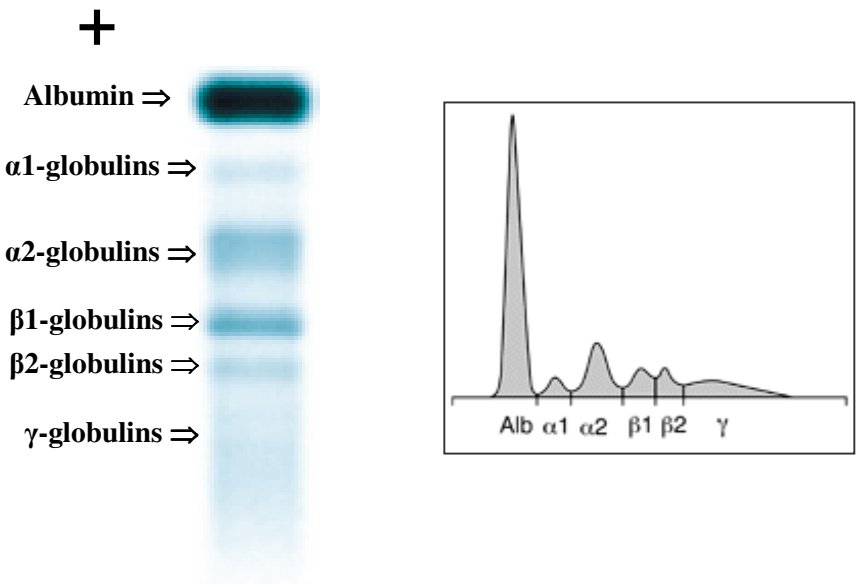
1 Electrophoresis of serum proteins in 0.5% agarose

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).

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 Coommassie 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. 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, insert a stirring bar, close with alumina foil, and heat to 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 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.

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 Comparison of native electrophoresis and SDS-PAGE

Discontinuous electrophoresis in polyacrylamide gel under denaturing conditions in the presence of anionic detergent sodium dodecylsulfate (SDS-PAGE) represents another type of protein electrophoresis, widely used nowadays especially in research laboratories. The following table summarizes the main differences between the native electrophoresis in agarose described above and SDS-PAGE (see the separate text Electrophoresis in Biochemistry for more information):

	Native agarose electrophoresis	***SDS-PAGE***
Action of gel as molecular sieve:	NO	YES
Focusing mechanism:	NO	YES
Resolution power:	Rather low	Fairly high
Condition of resolved proteins:	Native: 3D structure including bonds among subunits and biological activity preserved	Denatured: 3D structure is lost, coated with negatively charged SDS. Bonds among subunits disrupted
Proteins separate according to:	Surface charge density	Size of polypeptidic chains
Example of application:	Examination of human serum proteins	Detection of a specific protein in a tissue sample (if followed by Western immunoblotting)

Procedure:

The SDS-PAGE was performed with the same samples of serum proteins as used for native agarose electrophoresis. The stained gels are available for viewing. Compare the results of protein separation with both kinds of electrophoresis and try to explain any found differences.

3 Selected reactions of amino acids and proteins

Amino acids are characterized by a simultaneous presence of carboxyl group –COOH and amino group $–NH_2$. In addition, some amino acids contain in their side chains other functional groups such as –SH, –OH, guanidine group or aromatic ring. Presence of these structures enables various color reactions that can be employed for amino acid detection. Thus, for instance, free α-amino group of any amino acid reacts with **ninhydrin**. Aromatic rings in structures of tyrosine, phenylalanine or tryptophan give **xanthoproteic reaction**. Phenolic (tyrosine) and imidazole (histidine) groups can react with **diazonium salts**. -SH group of cysteine can be demonstrated by reaction with **Pb^{2+} ions**.

Amino acids are connected by peptidic bonds in peptides and proteins. Proof of peptidic bond by **biuret reaction** with Cu^{2+} ions is utilized for estimation of total protein concentration in a sample.

The functional groups in the side chains of amino acids joined to polypeptidic chain react in a similar way as the free amino acids do. It means that reactions specific only for certain amino acids can be employed as a test whether given peptide or protein contains the specific amino acid or not. In this way we will compare two proteins in our practical lesson: **egg albumin** and **gelatin**. The albumin is an example of 'full-blown' protein, containing all amino acids. On the other hand, gelatin is a product of denaturation and partial hydrolysis of collagen; its prevailing amino acids are glycine, proline, hydroxyproline and glutamic acid, whereas the content of aromatic amino acids phenylalanine and tyrosine is very low, and tryptophan as well as cysteine is absent.

3.1 Ninhydrin reaction

Ninhydrin is a reagent for ammonia and primary aliphatic amines; hence also for any amino acid that contains a free amino group. In dependence on the kind of amino acid its reaction with ninhydrin results in a blue-violet to brown product. The imino acids proline and hydroxyproline react with ninhydrin as well, but to a different product that has a yellow color. In peptides and proteins the amino acids amino groups are bound in the peptidic bonds, but the ε-amino groups of lysine or terminal $-NH_2$ groups can still give the ninhydrin reaction.

The reaction with ninhydrin is widely used for amino acid detection. In medicine it is employed in examination of disorders of amino acid metabolism, such as phenylketonuria. Ninhydrin reaction can be used for instance as a detection reaction in qualitative tests for amino acids in blood or urine by means of paper or thin-layer chromatography. Another example is a quantitative estimation of the amino acid composition of polypeptides. In this technique the analyzed polypeptide is first hydrolyzed, i.e., all its peptidic bonds are cleaved. The resulting mixture of amino acids is separated by ionex chromatography and the subsequent detection of particular amino acids is facilitated by reaction with ninhydrin.

Reagents:

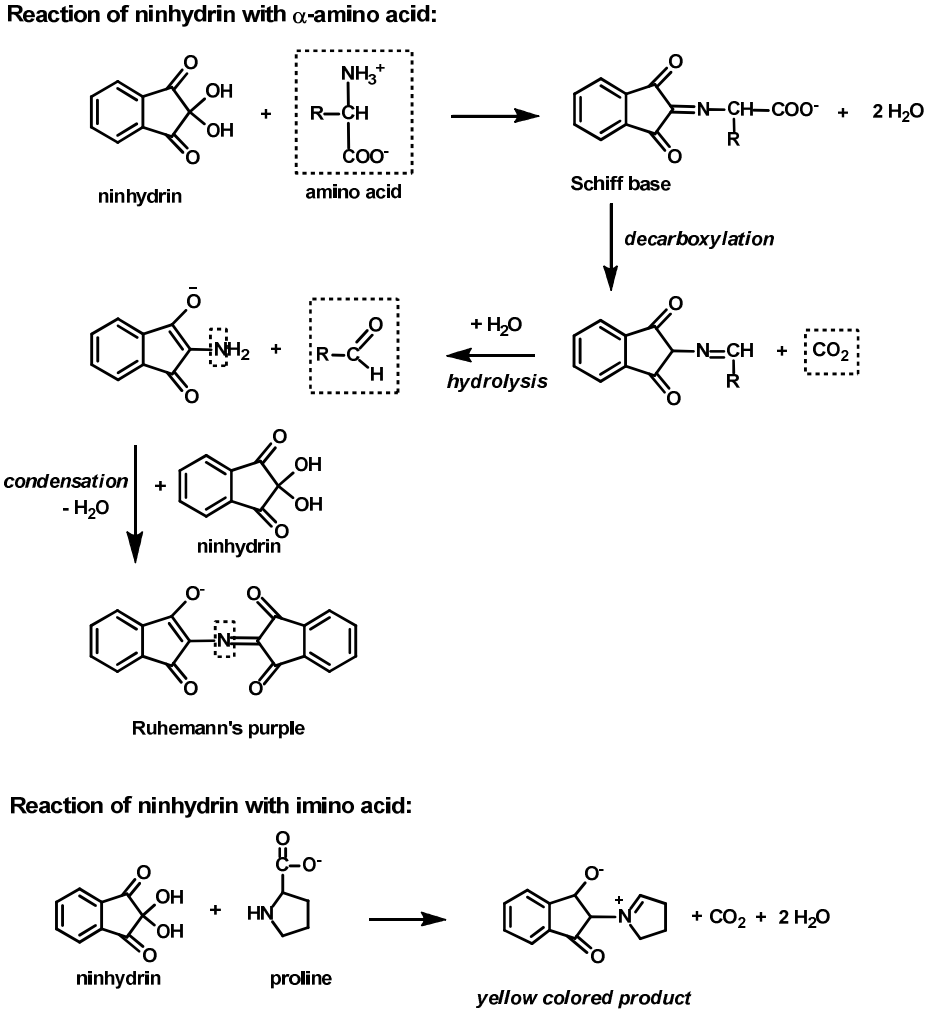
1. Tested samples: alanine 20 g/l, tyrosine 1 g/l, proline 20 g/l, solution of egg albumin 20 g/l, solution of gelatin 20 g/l
2. Ninhydrin 2 g/l in ethanol 

Procedure:

Into 5 test tubes prepare the reaction mixtures according to the table:

	1 Alanine	**2** Tyrosine	**3** Proline	**4** Albumin	**5** Gelatin
Alanine	cca 0.5 ml	–	–	–	–
Tyrosine	–	cca 0.5 ml	–	–	–
Proline	–	–	cca 0.5 ml	–	–
Egg albumin	–	–	–	cca 0.5 ml	–
Gelatin	–	–	–	–	cca 0.5 ml
Ninhydrin	several drops	several drops	several drops	several drops	several drops

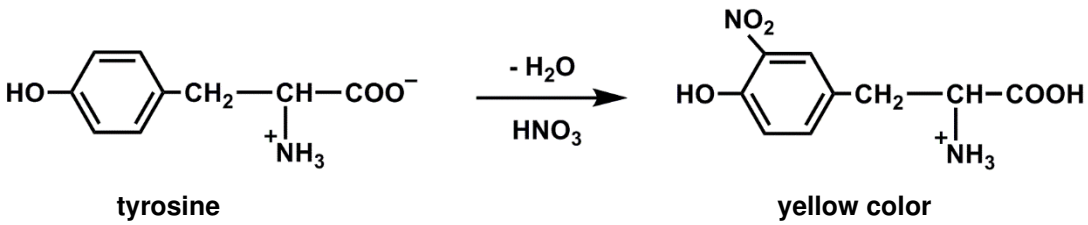
Mix contents of the tubes, heat the tubes in water bath and record any color changes.



Ninhydrin reaction with amino acids. In the first step the hydroxyl group of ninhydrin is replaced with the amino group of amino acid; a Schiff base intermediate results. Next, the attached amino acid is decarboxylated, and release of an aldehyde follows. The resulting unstable amine intermediate then combines with another molecule of ninhydrin to give a colored product called Ruhemann's purple. The only part of the product that comes from the original amino acid is actually the atom of nitrogen, whilst the rest of amino acid molecule has split off as carbon dioxide and an aldehyde. The yellow colored product resulting from ninhydrin reaction with imino acids has a different structure.

3.2 Xanthoproteic reaction

Nitration by concentrated nitric acid is a reaction typical for aromatic ring. The resulting nitro derivatives have an intense yellow color (Greek *xanthos* = yellow). Amino acids that possess an aromatic ring – tyrosine, tryptophan and (weakly) phenylalanine react in a similar way. Likewise, majority of proteins in dependence on their aromatic amino acid contents give a positive reaction with concentrated nitric acid. Following addition of concentrated nitric acid to a protein solution a white precipitate of denatured protein appears that turns yellow after boiling.



Yellow spots on the skin that develop after sullying with nitric acid are also due to xanthoproteic reaction, this time with aromatic amino acids present in epidermal proteins.

Reagents:

1. Tested samples: alanine 20 g/l, tyrosine 1 g/l, egg albumin 20 g/l, gelatin 20 g/l
2. Nitric acid concentrated 

Procedure:

Into 4 test tubes prepare the reaction mixtures according to the table:

	1 Alanine	**2** Tyrosine	**3** Albumin	**4** Gelatin
Alanine	cca 0.5 ml	–	–	–
Tyrosine	–	cca 0.5 ml	–	–
Egg albumin	–	–	cca 0.5 ml	–
Gelatin	–	–	–	cca 0.5 ml
Nitric acid	cca 0.5 ml	cca 0.5 ml	cca 0.5 ml	cca 0.5 ml

Mix contents of the tubes and heat carefully only the test tube with egg albumin. Record any color changes.

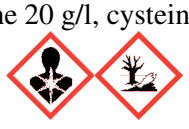
3.3 Reaction of cysteine – proof of sulfur in protein molecules

Cysteine, as well as proteins containing significant amount of this amino acid, in strongly alkaline medium release hydrogen sulfide, which can be demonstrated by reaction with lead(II) acetate. A brownish-black precipitate of lead(II) sulfide develops:

$$R\text{-}SH \xrightarrow{OH^-} R\text{-}OH + H_2S$$

$$H_2S + Pb(CH_3COO)_2 \longrightarrow PbS\downarrow + 2\,CH_3COOH$$

Reagents:

1. Tested samples: alanine 20 g/l, cysteine 20 g/l, egg albumin 20 g/l, gelatin 20 g/l
2. Lead(II) acetate 50 g/l 
3. Sodium hydroxide 2 mol/l (from the basic set) 

Procedure:

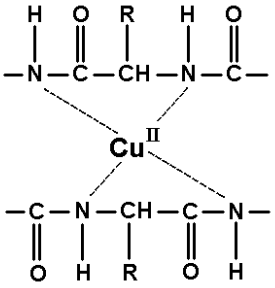
Into 4 test tubes prepare the reaction mixtures according to the table:

	1 Alanine	**2** Cysteine	**3** Albumin	**4** Gelatin
Alanine	cca 0.5 ml	–	–	–
Cysteine	–	cca 0.5 ml	–	–
Egg albumin	–	–	cca 0.5 ml	–
Gelatin	–	–	–	cca 0.5 ml
Sodium hydroxide	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Lead(II) acetate	2-3 drops	2-3 drops	2-3 drops	2-3 drops

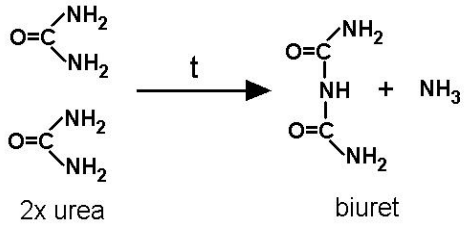
Mix contents of the tubes and boil in water bath about 5 minutes. Record any color changes.

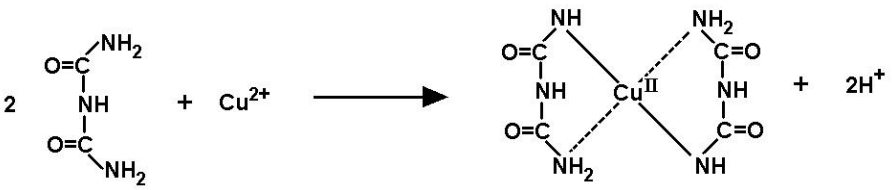
3.4 Biuret reaction

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-NH_2 (amide) or at least two peptide bonds -CO-NH-, will give the reaction. Thus, the simplest reacting compounds are oxamide H_2N-CO-CO-NH_2, 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. Urea (solid substance)
2. Tested samples: alanine 20 g/l, egg albumin 20 g/l, gelatin 20 g/l
3. Sodium hydroxide, 2 mol/l (from the basic set) 
4. Copper sulfate 70 g/l (from the basic set) 

Procedure:

a. **Preparation of biuret:** Into one long glass test tube get about 0.1 g (one measure) of urea and carefully heat by keeping the bottom of the tube in direct contact with the hot plate of an electrical cooker (can be held in hand). The heated urea melts and changes to biuret. Stop heating once the melt hardens to white solid. Allow to cool and dissolve the biuret in about 1 ml of deionized water.

b. **Biuret reaction:**

Into 4 test tubes prepare the reaction mixtures according to the table:

	1 Alanine	**2** Biuret	**3** Albumin	**4** Gelatin
Alanine	cca 1 ml	–	–	–
Biuret	–	cca 1 ml	–	–
Egg albumin	–	–	cca 1 ml	–
Gelatin	–	–	–	cca 1 ml
Sodium hydroxide	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Copper sulfate	1 drop	1 drop	1 drop	1 drop

Mix contents of the tubes and record any changes in color. Positive reaction manifests by violet coloration, better seen against a white background. Avoid excess of copper sulfate – it leads to a light blue precipitate of copper hydroxide that interferes with proper evaluation of the test.

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 $(NH_4)_2SO_4$. 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 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 2)
2. Lead(II) nitrate 5 g/l 
3. Copper sulfate 70 g/l (from the basic set) 
4. Nitric acid concentrated 
5. Trichloroacetic acid, CCl_3COOH 30 g/l 
6. Sulfosalicylic acid (2-hydroxy-5-sulfobenzoic acid) $HO_3SC_6H_3$-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

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

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 plastic dropper, 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

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 the proteins would interfere. 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)

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 of boiling eggs in contrast to boiling 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.

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