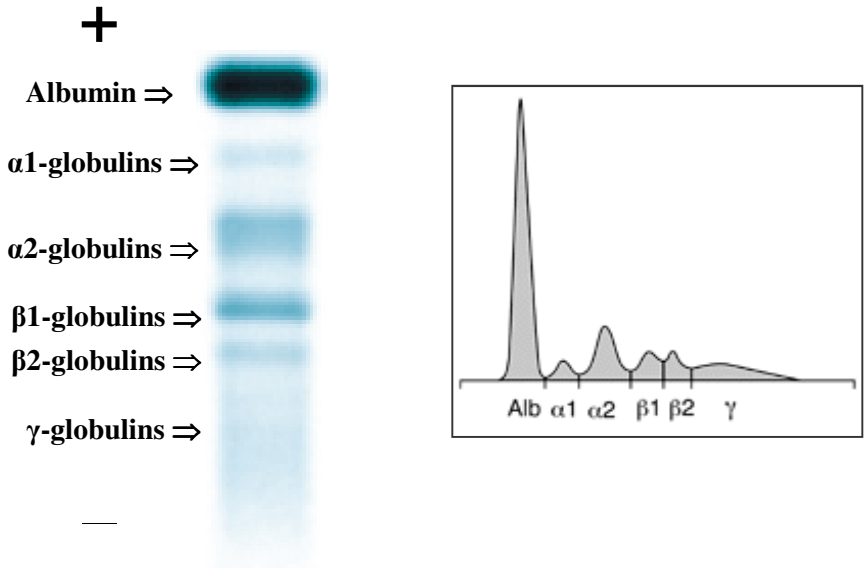
Task 1 - Electrophoresis of serum proteins in 0.5% agarose

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).

Task 3 - 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 classic 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.

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.

4.1 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).

4.2 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).

4.3 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.

4.4 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.