Proteomics: State of the Art in Post-Genomic Physiological and Biomedical Sciences

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Abstract

After the completion of the Human Genome Project, there was an immediate flooding of human genome information obtained from the high-throughput genomic approach. This big leap has led to the development and/or improvement of several biotechnologies to utilize the genomic information to explain biology and (patho)physiology of cells, tissue or organs during normal and abnormal (diseased) states. One of such post-genomic studies is proteomics, which can simultaneously examine a large number (or a set) of proteins encoded by the genome (proteome). Proteomics has been recently defined as "*the systemic analysis of proteins for their identity, quantity and function*". With its high-throughput capability and other advantages that overcome limitations in conventional methods, proteomics has become one of the most powerful tools for physiological and biomedical sciences. This article provides an overview of proteomics and some methodological aspects that are important for proteome analysis. There are two main types of proteomics study, including expression and functional proteomics. Bioinformatics serves as a bridge between expression and functional proteomics (i.e. to obtain additional information of proteins identified from expression analysis for further designing highly-focused functional study). The ultimate goals of a classical proteomics study are: 1) better understanding of biology and physiology of normal cells, tissues or organs; 2) unraveling the complexity of pathogenic mechanisms and pathophysiology of diseases; 3) identification of novel biomarkers and new therapeutic targets; and finally 4) drug and vaccine discovery.

During the past few decades, molecular genetics and genomics have been applied successfully to biomedical research to better understand molecular biology and expression profiles of genes in normal and diseased states.[1] Genetic/genomic analyses can be applied to define genes that are involved in the pathogenesis of many diseases; e.g., oncogenes and tumor-suppressing genes in several types of cancers.[2] However, several diseases are not gene-mediated. Therefore, applications of genetics/genomics are limited for some instances. Indeed, proteins, which are the translation products of genes, directly govern cellular functions. One gene can be translated to several different proteins by a process called *'post-translational modification'* (PTM), which determines differential functions of various forms of proteins derived from the same gene. Although the term *'functional genomics'* has been utilized recently in the study of differential expression of genes during experimental conditions, this type of the study cannot be applied to examine functional units of the cell, especially when PTMs occur. Therefore, extensive study of proteins is crucial to better understand the biology and physiology of cells, tissues and organs, which have a high degree of dynamicity.

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The need for a method to explore the protein universe

The dynamic information at the protein level will be complementary to the static information obtained from genetic/genomic approach. Previously, conventional immunological methods, such as Western blotting, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), were the main techniques employed in the study of proteins. However, only a few proteins can be examined in each experiment and the specific antibody must be existing and available. In addition, the proteins to be tested are based on previous findings. This type of protein analysis is, therefore, not suitable for the high-throughput study of proteins. Because of the complexity of micro- and ultra-structures of cell, there are a large number of various proteins that may act independently or interact with others to govern differential cellular functions. To better understand the cellular biology and physiology, an effective method for '*global analysis*' of proteins in the target cells, tissues or organs is required.

Proteomics: the post-genomic protein science

Previously, there was no effective tool to perform simultaneous analysis of multiple proteins until 1975, when O'Farrell[3] and Klose[4] first introduced two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to separate a large complement of proteins. This technique permits separation of proteins in a complex mixture into hundreds to thousands of components (or spots in 2-D gel) (Figure 1). Recent advance of 2-D PAGE (high-resolution 2-D PAGE) enables separation of $\geq$10,000 proteins in a single 2-D gel.[5] Even with 2-D

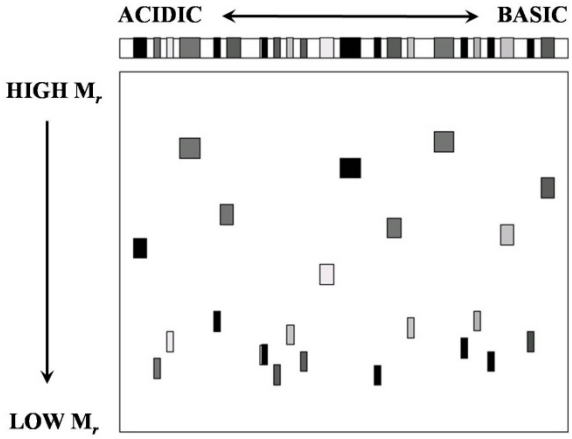

Figure 1. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Proteins are first separated along the horizontal axis by differential isoelectric points (pI). After completion of the isoelectric focusing (IEF), proteins are then separated by differential molecular masses (M_r) in the second dimension along the vertical axis.

separation, the global analysis of proteins during the past few decades was handicapped because of a limitation in protein identification at that time.

In the post-genomic era, several biotechniques have been developed or optimized to utilize the invaluable genomic information to explain the complexity of biology and physiology of cells, tissues and organs. Mass spectrometry (MS) has become an important tool to identify proteins using genomic information.[6] This step of development has made high-throughput, global analysis of proteins feasible and practical (the success of MS applications in biomedical research has been confirmed when John B. Fenn and Koichi Tanaka shared the Nobel Prize in Chemistry in 2002 from their great contributions in development of MS: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), respectively)).[7] With the strengths of 2-D PAGE for protein separation and MS for protein identification, Marc R. Wilkins first coined the term **'proteome'** (set of **prote**ins encoded by the gen**ome**) during the Siena electrophoresis conference in 1994.[8] The field of the proteome study that involves high-resolution protein separation and high-throughput protein identification has acquired the name **'proteomics'**[9] which has been recently defined by Peng and Gygi as "*the systemic analysis of proteins for their identity, quantity and function*".[10] Proteomics serves as a link between static information (gene expression) and dynamic aspects of those genes' products or proteins. Proteome analysis is a method to examine proteins based upon differential masses of peptide fragments and does not require any specific antibody for protein identification. Furthermore, both known (previously determined) and unknown (previously undetermined) components can be examined simultaneously. Proteome analysis is, therefore, an ideal method for the large-scale, high-throughput, post-genomic study of the protein universe in a given cell, tissue or organ.

Rapid growth of the proteomics arena

During the past decade, proteomics has been applied extensively to various fields of science and medicine. The proteomics arena has been growing rapidly as determined by a rapid increase in number of published proteomics-related articles. The first publication related to proteomics appeared in PubMed in 1995[11] and there have been >30,000 proteomics-related articles cited in the PubMed through the end of February 2009. The rapid increase in these numbers is shown in Figure 2.

A brief overview of proteomic technologies

The challenge in proteome analysis is that it involves the complex mixture of proteins, of which physicochemical properties vary and some components have relatively low amount (low abundant proteins). An ideal analytical technique used for proteome analysis should have high-resolution, high-sensitivity, and high-throughput capabilities of protein separation and identification. There are two main types of proteome analyses that are classified based on separation techniques: gel-based and gel-free proteome analyses (Figure 3).

Gel-based proteome analysis

The fundamental procedure of protein separation for gel-based method is 2-D PAGE (Figure 1). Proteins are first separated on the basis of differential pH or charges. During electrophoresis, acidic (anionic) proteins migrate towards the anode, whereas basic (cationic) proteins (cations) move towards the cathode. These proteins then gain opposing charges, which determine the protein dynamic net charges along the migration path. At a point where the dynamic net charge of a protein is zero (neutral), it will stop migration. This point of pH value is called '*isoelectric point*' (pI) and this process is called '*isoelectric focusing*' (IEF). After completion of the IEF, the resolved proteins are subjected to further separation in the second dimension, based on differential molecular sizes (M_r) using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein spots in 2-D gel are then visualized by various types of dyes (e.g., Coomassie Brilliant Blue, silver, fluorescence, etc.). Quantitative analysis is performed by comparing intensity levels of corresponding protein spots that are matched across different gels. The spots of interest are then excised from the gel and subjected to in-gel tryptic digestion. Thereafter, proteolytic peptide fragments are identified by MS analysis. The most common MS technique utilized to identify those peptide fragments (extracted from protein spots in 2-D gel) is MALDI-MS.

Tryptic peptide fragments are mixed with organic matrix, which facilitates ion activation by laser beam. The most common matrix used in MALDI is α-cyano-4-hydroxycinnamic acid.[12] The mixture of analytes and matrix is spotted onto the target plate and allowed to air-dry. Thereafter, crystals of matrix together with analytes are formed. After laser firing with the mass spectrometer, the matrix adsorbs the energy from laser and then

undergoes rapid solid-to-gas phase transition or the exciting state.[12] The analytes embedded in the matrix crystals are ionized by the excited matrix with a poorly understood process.[13] Most of ions formed in activated analytes are single-charged ions.[13,14] These ions then migrate from the target plate to the mass analyzer. The most common type of mass analyzer employed in MALDI analysis is time-of-flight (TOF).[15] The time to the target of activated ions differ because of the variety of mass per charge (*m/z*) values of the ionized peptides. Various peptide fragments can then be distinguished and identified by peptide mass fingerprinting (PMF), based on differential *m/z* units.[16,17]

Gel-free proteome analysis

The strategy of gel-free method is similar to that of gel-based analysis except for protein separation, which is based on liquid chromatography (LC),[18] capillary electrophoresis (CE),[19] and protein chip/microarrays technologies.[20] Most of gel-free studies employ high-performance LC (HPLC) to separate proteins or peptides in the mixture.[21] The resolved proteins/peptides can then be analyzed by various types of MS. The most common MS system used in gel-free analysis is ESI-MS/MS.[22,23] ESI is the process of ionization from electrospray source, whereas tandem MS (MS/MS) refers to a strategy for multi-step mass analyses. In contrast to MALDI analysis, in which analytes are prepared in solid phase, the analytes for ESI-MS/MS are generally in liquid phase. The solution of analytes is pumped through the high-voltage capillary.[24] The electrostatic force of this high-voltage field exceeds surface tension of the solution, leading to the spray of highly charged droplets from the capillary tip.[25] Desolvation process of these droplets can be accomplished by either passing through a heated capillary or passing across a stream of heated gas. The peptide ions then enter into the mass analyzer. There are several types of mass analyzers (e.g., quadrupole, ion trap, TOF) that can be used in tandem MS in various combinations.[12] Although these combinations operate differently, all of them have similar strategy to analyze peptide masses in the tandem manner (multi-step mass analyses) to produce more accurate data than those obtained from a single path of mass analyzer.

Classical versus alternative approaches for proteome analysis

There are two main approaches for proteome analysis as classified by the study purposes. The '*classical approach*' is to extensively and systematically examine protein expression and function to better understand the biology and (patho)physiology of cells, tissues or organs,[26] whereas the '*alternative approach*' is to examine proteome '*profiles*' or '*patterns*' of protein expression in various samples to differentiate types or groups of those samples (i.e. normal versus diseases) without any requirement for detailed characterizations of proteins.[27] The latter is more suitable for biomarker discovery or diagnostic/prognostic purpose.

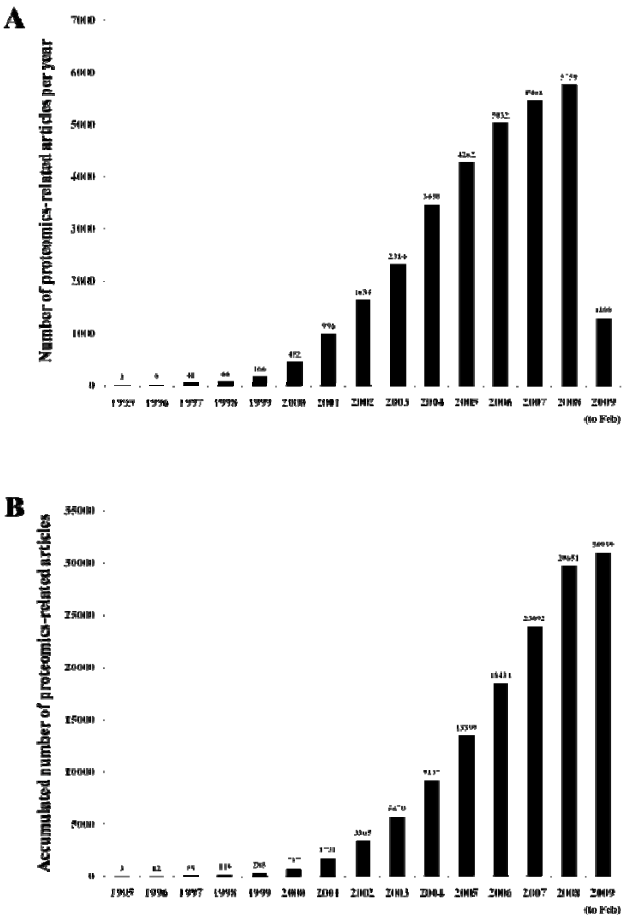

Figure 2. Number of published proteomics-related articles. A) Number of publications per year. B) Accumulated number of publications through February 2009. The PubMed search was performed using the keyword 'proteomics' or 'proteomic' or 'proteome'. The article with more than one of these keywords found was counted as only one.

Classical approach

This approach usually begins with protein extraction/isolation, followed by protein separation either by gel-based (mostly referred to 2-D PAGE) or gel-free (mostly referred to LC) method. Separated proteins are then identified by various types of MS. Bioinformatics is utilized to obtain additional protein information for deciding subsequent functional studies. The final results of this approach are new hypotheses, better understanding of normal physiology and disease mechanisms, identification of novel biomarkers and new therapeutic targets, and drug and vaccine discovery.[26] There are three main types of the study involved in the classical proteomics approach, including expression proteomics, bioinformatics of proteins, and functional proteomics.

Expression proteomics

Expression proteomics is an initial step of proteome analysis to identify differentially expressed proteins in different biological samples or in the same sample but with different interventions (e.g., (patho)physiological conditions, disease models,

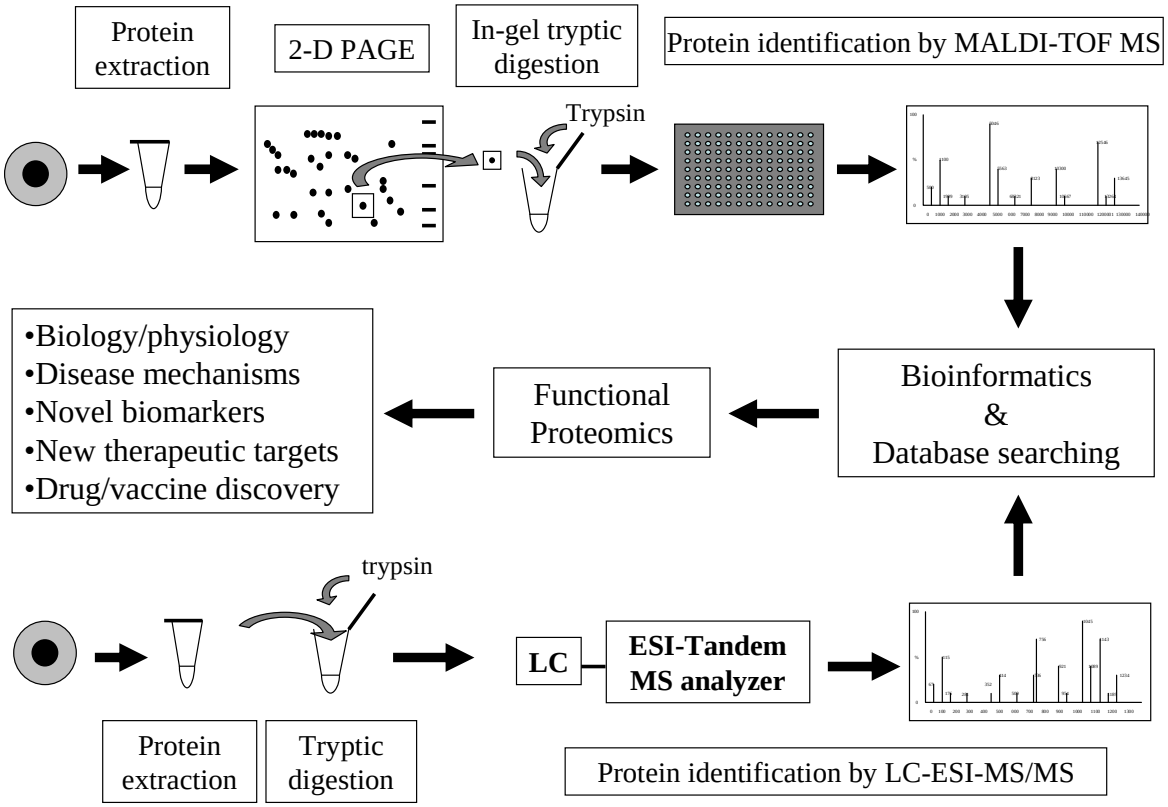

Figure 3. Proteomic methodology. In gel-based proteome analysis, proteins extracted from cells or tissues are separated by 2-D PAGE. Protein spots in 2-D gel can be visualized by various types of dyes and the spots of interest undergo in-gel tryptic digestion. The proteolytic peptide fragments are then analyzed by MALDI-TOF MS. Identification of a protein is performed by peptide mass fingerprinting (PMF) of peptide masses obtained from MALDI analysis. In gel-free proteome analysis, extracted proteins are first digested with trypsin (or other proteolytic enzymes), separated by LC, and identified ESI-MS/MS. For both gel-based and gel-free methods, bioinformatics serves as a bridge between the expression and functional analyses (to obtain additional information of the proteins identified from expression study to guide further functional studies).

treatments). Quantitative analyses are required to examine and compare expression levels of proteins in individual samples. For gel-based method, quantitation can be made by intensity analysis using 2-D gel image analysis software, of which the analytical strategy is similar to the strategy of densitometry.[28] For gel-free method, quantitation is performed by labeling peptides with isotopes (^{14}C, ^{15}N, and ^{18}O) or deuterium (for isotope-coded affinity tag; ICAT), comparing to non-radioisotopic or non-deuterated labeling in control samples.[29] The presence/absence or increase/decrease of protein expression may provide information for further functional study to better understand normal physiology and pathogenic mechanisms of diseases. Bioinformatics, therefore, plays an important role in linking the expression data to the functional study.

Bioinformatics of proteins

Bioinformatics can be applied to obtain additional protein information, e.g.: 1) physicochemical properties; 2) three dimensional structure; 3) sequence alignment and homology; 4) motifs and functional domains; 5) protein interactions and networks; 6) potential PTMs; 7) predicted transmembrane regions; 8) subcellular localizations; and 9) miscellaneous.[30] There are numerous on-line analytical tools that are freely accessible via websites for bioinformatic analyses of proteins. The data obtained from bioinformatic analyses are very helpful to link between expression and functional data, making functional studies more focused.

Functional proteomics

Alterations and/or modifications of a set of proteins, which work closely together with the others in the cell, determine the cellular response to a specific condition or stimulus. All of the involving proteins may form a multi-protein complex to carry out specific functions. Additionally, alternative splicing of transcripts and various PTMs cause multiple forms of products derived from the same gene,[31] leading to the more complexity of protein-protein interactions and their functions. Therefore, it is crucial to characterize components in the protein complex, protein-protein interactions, PTMs, and their functions in order to better understand the biology and physiology of cells, tissues and organs. Protein-protein interactions and protein complex can be analyzed on the basis of ligand fishing (e.g., co-immunoprecipitation, affinity chromatography, and pro-

tein chip) using specific antibody or ligand followed by either gel-based or gel-free technology.[32] PTMs can be analyzed by various methods on the basis of their chemistry or by MS-based methods. After characterization of components in the protein complexes, protein-protein interactions and PTMs, the next (and most important) step is to define functional significance of those proteins and modifications. There are several methods that can be applied to examine physiological role(s) of a protein of interest in the cell; e.g. using transgenic and/or knockout models, in which the specific gene can be modulated. The altered cellular function(s) can then be evaluated and the functional significance of such protein can be defined.[33]

Alternative approach

Alternative approach of proteomics can be described as the large-scale comparative analysis of proteome profiles or patterns of protein expression between different samples. The most common application of this approach is for diagnostic/prognostic purpose. Biological samples from patients can be differentiated from those of healthy individuals. Moreover, this approach can be applied to distinguish a specific disease from other related disorders, leading to biomarker discovery for earlier and more accurate diagnoses.[27,34] In addition, dynamic changes of proteome profiles during or after treatment are useful to predict therapeutic response, outcome and prognosis. The advantage of this application is that detailed characterizations of a specific protein are unnecessary. Additionally, the alternative approach examines overall expression profile rather than focusing on a specific protein. Therefore, this approach is suitable for clinical application, especially in multifactorial diseases in which a single biomarker may not be sufficient for effective detection or diagnosis. Commonly used analytical methods for this approach include microarrays,[35] protein chip,[36] and CE coupled to MS (CE-MS).[37]

Microarrays are produced by immobilizing multiple antibodies or other binding ligands onto individual arrays on a solid surface. The analytes are then incubated with antibodies- or ligands-assigned arrays. There are several types of microarrays that are named by ligands used; e.g., DNA, RNA, organic compound, peptide, protein, antibody, as well as tissue microarrays.[38,39] After incubation, unbound components of the analytes are removed and the bound components are examined using fluorescent or chemiluminescent detecting reagents. Protein chip is a subset of microarray technology of which proteins are bound onto the affinity surface. Surface-enhanced laser desorption/ionization' (SELDI)[40] is a typical model for protein chip technology, which is coupled to the MS instrumentation. CE-MS is another powerful method for proteome profiling.[41] The on-line coupling of CE with ESI-TOF MS is the non-chip method to generate proteome profiling. Proteins/peptides in the complex mixture are separated by CE and their *m/z* values are analyzed by ESI-TOF MS.[42] These techniques are able to examine a large number of proteins simultaneously, making comparison among different groups or subsets of biological samples more feasible.

Perspectives

It has been clearly demonstrated that proteomics holds a great promise in physiological and biomedical sciences, as demonstrated by the rapid growth of proteomic applications to several aspects of biology, physiology and medicine (Figure 2). Even with its strengths, proteomics also have some limitations. It should be emphasized that the major problem in global analysis of proteins by proteomics is that high abundant proteins may obscure the identification and/or characterizations of low abundant proteins, particularly when the entire proteome of a given cell, tissue or organ is examined. Prefractionation, enrichment of low abundant proteins, and removal of high abundant proteins prior to proteome analysis are the potential solutions, which gain a wide acceptance.

Another problem of the analysis of the entire proteome of a given cell, tissue or organ is that the global analysis does not provide precise information of each identified protein regarding its locale, although bioinformatics can be applied to predict its localization. In this case, the highly focused study is required. '*Subproteome analysis*' is an approach to study selected compartment of a given cell, tissue or organ, based on micro- or ultra-structures (e.g., plasma membrane, cytosol, individual organelles in cell, individual microstructures of an organ, etc.).[43,44] This type of analysis needs optimized protocols to isolate or enrich individual micro- or ultra-structures.

Proteomics of membrane or highly hydrophobic proteins also has some limitations, particularly in gel-based proteome analysis because of the difficulty in solubilizing hydrophobic components. Although some ionic detergents are very powerful to solubilize membrane proteins, they may not be compatible with 2-D PAGE. To date, several techniques have been applied to solubilize membrane or highly hydrophobic proteins to be compatible with 2-D PAGE, but its seems that the gel-free technology is the method of choice for analyzing membrane proteins[45,46] even though non-ionic detergents can be employed in gel-based study.[47]

Finally, most of the proteome studies, to date, have dealt mainly with expression proteomics. As a result, there are numerous sets of the expression data for some cells, tissue and organs, of which functional significance and roles remain unclear. Hence, the future direction of the proteomics arena will move onward to more functional studies.

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