



## Working with proteins: protein stability and storage - a brief guide

Proteins are highly complex biomolecules with specific functions. Their biological function, but even their mere molecular stability in terms of an intact primary structure depends on a variety of factors. If you are planning to use proteins in your work, you should be highly aware of those factors and work accordingly.

Proteins easily lose their biological function and/or overall stability. For some proteins, biological function can be lost almost completely if you unfreeze them from being stored at  $-20^{\circ}\text{C}$  for only a single time.

Furthermore, even when kept at  $4^{\circ}\text{C}$  but without the addition of protease inhibitors, proteins will be degraded significantly by proteases within hours.

Listed below are a number of factors which influence protein stability. **This list is not complete and only meant as a brief introduction for new chemistry students coming to our lab. We do not take any responsibility for the content of this document for use outside our lab.**

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### Protein structure

A brief introduction into protein structure is required to understand the principles for protein stability, applications and storage. Proteins have a primary, secondary, tertiary and quaternary structure. The primary structure is simply the basic, intact chain of amino acids which form the protein. A lot of applications only rely on this primary structure (SDS-PAGE, 2D gel analysis etc.). However, proteins are generally not active in this form. Activity requires an intact secondary, tertiary and sometimes quaternary structure (secondary structure: determined by the bond lengths and angles of its primary amino acid sequence and hydrogen bonds between them e.g. alpha helices and beta sheets / tertiary structure: three dimensional arrangement of secondary structure, the active form of the protein / quaternary structure: protein complex of several protein molecules which function as part of this complex). Active protein is required when your assay relies on a specific protein function (antibodies, enzymes etc.).

### Factors influencing protein stability

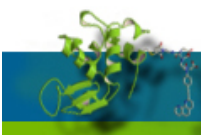
#### Temperature:

Most proteins from mammals have a temperature optimum for their biological function at around  $37^{\circ}\text{C}$ . Temperatures above  $43^{\circ}\text{C}$  will denature most mammalian proteins more or less quickly. At  $55^{\circ}\text{C}$ , complete denaturation takes place within one or two hours, at  $95^{\circ}\text{C}$  only a couple of minutes.

However, severe protein denaturation and destabilisation also occurs at room temperature, but mainly due to other factors. Proteins are protected in their normal cellular environment by other proteins (chaperones), which is not the case for pure proteins in solution. You have to expect, that proteins in solution quickly lose their biological function at room temperature. Always work on ice ( $4^{\circ}\text{C}$ ) when you have to ensure that your protein stays active.

#### Freeze/thaw cycles:

Repeated freeze/thaw cycles usually degrade proteins. As a rule of thumb, never freeze the same batch of protein twice. Some proteins may be more stable than others, but to be on the safe side, aliquot your samples and do not freeze them again after you have used one. If you need to use a protein very frequently, there are better ways to store it (see "Storage").



## **Proteases/Peptidases:**

Proteases and peptidases are enzymes which degrade proteins and peptides. Most of these enzymes have a temperature optimum at around 37°C. At this temperature, some proteins will be degraded by proteases within minutes. At lower temperatures, the activity of proteases will be reduced. However, working at 4°C does NOT mean that proteases are not active! If you want to make sure that proteases do not degrade your protein, you have to use protease inhibitors (see below). However, be aware that many protease inhibitors themselves are proteins or peptides! Proteases do not only occur on the human skin but also in the air, so samples are easily contaminated. Furthermore, proteases are produced by bacteria (see next point).

## **Bacteria:**

If you do not work in a sterile environment (which will not be the case unless you are working in a certified cleanroom), all of your samples will be contaminated with bacteria. If you want to keep your proteins in solution at 4°C, you have to add an antibacterial agent such as sodium azide (NaN<sub>3</sub>, final concentration of 0.02-0.05% (w/v)).

## **pH:**

Every protein has an optimal pH for its biological activity or function. Slight changes in pH affect this activity. A change from pH 7.4 to 6.4 can mean more than 90% loss of activity. Furthermore, a strong acidic or basic pH will denature proteins quickly. Physiological pH for most proteins is pH 7.2 to 7.4.

## **Protein concentration:**

The protein concentration will not directly affect your protein stability. However, when using low protein concentrations (<1mg/ml), low-level binding to the storage vessel material (for instance Eppendorf tube) may occur, resulting in a loss of protein from the solution. If it does not disturb your experiment, add a carrier protein to your solution (for example BSA) in a concentration of 2mg/ml to protect against the loss.

## **Salt conditions:**

Proteins are biomolecules which occur in a physiological environment with a delicate balance of salt concentrations. Too high or too low salt concentrations can lead to precipitation of a protein, suggesting its denaturation. PBS is commonly used as a physiological buffer for proteins. Do NOT use pure water to dissolve proteins.

## **Solvents:**

Most organic solvents will denature your protein. However, the primary structure will mostly be unaffected. This means, that a loss of biological function will occur, but you may still use the protein for other purposes e.g. for some labelling reactions or SDS-PAGE.

## **Formaldehyde/Glutaraldehyde:**

These reagents are used to crosslink proteins. They are susceptible to a nucleophilic attack of the amino groups in your protein and subsequent covalent bonding via the crosslinker occurs. Be aware of this when using these reagents.

## **Beta-Mercaptoethanol, DTT, TCEP:**

These reagents break disulfide bonds in proteins and therefore destroy the tertiary (and quaternary) structure of proteins. They are most commonly used in preparing samples for SDS-PAGE analysis. Be aware that they will denature every protein which contains disulfide bonds.

## **Urea:**

Urea is used in high concentrations (> 6M) to denature proteins. Other chaotropic agents include guanidinium chloride and lithium perchlorate. All of them destroy the tertiary (and secondary) structure of proteins by influencing hydrophobic effects, hydrogen bonds, and others.

## Storage of proteins

Please refer to the following table first. Some further comments are given below.

Storage condition	in solution, 4°C	-20°C, -80°C or liquid N <sub>2</sub>	lyophilized, 4°C or -20°C	in solution (with 50% glycerol), -20°C
Maximum storage time	1 day - 2 weeks	several years	several years	6 - 12 months
Number of times sample can be brought to room temperature	many	one time! (if necessary twice)	several times	many
Antibacterial agent	required	if possible, yes	none	none
Protease inhibitor	required	if possible, yes	none	none

The **maximum storage time in solution at 4°C** is highly dependent on your type of protein. Especially enzymes and antibodies can be very sensitive and some may already significantly lose activity after 2 hours at 4°C. If your application does not require biologically active protein but only an intact primary structure (e.g. SDS-PAGE, Western blot, 2D-gels), a storage time of one or two weeks should be ok.

If you add **protease inhibitor** to your sample, be aware that many protease inhibitors are small proteins (or peptides) themselves! They can interfere with your assay. You will find a list of protease inhibitors below.

As an **antibacterial agent**, you may add sodium azide (NaN<sub>3</sub>) at a final concentration of 0.02-0.05% (w/v) to your sample. When handling it, be aware of the fact that sodium azide is highly toxic.

When using **low protein concentrations** (<1mg/ml), low level binding to the storage vessel material (for instance Eppendorf tube) may occur, resulting in a loss of protein amount in solution. If it does not disturb your experiment, add a carrier protein to your solution (for example BSA) in a concentration of 2mg/ml to protect against the loss.

Protease inhibitor	inhibits	effective conc.	class	comments
PMSF	serine proteases	0.5mM	organic	not stable at pH 7.4 (half-life of 50min)
AEBSF	serine proteases	1.0mM	organic	more stable than PMSF, but only good stability at acidic pH
Pepstatin	aspartyl proteases	1.0µM	peptide	dissolve in methanol as stock solution
Leupeptin	thiol proteases	10.0µM	peptide	-
Aprotinin	serine proteases	0.1µM	polypeptide	58 amino acids long, contains disulfide bridges
EDTA / EGTA	metalloproteases	1.0mM	organic	complexes Ca <sup>2+</sup> which may be needed for enzyme activity
Benzamidine	serine proteases	1.0mM	organic	-