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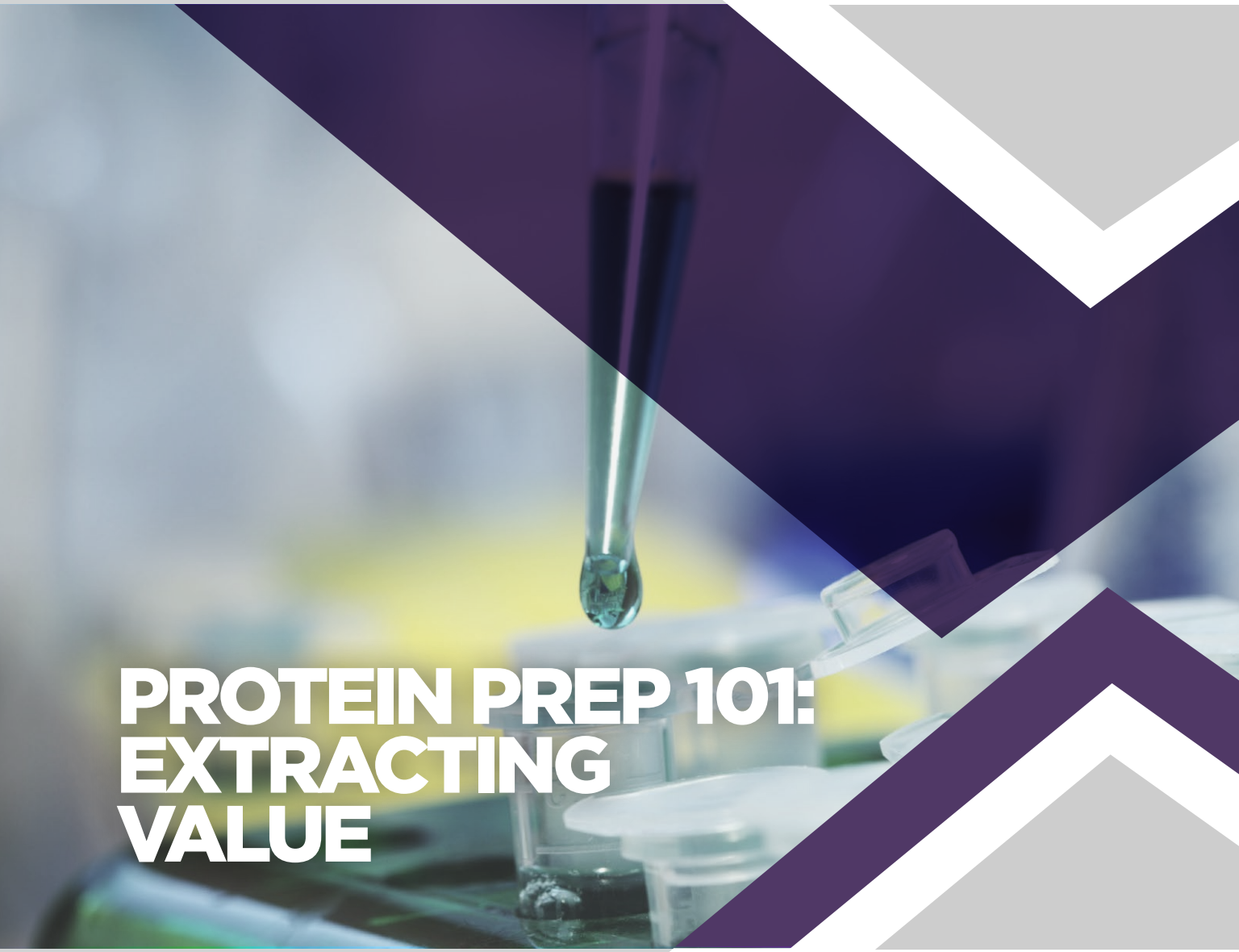

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Be Prepared: Protein Prep Applications & Common Challenges

"all scientists using protein preparation protocols need to be aware of common obstacles [...] and adjust their protocols accordingly"

If DNA is the blueprint of life, then proteins comprise the edifices themselves. In an organism, proteins serve structural purposes, facilitate transport and exchange, mediate intracellular and extracellular communication, provide fuel, enable protective responses, and much more.[1] Naturally, the study of proteins – their structures, abundance, function, and interactions – has proven integral to our understanding of biology. Additionally, protein abnormalities often contribute to disease development, thereby making protein-centered research pivotal to human health and disease.[1] Finally, proteins are important in biomanufacturing and bioproduction, as protein-based products are consumed commercially, used in academic research, and serve as biopharmaceuticals.

To effectively study proteins or manufacture protein-based products, researchers need to be able to detect, characterize, and quantify the protein(s) of interest. This is typically done by extracting proteins from their natural environments (e.g., cells, tissues, or fluids[2]) and preparing purified samples for further observation or experimentation. The actual steps involved in protein extraction and preparation protocols will depend on a host of factors, including the protein of interest, the natural environment, and the post-extraction application, but all protein preparation protocols strive to optimize two main parameters: yield and purity.

High Yield, Excellent Purity: The Best of Both Worlds

In general, protein preparation protocols strive for both yield and purity at the same time, as miniscule amounts of pure protein tend to be difficult to detect and/or insufficient for experimentation, while more abundant quantities of low-quality protein may not provide relevant data. Within a research framework, the precise balance between yield and purity required can vary for each scientist depending on their intended downstream application. For example, research projects exploring protein crystal structures or proteomics will prioritize higher sample purities, whereas those using Western blot or ELISA to quantify protein abundance may require larger sample quantities.

Balancing yield with purity is generally more straightforward for manufacturers of protein-based products: they require both. High yields are important for optimizing production efficiency, ensuring supply keeps up with demand, and maintaining commercial viability. High purity is necessary for regulatory compliance, batch/lot consistency, and public safety.

Bumps in the Road: Protein Prep Challenges

Regardless of the ultimate purpose of the extracted protein, all scientists using protein preparation protocols need to be aware of common obstacles impeding protein extraction and purification, and adjust their protocols accordingly to overcome these challenges. These include preventing protein loss or damage during the extraction process (e.g., caused by chemical agents or mechanical forces during lysis induction),[1] detecting low-abundance proteins (e.g., for single-cell or cellular sub-fractionation applications),[2] identifying and removing undesired conformations of the protein of interest (e.g., misfolded, truncated, or aggregated variants),[2] and the sensitivity and selectivity of the probe used to label/extract a protein of interest from a heterogeneous milieu.[2]

Scientists also face a variety of systemic challenges, ranging from the contamination of protein samples during preparation or by the laboratory environment or instrumentation to designing extraction and purification protocols capable of sufficient throughput to accommodate researcher needs.[1,3] Fortunately, advancements in technology have both augmented the capabilities of older techniques such as 2-D gel electrophoresis and provided new options such as HPLC/MS.[1,3] Today's scientist has more sensitivity, more throughput, more options, and more resources available to him or her than ever before, ensuring that with some care and some thought, every protein preparation can be a pure one.

For references, please see page 7.

THE PATH TO PURITY

A PROTEIN ISOLATION WORKFLOW

TISSUES

Tissues represent some of the most abundant – and difficult to access – sources of protein. Tissue-level protein expression is often indicative of homeostatic or pathological function.

HOMOGENIZATION

Tissue structures need to be broken down in order to maximize yield. Chemical reagents, digestive enzymes (e.g., proteinases), heat, and mechanical forces can all be used to degrade extracellular matrix and liberate individual cells.

CELLS

Cell lines are commonly used as protein sources for basic research and biomanufacturing applications alike.

FLUIDS

The extraction and purification of soluble proteins is important for many applications, from examining protein levels in biological fluids for research or diagnostic purposes to harvesting proteins secreted into culture media during bioproduction.

LYSIS

Cell membrane disruption is necessary for the liberation of cellular proteins. This can be done chemically (e.g., detergents), enzymatically, mechanically (e.g., sonication), or through application of osmotic pressure.

PRECIPITATION

Soluble proteins can be precipitated using salts, polymers, or solvents. Taking proteins out of solution allows the scientist to remove unwanted soluble components (e.g., nucleic acids, ions, amino acids) and reconstitute the purified protein at a desired concentration for downstream use.

PURIFICATION

Protein purification typically involves the removal of debris and other particles. Centrifugation is one of the most commonly used methods for this purpose, where centrifugal force can separate proteins from unwanted elements. Other popular techniques for protein purification include affinity purification, ultrafiltration, and dialysis.

QUANTIFICATION

It is important to quantify protein concentrations within any given sample. This identifies whether sufficient amounts for use have been extracted, as well as facilitates the standardization of downstream applications.

RESUSPENSION

After centrifugation, the pellet can now be washed to remove impurities and then resuspended at a desired concentration in a solution optimized for use or storage.

ANALYSIS

Work for it: Considerations for Challenging Samples

"each step of a workflow should be carefully tailored to the protein of interest"

Proteins come in all shapes, conformations, and sizes, and since no two proteins are exactly alike, no generalizations can be applied in protein purification.[1] Different proteins may respond differently to treatment with chemical, mechanical, or biological agents used during the extraction and purification process. As such, to optimize yield and quality, each step of a workflow should be carefully tailored to the protein of interest.

Starting with Quantity: Maximizing Yield

Maximizing yield starts with selecting the best technique for protein extraction and isolation based on protein source and endogenous location.[1] Choosing the appropriate extraction reagents and equipment for your sample type can be instrumental in maximizing yield.

When working with tissue samples, an extra homogenization step is required to liberate individual cells from tissue structures prior to protein extraction. This step can use enzymatic (e.g., proteinase digestion of extracellular matrix) or mechanical (e.g., shear stress) forces.[1] Enzymatic digestion is thorough, but the researcher is unable to exert direct control over the reaction, and the enzymes often need to be removed and/or inactivated prior to cell lysis so that they do not digest the protein of interest. Mechanical methods are easier to directly control, but the forces they impart can damage and lyse cells, rendering the proteins susceptible to heat-based denaturation from subsequent and continued mechanical homogenization.[2] Since researchers often have to work with samples composed of multiple cell/tissue types, a combination of multiple homogenization methods, such as the tandem use of chemical/enzymatic approaches with mechanical lysis, may be employed to take advantage of the respective strengths of each individual homogenization technique while compensating for their weaknesses, leading to increased protein yield.

For both cellular and tissue samples, the intracellular location of the protein also needs to be carefully weighed. In particular, membrane-and intraorganelle-located proteins may not be fully liberated by cell lysis alone. Liberating these proteins usually requires a detergent to facilitate the solubilization of hydrophobic membrane proteins and the destruction of organelle membranes.[1] However, detergents – particularly strongly ionic ones – can denature proteins, which is undesirable if investigating protein-protein interactions and/or conformation.[3] Furthermore, impurities resulting from detergent use can impact downstream processing steps.[4] Therefore, detergent usage and choice should be carefully made based on ionic strength, intended application, and ease of removal.[3]

Finishing with Quality: Maximizing Purity

While yield is important for the extraction phase of a protein preparation workflow, purity is important for the final product generated for downstream applications. Liquid chromatography or antibody-based methods such as co-immunoprecipitation are popularly employed to extract purified fractions containing a specific protein within a heterogeneous sample.[1] However, epitope- or size-based selection methods may not have the specificity to parse out variants of the protein of interest.

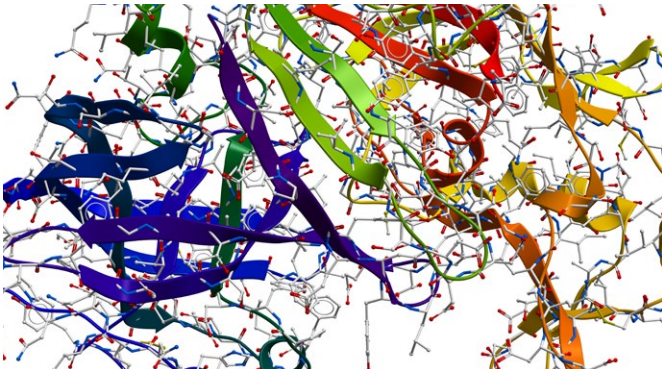
The presence of misfolded variants, truncations, dimers, multimers, aggregates, and complexes not only results in inaccurate quantification and characterization data, but can significantly affect the properties and function of protein-based products. Detergent- and heat-based methods can break up these multi-protein structures, but may cause protein inactivation and damage. Alternatively, amino acids such as arginine have been shown to be effective in solubilizing proteins, promoting stability, and preventing aggregations.[5] Advances in analytical ultracentrifugation now allow for the detection, quantification, and separation of proteins based on properties such as size and conformation, allowing researchers to remove sample impurities.[6] Finally, affinity tags, where peptide sequences are attached to the protein of interest to facilitate their retention and isolation during liquid chromatography, are popularly used to enhance protein solubility, detection, and/or purification.[7] In particular, tandem affinity purification (TAP), a two-stage process employing two different affinity tags, not only allows the purification of a protein of interest but also the isolation of protein complexes interacting with the protein of interest. The utilization of two purification steps results in a considerable reduction in non-specific background.[8]

On the surface, most protein preparation protocols share seven common fundamental steps. However, this belies the complexity of protein extraction and purification. Careful consideration of each step and each reagent used during protein preparation will aid in overcoming the different challenges each sample may present.

For references, please see page 7.

Keep it Together: Confirming Intact Protein Extraction

"as protein research moves towards the clinic, translational and clinical researchers will need to experiment with proteins extracted from banked clinical samples"



The protein extraction and purification process can involve significant amounts of physical, chemical, and enzymatic manipulation. Even if executed with care, these forces have the potential to cause damage and fragmentation to the proteins being isolated. Additionally, the protein source itself, whether cellular, tissue, or fluid in nature, may suffer degradation. This is not typically a problem for basic scientists working with fresh samples. However, as protein research moves towards the clinic, translational and clinical researchers will need to experiment with proteins extracted from banked clinical samples – obtained, processed, and stored under varying conditions.

Crumbling Away: Protein Fragmentation

Protein degradation and fragmentation is a natural process for removing and recycling proteins, and as such, every cell is equipped with proteases. Under basal conditions, protease activity is relatively low, as these enzymes are regulated by molecular elements and physically segregated in intracellular compartments.[1] However, cell lysis, which is necessary for most protein preparations, disrupts both of these regulatory mechanisms. Protease activity can be countered with protease inhibitors, which are widely available commercially. Cocktails containing multiple inhibitor agents are generally sufficient for most applications, but to optimize yield, it may be necessary to identify the predominant protease(s) present within the sample and include the corresponding inhibitor agent(s).[1]

Fragmentation can also occur due to non-enzymatic forces. High temperatures can be useful during protein preparation for denaturing folded proteins and disassociating protein complexes, but excess heat can cleave peptide bonds.[2] Bond cleavage can also result from the presence of metallic ions, free radicals, and both excessively low and high pH.[2]

Protein fragmentation presents a significant challenge to both researchers and manufacturers alike. For example, the inability to confirm whether a detected protein is a fragment or the intact form makes characterizing protein structure, conformation, function, and expression profile all but impossible, and likewise confers doubt regarding the integrity and functionality of a production batch/lot. It is therefore imperative that protein fragmentation be identified.

Identifying and Countering Protein Fragmentation

To identify and assess protein fragmentation, the various proteins within a heterogeneous mixture need to be separated. This can be done by size (e.g., gel electrophoresis, size-exclusion chromatography) or by structure/conformation (e.g., HPLC).[2] Size-based separation methods provide excellent fragment resolution. However, extracting separated protein fractions from commonly employed SDS-based gels can be difficult.[2,3] SDS is not only a denaturing agent, but also interferes with commonly used post-hoc analysis techniques such as MS.[3] Various strategies, including electroelution and passive elution, have been successfully devised to overcome this challenge.[3,4] In contrast, structure-based separation methods are better suited to identifying specific cleavage sites because they can be coupled with MS.[2] However, they are not as adept at characterizing and quantifying the overall fragmentation profile of an entire protein.[2] Finally, analytical ultracentrifugation (AUC) represents an interesting combination of size- and structure-based analysis. While the application of centrifugal force sorts proteins by size, AUC can delineate protein structure and conformation information through analysis of particle diffusion kinetics.[5]

Regardless of whether size- or structure-based separation methods are selected, antibody-based protocols (e.g., ELISA or Western blotting) can be subsequently applied to the extracted purified fraction to confirm the presence of the protein of interest. When it comes to either research data or manufactured product, don't take chances. Make sure that a purified sample of intact protein has been prepared for subsequent experimentation, manipulation, or storage.

For references, please see page 7.

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Article 1 - Be Prepared: Protein Prep Applications & Common Challenges

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Article 2 - Work for it: Considerations for Challenging Samples

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Article 3 - Keep it Together: Confirming Intact Protein Extraction

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