Assessing the Value of Temperature Control on Centrifugal Vacuum Concentration

Introduction

Temperature effects are one of the biggest issues researchers face during protein purification. The diversified nature of each protein makes potential temperature fluctuations a hazard to the protein's stability. It is well known that high temperatures can cause denaturation. Freezing can also cause issues as some proteins are cold labile and are sensitive to low temperatures. Using traditional practices of chilling the sample may result in loss of activity.

A common technique used in protein purification is vacuum concentration. The Labconco CentriVap Centrifugal Concentrator easily concentrates gel filtration fractions and dilute extracts through the removal of water. Samples are placed in the vacuum concentrator and centrifuged while a vacuum is applied. The centrifugation process prevents sample bumping from tubes or microwell plates as a result of the pressure decrease. Heating and cooling of the sample under moderate vacuum is also possible with the Labconco Refrigerated CentriVap. As noted below, temperature control can have an important impact on sample integrity.

Many samples that are vacuum concentrated contain inert or resilient solutes, as with inorganic ions or non-volatile organics. Many 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 there's still a risk that some of the desired biomolecules will be lost. Typically a combination of inhibitors and temperature control are used when manipulating samples with a temperature regulated centrifugal concentrator. Labconco's Refrigerated CentriVap offers protection of biomolecules during vaccum concentration.

Effects of Temperature Control During Protein Purification

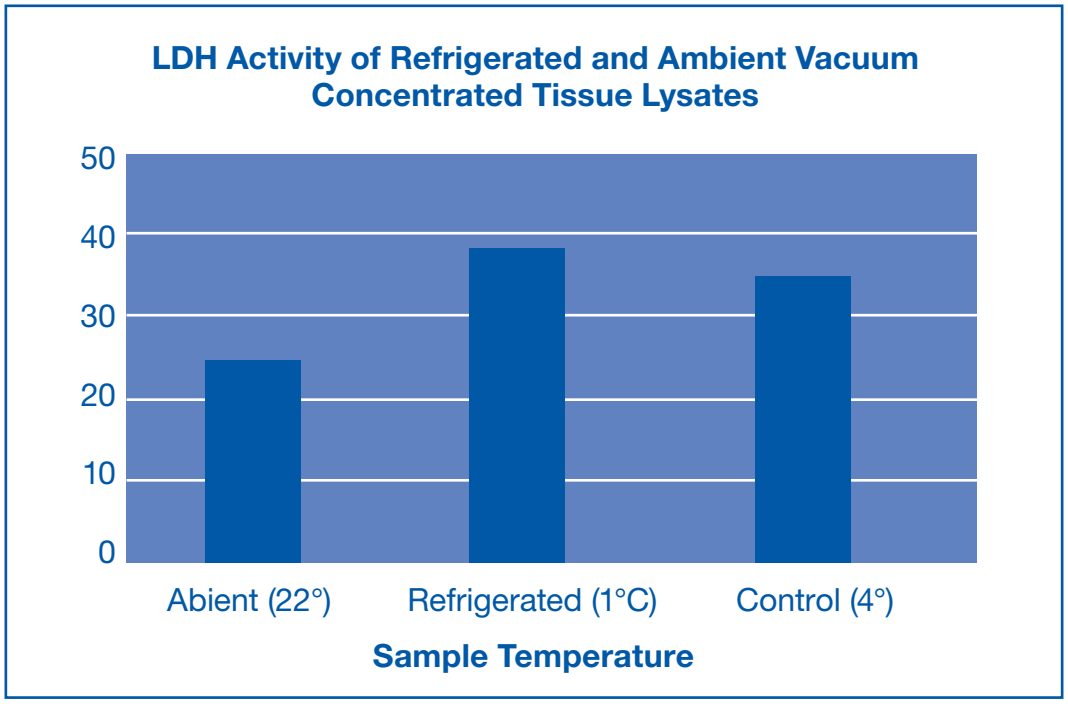
To illustrate the value of regulating sample temperature during protein purification, a liver tissue lysate was concentrated using a Labconco's Refrigerated CentriVap which maintained the sample at 1°C (just above freezing) and with a Savant SpeedVac operated at ambient temperature (22°C). A retained 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 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 microfuge tubes for vacuum concentration.

Samples were concentrated for one 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 35% of its activity compared to the refrigerated vacuum concentrated sample. 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 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.



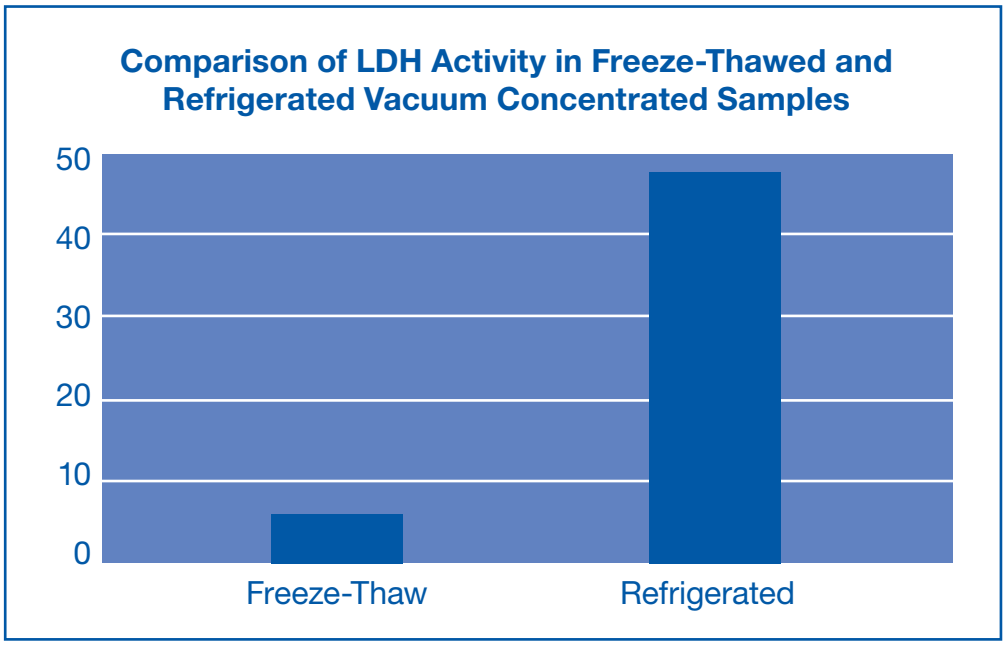
Effects of Freeze/Thaw During Protein Purification

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 liquid to gas. Heat loss can be so significant that solutions can freeze, 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.

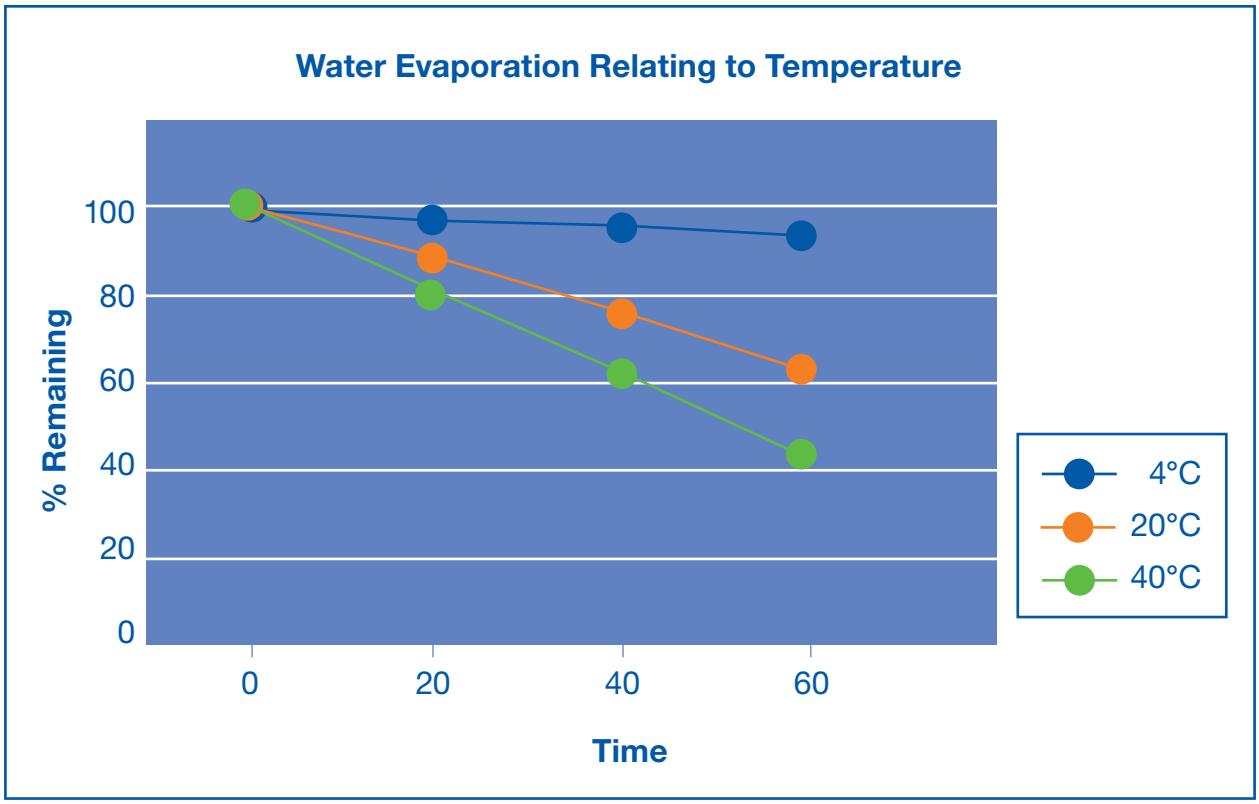
To demonstrate this concern, two tests were conducted. LDH samples were prepared as noted above. One was concentrated using a standard Labconco CentriVap and high efficiency rotary vane oil pump and the other using Labconco's Refrigerated CentriVap with a Welch DryFast Diaphragm pump (Model 2042). During the vacuum concentration, samples in the CentriVap froze (due to the high efficiency pump) while those in the Refrigerated CentriVap remained liquid. After one hour 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 85% 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.



Effects of Temperature on Evaporation Rate

Although controlling the temperature of a sample 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 expedites the speed of water loss. Vacuum concentration at 4°C results in much reduced water loss, and thus requires longer



processing times. The data presented above poses the question of when is it necessary to use refrigerated vacuum concentration, as it will require more processing time. The 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 or new proteins, it is advisable to concentrate using a device such as the Refrigerated CentriVap.

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 to protect proteins. The Labconco Refrigerated CentriVap Vacuum Concentrator can supply and remove heat during sample concentration. Using LDH as a model protein, activity was preserved in protein samples by both decreasing proteolytic activity and preventing sample freezing.

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