Protecting the Heat Sensitive Protein and Other Biomolecule Thermal Degradation during Sample Preparation

Introduction

With rapid development of proteins as therapeutic drugs, structural stability of proteins during various stages of sample preparation and analysis has become critical. Protein structures are very sensitive to temperatures, and these temperature adaptive changes in protein due to their low net free energies of stabilization could affect protein stability and functions.

The most familiar phenomenon related to protein unfolding, known as "thermal denaturation," occurs when protein solution heats up from ambient temperature to higher values. To contrary, "cold denaturation" the phenomenon where proteins unfolds when the protein solution is cooled from ambient temperatures to lower values. This creates a unique challenge for users to identify the ideal temperature conditions to prevent denaturation of protein. Maintaining ideal temperature conditions at which the proteins are processed and stored without losing sample integrity is critical.

A common technique used for sample preparation during protein purification is vacuum concentration. Gel filtration fractions and dilute extracts can be easily concentrated by the removal of water using products such as Thermo Scientific™ Refrigerated SpeedVac Centrifugal Vacuum Concentrator SRF110. Samples are placed in the vacuum concentrator and centrifuged while a vacuum is applied. The Refrigerated SpeedVac (SRF110) provides programs that can be selected to provide ultimate control of sample temperatures pre, post, and during sample concentration as the chamber can be heated or cooled based on application need.

Many samples that are vacuum concentrated contain inert or resilient solutes, as with inorganic ions or non-volatile organics. However, most biomolecules are susceptible to some type of degradation during processing. Major hazards are caused by proteases and nucleases which attack molecules in fresh lysates. Inhibitors can be used to suppress most enzymatic degradation, but a risk still exists that some of the desired biomolecules will be lost. Typically, a combination of inhibitors and temperature control are used while manipulating samples. Using a temperature regulated centrifugal concentrator, such as the Refrigerated SpeedVac SRF110, helps to protect biomolecules during vacuum concentration and offers users peace of mind.

Effect of Temperature control during protein purification

To illustrate the value of regulating sample temperature during protein purification, a liver tissue lysate was concentrated using a SRF110P1 kit which maintained the sample at 1°C (just above freezing) and with a competitor product operated at ambient temperature (22°C). A retained sample was stored at 4°C as an unprocessed control. Following concentration for one-hour, distilled water was added to the samples to bring them up to their original volume. The samples were then assayed for lactate dehydrogenase activity (LDH) as a marker of protein stability.

LDH activity is measured spectrophotometrically at 490 nm using the INT assay. INT (i.e., Iodonitrotetrazolium chloride) is reduced by electrons stripped from lactate by LDH and transferred through NAD and the intermediate PMS (phenazine methosulfate). The reaction components are 1 part buffer (0.2 M Tris, pH 8), 1 part lactate (49 mM lithium lactate in water), 1 part INT/PMS/NAD, and 1 part enzyme solution. The INT/PMS/NAD solution is prepared before use by mixing 2.3 ml NAD (8.6 mg NAD [Sigma N-0632] in 2.3 ml water), INT (3.3 mg INT [Sigma I-8377] in 100 µl DMSO), and PMS (0.9 mg PMS [Sigma P-9625] in 100 µl water).

Tissue sample was prepared from mouse liver. Approximately 0.5 gm of liver was homogenized in a conical tissue grinder with 5 ml of phosphate buffered saline. The tissue lysate was centrifuged to clarify the solution which was further purified by passing the lysate over a PD-10 column (Sephadex G-25). The lysate was then divided into 0.5 ml aliquots in 1.5 ml micro centrifuge tubes for vacuum concentration.

Samples were concentrated for 1 hour and then readjusted to 0.5 ml with water to standardize the samples. The samples were then assayed for LDH activity in a microplate reader.

After an hour of concentration, the sample processed at ambient temperature lost significantly when as compared to the sample prepared using SRF110P1 setup using temperature control. During this process, water loss from the ambient sample was faster due to the higher processing temperature, however proteolysis was also high. Reducing the sample to above freezing (1°C) resulted in slower water loss, but significantly higher LDH activity. Interestingly, the vacuum concentrated sample using SRF110P1 setup retained higher activity than the refrigerated control sample. This demonstrates the impact of slight temperatures differences on protein stability. The following graph summarizes this data.

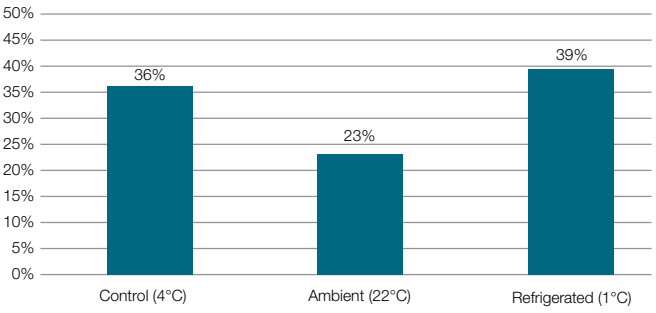

Figure 1. Comparing tissue lysate LDH activity refrigerated and ambient vacuum concentrated.

Effects of freeze-Thaw during Protein Purification

LDH is also recognized as an enzyme sensitive to freeze-thawing. The sensitivity of enzymes to repeated freezing and thawing of samples is well documented. However, some enzymes, such as LDH, can exhibit significant activity loss even after one freeze-thaw event. The significance of this problem as it relates to vacuum concentration is that aqueous solutions placed in high vacuums lose significant heat (molecular motion) to water as it changes phase from water to gas. Heat loss can be so significant that solutions can freeze, in effect resulting in lyophilization. Though freeze drying has many virtues, proteins that are frozen during concentration without chemical protectants (cryo or lyoprotectants) are often damaged. Furthermore, if the protein sample is subsequently thawed, i.e., concentrated but not to complete dryness, then the melting or collapse of the sample can further damage the solutes.

To demonstrate this concern, LDH samples prepared as noted above were concentrated in a Lyophilizer. During the vacuum concentration, samples in the Lyophilizer froze while those in the SRF110p2 kit setup remained liquid. After 1 hr. of concentration, the samples were readjusted to their original volume and assayed as described previously.

The effect of freeze-thawing on LDH activity is significant (see chart below). The single freeze-thaw event which occurred during concentration resulted in an 94% loss of activity. Though this denaturation of the LDH could have been prevented by the addition of cryo- and/or lyoprotectants, the addition of such excipients is not necessarily desirable during purifications and concentrations of solutes.

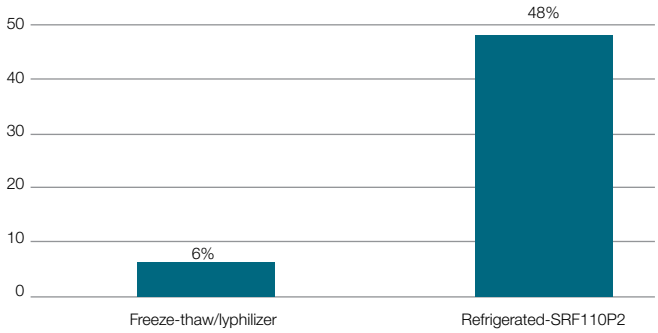

Figure 2. Comparing LDH sample activity freeze-thaw versus refrigerated vacuum concentrated.

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Effect of temperature of evaporation rate

Though controlling the temperature of samples during vacuum concentration has real and measurable value, it can also have a slowing effect on the concentration. The vaporization of solvent is accelerated as temperature increases, and inversely slowed by lowering temperature. With aqueous solutions, concentration is more effective at high temperatures and reduced as the samples cools. The following graph shows a comparison of water loss at 40°C, 20°C, and 4°C. This data illustrates that higher processing temperatures hasten the speed of water loss. Vacuum concentration at 4°C results in much reduced water loss, and thus requires longer processing times.

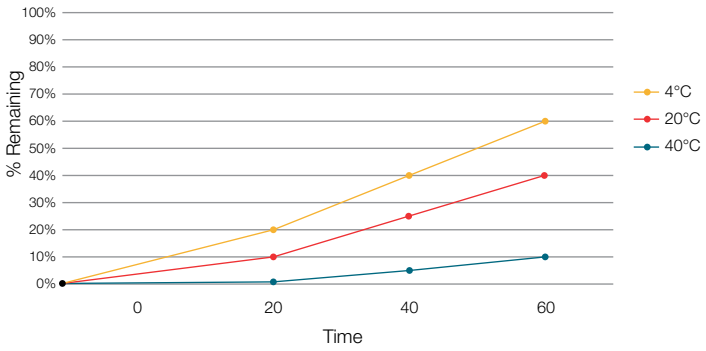

Figure 3. Temperature effect on Water Evaporation

The data presented above leads to the obvious question of when it is necessary to use refrigerated vacuum concentration as it will require more processing time. The most obvious answer is when needed. The degree to which a protein sample is sensitive to degradation or denaturation should dictate the use of heat and refrigeration in processing. For known proteins, the parameters used in handling are defined by experience. However, when handling unknown proteins, lack of activity or poor yields may be related to sample handling issues as described above. Thus, for unknown (new) proteins, it may be prudent to concentrate using a device such as the Thermo Scientific Refrigerated SpeedVac SRF110 (also available in kits SRF110P1 and SRF110P2- for complete solution to your vacuum concentration needs).

Summary

Protein samples can be adversely affected if vacuum concentrated at temperatures that allow proteolytic activity or result in denaturation. Both high and low temperatures can result in protein loss, and although inhibitors and protective agents can significantly reduce loss, regulating processing temperature can also serve to protect proteins. The Thermo Scientific Refrigerated Speedvac Vacuum Concentrator -SRF110 can supply and remove heat during sample concentration to meet the application need and ensure sample integrity preventing thermal degradation. Using LDH as a model protein, activity was preserved in protein samples by both decreasing proteolytic activity and prevent sample freezing when processed using SRF110.

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