Patología molecular

Proteomics techniques (I)

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INTRODUCTION

Marc Wilkins and Keith Williams coined the word "proteome" in 1996 (1). Proteomics is defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes and decipher the mechanisms of gene expression control". As such, proteomics focuses on the dynamic description of gene regulation and, by doing so, offers something much more powerful than a protein equivalent of DNA databases. In addition, proteome databases and associated technology are expected to address problems that cannot be approached by DNA analysis, namely, relative abundance of the protein product, post-translational modifications, subcellular localization, turnover, interaction with other proteins, as well as functional aspects. There are three main reasons for the proteome analysis to become an essential component in the comprehensive analysis of biological systems: i) protein expression levels are not predictable from the mRNA expression levels; ii) proteins are dynamically modified and processed in ways which are not necessarily apparent from the gene sequence; and iii) proteomes are dynamic and reflect the state of a biological system.

Proteins are usually the functional molecules and, therefore, the most likely components to reflect qualitative (expression of new proteins, post-translational modifications) and quantitative (up- and down-regulation, coordinated expression) differences in gene expression. Genes may be present, they may be mutated, but they are not necessarily transcribed. Some genes are transcribed but not translated, and the number of mRNA copies per cell does not necessarily reflect the number of functional protein molecules. Several studies have found a general trend but not a strong correlation between protein and transcript levels. These results suggest that even for a population of genes predicted to be relatively homogeneous with respect to protein half-life and gene expression, the protein levels cannot be accurately predicted from the level of the corresponding mRNA transcript (2).

Proteomics is an emerging area of research of the post-genomic era that deals with the global analysis of gene expression using a combination of techniques to resolve (high resolution two-dimensional polyacrylamide gel electrophoresis; 2-D PAGE), identify (peptide sequencing by Edman degradation, mass spectrometry, Western immunoblotting, etc.), quantitate (scanners, phosphorimager, etc), and

characterize proteins, as well as store in databases. Each one of these technologies can be applied independently, although their impact can be maximized when used in together in the study of complex biological problems.

PROTEIN STRUCTURE

One of the major areas of biological research today is how proteins, constructed from only 20 different amino acids, carry out the incredible array of diverse tasks that they do. Unlike the intricate branched structure of carbohydrates, proteins are single, unbranched chains of amino acid monomers. The unique shape of proteins arises from non-covalent interactions between regions in the linear sequence of amino acids. Only when a protein is in its correct three-dimensional structure, or conformation, is it able to function efficiently (3).

Many terms are used to denote the chains formed by polymerization of amino acids. A short chain of amino acids linked by peptide bonds and having a defined sequence is a peptide; longer peptides are referred to as polypeptides. Peptides generally contain fewer than 20-30 amino acid residues, whereas polypeptides contain as many as 4,000 residues. The term protein is reserved for a polypeptide (or a complex of polypeptides) that has a three-dimensional structure.

The structure of proteins is commonly described in terms of four levels of organization. The primary structure of a protein is the linear arrangement, or sequence, of amino acid residues that constitute the polypeptide chain. Secondary structure refers to the localized organization of parts of a polypeptide chain, which can assume several different spatial arrangements. A single polypeptide may exhibit all types of secondary structure. Without any stabilizing interactions, a polypeptide assumes a random-coil structure. However, when stabilizing hydrogen bonds form between certain residues, the backbone folds periodically into one of two geometric arrangements: an alpha helix, which is a spiral, rod-like structure, or beta-sheet, a planar structure composed of alignments of two or more beta-strands, which are relatively short, fully extended segments of the backbone. Tertiary structure refers to the overall conformation of a polypeptide chain, that is, the three-dimensional arrangement of all amino acids residues. Tertiary structure is stabilized by hydrophobic interactions between the nonpolar side chains and, in some proteins, by disulfide bonds (4). For proteins that consist of a single polypeptide chain, monomeric proteins, tertiary structure is the highest level of organization. Multimeric proteins contain two or more polypeptide chains, or subunits, held together by non-covalent bonds. Quaternary structure describes the number (stoichiometry) and relative positions of the subunits in a multimeric protein.

2-D ELECTROPHORESIS

P.H. O'Farrell (5) and J. Klose (6) first introduced two-dimensional electrophoresis in 1975. The power of 2-D electrophoresis has been recognized virtually since its introduction. Its application, however, has become significant only in the past few years as a result of a number of developments. A large and growing application of 2-D electrophoresis is "proteome analysis". Proteome analysis is the analysis of the protein (prote-) complement expressed by a genome (-ome) (7). The analysis involves the systematic separation, identification, and quantification of many proteins simultaneously from a single sample.

There are a number of inherent difficulties in protein analysis which complicate these tasks. Firstly, there is no amplification method for proteins, analogous to the PCR method for amplifying genes, and for this reason, it is frequently difficult to purify from the native source sufficient amounts of a protein for analysis. For the technological point of view this translates into the need for high-sensitivity analytical techniques or using time-consuming tricks, such as fractionating cells into their various organelles before running consecutive gels. Secondly, many proteins are modified and processed post-translationally. Therefore, in addition to the protein identity, the structural basis for differentially modified isoforms also needs to be determined. The complexity and dynamics of post-translational protein editing thus significantly com-

plicates proteome studies. Thirdly, proteins vary dramatically with respect to their solubility in commonly used solvents. This makes the development of protein purification methods particularly difficult. Lastly, the number of proteins in a given cell system is typically in the thousands. Therefore, a successful, general proteomics technology requires high sensitivity, high throughput, the ability to differentiate differentially modified proteins, and the ability to quantitatively display and analyze all the proteins present in a sample.

Electrophoresis is a technique for separating, or resolving, molecules in a mixture under the influence of an applied electric field. Dissolved molecules in an electric field move, or migrate, at a speed determined by their charge/mass ratio. For example, if two molecules have the same mass and shape, the one with the greater net charge will move faster toward an electrode. The separation of small molecules, such as amino acids and nucleotides, is one of the many uses of electrophoresis. Electrophoretic separation of proteins is most commonly performed in polyacrylamide gels.

Two-dimensional electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis, separates proteins according to their molecular weights (Fig. 1). Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different protein species can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained.

Sample preparation

Appropriate sample preparation is absolutely essential for good 2-D PAGE results. Because proteins vary in size, charge, and water solubility, no single method can be used to isolate all proteins. Due to the great diversity of protein sample types and origins, the optimal procedure must be determined empirically for each sample type (8). Ideally, the process will result in the preparation/purification of cells, cell lysis, complete solubilization, disaggregation, denaturation, and reduction of the proteins in the sample.

In general, extremely diverse preparation/purification techniques have been proposed for the preparation of tissues for 2-D electrophoresis. The majority of preparation methods deal with whole-tissue extracts (9). One of the main problems with protein profiles of tissues is their heterogeneous nature that exerts serious problems in terms of quality of 2-D

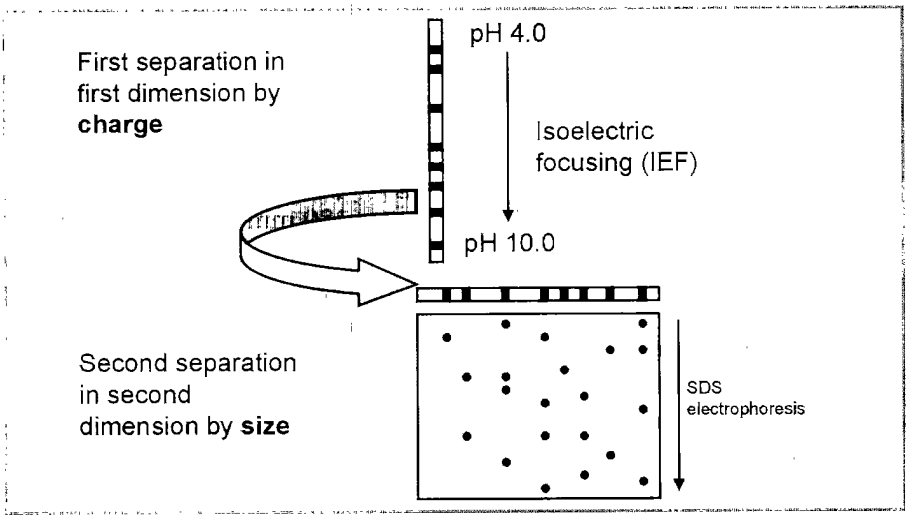

Figure 1. Separation of proteins by two-dimensional electrophoresis.

PAGE patterns. Various microdissection techniques have allowed for substantial enrichment in the targeted cell populations in a tissue.

Proteolysis greatly complicates analysis of 2-D results; thus, the protein sample should be protected from proteolysis during cell disruption and subsequent preparation (10). It is preferable to disrupt the sample material directly into a strong denaturing lysis solution (such as 8 M urea, 10% TCA, or 2% SDS) in order to rapidly inactivate proteases and other enzymatic activities that may modify proteins.

In order to characterize specific proteins in a complex protein mixture, the proteins of interest must be completely soluble under electrophoresis conditions (11). Different treatments and conditions are required to solubilize different types of protein samples: some proteins are found in complexes with membranes, nucleic acids, or other proteins; some proteins precipitate when removed from their normal environment; and some proteins form various nonspecific aggregates.

In general, it is advisable to keep sample preparation as simple as possible (12). The composition of the sample solution is particularly critical for 2-D, because solubilization treatments for the first dimension separation must not affect the protein pI, nor leave the sample in a highly conductive solution. In general, concentrated urea as well as one or more detergents are used. First dimension performed under denaturing conditions gives the highest resolution and the cleanest results.

FIRST-DIMENSION ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI) (13). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The isoelectric point is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. This is the focusing effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences.

The original method for first dimensional IEF depended on carrier ampholyte-generated pH gradients in polyacrylamide tube gels (13). Although this basic method has been used in hundred of 2-D electrophoresis studies, it has several limitations that have prevented its more widespread applications (14). Because of the limitations of the carrier ampholytes method, an alternative technique for pH gradient formation was developed: immobilized pH gradients, or IPG. Bjellqvist *et al.* introduced this technique in 1982 (15). For improved performance and simplified handling, the IPG gel is cast onto a plastic backing. Finally the gel is dried and cut into 3-mm wide strips. The resulting IPG strips can be rehydrated with a rehydratation solution containing the necessary components for first-dimension IEF and, optionally, the sample proteins (14).

The optimum amount of protein to be loaded onto a single IPG strip for optimum resolution, maximum spot numbers, and minimum streaking-background smear depends on parameters such as pH gradient (wide or narrow), separation distance, and protein complexity of the sample. For analytical 2-D PAGE followed by silver staining, 50-100 µg of protein of a total cell lysate per IPG strip being 180 mm long and 3-6 pH units wide, proved to be the optimum for the majority of samples. For micropreparative 2-D PAGE, up to 1 mg of protein can successfully be applied (14).

SECOND DIMENSION SDS-PAGE

After IEF, the IPG gel strips are equilibrated in the presence of SDS, DTT, urea, glycerol and iodoac-

etamide, and placed onto the surface of a horizontal or on top of a vertical SDS-gel (14).

SDS-PAGE is an electrophoretic method for separating polypeptides according to their molecular weight. The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. The bound SDS masks the charge of the proteins themselves, forming anionic complexes with constant net negative charge per unit mass.

During SDS-PAGE, all the proteins of the sample have the same net charge per gram, and movement through the gel is based solely on the molecular mass of the proteins. The second dimension can be calibrated using molecular weight marker proteins loaded to the side of the second dimension gel. Often there are abundant proteins in the sample for which the pI and molecular weight are known. These proteins can serve as internal standards. Second-dimension gels are made as either homogeneous gels, with constant acrylamide and crosslinker % or gradient gels, with increasing acrylamide % and usually constant crosslinker %. The choice of acrylamide % is determined by the molecular weight of the proteins that are to be separated. When separating proteins in a narrow molecular weight range, homogeneous gels generally give better separation. Gradient gels have two advantages: they allow proteins with a wide range of molecular weight to be analyzed simultaneously, and the decreasing pore size functions to sharpen the spots, *i.e.*, it improves resolution (16).

Visualization and analysis of results

When the bromophenol blue front had completely migrated out of the SDS gel, the resolved polypeptides were fixed in ethanol/acetic acid/water, normally overnight. Analytical gels are usually stained with silver nitrate, whereas micropreparative gels are preferably stained with Coomassie blue (14) (Fig. 2).

Silver staining of polyacrylamide gels was introduced in 1979 by Switzer *at al.*, and is the most sensitive nonradioactive method (17). The detection limit is as low as 0.1 ng of protein per spot. Silver staining has several drawbacks, including:

i) Silver staining is a complex, multistep process, and many variables can influence the results.

ii) High-purity reagents and precise timing are necessary for reproducible, high-quality results. Impurities in the gel and/or the water used for preparing the staining reagents can give poor staining results.

iii) Limited dynamic range.

iv) The fact that certain proteins stain poorly, negatively, or not at all (14, 18).

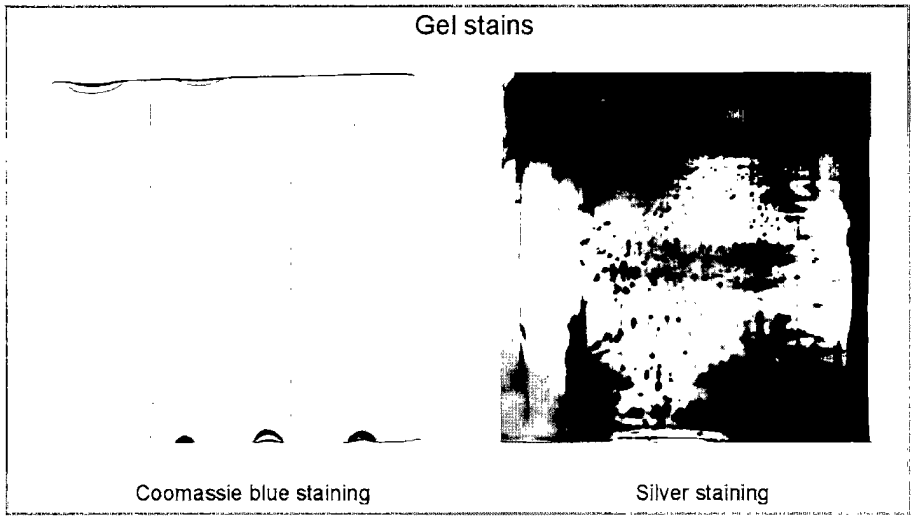

Figure 2. Staining with Coomassie blue and silver staining.

Coomassie staining, although 50-fold less sensitive than silver staining, is a relatively simple method and more quantitative than silver (19, 20). Coomassie blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of proteins are to be determined by densitometry. Micropreparative gels are preferably stained with Coomassie blue.

Autoradiography and fluorography are the most sensitive detection methods. To employ these techniques, the sample must consist of proteins radiolabelled *in vivo* using either 35S, 14C, 3H, or, in the case of phosphoproteins, 32P. For autoradiography detection, the gel is dried and exposed to x-ray film or a storage phosphor screen (21). Fluorography is a technique that provides extra sensitivity by impregnating the gel in a scintillant such as 2,5-diphenyloxazole (PPO) prior to drying (22).

Second-dimension gels can be transferred ("blotted") onto the surface of an inert membrane such as nitrocellulose or **PVDF**. When immobilized in this way, the proteins are readily accessible to interaction with probes, such as antibodies or other ligands specific for the protein being analyzed. The most efficient method for the transfer of proteins separated by 2-D electrophoresis to membranes is the application of an electric field perpendicular to the plane of the gel. This technique of electrophoretic transfer is also known as Western blotting (23).

IMAGE ANALYSIS OF 2-D GELS. PROTEIN BIOINFORMATICS

In theory, the analysis of up to 15,000 proteins should be possible in one gel; in practice, however, 5,000 detected protein spots means a very good separation.

While most standard imaging systems do the job of collecting images of 2-D gels, specialized software has been developed for analyzing the complex patterns of spots (Fig. 3). Packages are available that

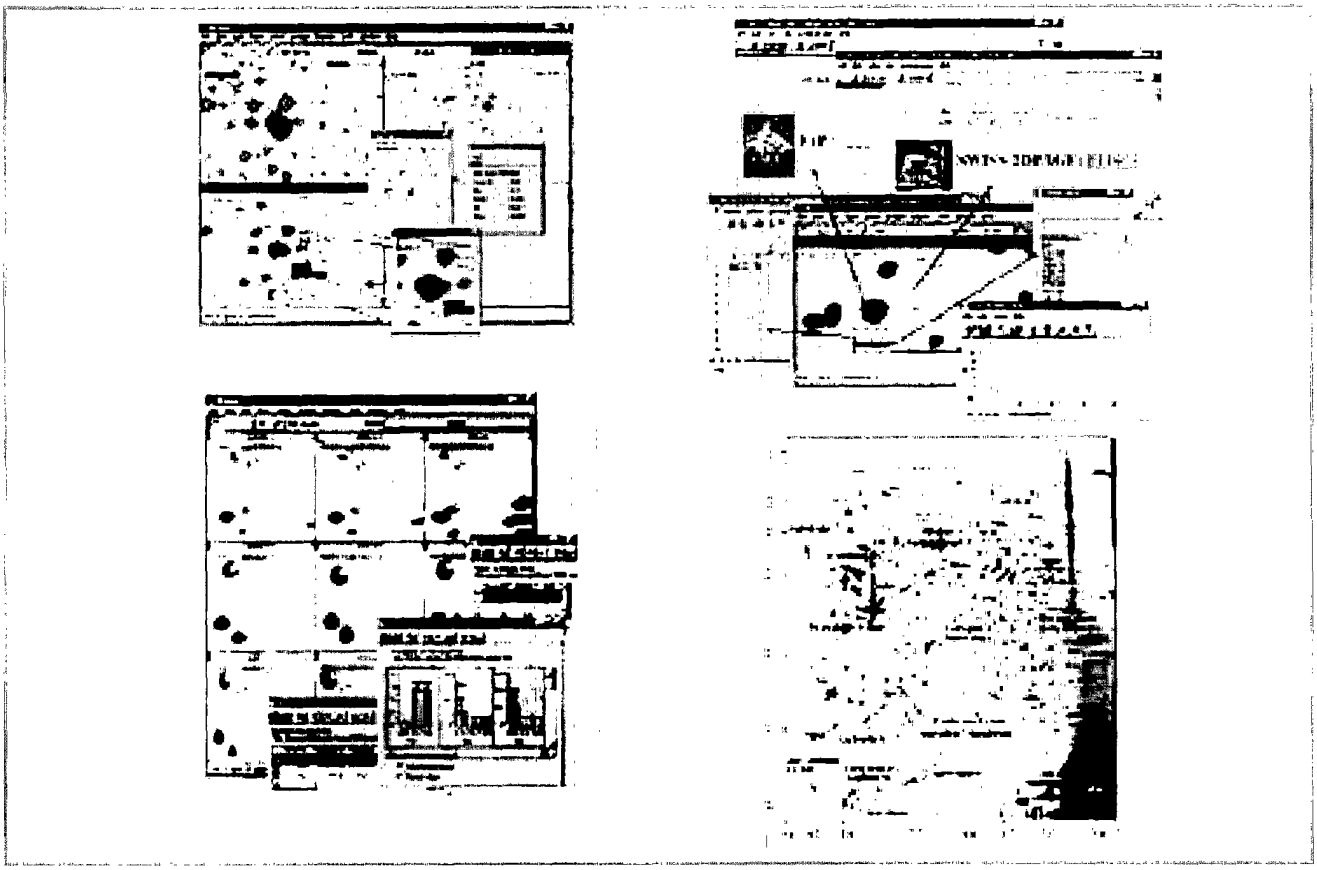

Figure 3. Specialized software developed for analyzing complex patterns.

will go from spot detection to database searches and, in one case, even to Web page construction for posting the 2-D gel pattern on the Internet. The three major software systems used in proteomics research are: Melanie-3 2-D (24, 25), PDQuest (26), and GELLAB II+ (27).

In addition to the software packages for analyzing the 2-D electrophoretic gels, bioinformatic tools have been developed. Some of these are available via the Internet. These allow not only identification of proteins but further characterization ranging from the calculation of basic physicochemical properties to the prediction of potential post-translational modifications and three-dimensional structures. These databases are the bioinformatic core of proteome research (28, 29).

PROTEIN IDENTIFICATION METHODS

High-resolution gel electrophoresis, of which 2-D PAGE is currently the most powerful protein separation method, was already used as an analytical tool in the late 1970s. This came only after the protein sample preparation methods and sequencing tools had improved considerably. 2-D PAGE has evolved into a preparative protein purification procedure. The analysis of gel-separated proteins has traditionally been performed by blotting the spots to a chemically inert **PVDF** membrane followed by amino acid analysis and protein sequencing to establish protein identity, purity, and quantity. Proteins of interest can be excised directly from the gel or blotted onto a suitable membrane and they can then be subjected to identification techniques.

Immunoaffinity identification

For immunoaffinity identification of 2-D electrophoresis separated proteins immunoblotting is now widely used in conjunction with 2-D PAGE (30). Specific gene products can be identified in 2-D protein maps using antibodies prepared with the help of modern biotechnology on the basis of gene and cDNA sequences (31). Immunoblotting results also complement with pI and molecular weight information to characterize post-translational modifications of proteins.

Amino acid analysis

Amino acid analysis is a powerful and sensitive technique for the determination of the amino acid composition of proteins. A demand for high sensitivity and high sample throughput has led to the development of precolumn derivatization of amino acids and subsequent separation by reverse phase-high performance liquid chromatography (RP-HPLC) (7).

^{N}-terminal sequencing

Electroblotting combined with the membrane-based Edman chemistry generated either ^{N}-terminal or internal peptide sequences. This combined technology is now referred to as microsequencing, allowing the analysis of proteins in the low pmole or microgram range (32).

Mass spectrometry

Edman sequencing is a slow process and lacks the required sensitivity. This method also requires unblocked amino-termini and so cannot be used for the identification of most 2-D gel spots. Amino acid analysis is more sensitive and relatively fast, but reliable identification requires other information such as pI, molecular mass, ^{N}-terminal sequence or peptide mass data. Mass spectometry (MS) in conjunction with software and automation techniques has become an important tool to identify and analyze virtually all proteins in a gel (33, 34). In protein analysis the majority of experiments are performed today with two types of instruments: matrix-assisted laser desorption/ionization (MALDI) MALDI-MS and electrospray ionization (ESI) ESI-MS (35).

MALDI-MS is the technique of choice for high throughput protein and peptide identification, as needed in proteomics. The sensitivity is routinely in the fmol range. In this approach, the protein of interest, which is purified by 2-D electrophoresis is either enzymatically or chemically cleaved and an aliquot of the obtained peptide mixture is analyzed by mass spectometry techniques. The obtained

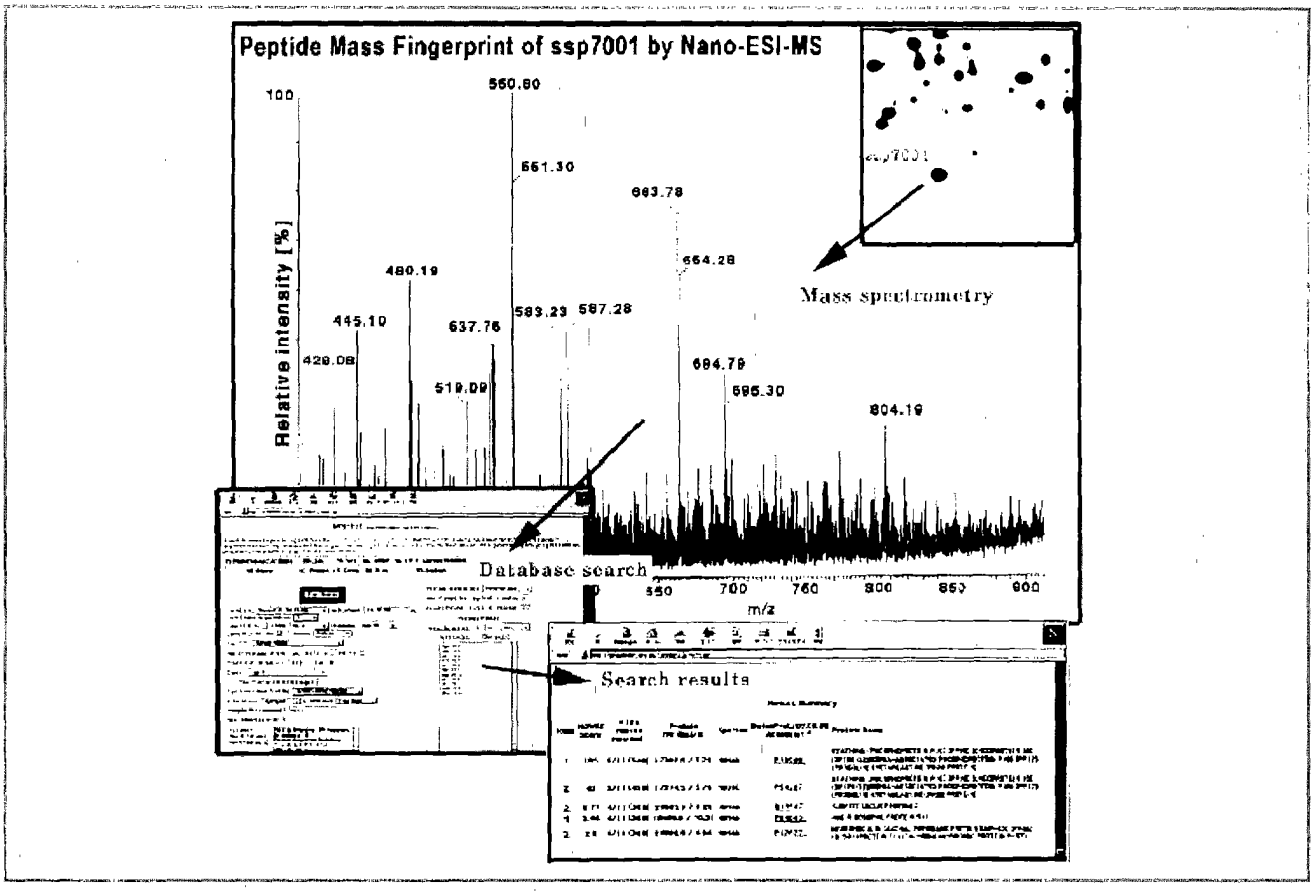

Figure 4. Identification of spot protein by mass spectometry.

peptide mass fingerprint is subsequently compared to "virtual" fingerprints obtained by theoretical cleavage of proteins sequences stored in databases and the top-scoring proteins are retrieved as possible candidate proteins (36) (Fig. 4).

LIMITATIONS OF PROTEOMICS

While proteomics offers a new approach to the study of many pathological conditions, there are still several technological limitations.

Archival tissue specimens are usually stored as formalin-fixed, paraffin wax-embedded blocks. Formalin fixation facilitates excellent morphological preservation, and the immunoreactivity of many antigens is preserved, but formalin-induced chemical cross-linking of proteins renders them insoluble and inaccessible to standard biochemical extraction and analytical methods such as 2-D electrophoresis. However, recently Ikeda *et al.* have described a method of protein extraction for Western blot analysis from formalin-fixed paraffin-embedded tissue sections (37). Also, polyacrylamide gel electrophoresis and immunoblotting of proteins can be performed using paraffin sections of tissues fixed in non-cross-linking fixatives (acetone, alcohol, or modified Carnoy's solution) (38).

A serious problem is the inability of 2-D PAGE to detect low abundance proteins; because there is no equivalent in protein biochemistry of PCR in molecular biology to increase the sensitivity and current protein staining techniques do not allow the visualization of low abundance proteins in 2D-gels. However, protocols to increase the resolving power to detect minor proteins in the presence of large quantities of housekeeping proteins are currently tested. Emmert-Buck *et al.* have found that methods such as radiolabelling or biotinylation dramatically increase the number of proteins visualized (39).

There are also several limitations because the lack of sensitive and reliable techniques for protein quantitation, although the use of sensitive fluorescent dyes has considerably improved this situation. Many staining techniques, such as silver staining, suffer from a limited dynamic range, so that the intensity of more abundant spots is not linearly correlated to that of less abundant spots (40).

In the early days of 2-D electrophoresis, there was substantial interest in the technique. However, enthusiasm waned because of the time-consuming and the complexity of the technique, which required as much art as science. It is obvious that currently the field of proteomic analysis is going through a renaissance thanks to the added capabilities of mass spectometry for protein identification. The challenge is to develop a system capable of simplification the technique, automation, high throughput, and high sensitivity. In conclusion, although, not being perfect, a method of 2-D PAGE with IPGs has been established that fulfills the basic requirements of proteome research and that is being continuously improved to meet the demands of the future.

FUTURE DEVELOPMENT OF PROTEOMICS

Protein chips or arrays

Analyses of proteins in microchips are now possible. In 1993, the concept of surface-enhanced laser desorption/ionization (SELDI) was introduced. This new strategy for mass spectometry analysis of macromolecules simplified sample extraction and facilitated effective on-probe investigation when compared to conventional MALDI (41, 42).

Optimizing and automation

Although proteomics is a relative young discipline, technology for increasing throughput in proteomic projects is rapidly being developed. Several steps of the technique can be optimized and automated to increase efficiency and save time.

The operating paradigm in proteome analysis today is the combination of 2-D PAGE (for protein resolution) with mass spectrometry (for protein identification). The entire intermediary steps in the procedure (gel staining, image analysis, protein spot excision, digestion and mass spectrometry) can be automated. While a degree of automation already exists in some stages of the protein identification process, such as automated acquisition of MALDI-TOF mass spectra, efficient interfaces between different stages are still lacking (43). Recently, an interesting solution to the problem of automation of protein identification was described by Bienvenut *et al.* (44).

CONCLUSION

The technologies of 2-D PAGE and mass spectometry are the driving force in contemporary proteomics and are likely to remain so in the immediate future, although new approaches including antibody-based techniques and protein chips may challenge their preeminence.

The development of proteomic techniques represents a new way to study pathological processes at the molecular level. These techniques are already leading to improvements in the understanding many processes. It will be possible in the near future to combine genomic and proteomic information to obtain more comprehensive picture of many pathological conditions. In this way, the molecular analysis of tissues at all three levels (genomic, transcriptomic and proteomic levels) has been referred to as operomics (45).

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