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# THE BIOLOGY OF THE POST-GENOMIC ERA: THE PROTEOMICS

## Review

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The complete identification of coding sequences in a number of species has led to announce the beginning of the post-genomic era, new tools have become available to study complex phenomena in biological systems. Rapid advances in genomic sequencing and bioinformatics have established the field of genomics to investigate thousands of genes' activity through mRNA display. However, recent studies have demonstrated a lack of correlation between the transcriptional profiles and the actual protein levels in cells, so investigation of the expressed part of the genome is also required to link genomic data to biological function. It is possible that evolutional development occurred by increasing complexity of regulation processes at the level of RNA and protein molecules instead of simple increase in gene number, so investigation of proteins and protein complexes became important fields of our post-genomic era. High-resolution two-dimensional gels combined with sensitive mass spectrometry can reveal virtually all proteins present in cells opening new insights into functions of cells, tissues and whole organisms.

*Keywords:* Post-genomic era – proteomics – *Caenorhabditis elegans*

## INTRODUCTION

It is now obvious that gene-based expression analysis is not enough to understand biological processes at the molecular level, because there is no direct correlation between gene and protein expressions. Differences can be explained by different stability and turnover of mRNA and proteins, or alternative mRNA splicing resulting various protein products as well as post-translational modifications [100]. It became clear that these processes occurring at the RNA and protein levels play an important role in expanding protein diversity and might be responsible for the apparent discrepancy between gene number and complexity of human cells. The "Kyoto

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Abbreviations: 2-DE: two-dimensional gel-electrophoresis, MS: mass spectrometry, IPG: immobilized pH gradient, SDS: sodium dodecyl sulphate, PAGE: polyacrylamide gel-electrophoresis, TCA: trichlororacetic acid, CBB: Coomassie Brillant Blue, CDD: charge-coupled device, MALDI-TOF: matrix-assisted laser desorption ionization-time of flight, ESI: electrospray ionization, PCD: programmed cell death, DSP: disease-specific protein, LCM: laser capture microdissection, SPR-BIA: surface plasmon resonance-biomolecular interaction analysis.

Encyclopedia of Genes and Genomes" (URL: http://www.genome.ad.jp/kegg/catalog/org_list.html) records 6 eukaryotic, 41 bacterial and 10 archeal complete genomes at the time of writing, and numerous genomes are expected to be released shortly. This is in addition to the eukaryotic organelle, phage and virus genome databases and the large number of individual gene sequences submitted to the nucleotide databases. These genome sequence data providing the usage of cross-species matching are important resources for polypeptide identification in proteomics. The main goal of proteomics is to answer specific questions by detecting qualitative and quantitative differences among biological samples. Analysis of proteins in complex samples is performed by two-dimensional gelelectrophoresis (2-DE) followed by gel staining, image analysis, protein spot excision, digestion and identification by mass spectrometry (MS) through sequence database searching.

## *Two-dimensional polyacrylamide gel electrophoresis*

Two-dimensional electrophoresis is the commonly used technique for separation of proteins, they are separated according to differences in their isoelectric points in the first dimension and the differences in their molecular mass in the second dimension.

Isoelectric focusing (IEF) is performed in individual immobilized pH gradient (IPG) gel strips, and they are applied to SDS/PAGE after equilibration in SDS buffers. The pH gradient is created by covalently incorporated acidic and basic buffering groups into a polyacrylamide gel during IEF, the gradient does not drift and cannot be distorted this way [48]. The acrylamide matrix is cast onto a GelBond backing sheet, polymerized, washed and dried. IPGs can be made and cut into individual strips manually [45], but they are also available from various manufacturers with different lengths and pH gradients. The pH separation range can be defined from broad (3–7 pH units) to narrow (1–1.5 pH units) according to the application's needs. IPG strips increase reproducibility and reduce the amount of effort and experience needed to start 2-DE work. IEF can be carried out either on Multiphor II or IPGphor horizontal electrophoresis units [47].

The current sample preparation procedures are the protein solubilization with original or modified O'Farell's lysis buffer [86], thiourea/urea mix [95], boiling with SDS sample buffer followed by dilution with excess urea lysis solution [54] or TCA/acetone precipitation and resolubilization in urea lysis buffer [49]. Some protocols are available to measure the protein content of samples in the presence of urea and other detergents [98]. Due to the high complexity of biological samples it is sometimes advisable to carry out a pre-fractionation step to enrich certain proteins by isolation of cell compartments [22], by sequential extraction procedures [119, 120], by precipitation [49] or affinity purification [77]. Application of narrow range pH gradient and/or pre-fractionation steps are usually required for detection of low abundance proteins. Samples with high ionic strength must be dialyzed to avoid higher than 10 mM salt concentration. Dry strips are usually rehydrated to their original size by incorporating the solubilized proteins with proteinase inhibitors into them (in-gel

rehydration) [46, 48, 96]. Sample preparation, amount and volume, separation distance, pH gradient and other running conditions must be optimized in order to get fine resolution, maximum number of spots with minimal streaking and background smear [47].

Prior to the second dimension, IPG strips are equilibrated in SDS buffers and stored at –80 °C or immediately applied onto SDS gels [48]. Either horizontal or vertical systems (DALT) may be used for the SDS/PAGE separation, the latter offers the opportunity of performing multiple slab gel separations simultaneously [5]. Protein markers for determining the pH and molecular weight ranges of a specific 2-D system are commercially available or can be prepared [6]. Applicability of 2-DE was demonstrated for both analytical and preparative separations.

It is generally assumed, that co-migrating proteins are functionally equivalent with amino acid homology. It is very important to run three or five parallel gels to prove equivalencies and check for possible contamination or false migration. Since its increasing resolution, reproducibility and simplicity, IPG-DALT has become the core technology of proteome research.

## *Visualization of proteins*

After fixation, separated proteins in analytical gels can be stained by silver nitrate without the application of glutaraldehyde to make possible the in-gel digestion and MS analysis [58]. Preparative 2-D gels are often stained by colloidal Coomassie Brillant Blue (CBB) [85] or imidazole-zinc negative procedure [88]. Proteins immobilized by electroblotting [30] are accessible to interaction with probes, such as monoclonal and polyclonal antibodies or other ligands specific for the protein being analyzed [41]. There are several methods for detection of total proteins on Western blots utilizing Ponceau S, amido black, CBB R-250, india ink, colloidal iron or gold [29]. Autoradiography is used to visualize and quantitate radiolabelled proteins that are resolved by 2-DE. Proteins are commonly labelled with either $^{3}H$, $^{14}C$, $^{35}S$, $^{32}P$ or $^{125}I$ *in vivo* [74]. The application of phosphor imaging [59] has been replacing the film-based autoradiography in the past years, and organic scintillates are often used to enhance the autoradiograms [70]. Numerous procedures for fluorescent derivatization of proteins in gels or on transfer membranes have been described [93], however, non-covalent fluorescent stains, for example Nile Red dye [4] or SYPRO Ruby [13] are more compatible with standard protein identification methods allowing low-background one-step staining of proteins in gels without long de-staining steps.

## *Image analysis*

The image on the visualized protein spots in gels must be obtained for further analysis. The charge-coupled device (CDD) camera is an excellent tool to acquire images of variety of stained gels, membranes or films [92], while document scanner is more

suitable for analysis of membranes [118]. Phosphor imagers and multichannel array detectors are commonly used with radioactive samples [108], fluorescently stained gels are analyzed using CDD camera combined with a UV light box [93]. The obtained gel image is processed using Photoshop or Corel programmes before applying an analysis system. Developments in computer-assisted image analysis softwares, PDQUEST [42] and Melanie [9, 10] have allowed the efficient evaluation of thousands of spots on 2-DE maps. It is possible to detect and quantify even the faintest spots, quantitatively compare 2-D gels among each other and identify protein expression changes across various sets of images [7]. The main strategy is the composition of master gels from parallel gels and comparison of the sample master gel to the reference one.

The next major step is the isolation of differentially expressed spots. It can be carried out manually, but there are few commercially available automated gel spot excision robots interfacing with the image analysis software [76, 116]. Gel slices containing proteins can be sent to major proteome centres for identification.

## *Identification of proteins*

The identification approaches require that the protein or its very close sequence homologue exists in the database. Commonly used methods are the determination of a partial amino acid sequence by Edman degradation [111], or a peptide mass profile [36, 75], or amino acid composition [121]. High portions of proteins are blocked at their N-termini in the course of the biosynthesis (acetylation, formylation, pyroglutamyl formation) or by manipulations during the isolation procedure [43], so chemical de-blocking [63, 83] or internal peptide sequence analysis [33] are often required in case of Edman-degradation.

Determination of peptide mass at femtomolar level is practicable using MS [44]. This time, proteins purified with 2-DE are either chemically or enzymatically cleaved (in-gel or on-membrane digestion) [104] and aliquots of the obtained peptide mixtures are analyzed by MS techniques [26]. Two main lines of the technological developments dominate this field: matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) [64] and electrospray ionization (ESI) MS [35]. The group of experimentally obtained peptide masses are compared to the theoretical mass fingerprints of protein sequences available in different databases (for example at Profound: http://prowl.rockefeller.edu/cgi-bin/ProFound) to identify the isolated protein by construction of a peptide mass map. These databases provide opportunities to consider possible modifications of amino acid residues to obtain the most accurate theoretical mass maps. There are other useful protein identification and analysis softwares, which are available through the ExPASy Web server [8] (http://www.expasy.ch/tools/#proteome).

In some cases amino acid composition analysis provides better results than peptide mass mapping, because single amino acid substitution might alter the construction of peptide mass map if it occurred at a protease cleavage site [24, 25, 117].

Amino acid analysis has remained the best way to quantitate proteins [69]. These procedures may be used alone, but their combinations are the best choice [20]. Monoclonal or polyclonal antibodies can be applied by immunoblotting [73], but it is also possible to compare the positions of proteins on a gel with known spots on another gel of a proteome database via the Internet [71] to identify individual proteins.

## *Proteomics of model organisms*

The success rate of protein identification is clearly improved when the genome of the investigated organism is fully sequenced. There are available protocols for sample preparation for 2-D gels from *Escherichia coli* [114], *Saccharomyces cerevisiae* [18], *Drosophila melanogaster* [34] and *Arabidopsis thaliana* [62]. The well-known model organism of genetics, the nematode *Caenorhabditis elegans* is an excellent candidate for proteomics research. The consistency of development, the complete transparency of the body at all stages of development and the small number of cells made the identification of individual cell nuclei by Nomarski optics and the complete description of developmental cell lineage possible [28, 106, 107]. Several hundreds of mutants were isolated and analyzed until now and it is possible to construct transgenic or knock-out animals for studying activities of genes *in vivo* [94]. Interesting problems with high biological significance are studied applying isolated mutant strains of model organisms for proteome analysis. For example, complex mammalian cell phenomena, such as programmed cell death (PCD) can be investigated in the nematode model system because of the evolutionary conserved molecular pathways [78, 79]. During the ontogenesis of hermaphrodite nematodes 1090 somatic cells are generated, from which 131 cells undergo PCD. Numerous mutant strains that are defective at various steps of this process have been isolated, wherein these cells do not die or die, but their corpses are not phagocyted and eliminated by the surrounding cells [32]. Organisms carrying real mass differences compared to the wild-type one can be easily studied by proteomics [15]. However, potentially almost all the mutant strains of a genetic model organism are suitable for comparative proteome analysis.

## *Medical applications of proteomics*

Important goals of medical microbiology is to understand the interactions between pathogenic microorganisms and their host leading to clinical disease, and to identify novel virulence factors that may serve as future targets for vaccine and drug development. A simple procedure is to compare the expressed genomic part of virulent and avirulent strains that grown under the same conditions in order to identify proteins linked to the virulence [53, 61, 105, 113]. This approach has some limitations, since protein differences unrelated to virulence can also be detected, and in addition,

bacteria grown '*in vitro*' do not express all of the proteins which are characteristic to its growth in the host '*in vivo*' [20]. However, combination of genetic and recombinant DNA technologies with proteomics and co-cultivation of the pathogen with eukaryotic cells under reproducible controlled conditions may solve most of these problems [1, 27, 67, 80, 97]. Sufficient resolution of bacterial proteins by 2-DE achieved the identification of novel immunogenic targets for new and improved vaccines after immunoblotting with polyclonal sera from patients infected with pathogen [72, 81, 109]. Moreover, valuable data were obtained on antibiotic and drug resistance in microorganisms by comparing protein pattern of the resistant and non-resistant strains by proteomics [19, 115].

Most of the human diseases are multifactorial and medical therapy has to be individualized by a molecular fingerprint of patients' phenotypes for the maximal effectiveness. Proteomics may assist in classification of a disease into mechanism-based subcategories that suitable to find the best target-based therapy. Technological advancements have led to the expansion of proteomic applications in the modern biomedical sciences to define disease-specific proteins (DSPs) to monitor progression of a disease or response to therapy. First studies included analyses of brain and cerebrospinal fluid proteins with neurological syndromes [31, 57, 68, 112, 122], heart disease [11, 66], kidney, bladder and urine associated diseases [23, 50, 101], the inner ear and associated disease [110]. Major pharmacoproteomics companies try to identify possible protein drug targets and measure the effect of different drugs in a range of diseases [55].

Many differences in gene expression that have been recorded between normal human tissues and tumors are due to post-translational modifications not detectable with RNA analyses [2]. Proteomics is an important approach to define predictive, diagnostic and prognostic polypeptide markers useful for tumor diagnosis and therapy [60, 99]. Important studies have been recorded on bladder [89], kidney [101], breast [37–39], lung [56, 87], ovarian [3] tumors and on leukemia [52] as well as on neuroblastoma [65]. There are some available methods on preparation of pure tumor cell populations free from contaminating tissue materials [40, 99] and infrared laser capture microdissection (LCM) technology has also been introduced that allows the isolation of proteins from specific cells of heterogeneous tissues [17]. The precise area of the section targeted with the laser can be lifted free of surrounding tissue and transferred onto a film. Samples can be pooled and loaded on 2-D gels for proteome analysis [12]. Further developments to increase throughput and to reduce sample requirement would enhance the proteomics to introduce into the clinical practice [51].

## *Proteome databanks*

Evidence of increased interest in proteomics includes the proliferation of Web-based databases of protein expression using 2-DE [21, 91, 102, 103]. Standardization of 2-DE has been resulted by the common application of IPG strips provided unique

opportunity to compare gels from different laboratories across the Internet using the Flicker computer-assisted visual method [71]. Several federated 2-DE databases containing pictures of master gels and data specific to the identified proteins can be reached from the Web-site WORLD 2-D PAGE at the ExPASy server in Geneva (http://www.expasy.ch/ch2d/2d-index.html).

## CONCLUSIONS

Automation in instrumentation and software has overcome some limitations of proteomics. High sensitivity electrophoresis methods are interfaced with automated gel stainers, image analysis workstations, robotic spot excision instruments, protein digestion workstations and MSes could enhance the introduction of proteomics into the biomedical field. However, there are still drawbacks for the routine application of the method. It must be mentioned that functionally equivalent proteins differing by only a single amino acid may appear as unique protein spots when analyzed by 2-DE and individual spots may contain different proteins [90]. The possible option to solve the problem of overlapping protein spots is to reduce the range of the pH gradient and to increase the gel size. However, this approach is labor-intensive and sample requirement also increases which is frequently limited in clinical laboratories.

Spots with their individual protein content will need to be achieved through a third dimension by MS that is independent of the two separation steps of 2-DE. There is a very effective and new approach, the MALDI-TOF-MS scanning, where all proteins are simultaneously digested in the 2-D gel *in situ* and electrotransfered onto a membrane which is directly scanned for protein identification [14,16]. There are other technologies under active improvement for replacement of 2-DE. Interfacing surface plasmon resonance-biomolecular interaction analysis (SPR-BIA) and MALDI-TOF is used for functional and structural characterization of proteins. SPR-BIA is an optical, chip-based technique able to monitor biomolecular interactions as they occur between an immobilized receptor and a solution-borne ligand. The analyte selectively retained on the chip during SPR-BIA can be investigated and identified directly from the sensor chip surface using MALDI-TOF-MS [84]. The similar concepts led to the construction of chips with several chemical or biochemical surfaces to selectively purify proteins from complex mixtures instead of 2-DE before MS identification [82]. Now it is clear that both the speed of MS analysis and the sensitivity have not reached their limits yet and the introduction of such methods promises to develop proteomics in the years to come.

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