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Review

Review

Proteomics: Technologies and Their Applications

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Abstract

Proteomics involves the applications of technologies for the identification and quantification of overall proteins present content of a cell, tissue or an organism. It supplements the other "omics" technologies such as genomic and transcriptomics to expound the identity of proteins of an organism, and to cognize the structure and functions of a particular protein. Proteomics-based technologies are utilized in various capacities for different research settings such as detection of various diagnostic markers, candidates for vaccine production, understanding pathogenicity mechanisms, alteration of expression patterns in response to different signals and interpretation of functional protein pathways in different diseases. Proteomics is practically intricate because it includes the analysis and categorization of overall protein signatures of a genome. Mass spectrometry with LC–MS-MS and MALDI-TOF/TOF being widely used equipment is the central among current proteomics. However, utilization of proteomics facilities including the software for equipment, databases and the requirement of skilled personnel substantially increase the costs, therefore limit their wider use especially in the developing world. Furthermore, the proteome is highly dynamic because of complex regulatory systems that control the expression levels of proteins. This review efforts to describe the various proteomics approaches, the recent developments and their application in research and analysis.

Introduction

The dynamic role of molecules to support the life is documented since the initial stages of biological research. To demonstrate the importance of these molecules, Berzelius in 1838 given the title "protein", which is originated from the Greek word, *proteios*, meaning "the first rank" (1). The "proteome" can be defined as the overall protein content of a cell that is characterized with regard to their localization, interactions, post-translational modifications and turnover, at a particular time. The term "proteomics" was first used by Marc Wilkins in 1996 to denote the "PROTein complement of a genOME" (2). Most of the functional information of genes is characterized by the proteome. The proteome of eukaryotic cells is relatively complex and exhibits extensive dynamic range. Moreover, prokaryotic proteins are responsible for pathogenic mechanisms; however, their analysis is challenging due to huge diversity in properties such as dynamic range in quantity, molecular size, hydrophobicity and hydrophilicity (3).

Proteomics is crucial for early disease diagnosis, prognosis and to monitor the disease development. Furthermore, it also has a vital role in drug development as target molecules. Proteomics is the characterization of proteome, including expression, structure, functions, interactions and modifications of proteins at any stage (4). The proteome also fluctuates from time to time, cell to cell and in response to external stimuli. Proteomics in eukaryotic cells is complex due to post-translational modifications, which arise at different sites by numerous ways (5).

Proteomics is one of the most significant methodology to comprehend the gene function although, it is much more complex

compared with genomic (6). Fluctuations in gene expression level can be determined by analysis of transcriptome or proteome to discriminate between two biological states of the cell. Microarray chips have been developed for large-scale analysis of whole transcriptome. However, increase synthesis of mRNA cannot measure directly by microarray (7). Proteins are effectors of biological function and their levels are not only dependent on corresponding mRNA levels but also on host translational control and regulation. Thus, the proteomics would be considered as the most relevant data set to characterize a biological system (8).

The conventional techniques for purification of proteins are chromatography based such as ion exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography (9–11). For analysis of selective proteins, enzyme-linked immunosorbent assay (ELISA) and western blotting can be used. These techniques may be restricted to analysis of few individual proteins but also incapable to define protein expression level (12, 13). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2-DE) and two-dimensional differential gel electrophoresis (2D-DIGE) techniques are used for separation of complex protein samples (14–16).

Protein microarrays or chips have been established for high-throughput and rapid expression analysis; however, progress of a protein microarray enough to explore the function of a complete genome is challenging (17). The diverse proteomics approaches such as mass spectrometry (MS) have developed to analyze the complex protein mixtures with higher sensitivity (18). Additionally, Edman degradation has been developed to determine the amino-acid sequence of a particular protein (19). Isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC) and isobaric tag for relative and absolute quantitation (iTRAQ) techniques have recently developed for quantitative proteomic (20–23). X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are two major high-throughput techniques that provide three-dimensional (3D) structure of protein that might be helpful to understand its biological function (22, 24).

With the support of high-throughput technologies, a huge volume of proteomics data is collected. Bioinformatics databases are established to handle enormous quantity of data and its storage. Various bioinformatics tools are developed for 3D structure prediction, protein domain and motif analysis, rapid analysis of protein–protein interaction and data analysis of MS. The alignment tools are helpful for sequence and structure alignment to discover the evolutionary relationship (25, 26) (Figures 1 and 2). Proteome analysis provides the complete depiction of structural and functional information of cell as well as the response mechanism of cell against various types of stress and drugs using single or multiple proteomics techniques. Therefore, this review will emphasized on current progress in proteomics techniques and their applications (Figure 3).

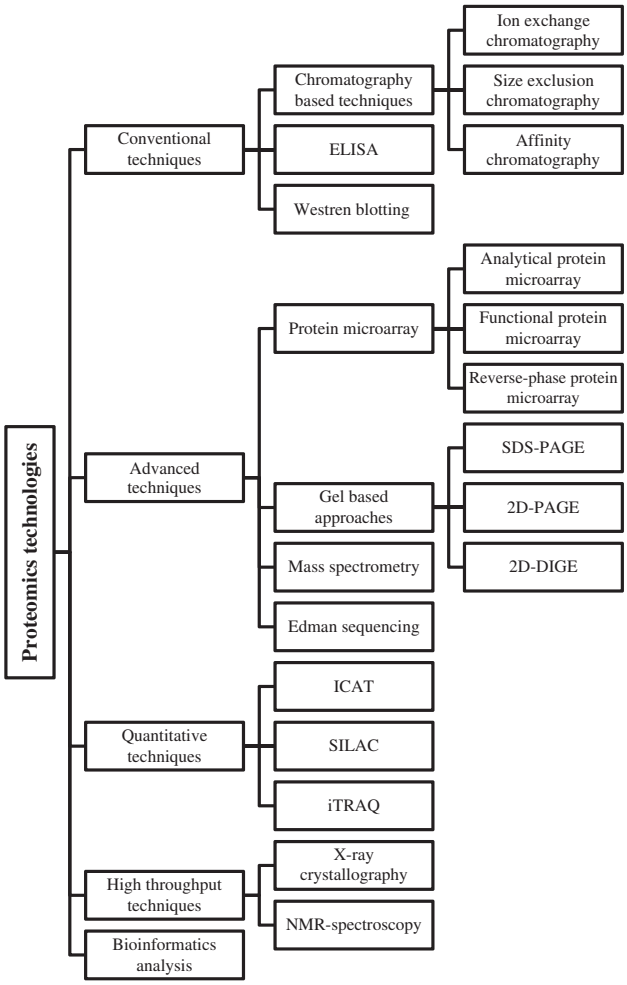

Figure 1. An overview of proteomics techniques.

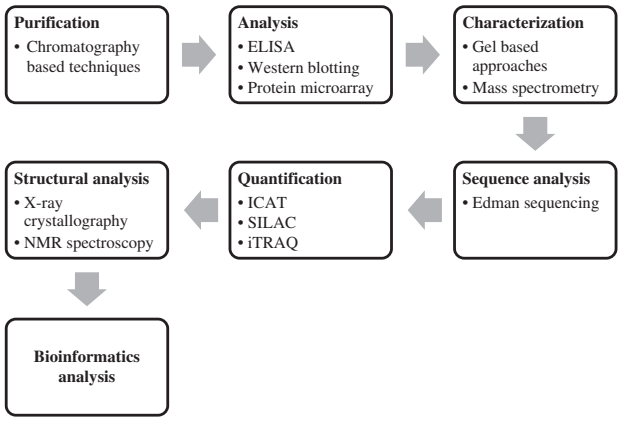

Figure 2. Applications of proteomics techniques.

Conventional techniques

Chromatography-based techniques

Ion exchange chromatography

The IEC is a versatile tool for the purification of proteins on the basis of charged groups on its surface. The proteins vary from each other in their amino-acid sequence; certain amino acids are anionic while others are cationic. The net charged contain by a protein at physiological pH is evaluated by equilibrium between these charges. Initially, it separates the protein on the basis of their charge nature (anionic and cationic), further on the basis of comparative charge strength. The IEC is highly valuable due to its low cost and its capacity to persist in buffer conditions (9).

A most important virulence factor of *Helicobacter pylori* is the Neutrophil Activator Protein (HP-NAP) that is able to activate human

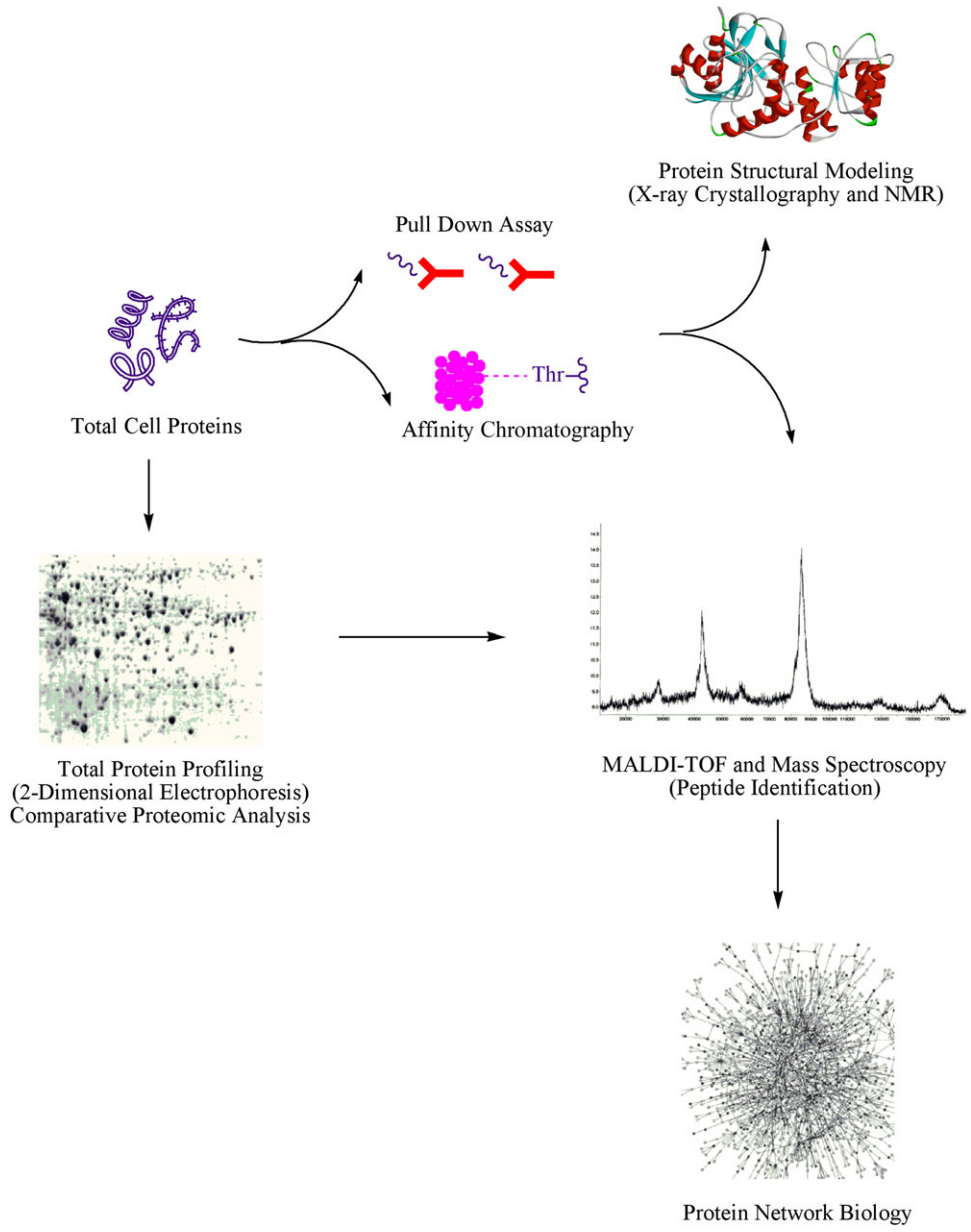

Figure 3. Schematic representation of protein analysis. The conventional methodology for protein analysis includes protein extraction, purification and structural studies. Cells or tissue are processed by various physical (sonication) and chemical (detergents) techniques for the extraction of total protein. Based upon physiochemical nature of polypeptides, the protein of interest can be separated out by different chromatographic techniques. Various methods including X-ray crystallography, NMR and MALDI-TOF are extensively used for structural elucidation and functional characterization of proteins. Nowadays, high-throughput techniques including total proteome analysis and MALDI-TOF are employed to study protein network biology.

neutrophil by secreting mediators and reactive oxygen species. The HP-NAP is a potential diagnostic marker for *H. pylori* and as well a probable drug target and vaccine candidate. One step anionic exchange chromatography has been designated by Shih *et al.* (27) to purify the recombinant HP-NAP expressed in *B. subtilis* with 91% recovery. The mussel adhesive proteins (MAPs) have distinctive biocompatible and adhesive properties that are useful for biomedical and tissue engineering (27). Choi *et al.* (28) expressed the recombinant MAPs in *E. coli* and successfully purified through IEC. Antifungal proteins from *B. subtilis* strain B29 were purified through IEC on diethylaminoethyl (29).

Nigella sativa proteins that retain immune modulatory action have been fractionated through IEC and four peaks were received in complete fractionation (30). Proteins expressed in transgenic plants commercially values in pharmaceutical products. An example is Aprotinin; an inhibitor of serine proteases that were expressed in corn seed and purified (31). Cysteine proteases are the key mediators of mammalian apoptosis and inflammation that are expressed in *E. coli* and purified by Garica-calvo *et al.* (32) for better understanding of catalytic properties. The serum consists of various chemokines, cytokines, peptide hormones and proteolytic fragments of large proteins that can be purified using strong cation exchange chromatography (33).

Size exclusion chromatography

SEC separates the proteins through a porous carrier matrix with distinct pore size on the basis of permeation; therefore, the proteins are separated on the basis of molecular size. The SEC is robust technique capable of handling proteins in diverse physiological conditions in the presence of detergents, ions and co-factors or at various temperatures. The SEC is used to separate low molecular weight proteins and is a powerful tool for purification of non-covalent multi-meric protein complexes under biological conditions (10).

The soluble factors produced by *Trichomonas vaginalis* have the ability to damage the target cells and involved in pathogenesis of trichomoniasis. The phospholipase A2-like lytic factor has been purified and further characterization exhibited 168 and 144 kDa two fractions (34). The antimicrobial peptides synthesized by marine bacterium *Pseodoalteromonas* have been purified from culture supernatant through SEC that possess strong inhibitory effect against pathogens involved in skin infections (35). Cytosolic proteins of *Arabidopsis thaliana* have been purified to understand how cell coordinates diverse mechanical, metabolic and developmental activities (36). Purification of intrinsically disordered proteins of *A. thaliana* was also carried out through SEC. These are expressed during advanced stage of seed development and have a significant role in transcription regulation and signal transduction (37).

Affinity chromatography

The affinity chromatography was a major breakthrough in protein purification that enables the researcher to explore protein degradation, post-translational modifications and protein–protein interaction. The basic principle behind the affinity chromatography is the reversible interaction between the affinity ligand of chromatographic matrix and the proteins to be purified (11).

The affinity chromatography has a wide range of applications in identification of microbial enzymes principally involved in the pathogenesis. Homodimer and heterodimer of HIV-I reverse transcriptase were rapidly purified by metal chelate affinity chromatography (38). The practical applications of bacteriophages in field of biotechnology and medicine persuade excessive requirement of the phage purification. The T4 bacteriophages have been purified from bacterial debris and other contaminating bacteriophages. The bacterial cells in 'competitive phage display' produced both fusion protein and wild-type proteins. The fusion proteins were integrated into phage capsid and permitted the effective purification of T4 bacteriophages (39).

A group of amyloid binding proteins interact with different forms of amyloidogenic protein and peptides, therefore modify their pathological and physical role. Affinity chromatography is potentially applied for the diagnosis of Alzheimer's disease by purification of Alzheimer's amyloid peptide from human plasma (40). The immobilized metal ion affinity chromatography purified the heterologous proteins comprising zinc finger domains. Hexa-histidine affinity tags displayed different affinities to the immobilized metal ions even though both contain same type of domain. However, zinc finger proteins vary in biochemical properties (41).

Plasma proteins such as factor IX, factor XI, factor VIII, antithrombin III and protein C have been purified through affinity chromatography at industrial scale for therapeutic use (42). Various ligands have been purified and applied in purification of antibodies. The examples include the lectins for IgM and IgA purification whereas proteins A and G for the purification of IgG molecules (43).

Enzyme-linked immunosorbent assay

In 1971, Engvall and Pearlmann published the first paper on ELISA and quantified the IgG in rabbit serum using the enzyme alkaline phosphatase. The ELISA is highly sensitive immunoassay and widely used for diagnostic purpose. The assay utilizes the antigen or antibodies on the solid surface and addition of enzyme-conjugated antibodies to and measure the fluctuations in enzyme activities that are proportional to antibody and antigen concentration in the biological specimen (12).

The diagnosis of paratuberculosis or John's disease was made possible by Ethanol Vortex ELISA. The assay distinguished the surface antigens of *Mycobacterium avium* subspecies *paratuberculosis* (44). Capture ELISA was established for detection of *Echinostoma caproni* in experimentally infected rats (45). This assay was based on recognition of excretory–secretory antigens by polyclonal rabbit antibodies. The detection limit was 60 ng/ml in fecal sample and 3 ng/ml in sample buffer. Deoxynivalenol (DON), a powerful mycotoxin produced by *Fusarium graminearum* is a major contaminant of barley and wheat and leads to Fusarium Head Blight. Indirect competitive ELISA for the identification of DON in wheat was developed with detection limit between 0.01 and 100 µg/mL in grains (46).

Wheat proteins causes allergic reactions in susceptible individuals that have been traced in foods to protect wheat-sensitive individuals using commercially available ELISA kits (47). Sandwich ELISA was used for the detection of Cry1Ac protein of *Bacillus thuringiensis* from transgenic BT cotton as their release adversely affect the environment (48). Indirect competitive ELISA was developed to detect *Botrytis cinerea* in tissues of fruits. *B. cinerea* is a phyopathogenic fungus responsible for gray mold and often present as latent infection and deteriorate the healthy fruits (49). Digital ELISA is capable of detecting single molecule in the blood. The assay was able to detect prostate-specific antigen (PSA) in the serum at low concentration of 14 fg/ml. This assay was capable to detect 1,1-Dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p'*-DDE); a metabolite of insecticide and persistent organic pollutant that accumulates in food chain and environment (50).

Western blotting

Western blotting is an important and powerful technique for detection of low abundance proteins that involve the separation of proteins using electrophoresis, transfer onto nitrocellulose membrane and the precise detection of a target protein by enzyme-conjugated antibodies (13). Western blotting is a dominant tool for antigen detection from various microorganisms and is quite helpful in diagnosis of infectious diseases. The seroprevalence of Herpes Simplex Virus type 2 (HSV-2) in African countries was investigated by measuring the specific immunoglobulin G in the sera of patients (51). *Leishmania donovani* is responsible for visceral leishmaniasis, which is classically diagnosed by the presence of Hsp83 and Hsp70 antigens in the bone marrow, spleen and liver (52).

Western blotting was carried out by Li *et al.* for identification and validation of 10 rice reference proteins. Elongation factor 1-α and heat-shock proteins were the most expressed proteins in rice (53). Kollerova *et al.* (54) identified the Plum Pox Virus (PPV) capsid proteins from infected *Nicotiana benthamiana* (54). The expression of PfCP-2.9 gene of *Plasmodium falciparum* in tomato was confirmed through western blot analysis (55). Specific IgE against Ara h1, Ara h2 and Ara h3 was determined in peanut allergic patients through western blotting (56).

Edman sequencing

Edman sequencing was developed by Pehr Edman in 1950 to determine the amino-acid sequence in peptides or proteins. The method comprises chemical reactions that eliminate and identify amino acids residue that is present at the N-terminus of polypeptide chain. Edman sequencing played a major role in development of therapeutic proteins and quality assurance of biopharmaceuticals (19).

Brucella suis survive and replicate in macrophage due to the acidification. The proteins that are involved in this acidification were identified. Edman degradation and comparison of 13 N-terminal amino-acid sequences revealed that these were signal peptides for its periplasmic location. The protein in *B. suis* that was involved in membrane permeability at acidic environment was Omp25 (57). The causative agent of hemorrhagic fever, Lassa virus belongs to family of *Arenaviridae*. The Lassa virus synthesis glycoproteins which are cleaved into GP-1 (amino-terminal subunit) and GP-2 (Carboxy-terminal subunit) after translation and are primarily involved in pathogenesis. The Edman degradation analysis of GP-2 revealed N-terminal tripeptide GTF_{262} (58).

The prevalence of sesame seed allergy has been increasing due to the use of bakery products and fast-food. The major allergic proteins of *Sesamum indicum* have been identified from allergic patients through 2D-PAGE and SDS-PAGE and then further analyzed through Edman sequencing. IgE binding epitopes of these proteins were identified that might be helpful in immunotherapeutic approaches (59). The proteins from leaf sheaths of rice were extracted and analyzed through MS and Edman sequencing to determine its function. The amino-acid sequence of majority of proteins analyzed by both techniques have similar results, therefore suggesting the use of these techniques in combination for the identification of plant protein (60).

Advanced techniques

Protein microarray

Protein microarrays also known as protein chips are the emerging class of proteomics techniques capable of high-throughput detection from small amount of sample. Protein microarrays can be classified into three categories; analytical protein microarray, functional protein microarray and reverse-phase protein microarray (17).

Analytical protein microarray

Antibody microarray is the most representative class of analytical protein microarray. After antibody capture, proteins are detected by direct protein labeling. These are typically used to measure the expression level and binding affinities of proteins (17, 61, 62). High-throughput proteome analysis of cancer cells was carried out through antibody microarray for differential protein expression in tissues derived from squamous carcinoma cells of oral cavity (63). Antibody array was also used for protein profiling of bladder cancer (64). Microarray immunoassay was used for detection of Staphylococcal enterotoxin B, cholera toxin, *Bacillus globigii* and *B. ricin* (65). Analytical and experimental approaches have been developed for identification of cellular signaling pathways and to characterize the plant kinases through protein microarray (66). Mitogen-activated protein kinases (MAPKs) from *Arabidopsis* have been characterized. MAPKs are highly conserved single transduction and universal molecules in plants that respond to wide range of extracellular stimuli (67).

Functional protein microarray

Functional protein microarray is constructed by means of purified protein, thus permits the study of various interactions including protein–DNA, protein–RNA and protein–protein, protein–drug, protein–lipid, enzyme–substrate relationship (17). The first use of functional protein microarray was to analyze the substrate specificity of protein kinases in yeast (68). Functional protein microarray characterized the functions of thousands of proteins. The protein–protein interaction of *A. thaliana* was studied and Calmodulin-like proteins (CML) and substrates of Calmodulin (CaM) were identified (69).

Reverse-phase protein microarray

Cell lysates obtained from different cell states are arrayed on nitrocellulose slide that are probed with antibodies against target proteins. Afterwards, antibodies are detected with fluorescent, chemiluminescent and colorimetric assays. For protein quantification, reference peptides are printed on slides. These microarrays are used to determine the altered or dysfunction protein indicative of a certain disease (17). The analysis of hematopoietic stem cell and primary leukemia samples through reverse-phase protein microarray was found to be highly reproducible and reliable for large-scale analysis of phosphorylation state and protein expression in human stem cells and acute myelogenous leukemia cells (70). Reverse-phase protein microarray approach was evaluated for quantitative analysis of phosphoproteins and other cancer-related proteins in non-small cell lung cancer (NSCLC) cell lines by monitoring the apoptosis, DNA damage, cell-cycle control and signaling pathways (71).

Gel-based approaches

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE is a high resolving technique for the separation of proteins according to their size, thus facilitates the approximation of molecular weight. Proteins are capable of moving with electric field in a medium having a pH dissimilar from their isoelectric point. Different proteins in mixture migrate with different velocities according to the ratio between its charge and mass. However, addition of sodium dodecyl sulfate denatures the proteins, therefore separate them absolutely according to molecular weight (14).

The protein profiling of *Mycoplasma bovis* and *Mycoplasma agalactiae* through SDS-PAGE has high diagnostic value as these species are difficult to differentiate with routine diagnostic procedures (72). The outer membrane proteins from *E. coli* strains in which ability to form K1 antigen is absent were analyzed through SDS-PAGE. It exhibited varied degree of susceptibility to the human serum. Extracellular protein profile of *Staphylococcus* spp. was also constructed and their characterization was achieved. The antigenic proteins of *Streptococcus agalactiae* have been characterized to test the immunogenicity of mastitis vaccine (73).

The *cleome* spp. are consumed as green vegetables in African countries and highly valuable for the treatment of cough, fever, asthma, rheumatism and many other diseases. The comparative analysis of leaf and seed proteins of *cleome* spp. was carried out by SDS-PAGE (74). The profiling of seed and leaf storage proteins of chickpea (*Cicer arientinum*) was conducted under drought stress and non-stress conditions (75). The seed storage proteins of *Brassica* species are also identified to evaluate the genetic divergence in different genotypes (76). The influence of heat treatment and addition of demineralized whey on the soluble protein composition of the skim milk was investigated [88]. High molecular weight complexes were formed during the addition of demineralized whey as well as heat

treatment which was determined by SDS-PAGE (77). Large-scale production of insulin is helpful for the management of diabetes, therefore different approaches and species have been used for the production of insulin. Elamin *et al.* (78) purified and characterized the pancreatic insulin from the *Camelus dromedaries*.

Two-dimensional gel electrophoresis

The two-dimensional polyacrylamide gel electrophoreses (2D-PAGE) is an efficient and reliable method for separation of proteins on the basis of their mass and charge. 2D-PAGE is capable of resolving ~5,000 different proteins successively, depending on the size of gel. The proteins are separated by charge in the first dimension while in second dimension separated on the basis of differences between their mass. The 2-DE is successfully applied for the characterization of post-translational modifications, mutant proteins and evaluation of metabolic pathways. Neidhardt and van Bogelen introduced the highly sensitive technique of 2-DE into the bacterial physiology (15).

The membrane proteins from the cell wall of *Listeria innocua* and *Listeria monocytogenes* involved in the host–pathogen interactions were analyzed with 2-DE and 30 different proteins of two strains were identified (79). This approach was useful for the comparative study of exotoxins and virulence factors released by enterotoxigenic strains of two food-derived *Staphylococcus aureus* strains (80). *Pseudomonas aeruginosa* secrete numerous proteins during different stages of infection as seen in isolates obtained from cystic fibrosis patients (81). Current improvements in the 2D-PAGE have been used to study the metabolic system of *B. subtilis* and a PyrR bacterial regulatory protein was characterized (82).

Large number of proteins were detected during the seed development in *Ocotea catharinensis*, and profile was constructed by characterizing these proteins during each developmental stage (83). Protein extraction from grapes is challenging due to the low concentration of proteins, high activity of proteases and high level of interfering compounds such as polyphenols, flavonoids, terpenes, lignans and tannins; however, Marsoni *et al.* (84) successfully extracted the proteins from grape tissue through 2-DE. Islam *et al.* (85) also extracted the proteins from mature rice leaves and applied in the proteome analysis.

Two-dimensional differential gel electrophoresis

2D-DIGE utilizes the proteins labeled with CyDye that can be easily visualized by exciting the dye at a specific wavelength (16). Cell wall proteins (CWPs) of toxic dinoflagellates *Alexandrium catenella* labeled with Cy3 have been identified through 2D-DIGE (86). Quantitative analysis of *Brucella suis* proteins has been carried out under long-term nutrient starvation and ~30 proteins were identified that vary in concentration among bacteria grown at stationary phase in medium with different nutrient levels. About 70% of regulated proteins showed an increase in expression. The proteins are also involved in regulation, adaptation to harsh condition and transportation (87). The characterization of proteins expressed in rat neurons have been carried to understand the pathogenesis of West Nile virus (88).

The plasma membrane responds to the biotic and abiotic stress in plants, therefore the characterization of plasma proteins provides new perception about the plant-specific biological functions. Komatsu (89) characterized the plasma membrane proteome of rice and *A. thaliana*. The role of apoplastic proteins of 10-day-old rice plants in salt stress response was investigated. For differential analysis, soluble apoplastic proteins from rice shoot stem were extracted and compared with untreated and were found to be involved in oxidation-reduction reaction, carbohydrate metabolism and protein degradation and processing (90). During ovule development of *Pinus tabuliformis*, female gametophyte cellularization is a vital process regulated by multiple proteins, which were first extracted in anaphase and prophase then separated through 2D-DIGE (91).

The biological drugs produced during cell culture technology constitute host cell proteins (HCP) as most important group of impurities. The HCP has diverse molecular and immunological properties and should be effectively monitored and removed during downstream processing. 2D-DIGE was used to screen the HCP composition in CHO cell culture and to compare HCP difference between null cell culture and monoclonal antibody producing cells (92). The quantitative changes in red blood cell membrane proteins in sickle cell disease were analyzed and the contents of 49 gel spots were found altered by 2.5-fold in comparison with normal cells (93).

The 2-DE remains a method of choice in proteomic research, though certain limitations enervate its potential as a principal separation technique in modern proteomics. Therefore, the state of the art instrumentation and techniques are rapid expanding as a new means of gel-free analytical techniques. The advancement of MS coupled with shotgun proteomics can find newer directions for sensitive and high quantity protein profiling with more accurate quantification. The chemical label-based approaches remained popular in quantitative proteomics, these methods also have certain drawbacks. The quantitative plant proteomics is more challenging due to problems associated with protein extraction, abundance of proteins in some plants tissues and the lack of well-marked genome sequences. The higher resolution power of MS, exact mass measurements, higher scanning rates and precise chromatogram alignment are essential feature for the successful use of MS in proteomics (94).

Quantitative techniques

ICAT labeling

The ICAT is an isotopic labeling method in which chemical labeling reagents are used for quantification of proteins. The ICAT has also expanded the range of proteins that can be analyzed and permits the accurate quantification and sequence identification of proteins from complex mixtures. The ICAT reagents comprise affinity tag for isolation of labeled peptides, isotopically coded linker and reactive group (20).

Mycobacterium tuberculosis is considered as a most important human pathogen that contain ~4,000 genes. The proteome analysis was carried out using a combination of Liquid Chromatography (LC), Tandem Mass Spectrometry (MS/MS) and ICAT (95). The combination of techniques offers comprehensive understanding of biological system and provides additional information. The systemic proteome quantification was carried out possible through ICAT during cell cycle of *Saccharomyces cerevisiae* that supported the cognition of gene functions (96). The levels of reactive nitrogen species and reactive oxygen species increase in living cells during abiotic and biotic stress.

The reversible oxidation of protein residues may assist as redox sensors and signal transducers for transmission of anti-stress responses. The thiol group on cysteine residue is sensitive to oxidative species and upon oxidation can modulate protein function. ICAT reagents precisely react with thiol group of cysteine residues, therefore the technique coupled with MS is useful to quantify the thiol-containing redox proteins (97). The tumor-specific proteins were analyzed through ICAT and MS from the aspirated fluid of

breast tumor patients at earlier stages. Beta-globin, hemopexin, lipophilin B and vitamin D-binding proteins were overexpressed while Alpha2HS-glycoprotein was under expressed. It seems that ICAT has potent applications to designate appropriate biomarkers for cancer diagnosis (98).

Stable Isotopic Labeling with Amino Acids in Cell Culture

SILAC is an MS-based approach for quantitative proteomics that depends on metabolic labeling of whole cellular proteome. The proteomes of different cells grown in cell culture are labeled with "light" or "heavy" form of amino acids and differentiated through MS. The SILAC has been developed as an expedient technique to study the regulation of gene expression, cell signaling, post-translational modifications. Additionally, SILAC is a vital technique for secreted pathways and secreted proteins in cell culture (21).

SILAC was used for quantitative proteome analysis of *B. subtilis* in two physiological states such as growth during phosphate and succinate starvation. More than 1,500 proteins were identified and quantified in the two tested states. About 75% genes of *B. subtilis* were expressed in log phase. Moreover, 10 phosphorylation sties were quantified under phosphate starvation while 35 phosphorylation sites under growth on succinate (99). Highly purified mutant adenovirus deficient in protein V (internal protein component), wild-type adenovirus and recombinant virus were quantified through SILAC. Viral protein composition and abundance were constant in all types of viruses except virus deficient in protein V which also resulted in reduced amount of another viral core protein (100).

SILAC was used by for quantitative proteome analysis of *A. thaliana*. Expression of glutathione S-transferase was analyzed in response to abiotic stress due to salicylic acid and consequent proteins were quantified (101). Salt stress response and protein dynamics in photosynthetic organism *Chlamydomonas reinhardtii* have been studied to establish the proteome turnover rate and changes in metabolism under salt stress conditions. RuBisCO was found as the most prominent protein in *C. reinhardtii* (102).

The intracellular stability of almost 600 proteins from human adenocarcinoma cells have been analyzed through "dynamic SILAC" and the overall protein turnover rate was determined. Tissue regeneration is imperative in many diseases such as lung disease, heart failure and neurodegenerative disorders. The tissue regeneration and protein turnover rate were quantitatively analyzed in zebra fish. Proteome analysis showed that fin, intestine and liver have high regenerative capacity while heart and brain have the lowest. The proteins in tissue regeneration were mainly involved in transport activity and catalytic pathways (102, 103).

Isobaric tag for relative and absolute quantitation

iTRAQ is multiplex protein labeling technique for protein quantification based on tandem mass spectrometry. This technique relies on labeling the protein with isobaric tags (8-plex and 4-plex) for relative and absolute quantitation. The technique comprises labeling of the N-terminus and side chain amine groups of proteins, fractionated through liquid chromatography and finally analyzed through MS. It is essential to find the gene regulation to understand the disease mechanism, therefore protein quantitation using iTRAQ is an appropriate method that helps to identify and quantify the protein simultaneously (22).

iTRAQ has been applied for quantitative analysis of membrane and cellular proteins of *Thermobifida fusca* grown in the absence and presence of cellulose. About 181 membrane and 783 cytosolic proteins were quantified during cellulosic hydrolysis. The quantified protein in cellulosic medium was involved in pentose phosphate pathway, glycolysis, citric acid cycle, starch, amino acid, fatty acid, purine, pyrimidine and energy metabolism. Consequently, these proteins have a functional role in cell wall synthesis, transcription, translation and replication (104). The huge amount of oxidative and hydrolytic enzymes is secreted by *Phanerochaete chrysosporium* that degrade lignin, cellulose and mixture of lignin and cellulose. The secretory proteins were quantified from *P. chrysosporium* and 117 enzymes were quantified including cellulose hydrolyzing exoglucanases, endoglucanases, cellobiose dehydrogenase and β-glucosidases (105).

The presence of soluble aluminum ions (Al^{3+}) in soil limits crop growth; however, *Oryza sativa* are highly aluminum tolerant; therefore, quantitative proteome analysis was carried out in response to Al^{3+} in roots of *O. sativa* at early stages. Out of 700 identified proteins, the expression of 106 proteins was different in Al^{3+} tolerant and sensitive cultivars (106). The role of hydrogen peroxide (H_2O_2) in growth of wheat was identified through iTRAQ-based quantitative approach that showed that the increased concentration of H_2O_2 restrained the growth of roots and seedlings of wheat. Out of 3,425 identified proteins, 44 were newly identified H_2O_2- responsive proteins involved in detoxification/stress, carbohydrate metabolism and single transduction. Several proteins such as superoxide dismutase, intrinsic protein 1 and fasciclin-like arabinogalactan protein could possibly be involved in H_2O_2 tolerance (107).

iTRAQ was a useful tool for determination of molecular process involved in development and function of natural killer (NK) cells. Membrane bound proteins of NK cells from CD3-depleted adult peripheral blood cells and umbilical cord blood stem cells were quantified. Ontology analysis exhibited that many of these proteins were involved in nucleic acid binding, cell signaling and mitochondrial functions (108). Protein profiling was carried out in mouse liver regeneration following a partial hepatectomy. A total of 827 identified proteins, 270 were quantified as well. Fabp5, Lactb2 and Adh1 were downregulated among these while Pabpc1, Mat1a, Oat, Hpx and Dnpep were upregulated (109).

X-ray crystallography

X-ray crystallography is the most preferred technique for three-dimensional structure determination of proteins. The highly purified crystallized samples are exposed to X-rays and the subsequent diffraction patterns are processed to produce information about the size of the repeating unit that forms the crystal and crystal packing symmetry. X-ray crystallography has an extensive range of applications to study the virus system, protein–nucleic acid complexes and immune complexes. Further, the three-dimensional protein structure provides detailed information about the elucidation of enzyme mechanism, drug designing, site-directed mutagenesis and protein–ligand interaction (24).

ZipA and FtsZ are the vital components of spatial ring structure that facilitates cell division in *E. coli*. ZipA is a membrane anchored protein while FtsZ is homologous of eukaryotic tubulin and their interaction is facilitated by C-terminal domains. X-ray crystallography revealed the structure of C-terminal fragment of FtsZ and binding complex of FtsZ-ZipA (110). The structure of Norwalk virus

that causes gastroenteritis in humans was determined through X-ray crystallography, which revealed that viral capsid consists of 180 repeating units of single protein. The two domains; shell (S) domain and protruding (P) domain of capsid protein are connected by flexible hinge. Eight-standard β-sandwich motif was present in Shell (S) domain while structure of Protruding (P) domain was similar to the domain of eukaryotic translation elongation factor. These domains are the key determinants responsible of cell binding and strain specificity (111).

The movement of phospholipids, glycolipids, steroids and fatty acids between membranes occurs due to non-specific lipid transfer proteins (nsLTPs). The comparative structure of maize nsLTP in complex with numerous ligands revealed variations in the volume of the hydrophobic cavity depending on the size of bound ligands (112). The microsomal cytochrome P450 3A4 catalyzes the drug–drug interaction in humans that induce or inhibit the enzymes and metabolically clear the clinically used drugs. The protein structure was analyzed through X-ray crystallography that exhibited a large substrate binding cavity capable to oxidize huge substrates such as statins, cyclosporin, macrolide antibiotics and taxanes (113). The X-ray crystallography revealed the 3D structure of recombinant horseradish peroxidase in complex with benzohydroxamic acid (BHA). The electron density for BHA was detected in active site of peroxidase along with hydrophobic pocket adjacent to aromatic ring of the BHA (114).

High-throughput techniques

Mass spectrometry

MS is used to measure the mass to charge ratio (*m/z*), therefore helpful to determine the molecular weight of proteins. The overall process comprises three steps. The molecules must be transformed to gas-phase ions in the first step, which poses a challenge for biomolecules in a liquid or solid phase. The second step involves the separation of ions on the basis of *m/z* values in the presence of electric or magnetic fields in a compartment known as mass analyzer. Finally, the separated ions and the amount of each species with a particular *m/z* value are measured. Commonly used ionization method comprises matrix-assisted laser desorption ionization (MALDI), surface enhanced laser desorption/Ionization (SELDI) and electrospray ionization (ESI) (18).

In clinical laboratories, bacterial identification depends on conventional techniques. However, identification of slow growing, fastidious and anaerobic bacteria through conventional techniques is expensive, complex and time consuming. Biswas and Rolain (115) used the MALDI-TOF for early pathogenic bacterial identification, which is useful for early disease control. MS has also became an significant tool in virus research at molecular level, and various viruses and viral proteins including intact viruses, mutant viral strains, capsid protein, post-translational modifications were identified (116). The study of the changes of viral capsid protein during the infection has allowed the researcher to develop new antiviral drugs. Electrospray ionization mass spectrometry (ESI-MS) coupled with PCR and rRNA gene sequencing provided the accurate and rapid identification of medically important filamentous fungi, yeast and *Prototheca* species (117).

Post-translational modification in plants including protein phosphorylation has been distinguished through MS (118). Top down Fourier Transform mass spectrometry was used to the characterize chloroplast proteins of *A. thaliana* (119). Hydrophobic properties and molecular mass of light harvesting proteins of photosystem-II of 14 different plants species were presented by Zolla *et al.* (120). ESI-MS was used for profiling of integral membrane proteins and detection of post-translational modifications (121). The most abundant proteins of tomato (*Lycopersicon esculentum*) xylem sap after *Fusarium oxysporum* infection were detected with mass spectrometric sequencing and peptide mass finger printing (122).

The blood proteins including the IBP2, IBP3, IGF1, IGF2 and A2GL have been proposed as biomarkers for the diagnosis of breast cancer. MS was used to characterize these blood proteins (123). PSA, human growth hormone and interleukin-12 were also analyzed from human serum (124). Imaging MALDI mass spectrometry was used for the analysis of whole body tissues. The distribution of drugs and metabolites was detected within whole body tissues following drug administration that was useful to analyze novel therapeutics and provide deeper insight into toxicological and therapeutic process (125).

NMR spectroscopy

The NMR is a leading tool for the investigation of molecular structure, folding and behavior of proteins. Structure determination through NMR spectroscopy typically involves various phases, each using a discrete set of extremely specific techniques. The samples are prepared and measurements are made followed by interpretive approaches to confirm the structure. The protein structure is fundamental in several research areas such as structure-based drug design, homology modeling and functional genomics (22).

The three-dimensional structure of transmembrane domain of outer membrane protein A from *E. coli* has been determined through heteronuclear NMR in dodecylphosphocholine micelles. The fold of protein consists of 19 kDa (177 amino acids) and the structure comprises larger mobile loops toward extracellular side and an eight-stranded β-barrel linked by tight turns on the periplasmic side (126). The interaction of iso-1-cytochrom *c* with cytochrome c peroxidase from yeast was investigated by NMR. Chemical shift was observed for both ^{1}H and ^{15}N nuclei arising from the interface of isotopically enriched ^{15}N cytochrome *c* with cytochrome *c* peroxidase (127).

Plant litter decomposition is essential in nitrogen and carbon cycles for the provision of necessary nutrients to the soil and atmospheric CO_2. ^{15}N- and ^{13}C-labeled plant materials were used to monitor the environmental degradation of wheatgrass and pine residues via HR-MAS NMR spectroscopy. The spectra revealed that condensed and hydrolysable tannin were lost from all plant tissues whereas the aliphatic components (cuticles, waxes) and aromatic (partly lignin) persisted along with a small portion of carbohydrate (128).

Holmes *et al.* described the variations between metabolic phenotypic from 4,630 participants belonging to 4 human populations through NMR spectroscopy. Metabolic phenotypes including in the study were the products of interactions between variety of factors such as environmental, dietary, genetic and gut microbial activities. Selective metabolites across populations were associated with blood pressure and urinary metabolites that offer the promising discovery of novel biomarkers (129).

The NMR can be coupled with various approaches like LC or UHPLC to increase the resolution and sensitivity for high-throughput protein profiling. In addition, the structural information can be generated is compared in relation to the identification of metabolites in complex mixtures (130). NMR coupled with ultra-high performance liquid chromatography (UHPLC) was developed to

characterize the metabolic disturbances in esophageal cancer patients for the identification of possible biomarkers for early diagnosis and prognosis. The study revealed considerable alterations in ketogenesis, glycolysis and tricarboxylic acid cycle and amino acid and lipid metabolism in esophageal cancer patients compared with the controls (131).

Bioinformatics analysis

Bioinformatics is an essential component of proteomics; therefore, its implications have been progressively increasing with the advent of high-throughput methods that are dependent on powerful data analysis. This new and emergent field is presenting novel algorithms to manage huge and heterogeneous proteomics data and headway toward the discovery procedure (25).

Endolysins are class of antibacterial enzymes that are becoming useful tool to control spreading of multi-resistant bacteria. The antibacterial property can be altered or expanded by domain swapping, mutagenesis or gene shuffling. The challenge of designing specific endolysins has been revealed *in-silico* analysis for protein domains present in prophage and phage endolysins. The combination of domains have been studied and sequence type with domain arrangement and conserved amino acids have been determined through multiple sequence alignment. The presence, number and types of binding domain with in endolysins sequence also have been studied (132). *In-silico* analysis approach was used to calculate the distribution of the plant food allergens into protein families and determination of conserved surface essential for IgE cross reactivity. The plant food allergen sequences were categorized into four families that indicate the role of conserved structures and biological activities in stimulating allergic properties (133).

A blood coagulation enzyme, Human Factor Xa (FXa) catalyzes the activation of prothrombin to thrombin and plays an important role in thrombosis and hemostasis. The imbalance in the activation of enzymes intrudes the hemostasis leading to the blood disorders. The safe and effective anticoagulants may be developed by direct inhibition of FXa without effecting thrombin activity essential for normal hemostasis. A study aided the design of more effective ligands through Discovery Studio. Docking studies and binding confirmations revealed that sulfonamide derivatives were inhibitors of FXa (134).

The use of Bioinformatics for proteomics has gain significantly affluent during the previous few years. The development of new algorithm for the analysis of higher amount of data with increased specificity and accuracy helps in the identification and quantitation of proteins therefore have made possible to achieve expounded data regarding protein expression. The management of such a high quantity of data is the main problem associated with these kind of analyses. Further, it is still difficult to find the association between proteomic data and the other omics technologies including genomics and metabolomics. The database technology along with new semantic statistical algorithms however are the potent tools that might be useful to overcome these limitations.

For MS, the proteins are extracted from the sample and digested using one or several proteases to produce definite set of peptides (135). Further steps including enrichment and fractionation can be added at protein or peptide level to decrease the complexity of sample or when the analysis of specific subset of proteins is desired (136–138). The obtained peptides are analyzed by liquid chromatography coupled with mass spectrometry (LC–MS). Common approaches include either the analysis of deep coverage of proteome by shotgun MS or quantitative investigation for a definite set of proteins through targeted MS (138, 139). The resulting spectra provide information regarding the sequence, which is important for the identification of proteins. The obtained data may be displayed in a form of 3-D map with mass-to-charge (m/z) ratio, retention time (RT) and intensities of peptides along with fragmentation spectra. The intensity of mass to charge ratio for a particular peptide is plotted along the RTs to get the chromatographic peak. The area under this curve can be used for quantification of peptides, whereas the proteins are identified by the fragmentation spectra. The proteomic data can be uploaded to the repositories that can also be helpful for searching the database (140). The largest proteome repositories including PRIDE proteomics identification database, Proteome Commons and PeptideAtlas project provide direct access to most of stored data and are valuable tools for data mining (141, 142).

The protein pathways are a series of reactions inside the cell that exert a particular biological effect. The proteins that are directly involved in reaction along with those that regulates the pathways are combined in pathway databases; therefore, a number of resources and databases are available for the protein pathways. The KEGG, Ingenuity, Pathway Knowledge Base Reactome and BioCarta are some of the pathway databases that include a comprehensive data regarding metabolism, signaling and interactions (143, 144). In addition to these comprehensive databases, the specific databases for signal transduction pathways such as GenMAPP or PANTHER have been developed (145–147). Moreover, databases such as Netpath have been developed, which involve the pathways active in cancer that are helpful for the identification of proteins relevant for a cancer type (148). These public databases possess higher connectivity that allows novel findings for proteins.

The proteins do not act independently in most of the cases and form transient or stable complexes with other proteins. The protein might be intricate as complexes of variable composition and it is essential to study the protein complexes along with the conditions that result in their formation or dissociation for the complete understanding of a biological system. The databases such as BioGRID, IntAct, MINT and HRPD contain the information with reference to protein interactions in complexes (138, 149, 150). STRING is not only a widely used database for protein interaction data, but it connects to various other resources for literature mining. Furthermore, protein networks can be drawn based on the list of genes provided and the available interactions using STRING database (138, 151, 152) (Table 1).

Sample preparation for proteomics

Preparation of sample is the most fundamental step in proteomics research that considerably affects the results of an experiment. Therefore, the selection of appropriate experimental model and sample preparation method is essential for reliable results, especially in comparative proteomics, that deal with the minor variances of experimental samples compared with the control (153). The major impediments associated with the analysis of complex biological materials are the wider range of protein abundance. A particular cell could have only few copies of a protein, but we may expect up to million copies of an abundant protein therefore these abundant proteins should be removed for most of the proteomic analysis. The Pre analytical samples treatment include various methods for fractionation and proteins enrichment could be helpful in this regard (154).

The animal tissue associated with the disease is often selected for proteomic analysis after the establishment of particular animal

Table 1. General Protein Sequence Databases, Sequence Similarity Search, Alignment Tools and Structural Analysis and Prediction Servers

Name	Type	Web links
GenBank	Database	http://www.ncbi.nih.gov/entrez/query.fcgi?db=protein
RefSeq	Database	https://www.ncbi.nlm.nih.gov/refseq/
nr	Database	http://www.ncbi.nlm.nih.gov/BLAST/
UniProt	Database	http://www.pir.uniprot.org/
UniRef	Database	http://www.pir.uniprot.org/database/nref.shtml
UniParc	Database	http://www.pir.uniprot.org/database/archive.shtml
TrEMBL	Database	http://kr.expasy.org/sprot/
SwissProt	Database	http://kr.expasy.org/sprot/
PIR	Database	http://pir.georgetown.edu/
OWL	Database	http://www.bioinf.man.ac.uk/dbbrowser/OWL/
BLASTP	BLAST	http://blast.ncbi.nlm.nih.gov
TBLASTN	BLAST	https://blast.ncbi.nlm.nih.gov
PSI-BLAST	Position Specific Iterated BLAST	https://blast.ncbi.nlm.nih.gov
PHI-BLAST	Pattern Hit Initiated BLAST	https://blast.ncbi.nlm.nih.gov
DELTA-BLAST	Domain Enhanced Lookup Time Accelerated BLAST	https://blast.ncbi.nlm.nih.gov
InterProScan	Protein domain servers	http://www.ebi.ac.uk/lnterProScan/
CD server	Protein domain servers	http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
ProWleScan	Protein domain servers	http://hits.isb-sib.ch/cgi-bin/PFSCAN
ScanProsite	Protein domain servers	http://us.expasy.org/tools/scanprosite/
PATTINPROT	Protein motif search tools	http://pbil.ibcp.fr/html/pbiljndex.html
SIRW	Protein motif search tools	http://sirw.embl.de/index.html
Match Box	Motif based alignment server	http://www.sciences.fundp.ac.be/biologie/bms/help.html
MEME	Motif based alignment server	http://meme.sdsc.edu/meme/website/meme.html
Gibbs	Motif based alignment server	http://bayesweb.wadsworth.org/gibbs/gibbs.html
Dialign	Motif based alignment server	http://bibiserv.techfak.uni-bielefeld.de/dialign/
BlockMakei	Motif based alignment server	http://blocks.fhcrc.org/make_blocks.html
PDB	Protein structure databases	http://www.rcsb.org/pdb/
SwissModel	Protein structure databases	http://swissmodel.expasy.org/repository/
SCOP	Protein structure databases	http://scop.mrc-lmb.cam.ac.uk/scop/
ModBase	Protein structure databases	http://alto.compbio.ucsf.edu/modbase-cgi/index.cgi
CATH	Protein structure databases	http://www.biochem.ucl.ac.uk/bsm/cath/
MMDD	Protein structure databases	http://www.ncbi.nlm.nih.gov/Structure/
ConSurf	Protein structure analysis server	http://consurf.tau.ac.il/
CASTp	Protein structure analysis server	http://sts.bioe.uic.edu/castp/index.php
ProtSkin	Protein structure analysis server	http://www.mcgnmr.ca/ProtSkin/intro/
LigandProtein	Protein structure analysis server	http://bip.weizmann.ac.il/oca-bin/lpccsu
PredictProtein	Protein structure prediction sever	http://www.embl-heidelberg.de/predictprotein
O-GlycoBase	Protein structure prediction sever	http://www.cbs.dtu.dk/services/NetOGIyc/
PhosphoBase	Protein structure prediction sever	http://www.cbs.dtu.dk/services/NetPhos
SwissModel	Protein structure modeling server	http://www.expasy.org/swissmod
Whatlf	Protein structure modeling server	http://www.cmbi.kun.nl/gv/servers/WIWWWI
ESyPred3D	Protein structure modeling server	http://www.fundp.ac.be/urbm/bioinfo/esypred
EBI	Protein structure modeling server	http://biotech.ebi.ac.uk:8400/

model. The tissue characteristics vary among the types, for example brain tissue have abundance of lipids that need to be eliminated for high quality results. The selective precipitation of proteins with acetone and trichloroacetic acid (TCA) is a widely used method for protein expression profiling in neuroscience (153). The fresh tissue samples are usually perfused with cold saline before excision and are used as unfettered from fat as well as connective tissue. The tissue is minced in freshly prepared lysis buffer that might contain detergents and/or protease inhibitors. The biopsy is frequently used as a source of tissue for expression analysis that is usually surgically obtained and need to freeze immediately using liquid nitrogen and stored at −80°C before analysis (155, 156).

The plant cells have the distinctive cell wall made up of cellulose mainly and its derivatives. The primary cell wall surrounds the young plant cells although some type of plants and cells contains a rigid secondary cell wall after developmental phase. The release of proteins as a result of cell wall disruption is essential for analytical success; therefore, different physical and chemical techniques are employed for the destruction of cell wall, for example, freeze thawing, sonication, high speed blending and use of lysing buffer (85). The CWPs constitute ~10% of the cell wall mass and are mainly involved in signaling, modification of cell wall constituents and communications with plasma membrane proteins. The extraction of CWPs is challenging and the available cell wall proteomes so far contain either labile or loosely bound proteins (157, 158).

The majority of research is conducted on model plants, i.e., rice (*O. sativa*) and Thale cress (*A. thaliana*) having a relatively small genome. Another problem associated with plants proteome analysis is the presence of contaminants other than proteins specific to the plant type including lipids, organic acids, polyphenols, terpenes and pigments that can impede in the separation procedures (159). The cleaning procedures are therefore desirable that frequently uses

acetone and TCA (85). It is established that TCA alone is insufficient to remove contaminants and therefore sonication and brief grinding are suggested along with TCA (85, 160).

The variable *pI* range of proteins, their relative abundance, hydrophobicity and solubility makes them difficult to separate through the classical 2-DE. The liquid chromatography technique connected with MS (LC–MS/MS) can be used as an alternative separation method (161). The sample preparation procedure in plant proteomics is generally dependent on the type of plants, its fragment (leaf, stem, fruit, etc.) and even on the stage of plant development. Fukuda *et al.* described the protocol for the preparation of sample from rice embryo and its analysis using 2D electrophoresis. The plant material was chemically homogenized with solution consisting of urea, thiourea, CHAPS (3-[(3-Cholamidopropyl)-dimethyl-ammonio] 1-propane sulfonate), Ampholine, polyvinyl lopolypyrrolidone and 2-mercaptoethanol. The mixture was boiled at 100°C, centrifuged and supernatant was discarded. Finally, the lipids were removed with the addition of hexane, and the samples were analyzed by 2D electrophoresis (162).

Conclusion

In the previous several years, tremendously useful advances are made in the field of proteomics. The technologies are rapid, sensitive and provide greater proteome coverage. Furthermore, combination of these technologies has achieved success in purification, analysis, characterization, quantification, sequence and structural analysis and bioinformatics analysis of large number of proteins in all types of eukaryotic and prokaryotic organisms. All fields related to biological sciences have been benefited with increasing use of proteomics techniques. However, further work is still required to improve the reproducibility and performance of well-known proteomics tools.

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