Proteomics and its impact upon biomedical science

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ABSTRACT

Proteomics is the protein equivalent of genomics and is the study of gene expression at a functional level. The proteome of an organism is the protein complement of its genome. However, unlike the genome, the proteome is dynamic: it varies according to the cell type and the functional state of the cell. In addition, the proteome shows characteristic perturbations in response to disease and external stimuli. Proteomics combines state of the art analytical methods with bioinformatics. Here, we review the concept and technology of proteomics with specific reference to applications in medical microbiology, cellular pathology, clinical chemistry, haematology/immunology, pharmacology and toxicology.

KEY WORDS: Bioinformatics.
Biomedical science.
Mass spectrometry.
Proteome.
Proteomics.
Two-dimensional electrophoresis.

Introduction

Proteomics is the protein equivalent of genomics and has captured the imagination of biomolecular scientists worldwide. This has resulted in the establishment of the Human Proteome Organisation (HUPO) in February 2001 to increase public awareness of the human proteome project and 'engender a broader understanding of the importance of proteomics and the opportunities it offers in the diagnosis, prognosis and therapy of disease' (http://www.hupo.org/). If proteomics fulfils its promise, it will have a major impact on biomedical science.

The proteome was first defined in 1995 as 'the entire PROTEin complement expressed by a genOME, or by a cell or tissue type[1,2]'. In unicellular organisms the proteome is the entire protein complement expressed by the genome.[1] In multicellular organisms the proteome is the summation of a number of subproteomes, each corresponding to an individual cell type.[3-5]

Proteomics is the study of proteomes and aims 'to examine the total protein complement encoded by a particular genome[4]' or, more specifically, 'seeks to identify and characterise the proteins present in a cell or tissue and define their patterns of expression[2]'. As individual cells express only a proportion of the genome, it is debatable whether or not proteomics can be applied effectively to multicellular systems in their entirety.[6]

In practice, the term proteomics has been more widely interpreted: for example, 'the large-scale study of proteins usually by biochemical methods[7]' and the determination of 'the salient properties of each protein (e.g. abundance, state of modification, involvement in multiprotein complexes, etc.)[8]'. Originally, proteomics could be interpreted as 'a surrogate name for the technology of two-dimensional electrophoresis (2-DE) and image analysis[2,9]'. However, proteomics is now perceived as the natural successor to genomics[10-14] and, as such, is more specifically defined as 'the use of quantitative protein-level measurements of gene expression to characterise biological processes (e.g. disease processes, drug effects) and decipher the mechanisms of gene expression control[15]'.

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Proteomics has triggered 'a renaissance in protein biochemistry[16]' and, as a consequence, its applications are wide-ranging. Distinctions already have been made between quantitative regulation proteomics,[15] which monitors changes in protein expression, and structural proteomics, which maps protein complexes.[17,18] The impact of proteomics can be gauged by the appearance in 2001 of the international journal *Proteomics* (http://www.wiley-vch.de/publish/en/journals/alphabeticIndex/2120/).

Historical note

The human proteome project and its advisory body HUPO are analogous to the human protein index (HPI) and its respective 'task force' established 20 years ago.[15] High-resolution 2-DE was introduced in 1975[19-21] and widely applied in biomedical science.[22,23] In response to this, the HPI was initiated to 'detect, characterise and catalogue all human proteins[24-29]'. As such, the aim of the HPI was similar to that of the current human proteome project.

Classical approach

Traditionally, proteins have been purified individually by the sequential use of a range of fractionation methods, each exploiting different physicochemical properties.[29] The fractionation methods (and separation parameters) include centrifugation (density), precipitation (solvation), ion-

exchange chromatography (electrostatic binding), gel filtration (size) and electrophoresis (charge). Subsequently, the purified protein is used to raise antibodies to develop immunoassays for quantitative measurement of the individual protein in biological fluids. This approach works when bulk amounts of starting material are available and the protein to be purified has a recognisable biological activity by which recovery can be monitored.

However, the classical approach is unsuitable for proteomics, in which the starting material tends to be scarce (particularly if human) and a high proportion of the protein complement is of unknown activity, thus making monitoring difficult. Furthermore, the number of proteins will exceed the separation capacity of the methods available[29] and the prospect of developing immunoassays for each protein is daunting.

Two-dimensional electrophoresis approach

High-resolution 2-DE[19-21] potentially can separate and simultaneously purify up to 10 000 polypeptides in a single analysis. Using only microgram amounts of sample, the resultant 2-DE map can indicate the relative amounts of each polypeptide.

Briefly, proteins are dissociated into their constituent polypeptides and separated by isoelectric focusing (IEF) – electrophoresis in a pH gradient – according to isoelectric point (pI; corresponding to a net charge of zero) in the first dimension. This is followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to relative molecular mass (Mr) in the second dimension (Figure 1).

The 2-DE patterns are visualised using ultrasensitive detection methods and analysed by computerised gel scanning.[2] The positions of the polypeptides are defined by their pI and Mr values, which can be used as spot coordinates on the resultant 2-DE map.[2] However, the aspect of 2-DE that defines proteomics is not the separation (which has changed little in ten years) but rather the microchemical methods used for protein identification.[30,31]

Proteomics is based upon these methods and its success is a measure of their development in recent years.

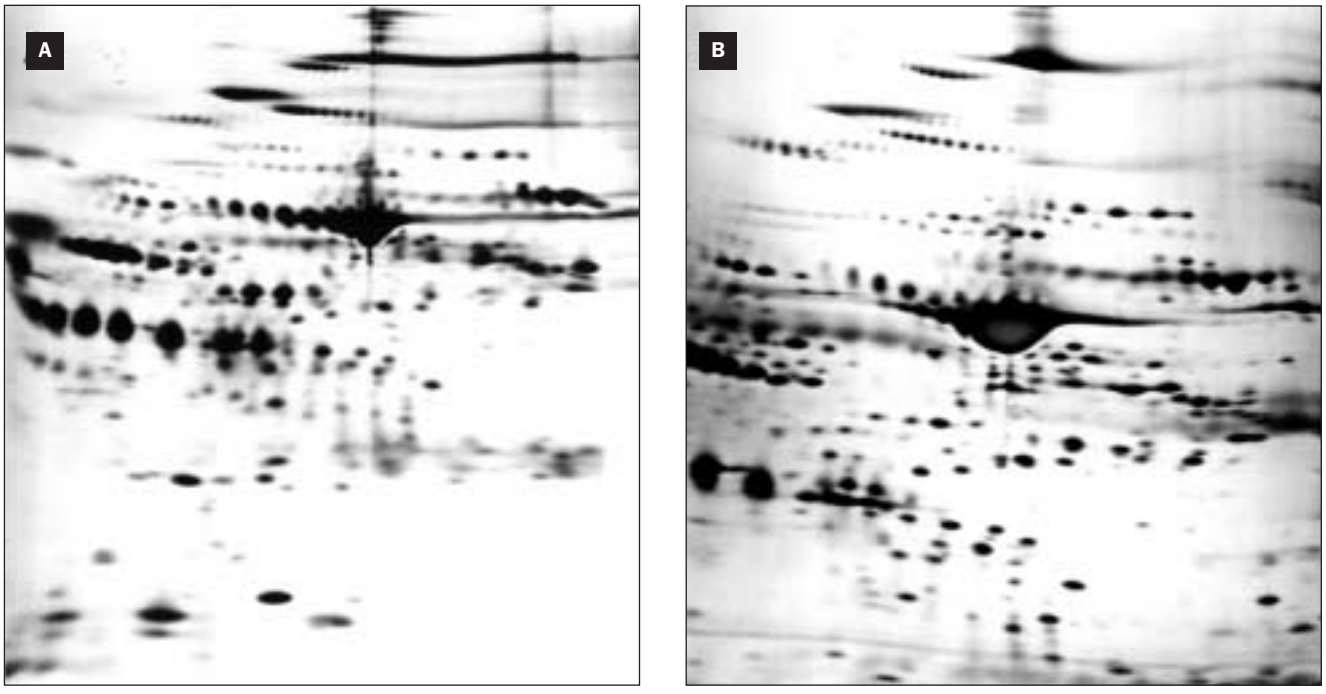
Protein identification

Polypeptides separated by 2-DE can be identified using amino acid analysis,[3,32-34] peptide-mass fingerprinting,[35-43] amino acid analysis/peptide-mass fingerprinting,[44] N-terminal sequence tag,[45] *N*-terminal Edman micro-sequencing,[46] internal peptide Edman microsequencing,[47-49] microsequencing by mass spectrometry (MS)[50] and ladder sequencing.[51]

Amino acid analysis involves acid hydrolysis of the polypeptide (transferred to blotting membrane) followed by derivatisation/chromatographic separation[32,52] and selective cross-matching of the amino acid composition with that of known proteins in databases.[3,33,34,53] The confidence of identification is improved by simultaneous cross- matching of both the amino acid composition and a 3-4 amino acid *N*-terminal 'sequence tag' determined by Edman degradation.[45,54]

Peptide-mass fingerprinting involves MS of the protease-cleaved polypeptide to generate peptide-mass profiles that are selectively cross-matched against profiles derived theoretically from known protein sequences or deduced from DNA sequences.[30,35-41] The MS involves electrospray

Fig. 1. Silver stained 2-DE patterns of human serum (0.2 μL) after isoelectric focusing (IEF, first dimension) with Ampholine, pH range 5 – 7 (A). The resolution of high- and intermediate-molecular-weight polypeptides was improved by prolonging electrophoresis (SDS-PAGE) in the second dimension (B). In this and subsequent 2-DE figures, the anode of the IEF gel is to the left and electrophoresis performed from top to bottom.



ionisation (ESI), MS or, more commonly, matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) MS, which is more sensitive and less prone to interference.[36,37,39,41]

In MALDI-TOF MS the polypeptide is co-crystallised in a matrix of weak aromatic acids on a sample probe and irradiated with a short-pulsed laser. Ionisation of the matrix results in energy transfer and the release of ionised polypeptides that are accelerated electrically (under vacuum) into a field-free flight tube.[41] Time of flight corresponds to the time interval from triggering to detection and is inversely related to peptide mass (i.e. the smallest arrives first).

In ESI-MS the sample is in solution (e.g. liquid chromatography [LC] effluent in LC-MS) and passes through a fine needle at high electric potential (5000 V) to generate a spray of highly charged droplets. These desolvate to eject the ions, which enter the inlet of a quadrupole scanning mass analyser.[41]

In tandem-MS, selected ions (following LC-ESI-MS) are fragmented by collision-induced dissociation and analysed further by MS. The resulting MS/MS spectra are then compared with predicted MS/MS spectra from protein sequence databases using cluster analysis algorithms for automated identification with high throughput.[55]

N-terminal sequencing is performed by determining the MS profiles of the residual truncated peptides (corresponding to sequential cycles of Edman degradation) and identifying the released amino acids from the mass differences of the ions.[41] The methodology includes 'post source decay' (PSD) MALDI-MS or ESI-MS/MS and the principle of ladder sequencing (or nested peptide sequencing) for analysis of isolated peptide fragments (internal sequencing).[51,56]

Alternative degradation reagents (to the Edman reagent phenylisothiocyanate) have been developed to permit detection at higher sensitivity.[41] In addition, sequentially truncated C-terminal peptide fragments (C-terminal sequencing) have been analysed by ESI-MS.[57-59] The use of MS for protein identification has been reviewed extensively, with the emphasis upon recent developments.[8,31,41-43,60-62] Briefly, the trend is towards automation, microchips and new MS configurations. These include MALDI quadrupole time of flight mass spectrometry (MALDI-Qq TOF MS),[63,64] tandem TOF[65,66] and surface-enhanced laser desorption ionisation (SELDI)-TOF MS, which exploits affinity capture of selective proteins by using MS probes with derivatised protein chip arrays.[62,67]

Fig. 2. Home page (http://www.expasy.org/) of the Expert Protein Analysis System (ExPASy) Molecular Biology Server of the Swiss Institute of Bioinformatics. This proteomics server is dedicated to protein analysis and provides hypertext links to protein databases, tools/software packages and major molecular biology servers. Reproduced with permission. Copyright: Swiss Institute of Bioinformatics, Geneva, Switzerland.

Fig. 3. SWISS-2DPAGE reference map of cerebrospinal fluid (http://www.expasy.org/cgi-bin/map2/def?CSF_HUMAN). This annotated database includes different formats of the reference map. The highlighted spots (+) can be clicked to reveal detailed information on selected proteins. Reproduced with permission. Copyright: Swiss Institute of Bioinformatics, Geneva, Switzerland.

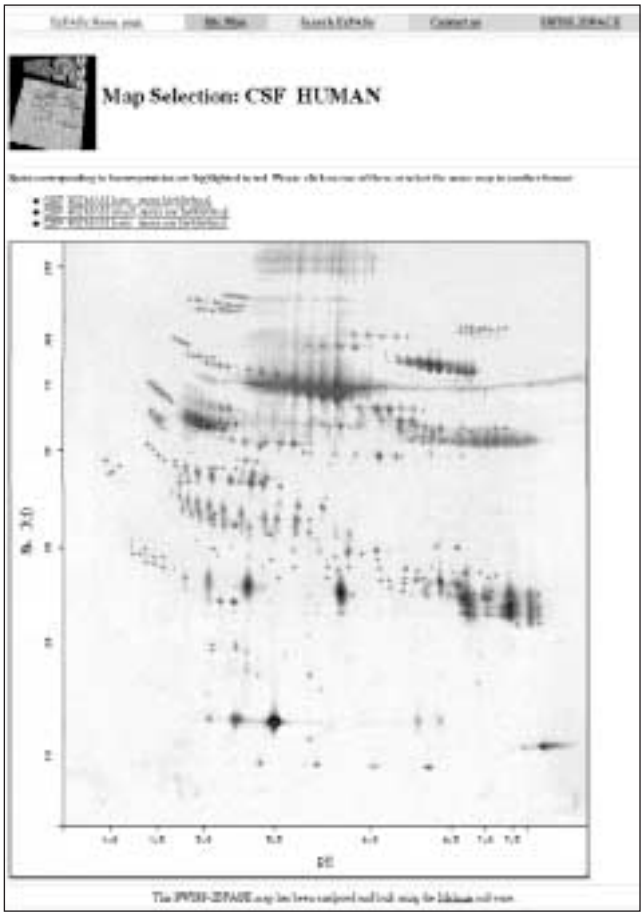


Table 1. Software tools for protein identification

Software	Key features	URL	Ref.
PepSea	Protein identification by MS based on peptide fragments and sequence tags	http://195.41.108.38/PepSeaIntro.html	38,45
MOWSE	Molecular weight search peptide mass database	http://srs.hgmp.mrc.ac.uk/cgi-bin/mowse	36
SEQUEST	Matches the tandem mass spectra of fragmented covalently modified peptides	http://fields.scripps.edu/sequest/	55
MS-Fit MS-Tag	Peptide mass and sequence tags with access to a range of tools for 'mining' sequence databases	http://prospector.ucsf.edu	90,91
PepFrag	Combines different types of mass spectrometric information	http://www.proteometric.com	92
TagIdent PepIdent MultiIdent	Multiple protein parameters for cross-species identification	http://www.expasy.ch/tools/	93-96
Mascot	Probability-based scoring algorithm combining mass and sequence data	http://www.matrixscience.com	97
ProFound	An expert system using a Bayesian algorithm to rank proteins according to multiple parameters including information relevant to peptide mapping experiment	http://www.proteometric.com	98

Protein quantification

The 2-DE polypeptide patterns are visualised by protein stains that include silver, Coomassie blue R-250, colloidal gold, zinc imidazole, ponceau S, amido black, India Ink and Stains-all.[2] Proteins display a variable and non-linear response. Consequently, staining is semi-quantitative and does not indicate absolute protein amounts.

Silver staining is used commonly for 2-DE reference maps but is particularly problematic due to poor reproducibility and variations in the kinetics of silver deposition.[68,69] Fluorescent stains based on SYPRO dyes (and the formation of luminescent ruthenium complexes) combine ultrasensitive detection of protein with improved performance characteristics and are fully compatible with protein microchemical techniques.[70-72]

More accurate methods of determining relative protein amount include radiolabelling. The proteins are labelled prior to 2-DE and the polypeptides quantitated by liquid scintillation counting or autoradiography.[73-75] More recently, the principle of stable isotope dilution has been exploited[76] using whole-cell stable isotope labelling,[77] isotope-coded affinity tags (ICAT)[78] or isotopic *N*-terminal labelling.[79] The protein samples to be compared are synthesised[77] or tagged[78,79] so that one contains a heavy isotope and the other a light one.[76] The two samples are then mixed, fractionated (by electrophoresis or affinity isolation), digested with protease and then analysed by MS. The relative abundance of the original proteins in the two samples is indicated by the ratios of the lower and upper mass components of the analyte pairs.[76]

Protein bioinformatics

Proteomics exploits bioinformatics for the manipulation of the 2-DE images, identification of the proteins and the construction of interactive databases accessible on the internet. The **E**xpert **P**rotein **A**nalysis **S**ystem (ExPASy) is an excellent example of a proteomics server (Figure 2) and provides links to many software tools (http://www.expasy.org/)[80,81].

The 2-DE patterns (visualised by staining) are digitised using a scanner (or camera) and the images manipulated with software analysis packages such as TYCHO,[82] ELSIE,[83] GELLAB,[84,85] QUEST[86] and MELANIE.[87,88] The software removes streaking, adjusts background, enhances spot detection, indicates relative amounts and facilitates cross-matching (using 'landmark' spots) to generate the reference maps and associated databases available on the internet. Third-generation 2-DE software packages are user friendly, run on low-cost general-purpose personal computers and facilitate state-of-the-art image comparisons with statistical analysis.[87,88]

Software tools for protein identification are based upon peptide mass spectra (e.g. MOWSE), peptide fragmentation mass spectra (e.g. SEQUEST) and sequence tags (e.g. TagIdent). These are available on the internet[89-98] and itemised in Table 1. Identification based upon cross-referencing amino acid composition, protein sequence and mass profile is indicated by a ranked list of candidate proteins. Consequently, there is a risk of false-positive identification and automated high-throughput analysis incorporates quality control[89] and simulation-based significance testing[99] to evaluate the search result.

Proteomic databases incorporate annotated 2-DE reference maps with interactive displays of information relating to the identity, structure/function and characterisation of individual polypeptide spots. SWISS-2DPAGE[100] is an excellent example of a 2-DE database (http://www.expasy.org/ch2d/). It includes protein maps of *Escherichia coli*, yeast, slime mould, *Arabidopsis thaliana*, mouse tissues (liver, muscle, pancreas and adipose tissue) and human tissues, body fluids and cell lines (e.g. blood plasma/cells, cerebrospinal fluid [CSF], liver, kidney, leukaemia cells and colorectal cells).

The site is updated regularly, contains comprehensive data on a wide range of 2-DE reference maps (e.g. CSF; Figure 3) and provides a gateway for direct access to protein identification tools (via ExPASy) and federated 2-DPAGE databases (via WORLD-2DPAGE). The latter includes a wide

Table 2. Human 2-DE databases

Website	Key features	URL	Ref.
SWISS-2DPAGE	Plasma, CSF, liver, kidney, blood cells and a range of other cells/cell lines with direct access to WORLD-2DPAGE databases and services.	http://www.expasy.org/ch2d/	100
HEART-2DPAGE	Ventricle, atrium in dilated cardiomyopathy	http://userpage.chemie.fu-berlin.de/~pleiss/	101
HSC-2DPAGE	Ventricle with Flicker comparison facility	http://www.harefield.nthames.nhs.uk/nhli/protein/index.html	102
MDC 2-DE database	Ventricle resolved by high performance large format gels	http://www.mdc-berlin.de/~emu/heart/	103
Danish 2-D PAGE database	Bladder cancer (including urine), keratinocytes and fibroblasts with human 2-DE gel gallery	http://biobase.dk/cgi-bin/celis/	104
SIENA-2DPAGE	Breast carcinoma and amniotic fluid	http://www.bio-mol.unisi.it/2d/2d.html	105,106
PMMA-2DPAGE	Colorectal carcinoma	http://www.pmma.pmfhk.cz/	107
JPSL proteomic databases	Breast carcinoma cell line	http://www.ludwig.edu.au/jpsl/jpslhome.html	108
BPP 2-DE database	Haematopoietic and lymphoid cell lines	http://www-smbh.univ-paris13.fr/lbtp/Biochemistry/biochimie/bque.htm	109
TMIG-2DPAGE	Fibroblasts in studies on ageing	http://proteome.tmig.or.jp/2D/header.html	110
BALF 2D database	Bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis	http://www.umh.ac.be/~biochim/BALF2D.html	111
Inner ear protein database	Perilymph	http://oto.wustl.edu/thc/innerear2d.htm	112
Mitochondrial proteome	Isolated mitochondria	http://www-dsv.cea.fr/thema/MitoPick/Mito2D.html	113

range of human 2-DE databases,[100-113] a selection of which are itemised in Table 2.

Biomedical applications

The present era has been described as the 'decade of proteomics[18]', and a billion dollar business is anticipated.[14] Much of this investment will be in diagnostics and drug development, with funding from biotechnology and pharmaceutical companies. Consequently, proteomics is likely to have a major impact on biomedical science and the following sections review its application in medical microbiology, cellular pathology, clinical chemistry, haematology/immunology, pharmacology and toxicology.

Medical microbiology

Proteomics has been described as functional genomics and, as such, is particularly well suited to the study of microorganisms because a large number of microbial whole genome sequences are now available (The Institute for Genomic Research [TIGR], URL: http://tigr.org/dtb/). Microbial 2-DE databases of medical significance have been established[100,114-118] (Table 3) but reference maps based upon well-characterised laboratory strains are of limited value in studies of clinical isolates.[119] The 2-DE patterns vary according to the laboratory 'type', the growth phase and the choice of growth conditions.[119,120]

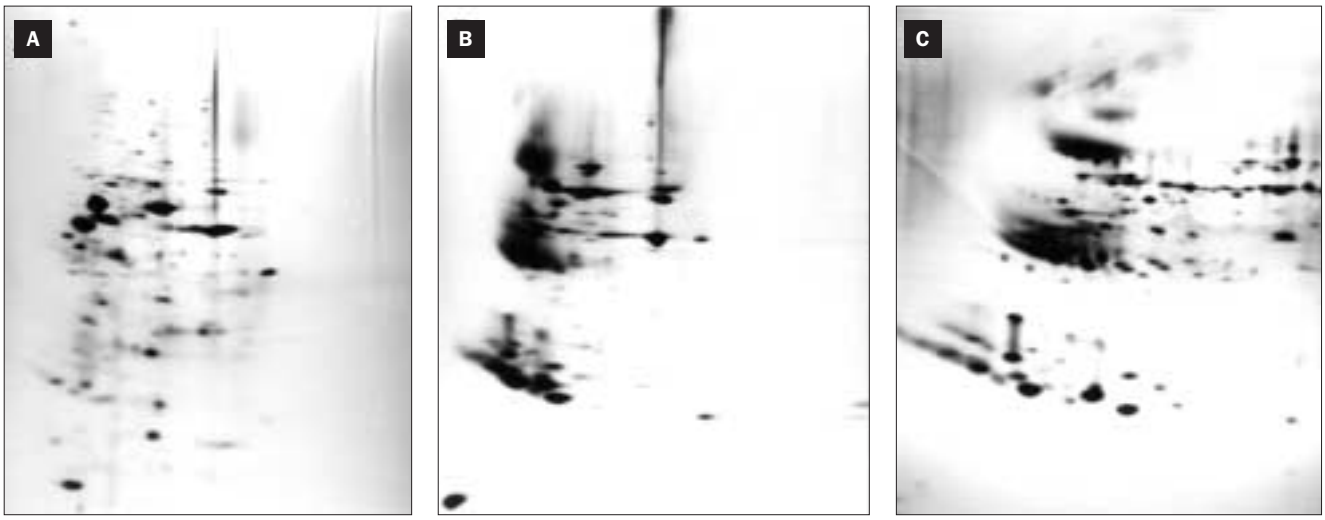
A good example of the application of proteomics has been the detection and annotation of one-third of *Haemophilus influenzae* genome within a three-year period.[121,122] Such studies indicate that microbial proteomes are unlikely to be viewed on a single 2-DE gel and the completion of microbial reference maps will require recourse to subcellular fractionation and the use of overlapping narrow-range ('zoom') immobilised pH gradients.[121,122] The need for narrow-range pH gradients was evident from early 2-DE comparisons of the protein constituents of inner and outer bacterial membranes[123] (Figure 4). Bacterial outer membranes are of particular biomedical significance as they interface the organism/host, define pathogenicity and contribute to antibiotic resistance.[120]

Proteomics has been applied in medical microbiology to investigate taxonomy, identify virulence factors, evaluate the host response and to study drug resistance.[119,120] Early applications of 2-DE to *Neisseria gonorrhoeae*,[124] *Mycoplasma capricolum*,[125] *M. pulmonis*[126] and *Campylobacter pylori*[127] demonstrated a high level of discrimination, consistent with taxonomic classification.[124,125,127] Such studies included subcellular fractionation for analysis of outer-membrane proteins and flagellum-associated antigens.[127] More recently, proteomics has been applied to isolates of *H. influenzae*,[116,128] *Listeria* spp.[129] and *Helicobacter pylori*,[130,131] with emphasis upon computerised gel analysis[129] and 2-DE databases[116,131] (Table 3).

Virulent and avirulent strains have been compared to detect virulence factors, in order to develop more effective vaccines. Early 2-DE studies involved *M. pneumoniae*[132] and *Brucella abortus*.[133] More recently, however, proteomics has been used to compare virulent tuberculosis strains with BCG vaccine strains of *M. bovis*.[115,134-136] This work incorporated the use of narrow pH ranges[135] and resulted in the detection of 1800 spots[136] and the establishment of a mycobacterial proteome database[115] (Table 3).

Using 2-DE, the expression of an additional 10 proteins in

Fig. 4. Silver stained 2-DE patterns of the inner (A) and outer (B,C) membrane fractions of *Methylobacterium organophilum* (2.5 μg protein) after isoelectric focusing (first dimension) with Ampholine, pH range 3 – 10 (A,B). The resolution was improved by use of a narrow Ampholine range (pH 4 – 6) for IEF in the first dimension (C).



BCG vaccine was indicated relative to virulent *M. bovis* and *M. tuberculosis*.[134] Subtractive genomic hybridisation then was used to locate genetic differences between the three. The introduction of a virulence-associated genome segment (RD1) into the BCG genome resulted in expression of a virulent-type 2-DE *M. bovis* profile, suggesting that RD1 suppresses protein synthesis in virulent mycobacteria.[134]

Proteome analysis of culture filtrate, cell wall and cytosol has been used recently to establish a second proteome database for *M. tuberculosis*[117] (Table 3). Model systems involving *in vitro* co-cultivation of bacteria and eukaryotic cells[137] provide a step towards the identification of specific virulence determinants expressed only *in vivo*.[119]

Viral infection (and transformation) has been investigated by 2-DE of tissue culture cell lines[138-142] and proteomics used to characterise ribosomal basic proteins associated with herpes simplex virus type 1 infection.[142]

Proteomics has also been used to study the host response to infection and identify new bacterial antigens for vaccine development. The cellular and/or outer-membrane proteins of *H. pylori*,[143-145] *Streptococcus pyogenes*,[146] *Borrelia burgdorferi*[147] and *Toxoplasma gondii*[148] have been separated by 2-DE and immunoblotted with sera from infected patients to characterise the antibody profile and locate the bacterial antigens. In similar studies, proteomics has been applied to culture filtrates to investigate the immunogenicity of *M. tuberculosis*.[149-151]

Drug resistance in microorganisms has been investigated by comparing the protein expression patterns of drug-sensitive and drug-resistant strains.[152-155] Imipenem-resistant *Pseudomonas aeruginosa* was characterised by reduced amounts of an outer- membrane protein;[152] rifampin-resistant *Neisseria meningitidis* showed an acidic shift in the pI of a protein Mr 18 900;[119,153] while erythromycin-resistant *S. pneumoniae* (M phenotype) showed increased expression of a basic isoform of glyceraldehyde-3-phosphate dehydrogenase.[154] These changes may be linked to membrane-associated reduced permeability.[152,153] 2-DE of *Candida glabrata* indicated that resistance to azole antifungal agents may be associated with chromosome duplication, increased expression of 25 proteins and down-regulation of a further 76.[155]

Cellular pathology

Eukaryotic cells present a major challenge to proteomics and this is of particular significance to the human proteome project. The task of integrating the protein complement and expression of 252 different human cell types[11] at all levels of structural organisation and development, both in health and disease, is daunting! To date, human proteomics has been limited to specific applications in molecular anatomy and pathological investigations of cancer or heart disease using biopsies and tissue culture cell lines.

Molecular anatomy is the structural organisation of cells at a molecular level in health and disease, and is a concept that is ongoing. A human molecular anatomy (MAN) programme was conceived in 1960,[11] an HPI taskforce was established in 1980,[11] and now the HUPO has been set up to promote the human proteome project (http://www.hupo.org/).

The human proteome is complex and best considered as the summation of the subproteomes corresponding to all individual cell types.[5] The problem is compounded, however, by the fact that each subproteome comprises fractions corresponding to the subcellular organelles,[156] which contain subfractions corresponding to multiprotein complexes.[18]

2-DE databases of isolated human mitochondria have been established (Table 2 and http://www.mips.biochem.mpg.de/proj/medgen/mitop/)[113,157] and these incorporate search facilities that include a 'human disease catalogue' specifying 110 human diseases associated with mitochondrial protein abnormalities.[157] A 2-DE database of nuclei isolated from human liver cells has been established[158] and can be accessed via the ExPASy server (Table 2). Likewise, the nuclei of Burkitt's lymphoma (BL60) cells have been studied to identify apoptosis-associated proteins.[159]

Application of proteomics has shown similarities between the nuclear matrix proteins of nuclei isolated from human lymphocytes, cultured amniotic cells and liver tissue cells,[160] but differences (including filament-forming components)

Table 3. Microbial 2-DE databases of medical significance

Website	Organisms	URL	Ref.
SWISS-2DPAGE	*Escherichia coli*	http://www.expasy.ch/ch2d/	100
SIENA-2DPAGE	*Chlamydia trachomatis*	http://www.bio-mol.unisi.it/2d/2d.html	114
Max Planck 2-DE Database	*Borrelia garinii, Helicobacter pylori, Mycobacterium bovis, Mycobacterium tuberculosis*	http://www.mpiib-berlin.mpg.de/2D-PAGE/	115
2-D PAGE Aberdeen	*Haemophilus influenzae*	http://www.abdn.ac.uk/~mmb023/2dhome.htm	116
SSI-2DPAGE	*Mycobacterium tuberculosis*	http://www.ssi.dk/en/forskning/tbimmun/tbhjemme.htm	117
Toxoplasma 2D database	*Toxoplasma gondii*	http://www-public.rz.uni-duesseldorf.de/~hfischer/	118

between those of isolated subtypes of human haematopoietic cells and cultured leukaemia cells.[161,162]

Lysosomes isolated from human placenta have been studied to compare the luminal and membrane proteins and to establish a 2-DE map[163] (http://www.health.adelaide.edu.au/2Ddatabase/front2.htm). In novel applications, the spliceosome complex of a human HeLa cell line has been completely characterised (50% of the proteins being identified via protein sequence databases and the remainder via sequence tag databases)[164] and sucrose density gradient centrifugation used as 'a third dimension' prior to 2-DE.[165]

Such studies are of fundamental importance in elucidating the human proteome but subcellular fractionation and the purification of individual organelles are notoriously problematic.[166] In addition, 2-DE fails to detect structurally important high-molecular- weight proteins (Mr >200 000)[4] and the isolated organelle approach does not reveal the subcellular redistribution of proteins (e.g. kinases) that is characteristic of the physiological response to stimuli and the activation of regulatory pathways.[4]

A complementary approach is differential detergent fractionation, which partitions cellular proteins into cytosol, membrane/organelle, nuclear and cytoskeletal fractions prior to electrophoretic analysis.[4]

Pathological investigations of cancer have involved 2-DE comparison of benign and malignant tumours to improve screening (i.e. tumour classification) and to identify specific protein markers for early clinical diagnosis.[167,168] Squamous cell carcinoma (SCC) of the bladder has been studied widely[168,169] and a 2-DE database established[104,170] (Table 2). Analysis of the bladder urothelium (following cystectomy) was used to investigate progressive differentiation from the early stages of metaplasia to premalignant lesions and invasive disease.[171] This revealed three types of non-keratinising metaplastic lesion that showed abnormal protein expression (when compared to normal urothelium) and these could be distinguished by immunowalking (i.e. immunostaining of serial cryostat sections).[171]

Related studies have involved detection of an SCC-associated calcium-binding protein, psoriasin (Mr 11 000; pI 6.2), as a potential biomarker,[172,173] and comparison of superficial transitional cell carcinomas with their primary cultures.[174]

In addition, proteomics has been used to grade breast tumours[175,176] in order to investigate down-regulation of high-molecular-weight tropomyosin isoforms,[175] and to demonstrate a highly heterogeneous pattern of gene expression in malignant human breast carcinoma.[176] 2-DE databases of human breast carcinoma and related cell lines can be accessed on the internet[177] (Table 2 and http://www.anl.gov/CMB/PMG/projects/index_hbreast.html).

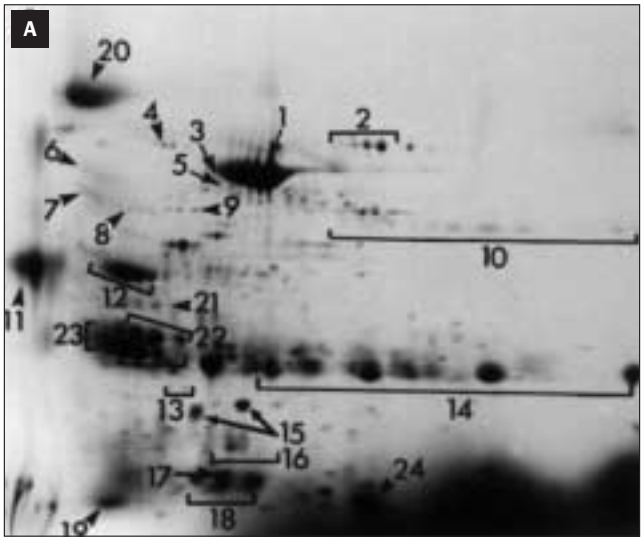
2-DE has also been used to compare benign and malignant tumours of ovarian[178,179] or prostatic origin.[180,181] These studies indicate that, in common with breast cancer, malignancy is associated with increased expression of proliferating-cell nuclear antigen (PCNA) and stress proteins (HSP90, pHSP60 and calreticulin). There is also a malignancy-associated down-regulation of high-molecular-weight tropomyosin isoforms.[178-181]

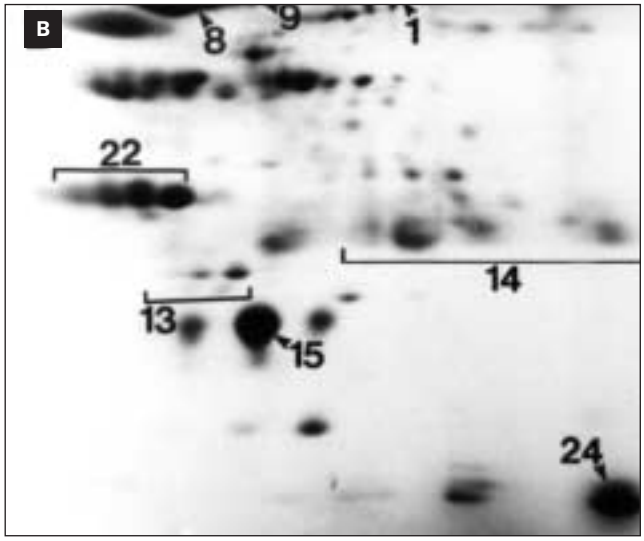
Proteins associated with individual cancers include calgranulin B (Mr 13 000; pI 5.6) in colorectal cancer[182] and TAO1/TAO2 (Mr 35 000; similar pI) in lung adenocarcinoma.[183,184] The latter may prove valuable for histocytological differentiation, with the potential to distinguish primary lung malignancies from distant metastases.[183,184] Studies of renal cell carcinoma indicated a malignancy-associated absence of ubiquinol cytochrome reductase (UQCR) and mitochondrial NADH-ubiquinone oxidoreductase complex I.[185] 2-DE of ovarian tumours followed by multivariate analysis has indicated that this approach has the potential to classify tumours using artificial intelligence.[179]

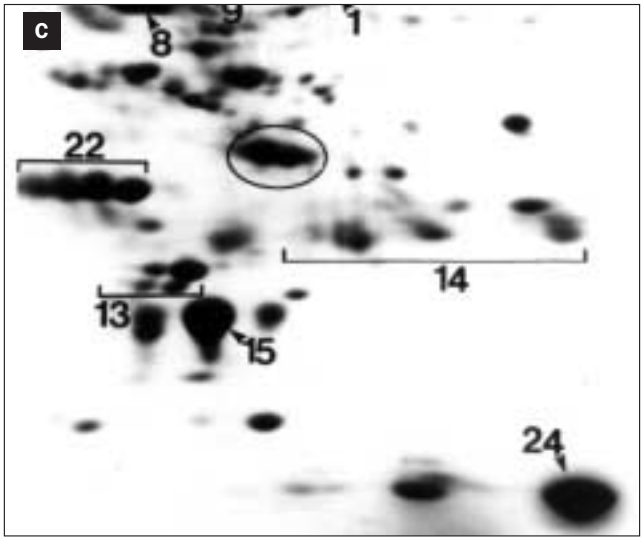
Pathological investigations of heart disease have involved 2-DE comparison of healthy and diseased myocardium. Early studies of normal and infarcted myocardium indicated proteins (including myosin light chain) that were depleted after acute myocardial infarction (AMI).[186] More recently, dilated cardiomyopathy (DCM) has been studied extensively[187,188] to establish the HEART 2DPAGE[101] and HSC-2DPAGE[102] databases (Table 2). Comparison of DCM biopsies with both donor heart samples and explanted hearts from patients with ischaemic heart disease (IHD) has revealed prominent changes in myosin light chain 2 and desmin,[188] heat shock proteins hsp60[189] and hsp27,[190] and the apoptosis-associated Bcl-2 family of proteins[191] – findings which indicate a future potential for proteomics in the study of heart disease.[192]

Biopsies are an important source of material for development of the human proteome project but the protein complement is a composite of the subproteomes of the constituent cell types and will be contaminated with plasma proteins. An exciting development, however, is the analysis

Fig. 5. CBB-stained 2-DE patterns of pooled human urine (n = 9) corresponding to normal healthy individuals (A, 125 μg protein) and patients with chronic renal failure (B, 160 μg protein) or end-stage renal failure (C, 160 μg protein). With B and C the central gel area of interest is enlarged to enhance clarity. The identified proteins are: 1, albumin; 2, transferrin; 3, hemopexin; 4, α_1-ß-glycoprotein; 5, Ig α chains; 6, α_1-antichymotrypsin; 7, α_2-HS-glycoprotein (fetuin); 8, α_1-antitrypsin; 9, GC-globulin; 10, Ig γ chains; 11, α_1-acid glycoprotein (orosomucoid); 12, haptoglobin ß chain; 13, apolipoprotein A-I; 14, Ig λ, κ light chains; 15, retino-binding protein; 16, haptoglobin α_2 chain; 17, prealbumin; 18, haptoglobin α^{IF} and α^{IS} chains; 19, apolipoprotein A-II; 20, Tamm-Horsfall mucoprotein (uromodulin); 21, Zn-α_2-glycoprotein; 22, α_1-microglobulin; 23, human chorionic gonadotrophin (hCG) chain; and 24, ß$_2$-microglobulin. Note: renal failure is associated with a progressive increase in the proportion of apolipoprotein A-I (including abnormal isoforms), retinol binding protein, α_1-microglobulin, ß$_2$-microglobulin and an unidentified cluster of spots (circled, C).







of individual cell types by laser capture microdissection (LCM),[193] which allows cells visualised in a tissue section to be bound selectively to a thermoplastic film overlay following activation of the film by a laser directed at the cell.[193]

The technique's potential has been investigated by proteomic analysis of renal and cervical tissue,[194] and in a comparative study of normal squamous epithelium with tumour cells in oesophageal cancer, 2-DE of 50 000 microdissected cells revealed 675 proteins which showed tumour-specific changes and proteins present uniquely in either the normal or abnormal material.[195] This new LCM technology (for laboratory protocols see http://cgap-mf.nih.gov/) has a unique ability to detect tumour-specific marker proteins[195,196] and promises to be a key feature of the proteomic analysis of tissues.[197,198]

Clinical chemistry

2-DE has been applied widely to human body fluids to detect protein markers of disease.[22,23,199] Proteomic databases have been established for blood plasma, CSF, urine and amniotic fluid[100,104,105] (Table 2). However, it is unlikely that the protein complement of a body fluid constitutes a proteome, in that it represents a varying proportion of incomplete subproteomes (corresponding to different cell types) modified by ageing effects, turnover and clearance mechanisms. Body fluids show wide biological variation and it is difficult to define a 2-DE reference map – of urine, for example – that is representative of healthy individuals.

Early clinical applications of 2-DE have been reviewed extensively.[199] Our own work includes the demonstration in human serum of subnormal amounts of mature apolipoprotein A-I isoforms in familial dyslipoproteinaemia,[200] heterogeneity of paraproteins in myelomatosis,[201] and time-course changes associated with either abstinence following alcohol abuse[202] or AMI.[186]

Alcohol abuse induced an abnormal heterogeneity of α_1-acid glycoprotein and α^1-antitrypsin and enhancement of an unidentified string of spots (Mr 30 000; pI 4.4-4.8).[202] AMI resulted in the appearance (and normalisation over a five-day period) of abnormal polypeptides (Mr 13 000; pI 6.2-7.5) tentatively identified as apo-serum amyloid A protein (an acute phase reactant) and myosin light chain (Mr 27 000; pI 5.2).[186]

More recently, proteomics has been applied to perinatal human plasma to characterise an acute-phase time-course

Fig. 6. CBB-stained 2-DE pattern of urine (30 μg protein) indicating light chain heterogeneity in Bence-Jones proteinuria. Arrowheads indicate polypeptide spots corresponding to free immunoglobulin light chain (L) and light chain fragments (F) as identified by immunoblotting. The pattern of light chain heterogeneity (A) varies from patient to patient and can be further characterised by recourse to narrow pH range 'zoom' gels (B).

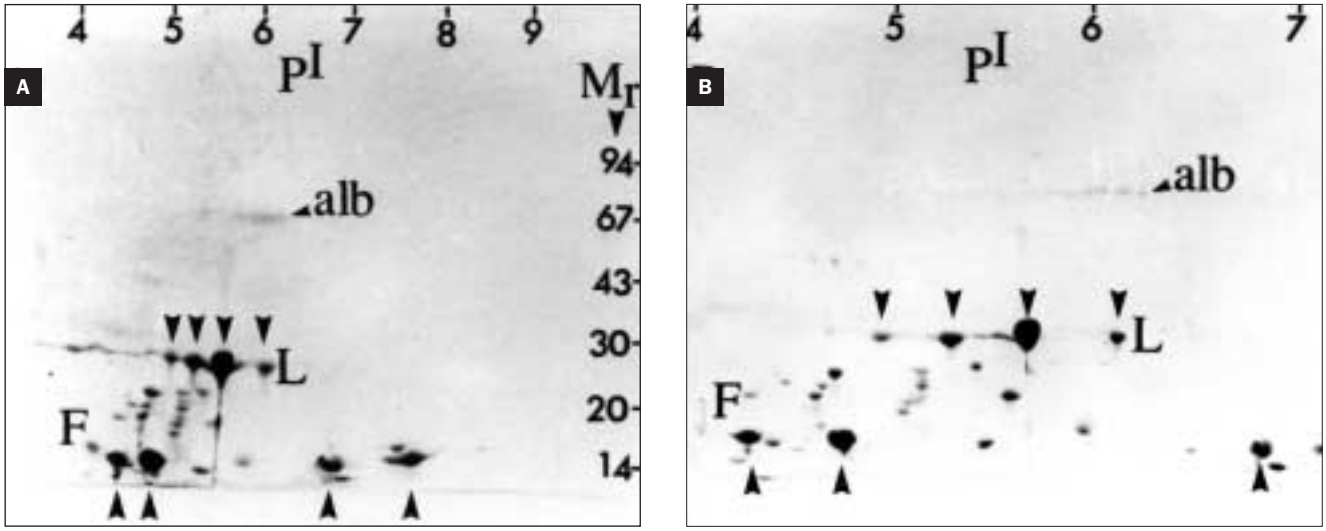
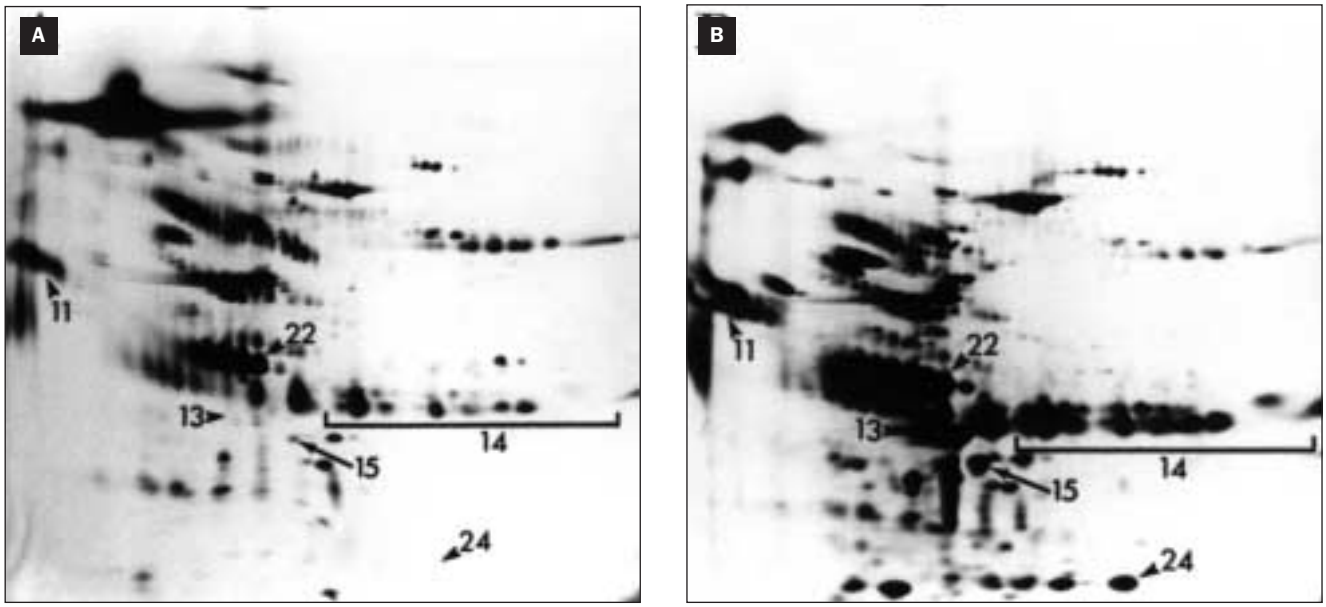


Fig. 7. Silver stained 2-DE patterns of 75 μL unconcentrated pooled urine (n = 10) from controls (A) and workers occupationally exposed to cadmium for 5 – 24 years (B). Nephrotoxicity associated with tubular damage is characterised by an increase in the amount of a range of low molecular weight proteins including 11, α_1-acid glycoprotein (orosomucoid); 13, apolipoprotein A-I; 14, Ig λ, κ light chains; 15, retino-binding protein; 22, α_1-microglobulin; and 24, β_2-microglobulin.



response of serum amyloid A protein and the haptoglobins in early-onset (<72 h) sepsis.[203] Comparison of the serum protein profiles of prostatic cancer patients with those of benign prostate hyperplasia indicates a greater proportion of protease inhibitor-free prostate-specific antigen, a potential early marker for prostatic cancer.[204] Proteomics also has revealed that fibrinogen δ-chain dimer (cross-linked by transglutaminase) is present in the blood plasma of tumour patients, suggesting an association between cancer and transglutaminase activity and a possible correlation between plasma levels of the dimer and tumour-associated fibrin deposition.[205]

Human CSF has been studied extensively by 2-DE and is well represented on the SWISS-2DPAGE database (Table 2, Figure. 3). Abnormal proteins have been detected in various neurological diseases, including schizophrenia[206] and Creutzfeldt-Jacob disease (CJD).[207,208] However, the clinical specificity of these changes is doubtful as the schizophrenia-associated proteins were detected in patients with herpes simplex encephalitis, CJD, multiple sclerosis and Parkinson's

disease,[206] and the two CJD-associated proteins (Mr 26 000; pI 5.2 and Mr 29 000; pI 5.1), were detected in 50% of patients with herpes simplex encephalitis.[207]

The CJD-associated proteins (denoted 130/131) are members of the 14-3-3 family of proteins and have been exploited to develop a highly sensitive and specific immunoblotting assay for CJD.[208] The test was strongly recommended for the diagnosis of CJD[209],[210] as it showed a positive predictive value of 95% (and a negative predictive value of 92%)[209] and was favourably assessed relative to other protein markers when applied to CSF samples from suspected CJD cases.[210] However, the validity of the 14-3-3 test has been questioned recently[211,212] as it failed to discriminate between CJD and non-CJD when false-positive results were obtained with various degenerative and secondary dementias unrelated to prion disease.[211] Furthermore, 14-3-3 was only detected in 22 of 45 patients with variant CJD.[212]

Nevertheless, CSF is known to contain many disease-specific proteins[213] (including tau, soluble amyloid protein precursors, apolipoprotein E, acetylcholinesterase, neuron-specific enolase and S-100 protein associated with Alzheimer's disease, Parkinson's disease and depression) and proteomics is likely to play a major role in the future investigation of neuropsychiatric disorders.[213,214]

Human urine has been analysed extensively by 2-DE[215] (Figure 5). Proteomics has been used to monitor and characterise psoriasin (S100A7; Mr 11 000; pI 6.2), a calcium-binding protein expressed by bladder SCCs, which is detected in urine and provides a potential biomarker for non-invasive follow-up of patients.[168,169,172,173] Recently, SELDI ProteinChip array-time of flight MS has been used as an alternative to 2-DE for proteomic analysis of urine to detect and characterise proteins (including β2-microglobulin) as biomarkers of impending nephropathy.[216]

Surprisingly, proteomics has not been applied to characterise the nephrotoxic effects of free immunoglobulin light chain (LC) in Bence-Jones proteinuria (BJP).[217] Nephrotoxicity is currently unpredictable; however, proteomics could be exploited to correlate the physicochemical characteristics of individual LCs (Figure 6) with prognostic data to provide a predictive index.

Haematology and immunology

2-DE has been used to map the proteins of erythrocytes, leucocytes and platelets, and to detect the protein changes in lymphocytes, lymphoblasts and myeloblasts associated with Huntingdon's disease, infectious mononucleosis, acute myeloid leukaemia (AML), acute lymphoid leukaemia (ALL) and chronic lymphocytic leukaemia.[22,23,68] A similar approach has been used to study differentiation of T-cell clones and to study dysfibrinogenaemia and the heterogeneity of paraproteins in myelomatosis and Waldenström's macroglobulinaemia.[22,23,201] Proteomics has been used to establish 2-DE databases for blood cells,[100] haematopoietic and lymphoid cell lines[109] (Table 2) and Jurkat T-cells (http://www.mpiib-berlin.mpg.de/2D-PAGE/)[218] and to catalogue tyrosine-phosphorylated human platelet proteins.[219]

Pharmacology

Proteomics is important in preclinical drug development.[220] Proteins are the functional units of the cell and the prime targets of most drugs.[15] Proteomics involves global analysis of the cellular response to drugs and is ideal for testing the efficacy of novel compounds and detecting possible side effects.[15,221] A potential drug should reverse disease-associated changes in protein expression and/or induce changes consistent with a desired mode of action. Proteomics offers an holistic approach to pharmacological investigation[222] and should rapidly promote drug development while greatly improving our understanding of the molecular basis of drug action.[220,221]

A 2-DE database of rat liver proteins has been established[223] and proteomics used to investigate the effects of the cholesterol lowering drugs lovastatin[224] and fluvastatin.[225] The affected proteins reflected changes in cholesterol biosynthesis and carbohydrate metabolism but also induced a stress response indicative of toxicity.[224,225] In similar studies, oltipraz (a cancer chemoprevention agent) was shown to induce a detoxifying enzyme (aflatoxin B_1 aldehyde reductase),[226] and etomoxir (a potential antidiabetic agent) induced adipose differentiation-related protein to levels which correlated with the degree of liver steatosis.[227] SDZ PGU 693 (a hypoglycaemic agent) caused mitochondrial protein changes consistent with down-regulation of fatty acid metabolism.[228]

Proteomics has been used to investigate the effects of peroxisome proliferators (PPs) on mouse liver[229-231] to identify a protein (correlating with peroxisomal β-oxidation) for potency testing,[229] and to demonstrate decreased abundance of selenium-binding protein 2, a cell growth regulation factor.[231] It has also been used to investigate acetaminophen (APAP, paracetamol)-induced hepatotoxicity by comparison of the effects of APAP and 3-acetamidophenol (AMAP, a non-toxic regioisomer) on the 2-DE patterns of mouse liver proteins.[232]

Such studies have resulted in the compilation of a comparative drug effects database (currently incorporating 51 pharmaceutical agents) used to investigate the correlation between chemical structure and reactivity, and identify sets of proteins co-regulated (or anti-co-regulated) in response to such agents.[15]

In a comprehensive study co-ordinated by the US National Cancer Institute, the 2-DE protein expression patterns of 60 different cancer cell lines were screened against 3989 compounds to establish a database for the molecular pharmacology of cancer (http://discover.nci.nih.gov/).[233]

More recently, the anticancer activity of bohemine (an olomoucine-derived synthetic cyclin-dependent kinase inhibitor) has been studied using a lymphoblastic leukaemia cell line[234]. Five down-regulated proteins (α-enolase, triosephosphate isomerase, eukaryotic initiation factor 5A and α and β subunits of Rho GDP-dissociation inhibitor 1) were identified, suggesting a mode of action related to inhibition of protein synthesis (and/or glycolysis) rather than inhibition of cyclin-dependent kinases.[234] The response of pancreatic carcinoma cells to the cytotoxic agent daunorubicin has been investigated to demonstrate a dose-dependent up-regulation of proteins.[235]

Other pharmacological studies incorporating proteomics have been directed more specifically towards the chemical characterisation of drug-related protein adducts[236] and post-translational modifications[237] or the efficacy of drug delivery using model carriers *in vitro*.[238,239]

Toxicology

2-DE and proteomics have been used to study the toxic effects of chemical pollutants in order to investigate occupational or environmental exposure. Early 2-DE work demonstrated serum protein changes in rats following exposure to carbon tetrachloride, trichloroethylene or dimethylformamide.[240,241] More recently, proteomics has been used to investigate inflammation induced experimentally in rats by intramuscular injection of turpentine, with or without daily doses of indomethacine.[242,243] The resulting acute-phase response affected a number of proteins (including C-reactive protein, serine protease inhibitor-3 and thiostatin) and these have been catalogued on a rat serum database (http://linux.farma.unimi.it/index.html).[242,243] In a follow-up study of rat serum proteins, arthritis was shown to mimic the acute-phase inflammatory response and the effects of non-steroidal anti-inflammatory drugs (indomethacine and ibuprofen) were investigated.[244]

2-DE has been used to investigate nephrotoxicity by monitoring changes in the protein composition of human urine following occupational exposure to cadmium[245,246] (Figure 7). However, most proteomic studies have been directed towards animal model systems. Thus, the renotoxic effects of lead have been investigated and a more marked effect demonstrated on the cortex (76 affected proteins) than the medulla (13 affected proteins) of rat kidney.[247] Occupational exposure to lead (at subchronic blood levels) has been simulated in rabbits and kidney protein expression investigated to demonstrate a dose-related response of a range of proteins (possibly including variants of glutathione-S-transferase).[248]

Occupational exposure to JP-8 jet fuel has been simulated in mice[249] and rats,[250] and proteomic analysis of kidney (and other tissues) used to demonstrate a moderate nephrotoxic effect[249] and significant alterations in 10-formyltetrahydrofolate dehydrogenase.[250] In addition, proteomics has been used to investigate the nephrotoxic effects of puromycin aminonucleoside in rats[251] and cyclosporine A (Cs-A) in different species.[252,253] Cs-A down-regulated a protein identified as calbindin-D-28kDa (a vitamin D-dependent calcium-binding protein) in rat kidney[252] and in human kidney biopsy sections from Cs-A-treated transplant recipients.[253] Dogs and monkeys (which are generally free of Cs-A-mediated nephrotoxicity) did not show this effect.[253]

Future prospects

High-resolution two-dimensional electrophoresis (2-DE) is a very powerful technique for the separation of proteins and will continue to underpin proteomics within the foreseeable future.[254,255] However, it is a semi-quantitative, technically demanding method and relatively few studies have included clinically significant sample numbers with adequate quality control. While immobilised pH gradients have improved reproducibility and the detection of basic proteins,[256] 2-DE still fails to detect high-molecular-weight or low abundancy proteins.[4,6,255] Recourse to prefractionation[16,255] or narrow pH range 'zoom' gels[257,258] complicates sample preparation and reduces throughput. Consequently, it has been suggested that 2-DE be replaced by alternative methods[9,255] such as ICAT,[78] protein arrays[259,260] or the multi-dimensional protein identification technique (MudPIT).[261]

If proteomics fulfils its promise, our understanding of gene expression, post-translational modifications[2,262-264] and protein function[11] will be enhanced considerably, with better appreciation of the regulatory importance of subcellular compartmentation,[156] subcellular redistribution of proteins[4] and protein-protein interactions.[17]

Within biomedical science, cytology screening[167,168] and the typing of microorganisms[119,120] will be vastly improved, and there will be a proliferation of new drugs[220,221] and more effective vaccines.[119] Clinical diagnosis will be revolutionised,[5] with early detection of disease by analysis of new, more specific biomarkers[10,14,265,266] using 2-DE clinical molecular scanners[267,268] and protein expression profiling by microarray analysis.[259,260] Sample throughput will be improved by automation and miniaturisation through the application of microchip technology.[259,260,269,270]

The human proteome project provides a focal point for the future development of proteomics and a strong justification for research funding. However, the level of funding required will be colossal and it is impossible to predict whether or not it will ever achieve its goal. Given the limited availability of public funds, it is possible that human proteomics will evolve within the private sector and, as a consequence, commercial interests may dictate that it never be freely accessed in its entirety. □

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