

Proteomics Techniques in Focus: From Basics to Advanced Applications

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September 25, 2023

Abstract

Proteomics is a collective approach using other “omics” technologies for analyzing and identifying the function and structure of proteins with greater precision. Along with the revolution in technologies and increase in statistical computing models, proteomics has been cognate promptly over the last ten years and enlightened on resolving complex diseases. Identification of various protein biomarkers, expression patterns of proteins in diseases, and the mechanism of pathogenicity, there is a demand for Proteomics-based technologies. This article profound impact on proteomics across diverse scientific domains, including disease biomarker discovery, drug development, structural biology, and functional genomics, complex biological systems, and underscores the significance of various techniques, including mass spectrometry, protein microarrays, SILAC, and iTRAQ. This review aims to illustrate several technologies of proteomics with their principle, the latest advancements, and their role in research.

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Abstract

Proteomics is a collective approach using other “omics” technologies for analyzing and identifying the function and structure of proteins with greater precision. Along with the revolution in technologies and increase in statistical computing models, proteomics has been cognate promptly over the last ten years and enlightened on resolving complex diseases. Identification of various protein biomarkers, expression patterns of proteins in diseases, and the mechanism of pathogenicity, there is a demand for Proteomics-based technologies. This article profound impact on proteomics across diverse scientific domains, including disease biomarker discovery, drug development, structural biology, and functional genomics, complex biological systems, and underscores the significance of various techniques, including mass spectrometry, protein microarrays, SILAC, and iTRAQ. This review aims to illustrate several technologies of proteomics with their principle, the latest advancements, and their role in research.

Key Word: Proteomics, Protein, Microarray, Technologies, Disease.

Introduction

After the Human Genome Project, numerous gene markers were identified for treating complex disorders. However, these markers have limitations. Genomic and transcriptomic analyses only reflect cellular conditions and don't account for post-translational modifications (PTMs) like phosphorylation or protein degradation [1]. Proteomics, which combines experiments and data analysis, offers a complementary view of protein structure, composition, expression, alterations, and interactions. It plays a crucial role in mapping intricate molecular pathways and networks that regulate vital processes such as cell division, proliferation, and apoptosis [2].

Proteomics, while more complex than genomics, is crucial for understanding gene function and distinguishing cellular states. Unlike microarrays for transcriptome analysis, proteomics directly assesses proteins, which are influenced by mRNA levels, translational control, and other mechanisms, providing a comprehensive view of biological systems [4].

Traditional protein purification methods include chromatographic techniques like affinity chromatography, ion exchange (IEC) and size exclusion chromatography (SEC) [5]. Proteins analyzed by Enzyme-linked immunosorbent assay (ELISA) and Western blotting methods have limitations to individual assessments and can't determine expression levels [6,7]. Protein assortments with complex symmetry are sorted using 2D gel electrophoresis (2D-E), 2D differential gel electrophoresis (2D-DIGE), and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [8] as shown in Figure 1.

Protein microarrays provide quick expression-level insights but lack whole-genome functionality. Mass spectrometry (MS), a proteomics technique, enhances the sensitive evaluation of complex protein mixtures [9]. Edman degradation can identify specific protein amino acid sequences [10]. Recent advances in quantitative proteomics include isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC), and isobaric tag for relative and absolute quantification (iTRAQ) [11]. Three-dimensional (3D) protein structures, which help to understand protein function and its binding affinities to other molecules, are determined by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [12] are shown in Figure 1.

Figure 1: A Flow chart of proteomics technologies

High-throughput proteomics generates extensive data stored in bioinformatics databases. These tools cover diverse applications, including protein structure prediction, interaction analysis, and evolutionary relationships [13]. Combining proteomic approaches provides comprehensive insights into cell function and responses to stress and medication. This review explores proteomic techniques and their applications.

3. Conventional methods

3.1 Chromatography-based methods

3.1.1 Ion exchange chromatography

Ion Exchange Chromatography (IEC) separates ionizable compounds by immobilizing the ions reversibly to adsorb to oppositely charged molecules. IEC is a broadly employed liquid chromatography (LC) technique due to its relatively simple and adaptable procedures along with notable sample-handling capacity, immense applicability, specifically concerning proteins and enzymes, cost-effectiveness, and remarkable resolving capability, making it more adaptable [14].

IEC depends upon Van der Waals forces among proteins containing charged amino acids and a solid matrix. Matrix holds ion-load opposite to a specific protein that is being extracted and the protein's affinity for the column is achieved through ionic interactions. By altering the pH, concentrating ion salts, or increasing the amount of ions in the buffer solution, proteins are capable of being removed from the column. Anion exchange matrices (positively charged) readily adsorb to the proteins carrying a negative charge and cation-exchange matrices (positively charged) adsorb to the proteins having a positive charge [15]. Ion Exchange Chromatography (IEC) has two types: anion exchange chromatography (AIEC) and cation exchange chromatography (CIEC). AIEC is commonly used for acidic proteins and for removing negatively charged molecules like DNA

and RNA during virus purification and endotoxin removal. IEC is versatile for capturing, purifying, and polishing recombinant proteins. It helps with capturing capacity and yield in the initial stages and prioritizes purity in later purification steps. It can also concentrate low-molecule concentrations before purification. In monoclonal antibody (mAb) purification, IEC assists in at least one of two polishing steps after the protein an affinity step [16].

RNA, DNA, host cell proteins, leached Protein A, and endotoxins being negatively charged are removed by blind and elute mode in CIEC during mAb polishing. Dissociate charged Ab variants are also eluted with CIEC. AIEC can efficiently purify simpler mAbs from impurities like DNA, RNA, acidic HCPs, and leached protein A in flow-through (FT) mode even in case of loose binding to resins. [16].

3.1.2 Size exclusion chromatography

Proteins in Size exclusion chromatography (SEC) are separated based on the size in a porous matrix, useful for diverse protein conditions and purifications [17]. SEC employs materials like dextran, reducing salt and determining molecular weights, using materials like Sephadex G type, polyacrylamide, or agarose [15]. Conventional SEC columns are valuable for proteins like erythropoietin and monoclonal antibodies (mAbs) in high-performance size exclusion chromatography (HP-SEC), assessing glycoprotein quality [18]. SEC-HPLC/UV efficiently monitors protein monomers, aggregates, and nitration degrees (NDs), directly quantifying bovine serum albumin (BSA) [20].

High-molecular-weight aggregates, significant in mAb and bispecific mAb production, increase immunogenicity. Silica-coated SEC columns with hydrophobic silica particles enhance reliability in identifying key protein aggregates [19]. Recent research combines UHPLC-SEC with techniques like UV-MALS-QELS-RI, accurately measuring molar mass for biotherapeutic proteins [21]. SDS-PAGE, Fast-SEC, and DLS analyze 18 kDa heat shock proteins, assessing their potential for generating aggregates under various conditions. MALS-UV-RI/SEC-HPLC distinguishes aggregation levels in protein antigens for a *Clostridium difficile* vaccine, a protein-based vaccine [19].

3.1.3 Affinity chromatography

Affinity chromatography, a significant protein purification advancement, enables the study of protein degradation, post-translational changes, and interactions. It relies on an interaction between an immobilized affinity ligand and target proteins needing purification. Ligands like dextran, polyacrylamide, or cellulose attach to the column-filling material. Specific proteins form complexes, binding to the matrix and adhering to the column. Unbound proteins pass through, and the bound protein elutes with ionic strength changes due to pH or salt addition [15].

In recent decades, various affinity chromatography techniques, including IMAC, immunoaffinity, heparin, and lectin, have been explored for lab-scale vaccine purification, offering promising results and higher yields than alternatives like ultracentrifugation. Additional ligands like dye ligands for virus and toxin purification, sugar ligands for toxin purifications, and novel methods for pDNA and vaccine antigen purification have been investigated [23].

However, challenges hinder industrial-scale implementation, including large-scale ligand production, composition limitations, raw material availability, lack of high-productivity chromatographic media, and standards for column disinfection and cleaning [23].

3.2 Enzyme-linked immunosorbent assay

This method can be classified into several distinct ELISA types

Direct ELISA: Involves the primary antibody-enzyme conjugate interacting directly with an antigen-coated plate.

Indirect ELISA: Utilizes enzyme-linked secondary antibodies specifically designed to interact with the primary antibodies that have already bound to the antigen-coated plates.

Competitive ELISA: Engages in a competition of sample antigen with plate-coated antigen for binding to the primary antibody, followed by the introduction of a secondary antibody-enzyme conjugate.

Sandwich ELISA: ELISA involves adding the antigen to an antibody-coated plate, followed by attaching a detection antibody and a secondary antibody-enzyme conjugate to the antigen recognition site. ELISA offers versatile and precise antigen detection methods [26], used in clinical practice for various analytes in diverse sample types. Since its invention, ELISA has found applications in fields like food quality, environmental monitoring, biotechnology, microbiology, and chemistry. Engvall and Perlmann pioneered ELISA research in 1971 by using enzyme marker, alkaline phosphate, to quantify IgG levels in rabbit serum [27]. ELISA assays detect allergic proteins in wheat that cause hypersensitive reactions in sensitive individuals [28], identify *Bacillus thuringiensis* (Cry1Ac) protein in recombinant BT cotton [29], and detect *Botrytis cinerea* in fruit tissues [30].

Digital ELISA detects mono molecules in the blood, like prostate-specific antigen (PSA) at low concentrations (14 fg/ml). It's also used to identify p, p'-DDE, an insecticide metabolite, aiding in persistent organic pollution detection [31]. However, ELISA has limitations, including time-consuming testing and limited sensitivity for challenging biomolecules.

3.3 Western blotting

Western blotting is a widely used technique to detect target protein presence, size, abundance, and modifications in research. It's valuable for diagnostics, therapeutics, and academic studies. This method transfers protein patterns from a gel to a membrane and has evolved since its introduction in 1979 [32]. Western blotting is also essential for identifying microorganism-related antigens and infectious diseases. For example, it assesses HSV-2 seroprevalence by measuring specific immunoglobulin G levels [33] and identifies *Leishmania donovani* through Hsp83 and Hsp70 antigen detection [34]. This technique has diverse applications, including protein quantity, kinase function, cellular localization, protein-protein interactions, and post-translational modifications. Its use in skeletal muscle and exercise physiology research is growing, but maintaining quality control is crucial to avoid misleading data [35].

Cell uniqueness is a fundamental aspect of biology, impacting development, stem cell biology, and cancer. Conventional bulk measurements, however, mask the intricate biology within individual cells. Single-cell measurement techniques are essential to unveil cell-to-cell variation, and microfluidic instruments are indispensable for precise biochemical experiments at this level. Single-cell western blotting (scWB), akin to traditional western blotting, proves especially advantageous when dealing with protein targets lacking specific antibodies or when background signal from intact cells poses challenges. This method is invaluable for direct protein quantification in single cells, with applications spanning basic bioscience research to practical biomedical purposes [36].

3.4 Edman sequencing

In 1950, Pehr Edman pioneered Edman sequencing, a method to determine protein amino acid sequences by reacting with and identifying N-terminal residues of polypeptide chains. This technique has played a vital role in biological drug development and quality control for therapeutic proteins [10]. It continues to find applications in various life science research fields. Edman's PITC reaction was successfully used to modify peptide amines and identify proteolytic protein cleavage in cell signaling processes with high throughput [37]. It helped identify the N-terminal tripeptide GTF262 of GP-2 in the Lassa virus, which causes hemorrhagic fever [38].

In plant protein research, both MS and Edman sequencing were employed to analyze rice leaf sheath proteins, revealing shared amino acid sequences and showcasing their complementary use [39]. Moreover, Edman degradation has contributed to the development of a photo thermally responsive Nanoprobe. The probe releases the fluorescent dye Cy5.5 upon exposure to an 808 nm laser, enhancing near-infrared (NIR) fluorescence under slightly acidic conditions. This Nano probe enables fluorescence imaging of v3 over-expressing U87MG cells in vitro and in vivo, presenting a novel tool for biomedical imaging, drug delivery, and gene

administration [40].

4. Advanced methods

4.1 Protein microarray

DNA microarrays were a significant genomics development but couldn't predict protein structures or dynamics accurately. Protein microarrays analyze thousands of proteins or peptides and are valuable for clinical and functional research. They assess PTMs, quantify target proteins in fluids, and study protein interactions and autoantibodies in disease conditions [41]. Protein microarrays allow active exploration of the human proteome, identifying biomarkers, immune profiles, enzymes, and quantifying proteins [42]. They offer multiplexed and sensitive protein analysis, addressing complex proteomes with limited specimens [43].

Nucleic Acid Programmable Protein Arrays (NAPPA) is a potent method for biomarker screening and protein-protein interaction investigation. Unlike traditional protein microarrays, NAPPA uses in vitro transcription/translation, eliminating the need for costly protein purification. Proteins are synthesized directly on the array using a DNA template and coupled with an affinity reagent [43]. Various in situ expressed microarrays, like PISA and DAPA, exist alongside NAPPA technology. PISA differs from NAPPA as it uses free DNA templates, eliminating the need for DNA immobilization [44].

Proto Arrays, designed for analyzing numerous proteins, require small sample volumes, around 10 μ L of serum, making them ideal for high-throughput screening. They are used in studies like the Parkinson's disease biomarker search (ParkCHIP) and systemic erythematosus lupus (SLE) autoantibody investigations. SLE patients had significantly higher levels of 446 IgG and 1218 IgM autoantibodies against 9500 antigens, including novel ones related to the nucleus, cytoplasm, or membrane [45,46]. Protein microarrays fall into analytical, functional, and reverse-phase categories [Figure 2].

Figure 2: Technologies and applications of protein microarray.

4.1.1 Analytical protein microarray

Expression levels, binding affinities and specificities of complex proteins assortments are studied using Analytical microarrays. These microarrays, often made on glass slides, consist of antibodies, aptamers, or affibodies. They are mainly employed for capturing specific proteins, including clinical applications such as diagnosing diseases and monitoring expression changes due to environmental stress [47]. For instance, antibody arrays have been used for profiling proteins in bladder cancer and squamous carcinoma, as well as detecting toxins like B. ricin, cholera toxin, Bacillus globigii, and Staphylococcal Enterotoxin B [48].

4.1.2 Functional protein microarray

The emergence of protein domains with full-length functionality provides a way forward for Functional protein microarrays. The protein chips are utilized in a single experiment to examine the biochemical functions of the whole proteome [47]. Purified proteins are now being used to create functional protein microarrays, that allows to study the protein interactions with protein, lipid, drug, DNA and RNA. In yeast, protein kinases substrate specificity was first analyzes using functional protein microarray. Protein functions and disease biomarkers are now identified via functional protein microarray [49].

4.1.3 Reverse-phase protein microarray

Reverse phase protein microarrays (RPA) are a subtype of analytical microarrays. RPA involves lysing cells from different tissues, arranging them on a nitrocellulose slide, and probing specific proteins with antibodies. Detection is typically done using chemiluminescence, fluorescence, or colorimetry, with reference peptides enabling protein quantification [47]. RPA excels at comprehensive protein profiling, including the detection of various modifications like phosphorylation, glycosylation, ubiquitylation, cleavage, and alteration. This makes RPA valuable for identifying disease-related protein variations and potential targeted therapies [50]. It's instrumental in identifying defective protein pathways, potentially leading to targeted drug development for disease treatment.

4.2 Gel-based approaches

4.2.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Analytical electrophoresis uses polyacrylamide gels to separate proteins without aggregation. It denature proteins using a reducing agent along with sodium dodecyl sulfate (SDS) as an anionic detergent, allowing separation based on molecular weight during electrophoresis [51]. SDS effectively denatures proteins by unfolding them and coating them in negatively charged SDS molecules. This masks the protein's native charge. β -mercaptoethanol cleaves disulfide bonds. Uniformity in homology and charge to mass ration is attained after SDS and mercaptoethanol treatment of proteins. Electrophoretic mobility is determined by size, with smaller complexes moving faster due to the gel's sieving effect. Mobility is inversely proportional to protein mass [52].

4.2.2 Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates proteins based on both mass and charge, allowing precise protein analysis. It can characterize around 5,000 distinct proteins depending on gel size. Proteins are dissociated based on their charge followed by their mass in first and second dimensions respectively. This technique is used for studying biochemical processes, post-translational modifications, and protein alterations [53]. The process involves five steps: sample preparation, first separation, linking, second separation, and protein identification. Isoelectric focusing usually precedes SDS electrophoresis. The traditional order is preferred for practical reasons, as SDS gels are easier to work with and compatible with downstream analysis methods like mass spectrometry [54].

5. Quantitative techniques

5.1 ICAT labeling

Proteins from cancer cells are tagged with isotope-coded affinity tags (ICAT reagents) and analyzed with a mass spectrometer, efficiently identifying disease-related proteins. This method allows simultaneous detection and assessment of various proteins, aiding researchers in pinpointing disease causes for potential targeted therapies [55]. ICAT, an early mass spectrometry-based labeling method, targets cysteine residues with three components: a biotin affinity tag, a thiol-reactive group, and a linker with specific isotopes. It labels cysteine residues with either 1H or 2H atoms (or 12C and 13C) using ICAT reagents. Afterward, proteins are purified with avidin affinity chromatography, digested by trypsin, and analyzed using LC-MS/MS to generate a spectrum. ICAT specifically tags cysteine residues, making up 3.3% of vertebrate amino acids, potentially missing proteins without Cys residues and posing quantitation challenges for those with a single Cys residue [56].

5.2 Stable Isotopic Labeling with Amino Acids in Cell Culture (SILAC)

SILAC is a quantitative proteomics method that uses mass spectrometry (MS) for labeling and distinguishing proteins within cells or samples. It employs "light" and "heavy" amino acid labels to differentiate proteins in cultured cells. SILAC is valuable for studying post-translational modifications, cell signaling, gene expression, and secretory protein pathways in cell culture [57]. Stable amino acids (like 13C or 15N-labeled arginine or lysine) are integrated into the entire proteome during cell culture with isotope-labeled residues using SILAC. Two cell populations are grown in different media: "light" with natural amino acids and "heavy" with stable isotope-labeled amino acids. After multiple cell divisions (usually at least five cycles in mammalian cells), proteins in heavy media are entirely labeled. The labeling efficiency is checked before quantification, and then equal amounts of labeled and unlabeled cells or protein extracts are combined. Peptides are generated from these samples and analyzed using LC-MS/MS. SILAC quantification is based on comparing the ratio of isotope-labeled peptides to unlabeled peptides, allowing for quantitative comparison of light and heavy samples using signal intensities [58].

5.3 Isobaric tag for relative and absolute quantitation

iTRAQ is a mass spectrometry-based method using isobaric tags (8-plex or 4-plex) to label proteins for quantification. Labeling targets N-termini and amine groups on side chains. Liquid chromatography separates labeled proteins, which are then analyzed by mass spectrometry. iTRAQ allows simultaneous protein identification and quantification [59].

Shotgun proteomics involves extracting and digesting protein mixtures from diverse samples. Multiple iTRAQ reaction mixtures, capable of labeling up to 8 samples, differentially label the proteins. After labeling, the samples are combined in a 1:1 ratio for multiplexing. Subsequently, they undergo high-performance liquid chromatography separation and mass spectrometry analysis. The 8-plexed sample can be separated using one of three methods: (1) IEF separation on gel followed by MALDI mass spectrometry (MS and MS/MS of the spots), (2) Gel-C-MS separation followed by high-resolution mass spectrometry, or (3) High-performance liquid chromatography separation coupled with high-resolution mass spectrometry [60].

5.4 X-ray crystallography

X-ray crystallography is a method for determining crystalline 3D structures of protein. Crystallized samples are exposed to X-rays, and the resulting patterns yield information about repeating unit sizes and crystal packing symmetry. This technique is applied to study various processes, including protein-DNA complexes, virus systems, and immunological functions. Moreover, it provides valuable insights into enzyme mechanisms, drug development, mutagenesis, and protein-ligand interactions [61].

6. High-throughput techniques

6.1 Mass spectrometry

Mass spectrometry (MS) determines protein molecular weights by analyzing mass-to-charge ratios (m/z) in a three-step process: converting molecules into gas-phase ions, separating ions based on m/z ratios, and quantifying ion quantities for specific m/z values. Common ionization methods include electrospray-ionization, matrix-assisted laser desorption ionization (MALDI), and surface-enhanced laser desorption/ionization (SELDI) [62].

GF1, IGF2, A2GL, and other blood proteins have potential as breast cancer biomarkers, with MS used to detect and quantify them along with interleukin-12, prostate-specific antigen (PSA), and human growth hormone in human samples. MS also reveals drug administration and metabolite distribution within the body, aiding in analyzing novel treatments and therapeutic processes [63].

6.2 NMR spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) efficiently determines protein molecular structure, function, and folding. The process involves sample preparation and specific measurements to validate structural information. NMR complements X-ray crystallography, particularly for smaller proteins (<30 kDa), and has resolved 17% of Protein Data Bank structures, often when crystallography data is unavailable. Despite being less prominent than X-ray techniques, NMR remains valuable for mapping cofactor binding sites, assessing protein dynamics, detecting different conformations, exploring protein forms, and determining Ionizable group pKa values [64,65].

7. Bioinformatics analysis

Over the past few years, the application of bioinformatics for proteomics has become much more prominent. The production of a new algorithm that allows the analysis of larger datasets (Figure 3) with greater specificity and accuracy aids in the detection and quantifying of proteins, making it possible to acquire detailed information about the protein's expression.

Figure 3: Some Bioinformatics tools for proteomic data analysis.

There are many bioinformatics tools that are used for primary sequence analysis and structure-function analysis of protein (Figure 4). The main problem is to manage such a large amount of data. Finding connections between proteomic data and other omics technologies, such as genomics and metabolomics, is

still challenging. However, new semantic statistical algorithms are an effective tool that could be helpful to get beyond these restrictions [66].

Figure 4: Some Bioinformatics tools for primary sequence and structure-function analysis of protein.

8. Conclusion

Proteomics has made several immensely helpful advances over the past few years. These technologies provide sensitive and expeditious coverage of proteome. Additionally, combining these technologies helps in structural and functional analysis, purification, quantification, and bioinformatics analysis of several proteins. The emerging field of proteomics has been beneficial for all biological fields of science. The article underscores proteomics' impact in fields like disease biomarker discovery, drug development, structural biology, and functional genomics. Despite its successes, the review acknowledges existing challenges, particularly in quantification accuracy and proteome coverage. Overall, this review is a valuable resource for researchers, offering insights into proteomic techniques and their applications, with the potential to further enrich our knowledge of proteins and their roles in biology and medicine. However, much work is still needed to enhance the efficacy of prominent proteomics techniques.

Acknowledgments:

We would like to acknowledge the contributions of all the researchers, and scientists, whose work and studies have been referenced in this article.

We also extend our appreciation to the Alliance International Organization (ANSO) Institute Pasture of Shanghai Chinese Academy of Sciences supported the research. Without their financial support, many of these studies would not have been possible.

conflicts of interest

The authors declare no conflicts of interest related to the research, writing, or publication of this article.

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