PROTEIN ANALYSIS: KEY TO THE FUTURE

NAWSHEEN BOODHUN EXPLORES WHAT LIES ON THE HORIZON FOR PROTEIN ANALYSIS

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Proteins are a very versatile group of macromolecules and they form the fundamentals of critical processes in the body. At the cellular level, they control cell division, metabolism and the movement of a variety of material and information into and out of cells. This ultimately has an impact on the structure, function and regulation of the tissues and organs in the body. Each protein consists of a linear sequence of amino acids. There are around 20 amino acids that are naturally occurring and each has a particular characteristic defined by its side chain, known as the R-group; this imparts chemical individuality to the molecule. The R-groups vary in shape, size, charge and reactivity, and their features dictate the physical properties of amino acids. The propensity of the side chain to interact with water determines the classification of the amino acid as either hydrophobic, polar or charged. Examples include valine and phenylalanine, which are hydrophobic amino acids meaning that they are essentially 'water-fearing' and are repelled by water, whilst those with a polar side chain, such as serine and asparagine, are soluble. Therefore, the specificity of the sequence of amino acids and interactions between the side chains determines the manner in which the polypeptide folds into a particular conformation, giving the protein a specific three-dimensional structure [1].

The differences that exist between macromolecules allow for protein separation to be carried out and ultimately analysis and characterization of the entities. Proteins can be separated by various means that exploit the differing characteristics of the proteins – for instance, by methods that include isoelectric

precipitation and denaturation of contaminating proteins. This is done based on the different solubility characteristics of the proteins. Ion exchange chromatography and affinity chromatography allows for separation of proteins based on different adsorption characteristics whilst separation by electrophoresis relies on the differences in the migration of charged molecules. Western blotting, a technique first described in 1979 [2], is the immunodetection of electrophoretically separated proteins on a membrane. The method remains the most commonly used to detect specific protein molecules from a sample of tissue homogenate or extract. Identifications are usually achieved by two common methods; mass spectrometry and Edman degradation.

There are a number of methods that are utilized to determine the structure of macromolecules and these include x-ray diffraction, cryo-electron microscopy and nuclear magnetic resonance. To-date, there are over 139,000 biological macromolecular structures that have been deposited in the Protein Data Bank, with protein-only structures accounting for over 129,000 of this figure. This allows for better understanding of changes that occur in diseases, ultimately leading to the development of appropriate therapeutics.

In last month's issue of *BioTechniques*, we explored the ways in which protein structure can be elucidated. In this feature of Tech News, we will take a look at the state of protein analysis with a focus on current approaches and implications for future strategies.

Protein separation

Several forms of polyacrylamide gel electrophoresis (PAGE) exist, and each can provide different types of information about the proteins of interest. Sodium dodecyl sulfate (SDS)-PAGE is a technique that was first described by Laemmli in 1970 and was initially known as the Laemmli method [3]. SDS-PAGE is an electrophoretic methodology that is now widely used in a number of disciplines including biotechnology, molecular biology, genetics, forensic science and other life science fields, and it is the most commonly used technology to achieve high-resolution analytical separation of mixtures of protein [4]. This method separates proteins in a polyacrylamide gel mainly based on their molecular weight.

Due to the complex conformation and different charges of proteins, a unique challenge is posed for electrophoresis as these features affect migration through the gel. Therefore, to ensure that proteins are accurately separated based upon their molecular weight, as opposed to their charge or folding, SDS, an anionic detergent, and a reducing agent such as dithiothreitol (DTT) are utilized to denature the native protein [5]. The SDS molecules form a complex with the protein coating it with a uniform negative charge ultimately masking the intrinsic charge of the R-groups. The reducing agent disturbs the noncovalent forces that are responsible for the 3D structure of the protein and causes the protein to unfold, forming linear molecules. The denatured linear protein molecules are loaded into wells on the polyacrylamide gel. There are two aspects of this: a stacking gel and a separation gel. When an electrical current is applied, due to the strong negative charge of the SDS-protein complex, the current drives the proteins through the gel towards the positive electrode. Molecules of different molecular sizes travel at varying speed whereby the mobility of the molecule is inversely proportional to its size. They separate on the gel and this is viewed as bands when a stain or dye solution such as Coomassie Brilliant Blue or ethidium bromide is utilized.

Agarose gel electrophoresis (AGE) can also be used for DNA and protein separation. The agarose gels are more easily and rapidly prepared compared with polyacrylamide gels. Although they have a wide separation, they have low resolving power as the bands formed in the gel tend to be fuzzy and spread apart due to the pore size. This is an established method used to separate medium-to-large biomolecules and considered to be the most effective way of separating DNA fragments ranging from 100 base pair to 25 kb [6]. PAGE on the other

hand has a greater resolving power, and can accommodate a larger quantity of DNA without compromising on resolution, thus making it a good technique for analysis of low-molecular-weight fragments.

Collaboration is key

Isoelectric focusing electrophoresis (IEF) is a high-resolution technique and a common method for analysis of the isoelectric point (pI) of proteins within a continuous pH gradient. The technique is based on the mechanism that for any individual protein, there is a pH point at which the molecule will have a net charge of zero, thus it will not migrate further in an electric field. IEF allows detection of charge heterogeneities in addition to impurities. Combining IEF and SDS-PAGE in consecutive steps brings about a powerful and sensitive technique for separating and analyzing protein mixtures known as two-dimensional gel electrophoresis (2-DE). It is considered a key tool for comparative proteomics research and allows for the separation of the protein sample by charge (pI) via IEF in the first dimension. The sample is further separated by its molecular weight via SDS-PAGE in the second dimension [7]. Although the method was first described in 1975 by O'Farrell [8], its applicability and adoptability have been enhanced by the introduction of immobilized pH gradient strips to allow for reproducible results with easier handling. However, 2-DE is still laborious and time-consuming. Conventional protocols are also restricted to detecting denatured proteins within the size range of 10–200 kDa at pH 3.5–11.5 [7]. Despite these limitations, 2-DE gels have been successfully utilized in biomarker discovery in neurodegenerative diseases [9].

Over the years, advances have been made at various stages of the 2-DE technique, which now has the capability of separating up to 10,000 proteins in a single gel and allowing high-resolution proteomic analysis to take place [10]. The advantage of 2-DE is not limited to the separation of large numbers of proteins, but it also allows their relative abundances to be determined. For instance, proteins that are extracted from a serum representing healthy and diseased state can be separated and stained, ultimately allowing for the protein spots to be aligned and scanned to measure their individual intensities. Although software alignment tools have seen various advancements, there are challenges carrying out direct spot-to-spot intensity comparison. This is due to the differences that exist in the gel composition involved in the technique, the pH

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gradient and the applied voltage. These factors also contribute towards the reproducibility difficulties encountered with the 2-DE technique [11].

The challenges seen in the 2-DE technique have been overcome with the development of two-dimensional difference in gel electrophoresis (2D-DIGE). This technique addresses the concerns of reproducibility and enables better protein comparison and quantification to take place. It is a modification of the 2-DE process and requires only a single gel to reproducibly compare two different protein samples. The principle of the method is based on the labelling of each protein mixture with a different, spectrally resolvable fluorophore agent (Cy2, Cy3 and Cy5) [12]. Following the labelling stage, the proteomes are pooled together with an internal standard consisting of the mixture of all samples, leading to a more accurate normalization of protein spots from all sample gels. The proteins are then visualized separately by use of a different emission wavelength and each particular fluorophore is excited. This generates distinct gel images corresponding to each prelabelled sample that can be examined using an image analyzer software such as DeCyder [13]. The abundance of each protein spot is measured as a ratio to its corresponding spot present in the pooled internal standard by use of software programs.

Combining mass spectrometry with 2D-DIGE is a powerful tool in characterizing differential proteome expression. In a study published in *Respiratory Research* in 2017, Lakhdar and colleagues utilized 2D-DIGE combined with mass spectrometry to identify proteins that could be involved in the process of muscle wasting in chronic obstructive pulmonary disease. By using this technique, they elucidated differences in the expression of various proteins in the wasted *vastus lateralis* muscle that are relevant to the disease process [14].

Mass spectrometry

Mass spectrometry is a core analytical technique for protein research and for the study of biomolecules as a whole. This technique can be utilized to measure the mass-to-charge ratio of ions for the identification and quantification of molecules in simple and complex mixtures of protein. In general, ionization, acceleration, deflection and detection are the key stages to mass spectrometry. The process involves a sample being injected into the mass spectrometer. The molecules are then ionized and accelerated, which means that the positive ions created at the ionization phase are accelerated towards the negative plates, the speed of which is dependent on their mass. Under an ultra-high vacuum, the ions are separated by the mass analyzer via electromagnetic deflection based on their mass-to-charge value and recorded by a detector [15]. The ions that are properly aligned are detected and amplified. This technique has become invaluable in a range of disciplines.

The last two decades has seen immense development with regards to high-throughput and quantitative mass spectrometry proteomic workflows, allowing knowledge to be extended about protein structure and function. Nakamura and colleagues have demonstrated, in a study published in *Nature*, the development of the first blood test to detect amyloid- β protein build-up in the

brain, one of the earliest pathological signatures of Alzheimer's disease [16]. Currently, the only validated methods for detecting amyloid- β deposition in the brain are costly and can be highly invasive. Examples of such methods include utilization of PET imaging or measurement of amyloid- β presence in cerebrospinal fluid. Through use of immunoprecipitation coupled with mass spectrometry, Nakamura and colleagues demonstrated the measurement of high-performance plasma amyloid- β biomarkers. They showed that blood samples and tests could be used to predict brain amyloid- β burden with an accuracy approximately equal to 90% when using PET imaging as a standard of truth. This process provides advantages for the future in terms of cost-benefit, scalability and an alternative to highly invasive processes. Future potential implications include broader clinical access, efficient population screening of plasma amyloid- β biomarkers for Alzheimer's disease and detection of early signs of Alzheimer's disease even in those who have not exhibited clear symptoms.

Protein analysis methodologies and techniques have come a long way. Particular advances include the discovery of new diagnostic markers, approaches for multiplex analysis of proteins and better visualization and interpretation of results. Advancements in protein analysis technology, methods and processes allow researchers to improve knowledge and understanding about protein structure and function as well as presence and

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abundance in healthy and disease states. This can ultimately provide better opportunities to detect biomarkers of particular conditions and development of treatments.

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