Amino Acids (2001) 21: 363–381

Amino
Acids

Proteomics: Current technologies and applications in neurological disorders and toxicology

Review Article

M. Fountoulakis

Pharmaceutical Research, Genomics Technologies, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Accepted September 13, 2001

Summary. Proteomics is the science that studies the proteins in general and in particular their changes, resulting from various disorders or the effect of external factors, such as toxic agents. It has as goal the detection of novel drug targets, diagnostic markers and the investigation of biological events. Proteomics has emerged the last few years and its major difference from the previously existing protein analytical techniques is that it does not analyze the proteins one by one, but in a possibly automated, large-scale mode. In this article, the state of the art of proteomics in our laboratory is presented, as well as selected applications of proteomics in the study of disorders of the central nervous system and of toxic events.

Keywords: Amino acids – 2-D electrophoresis – Neurological disorders – Matrix-assisted laser desortpion ionization mass spectrometry – Proteomics – Toxicology

What is proteomics

In addition to the existing classical terms *genome* and *genomics*, the last years two new terms appeared, *proteome* and *proteomics*. Proteome was originally defined as the *protein* complement of a *genome*, but it now means the total proteins expressed in an organism, an organ or a cell line at a given time. Proteomics is the technology-driven science which studies the proteomes, i.e. the proteins, their post-translational modifications, their interactions and in particular the changes in their levels and their modifications, which result on account of specific diseases or from various external factors, such as toxic agents and has as goal the detection of novel drug targets and diagnostic markers.

The genomic analysis quickly provides us with data about the mRNA levels (here the corresponding terms are *transcriptome* and *transcriptomics*).

However, knowledge of the transcription levels does not necessarily informs us about the protein levels or the number of modified protein forms, information, which can only be provided by the proteome analysis. Life is a dynamic process and not all-possible proteins are expressed at any time point. Moreover, the pattern of protein expression changes depending on factors, such as the stage of development of an organism and the organism's physiological state. It seems that the more complex the genome, the less of the total possible proteins will be expressed at any particular moment. Thus, proteomics supplements the genomics data with information on what gene products are being made, in what cell type, at what amounts and under what conditions and how protein levels and modifications change as a result of specific diseases or external challenges (Abbott, 1999).

Proteomics is a member of the New Technologies and it enjoys a remarkable bloom today. This can be attributed to developments in the computer and software sciences, developments in mass spectrometry and the enormous amount of information, which became available from the sequencing of the whole genome of many organisms. The genomes of about 30 microorganisms have been sequenced so far and the sequencing of the human genome has been almost completed. Much more information is expected to be released in the near future. The era of the human proteome analysis has already started, newly established companies as well institutions have undertaken the task of analyzing various proteomes under healthy and diseased states for the detection of drug targets and diagnostics. This effort is probably going to revolutionize protein science, in particular the analytical techniques and to increase their efficiency and capabilities.

The steps of proteomics

The aim of proteomics is the high throughput analysis of proteomes. Proteomics mainly comprises two steps: (i) protein separation usually by two-dimensional gel electrophoresis and (ii) protein analysis and identification by efficient analytical techniques, mainly by mass spectrometry, but also by amino acid composition analysis, N-terminal sequencing and immunoblot analysis (Fountoulakis, 2000). The whole process is supported by highly sophisticated software for protein identification, image analysis, comparison and quantification and data storage. Some of the techniques used by proteomics have existed for many years. The novel contribution of proteomics is the large-scale analysis, which was not possible before and which was enabled by the factors mentioned at the beginning. The proteome analysis appears to be more complex than the genome analysis. There are usually more than one product resulting from one gene. The protein modifications change constantly, depending on the influence of the environment and the disorders. Moreover, the protein detection and in particular the analysis of the modifications are not very efficient at present.

Recently the term proteomics has been used in a much broader sense, meaning roughly "Protein Science". Under this newer definition, it comprises

protein production and enrichment, study of protein changes, protein-protein interactions, protein function and protein structure analysis. These proteomics technologies comprise the yeast two-hybrid system, which provides information on protein-protein interactions, microarrays, in which chips coated with specific ligands or peptides are used for protein screening, antibody chips and large-scale crystallography (Borman, 2000; Dove, 1999; Eisenberg et al., 2000; Pandey and Mann, 2000; Wang and Hewick, 1999). Most of these technologies are in the early stages of development and only the classical proteomics, 2-D electrophoresis followed by mass spectrometry, although itself under development, is more mature than the other approaches. Here we will further deal with the classical definition of proteomics.

Two-dimensional electrophoresis

Figure 1 shows the work flow in proteomics. The first major step of proteomics is the analysis of protein mixtures by two-dimensional (2-D) electrophoresis,

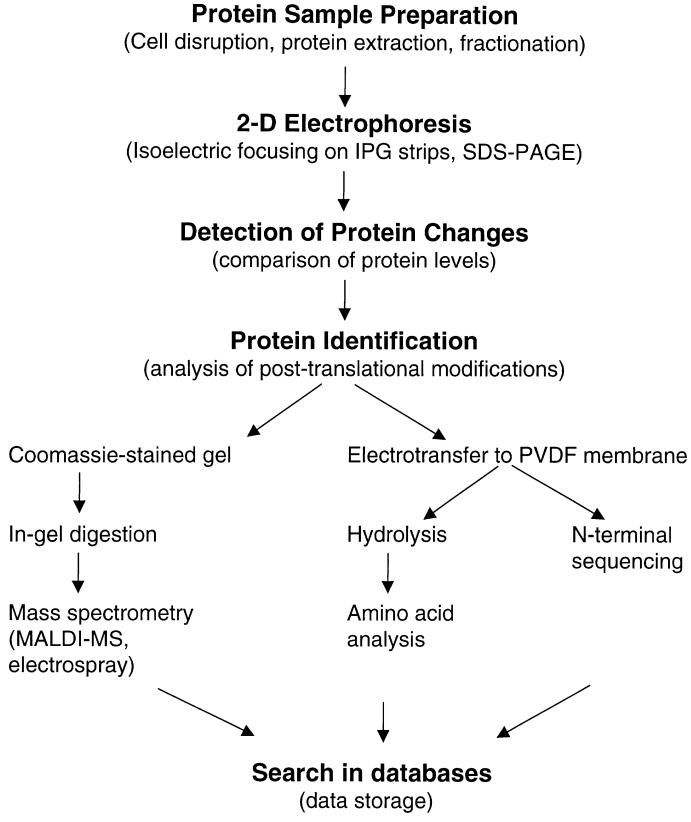

Fig. 1. Workflow in proteomics. Proteomics in its classical definition usually involves separation of a protein mixture and protein identification as well as detection of changes in the protein level and of post-translational modifications. *IPG*, immobilized pH gradient; *SDS*, sodium dodecyl sulfate; *PAGE*, polyacrylamide gel electrophoresis; *PVDF*, polyvinylidene difluoride; *MALDI-MS*, matrix-assisted laser desorption ionization mass spectrometry

which involves: (i) separation of the proteins on the basis of differences in their net charge, called isoelectric focusing (IEF) and (ii) separation of the focused proteins on the basis of differences in their molecular masses. IEF is an equilibrium process, during which, under the influence of a high voltage field, proteins move along a stable pH gradient and focus at a position where they have no net charge. There are two ways to form a pH gradient: (i) with the use of carrier ampholytes, that are amphoteric compounds of low molecular mass, which upon application of the electric field move and align themselves between the electrodes (O'Farrell, 1975; Klose, 1975). In this system, both the pH gradient and the proteins move; (ii) with the use of an immobilized pH gradient (IPG), which has been formed prior to the IEF run (Bjellqvist, 1982). The IPG is formed by acrylamide derivatives, called immobilines. They are weak acids and bases with a buffering capacity, copolymerized with acrylamide and fixed on a plastic sheet. In this system, under the influence of the electric field, the pH gradient does not move, only the charged protein molecules are moving and are focused along the IPG.

The carrier ampholytes technique was the first IEF approach. It has a high reproducibility and allows a reliable protein quantification. However, because the pH gradient is affected by the amount of the total proteins loaded, that have themselves a buffering capacity, only relatively low amounts of protein can be applied. Also the range of the pH gradient is limited (usually between pH 4 and 8). The IPG strips are commercially available in broad and narrow pH ranges, can be stored frozen for long times before use and after the first dimensional separation and a relatively large amount of protein can be applied to them. The latter is compatible with the subsequent protein identification, as with today's status of the various large-scale analytical techniques, a minimal protein amount is required for an unambiguous identity assignment. The bloom of proteomics is to a large extent due to the introduction of the IPG strips. We use exclusively IPG strips in our laboratory for the first dimensional protein separation. There are several ways of applying the protein sample on the strips (Langen et al., 1997b). The application method, the protein quantity applied and the voltage and time of IEF affect the protein resolution (Görg et al., 2000).

In the second dimensional separation, the proteins are separated according to their size on porous polyacrylamide gels. During this nonequilibrium step, the anionic detergent sodium dodecyl sulfate (SDS) is added, so that the proteins are negatively charged. In comparison with IEF, the second dimensional separation on SDS-gels is relatively easy to control. Gels of a constant acrylamide concentration or gradient gels can be used. The 9–16% linear gradient gels represent a good compromise to visualize proteins with a wide spectrum of molecular masses (5–200 kDa). However, for the efficient separation of low or high molecular mass proteins, gels of proper acrylamide concentration have to be used (Langen et al., 1997b). Use of gels of a large format (usually 18×20 cm) can facilitate the efficient resolution of the thousands of protein spots. There exist various stain methods for the spot visualization. More sensitive stains, such as silver stain, are preferentially employed for gel comparison, whereas stain with Coomassie is preferred when the gels are

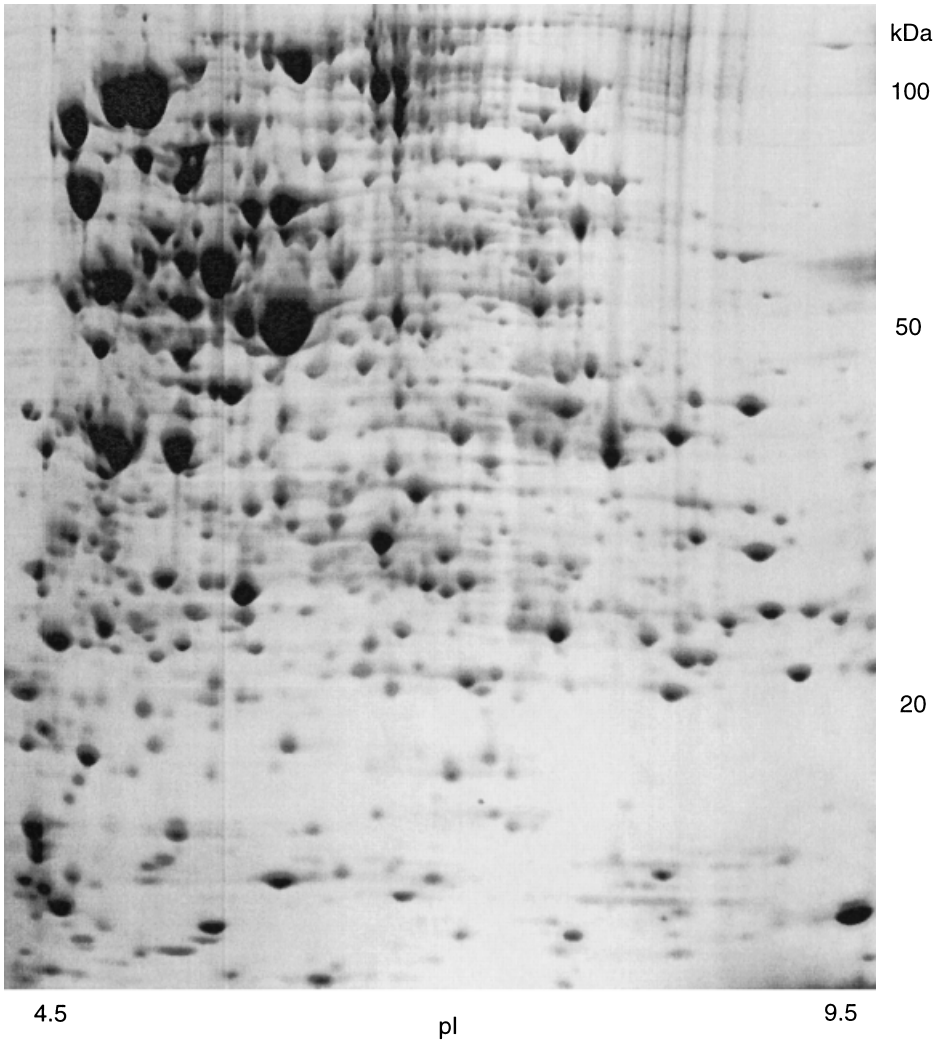

Fig. 2. Two-dimensional gel analysis of the soluble proteins of *Haemophilus influenzae*. The microorganism was cultured as described (Langen et al., 2000). Cells from the stationary phase were broken and the lysed cell suspension was centrifuged at 150,000 × *g* for 90 min. One mg of the supernatant was used for the 2-D gel analysis of the cytoplasmic proteins. The proteins were separated on a pH 3–10 nonlinear IPG strip followed by a 9–16% linear gradient polyacrylamide gel. The gel was stained with colloidal Coomassie blue

intended for protein identification by mass spectrometry. Figure 2 shows an example of 2-D electrophoresis where the proteins of the bacterium *Haemophilus influenzae* have been separated on a broad range pH 3–10 IPG strip, followed by a 9–16% SDS-gel. More than 1,000 spots can be seen on this gel.

On 2-D gels, the proteins are often represented by more than one spot, so that the number of expressed products is much higher than the number of the corresponding encoding genes. In prokaryotes, usually 1–2 proteins

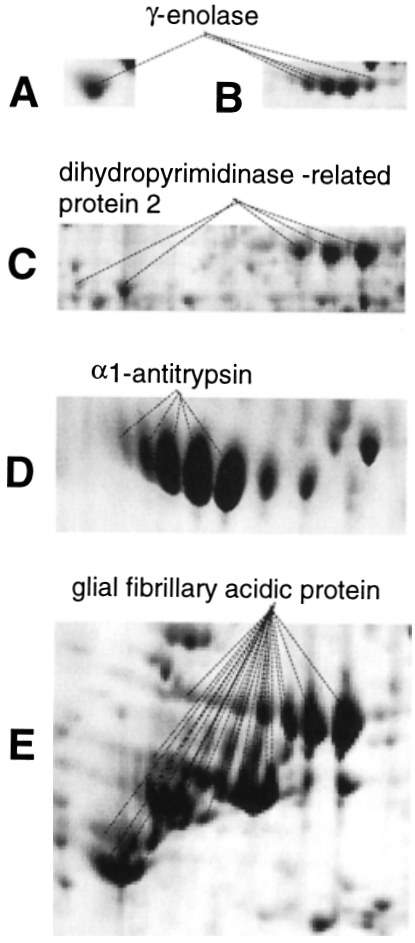

Fig. 3. Partial 2D-gel images showing examples of protein heterogeneity. **A**, **B** γ-enolase from human brain. The protein is represented by one spot when IEF was performed on a broad pH range 3–10 nonlinear IPG strip (**A**), and by at least five spots when IEF was performed on a pH 4–7 strip (**B**). **C** Dihydropyrimidinase-related protein 2 from human brain shows a high heterogeneity, represented by at least five spots, localised into two regions on the gel. The two shorter, acidic forms are most likely generated post-mortem from the larger forms (Fountoulakis et al., 2000b). **D** α1-antitrypsin from human cerebrospinal fluid is represented by many spots, most likely denoting different glycoforms of the protein. **E** glial fibrillary acidic protein from human brain. The protein is represented by at least 50 spots with molecular masses between 35 and 60 kDa

correspond to one gene and in eukaryotes 5–20 proteins may be derived from one gene (Fountoulakis et al., 2001). Figure 3 shows examples of protein heterogeneity. Presently, in most of the cases, we do not know the reasons or the biological meaning of the observed heterogeneity. One major task of proteomics is to identify and correlate cases of heterogeneity with eventual disorders. Post-translational modifications or changes in them may carry more significant messages than changes in the protein level.

The major advantage of 2-D electrophoresis is that it enables the simultaneous separation and visualization of thousands of unknown proteins. No

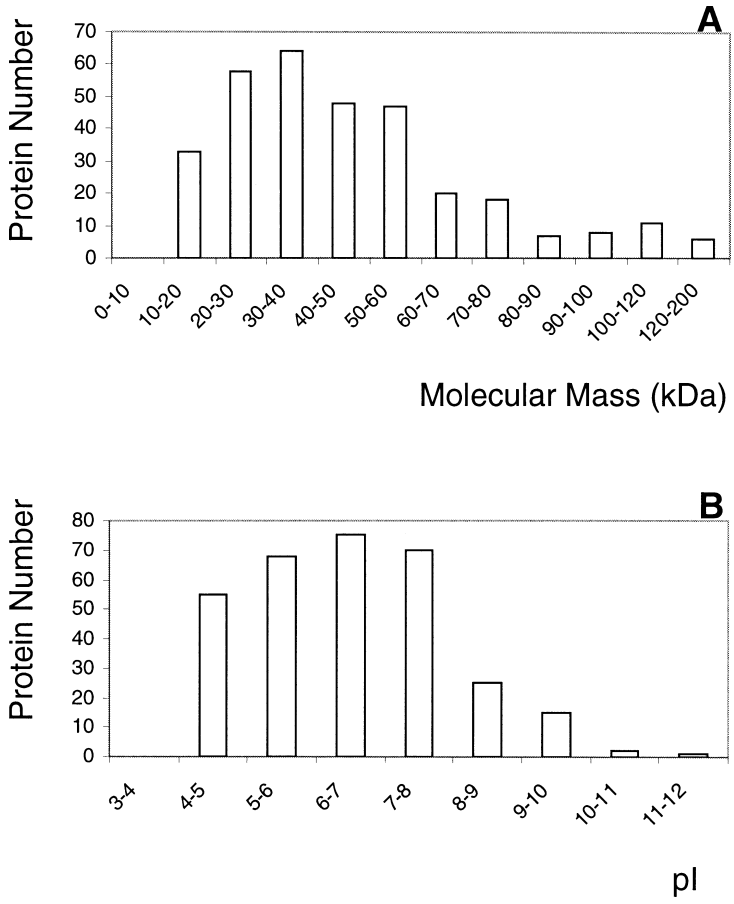

Fig. 4. Distribution of mouse liver proteins identified by proteomics technologies in relation to their molecular mass and pI. The about 330 mouse liver proteins were sorted according to their molecular mass (**A**) and pI values (**B**). The number of the proteins found in the indicated molecular mass and pI intervals are shown

other method can do that for the time being. However, it shows certain limitations: (i) only the major components of a protein mixture can be visualized (Gyri et al., 2000). For the detection of the low-abundance proteins, specific protein enrichment steps have to be employed prior to the 2-D analysis; (ii) the detection of the low and high molecular mass, of basic and hydrophobic proteins is inefficient (Fountoulakis et al., 1998a and 1998c). Figure 4 shows the mouse liver proteins identified in our laboratory, sorted according to their theoretical molecular mass (Fig. 4A) and pI (Fig. 4B) values. Most proteins have masses between 10 and 80 kDa. No protein with a mass below 10 kDa and only a small number of proteins with masses above 80 kDa were found. Similarly, no protein with a pI below 4 and only a few with pI values higher than 10 were detected. Improvements of the technology are urgently required for an efficient proteome analysis (Corthals et al., 2000).

The preparation of the protein sample to be analyzed is of a particular significance. Careful sample preparation is a prerequisite of a successful

analysis. Until now, in most cases of a proteome analysis, a sample of the total proteins has been analyzed. This provides a general image of the proteome, however, it is insufficient for the analysis of the difficult protein classes mentioned above. On a 2-D gel stained with colloidal Coomassie blue, 1,000–3,000 protein spots can be detected, which, however, are the products of 200–400 different genes only. For the construction of the 2-D database for mouse liver proteins, we analyzed about 5,800 spots from 14 gels. The analysis resulted in the assignment of 2,500 identities. The identified proteins were the products of 328 different genes. Some of the proteins were detected in the various gels with a higher frequency than others. Thus, three heat shock proteins were found in all gels and three housekeeping enzymes were found in 13 out of the 14 2-D gels. About 18% of the proteins were detected once and 15% were found twice (Fountoulakis et al., 2001). It is more likely that the low-abundance proteins could be potential drug targets rather than their high-abundance counterparts.

The detection of additional gene products can be facilitated by a pre-fractionation of the sample and a protein enrichment by application of centrifugation and chromatographic techniques. Chromatography prior to the 2-D electrophoresis is a very efficient approach to enrich several classes of low-abundance proteins. We have used chromatography steps to enrich cytosolic proteins of the bacteria *H. influenzae* (Fountoulakis et al., 1997b, 1998b and 1999d) and *Escherichia coli* (Fountoulakis et al., 1999c) and of human brain (Karlson et al., 1999). Multicompartment electrolyzers (Righetti et al., 2001) and other preparative electrophoretic techniques also represent strong tools in detecting low-copy number gene products. The identification of the proteins enriched by the various chromatography steps is useful for the construction of master purification maps, i.e. lists of the proteins enriched by the corresponding chromatography step (Fountoulakis and Takács, 1998). Biochemists could consult such lists and save a lot of labor, which is otherwise spent in "trial and error" approaches during protein isolation. The master purification steps are in particular useful for the isolation of "hypothetical" or "unknown" proteins. Such proteins have been found in large numbers from the sequencing of the various genomes and today no information about these proteins exists.

A protein can be only visualized and analyzed if it can be brought into and kept in solution during the whole two-dimensional separation process. In general, the solubility question appears at two time points along performance of the 2-D electrophoresis: (i) during the initial extraction step to solubilize the proteins of the sample to be analyzed using agents that are compatible with isoelectric focusing, such as solutions of urea and CHAPS, and (ii) during the performance of the first dimensional separation, when hydrophobic proteins or proteins present in large amounts could precipitate at their application positions. The detection of many proteins might not be possible, not because they are hydrophobic and consequently do not enter the immobilized pH gradient strips during the first dimensional separation, but because they remain insoluble during the extraction step. In addition to urea and CHAPS, which are usually employed during IEF, many other solubilizing agents are in

use, chaotropes, such as thiourea (Chevallet et al., 1998), novel detergents, such as amidosulfobetaines (ASB-n) (Rabilloud, 1999) and reducing agents, such as tributyl phosphine (Herbert et al., 1998). It seems that no one, single solubilizing agent is sufficient for the visualization of all proteins of a proteome (Fountoulakis and Tákacs, 2001).

Matrix-assisted laser desorption-ionization mass spectrometry

The second major step of proteomics is the protein identification. Nowadays, this is mainly performed by mass spectrometry techniques and in particular by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF-MS), but also by ion spray approaches (Lahm and Langen, 2000). Another protein identification technique of significantly lower throughput in comparison with MALDI-MS but of high confidence is the N-terminal sequencing analysis. We have previously employed other analytical methods as well, such as amino acid composition analysis for protein identification (Fountoulakis et al., 1997a; Fountoulakis and Lahm, 1998; Weiss et al., 1998). The latter has a relatively high throughput, but it is complex and the identification is often ambiguous. Today we mainly use MALDI-TOF-MS for protein identification and only in cases where this method does not deliver an identity we apply electrospray techniques.

The protein identification using MALDI-TOF-MS is performed on the basis of the peptide mass fingerprinting (Henzel, 1993). The peptide masses are compared with the theoretical peptide masses of all known proteins of all databases available. For the peptide production, the protein spots are automatically excised from the Coomassie stained 2-D gels and the proteins are in-gel digested with a protease, usually trypsin. We perform the digestion in the presence of a low concentration of Tris buffer which is compatible with a high throughput analysis, allowing the daily measurement of more than 1,000 spots per instrument by one person (Fountoulakis and Langen, 1997). The masses of the peptides generated during digestion are automatically measured in the MALDI-TOF-MS instrument. At Hoffmann-La Roche, Basel Peter Berndt and Hanno Langen have developed specific software for the automatic mass assignment, mass correction, considering the mass values of standard peptides that are included in each mass measurement, and the automatic search in the databases (Berndt et al., 1999). In this way, the labor has been significantly reduced and because of the stringent searching conditions, the automatic identity assignment is usually more confident than individual search even with the use of molecular mass and pI restriction windows. The results are stored in specific servers and can be called up on the computer desktop of each researcher. The software shows which spots from a particular gel have been analyzed, which proteins have been successfully identified as well as the confidence of the identity assignment. Usually, an identity is assigned to 60–80% of the spots analyzed, following the MALDI-TOF-MS approach.

The strength of MALDI-TOF-MS is that it enables the automatic analysis of thousands of samples per day with a minimal involvement of the operator.

Its drawback is that the identifications can occasionally be ambiguous and that it does not deliver amino acid sequence data. When sequence analysis is required, post-source decay or electrospray MS analysis can provide such information. This however demands a higher personnel engagement.

Both genomics and proteomics represent new tools in the drug discovery process and in the investigation of biochemical problems. In relation to proteomics, the comparison of nucleotide levels using genomics technologies, for example by high density oligonucleotide arrays, is usually more sensitive, has a higher throughput and requires a shorter analysis time. Comparison of protein levels on the other hand by proteomics, provides us with information on changing protein levels, post-transional modifications, subcellular protein location and turnover, data that can not be delivered by the genomics technologies. In most cases, the two technologies are complimentary, each delivering unique information not provided by the other.

Using the proteomics technologies described, we constructed 2-D databases for several bacterial and eukaryotic proteomes. The 2-D database for the *H. influenzae* proteins is one of the largest published databases today. It comprises 502 identified different gene products from the 1742 possible gene products of the microorganism (Langen et al., 1997a and 2000). All possible heat shock proteins and almost all possible proteins involved in some pathways, such as glycolysis and pentose phosphate, were identified. The major components of the *H. influenzae* proteome are elongation factors, heat shock proteins and certain enzymes such as aspartase and phosphoenolpyruvate carboxykinase, outer membrane proteins and hypothetical proteins. Gene products not included in the database are most likely expressed at low levels and the chromatographic steps applied were not sufficient for an efficient enrichment or the proteins could not be identified on account of limitations of the technology, because they are too small, large, basic or hydrophobic. The human and rat brain 2-D protein databases we prepared were the first and up to now the largest of their kind (Fountoulakis et al., 1999b; Langen et al., 1999; Lubec et al., 1999a). Each comprises approximately 200 unique gene products. In the meantime we have identified more than 700 human brain proteins (unpublished results). Also the mouse and rat liver 2-D protein databases are the most detailed today (Fountoulakis et al., 2000a, 2001, 2002).

Applications of proteomics

Neuronal diseases

Proteomics is a tool that can lead to drug discovery and as member of the New Technologies family, it belongs to the preclinical research sciences. The identification of disease-related differences is usually a difficult task. Proteomics can relatively easily identify changes in bacterial proteomes and also in cell lines. Analysis of human tissues, such as brain, is more difficult, as many factors are involved, such as differences in age, sex, possibly other diseases, treatment with medicines, as well as technical, disease-unrelated factors, such

as post-mortem time, improper treatment of the samples etc., which can affect a clear discrimination between healthy and diseased states of interest. Indeed, the expression levels of many proteins vary depending on the age of the subject (Fountoulakis et al., 2000c). Post-mortem time also strongly affects protein levels, in particular at 48 h post-mortem (Fountoulakis et al., 2000b).

Proteomics is routinely used in clinical diagnosis today and many changes in protein levels resulting from specific disorders have been identified. It has been applied in the investigation of infectious diseases, cancer, heart (Jungblut et al., 1999; Alaiya et al., 2000; Banks et al., 2000; Cash, 2000) and diseases of the central nervous system (Rohlff, 2000). We applied proteomics in the investigation of protein changes in disorders of the central nervous system, such as Alzheimer's disease (AD) and Down syndrome (DS). Alzheimer's disease is a well-studied dementia affecting mainly elderly people, for which no reliable premortem diagnosis marker exists today. Down syndrome is the most frequent genetic cause of dementia. Although the trisomic state is responsible for the phenotype, the pathomechanisms are not well understood. Almost all subjects with Down syndrome over 40 years show neuropathological and neurochemical abnormalities on post-mortem brain examinations indistinguishable from those seen in Alzheimer's disease. Thus, the results from the DS may be useful in the AD studies. The controls were individuals with no history of neorological disease. Samples from five regions of the brain of the control and the disease groups were analyzed by 2-D gels and the proteins with different levels in the disease groups were identified by MALDI-MS.

In the disease groups, we found about 4-fold increased levels in the AD brain of the glial fibrillary acidic protein (GFAP), a known marker of neuronal decay and brain damage, which distinguishes astrocytes from other glial cells during development of the central nervous system (Fig. 5) (Greber et al., 1999). We also observed increased levels for the 14-3-3γ and 14-3-3ε proteins in the Alzheimer's and Down syndrome groups by about 1.5-fold (Fountoulakis et al., 1999a). The 14-3-3 proteins exert complex functions in signal transduction pathways. Deranged levels may reflect impaired signaling and apoptosis in the brain. The levels of synaptosomal associated protein 25 kDa (snap-25) decreased in the two disease groups to approximately 40% (Greber et al., 1999). Snap-25 is widely distributed in the brain, is an integral constituent of the synaptic core complex, participates in synaptic vesicles exocytosis and is involved in the formation of presynaptic sites. Decreased snap-25 levels may lead to deranged functions in exocytosis and neurotransmission. Figure 6 shows examples of changed levels of the 14-3-3 and snap-25 proteins in the Alzheimer's disease and Down syndrome groups.

The proteomic analysis revealed changes in the levels of several other brain proteins as well, such as glycerol 3-phosphate dehydrogenase (Lubec et al., 1999b), dihydropyrimidinase-related protein (DRP) 2 (Lubec et al., 1999c), synaptotagmin (Yoo et al., 2001a), voltage-dependent anion channel proteins VDAC1 and VDAC2 (Yoo et al., 2001b), and others. Dihydropyrimidinase-related proteins show homology to chicken collapsin 62 kDa and the rat turned on after division protein. DRPs are expressed in

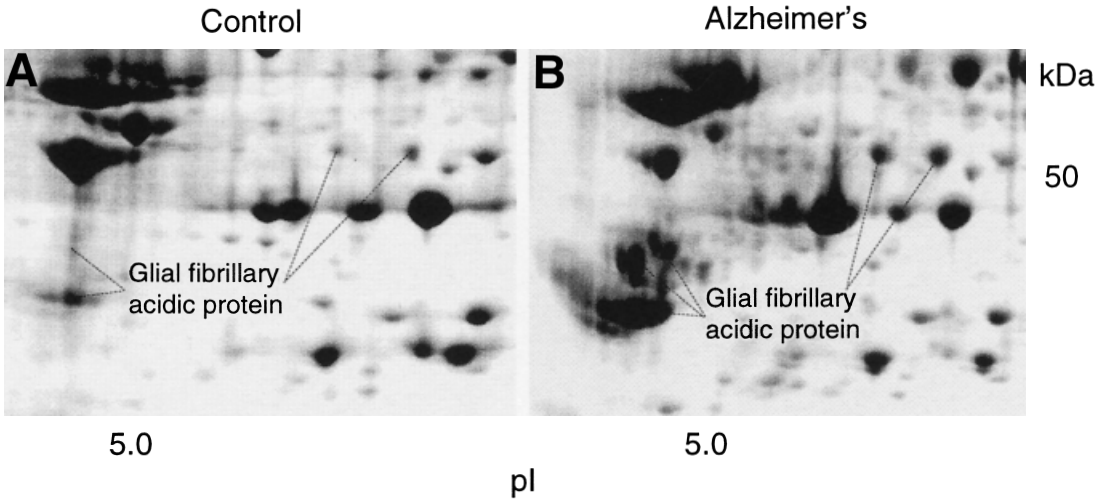

Fig. 5. Partial two-dimensional gel images of human brain proteins from the parietal cortex lobe of a control (**A**), and a patient with Alzheimer's disease (**B**). The proteins were separated on pH 3–10 nonlinear IPG strips, followed by 9–16% SDS-polyacrylamide gels. The gels were stained with Coomassie blue. The spots representing glial fibrillary acidic protein (GFAP) are indicated

relatively large amounts in the brain, DRP-1, -3 and -4 mainly in the neonatal brain and DPR-2 in both neonatal and adult brain (Fountoulakis et al., 2000c; Karlsson et al., 1999). The functions of DRPs are not clear. It is possible that these molecules are involved in neuronal migration during brain development. When the latter becomes less pronounced in the adult brain, DRP-2, which is almost equally expressed in both, adult and neonatal brain, may serve as a guidance cue in repair and remodeling in adult brain. Reduced levels of DRP-2 in the Alzheimer and Down syndrome brain may have consequences in the functions of the protein (Lubec et al., 1999c). We have also used the technology to study changes in the brain proteins of animals serving as models for neurological diseases, such as ischaemia, anxiety and pain. This research is still ongoing.

Toxicology

Proteomics has also been employed in the investigation of toxic events (Qiu et al., 1998; Steiner and Anderson, 2000). We studied liver toxicity in mice treated with acetaminophen (APAP), the known antipyretic and analgesic agent, which is toxic at high doses. The mechanism of the toxicity is not well understood (Cohen et al., 1997). The animals were treated with two doses of APAP of 100 and 300 mg/kg and with its non-toxic stereoisomer 3-acetamidophenol (AMAP) at 300 mg/kg (Fountoulakis et al., 2000a). The levels of about 30 proteins changed following administration of the two agents. The changes were in the order of 10–50% of the control value and appeared mainly in the high- than in the low-dose of APAP-treated animals.

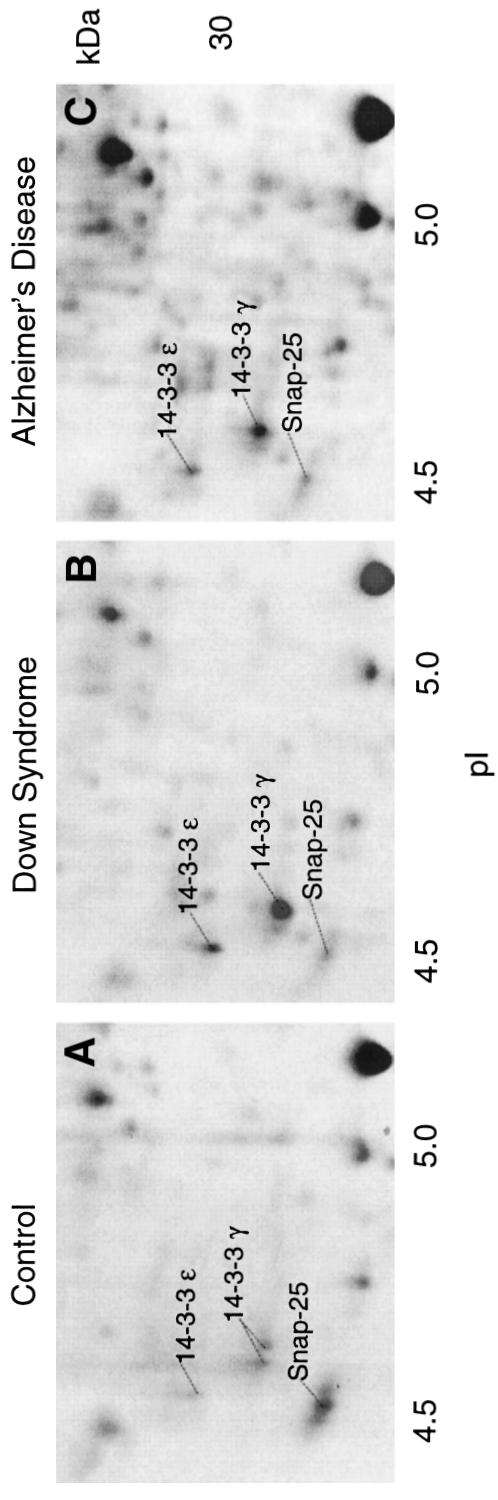

Fig. 6. Partial two-dimensional gel images of human brain proteins from the parietal cortex lobe of a control (**A**), a patient with Down syndrome (**B**) and a patient with Alzheimer's disease (**C**). The proteins were separated on pH 3–10 nonlinear IPG strips, followed by 9–16% SDS-polyacrylamide gels. The gels were stained with Coomassie blue. The spots representing the 14-3-3 γ and ε proteins and snap-25 are indicated

Figure 7 shows examples of changed protein levels following treatment with APAP and AMAP and the corresponding quantifications of the protein levels. We also applied proteomics to study the effect of administration of carbon tetrachloride in rat liver which resulted in significant changes in protein levels (manuscript in preparation).

We further studied the effect of systemic administration of kainic acid, a potent neurotoxin and excitatory amino acid, on the levels of brain proteins. Heat shock protein HSP 27 was exclusively detected in brains of animals treated with kainic acid. The levels of neurofilaments and alpha-internexin were significantly decreased and a fragment of tubulin alpha-1 chain was manifold increased in kainic acid-brains. The proteomic analysis indicates altered regulation of heat shock proteins, neuronal death, cytoskeletal disruption and mitochondrial derangement by systemic kainic acid administration (Krapfenbauer et al., 2001a). The cytosolic proteins from control and animals treated with kainic acid were fractionated over an ion exchange column prior to proteomic analysis and changes in the levels of low-abundance brain proteins were studied. We found a manifold decrease of annexin VII, heat shock co-factor HOP/p60 and SP-32 and a manifold increase of heparin-binding protein P30. The results suggest, respectively, for the involvement of an apoptotic pathway, the recruitment of the heat shock protein machinery, the generation of an antioxidant response and probably the induction of repair mechanisms. Three out of the four proteins with changed levels had not been detected in the cytosolic fraction before and the detection of the changed levels was possible only after the protein enriching step (Krapfenbauer et al., 2001b).

Conclusions

Proteomics studies changes in the level and the modifications of proteins and is a tool in drug discovery, diagnosis and the investigation of toxic events. It has two major steps, 2-D electrophoresis and protein analysis, performed mainly by mass spectrometry, and quantification. Recently, the term proteomics has been used in a broader sense, comprising many disciplines of the protein science. The technology is still under development. Improvements are required to increase sensitivity and visualization of certain classes of proteins, like the small, large, basic and hydrophobic proteins and in the detection of post-translational modifications.

In our laboratory, we have established the technology for the preparation of about 50 2-D gels per week and the analysis of about 1,000 spots per day and person. Applying this technology, we constructed 2-D databases for bacterial and eukaryotic proteomes, which are of the largest existing today. We identified many differences in the protein level in the brain of patients with Alzheimer's disease and Down syndrome, in the brain of animals serving as models for human diseases and in the liver and brain of animals subjected to treatment with toxic agents. This high throughput analytical system is becoming a powerful tool in pathological, biological and toxicological studies.

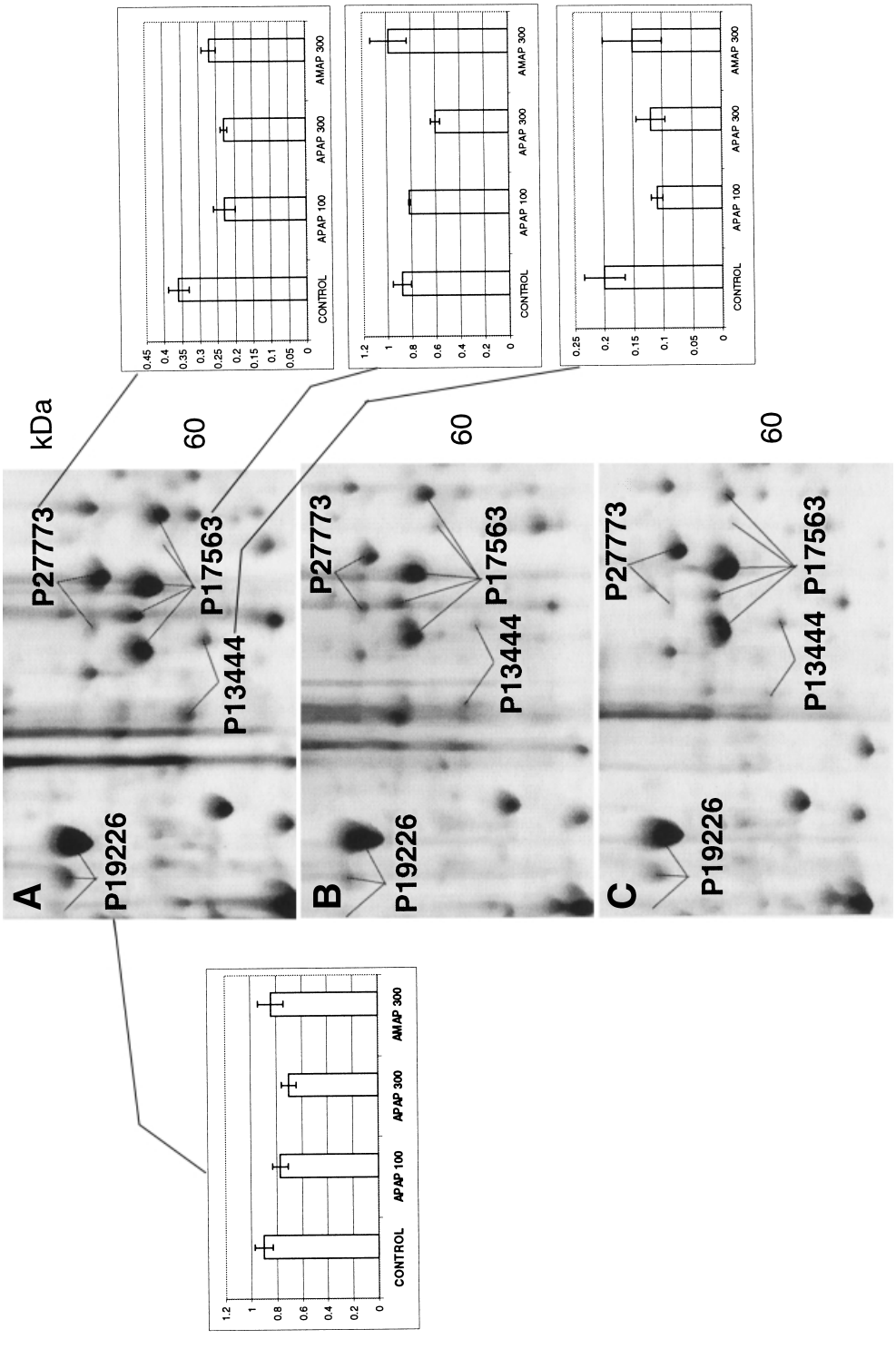

Fig. 7. Partial 2-D images of mouse liver proteins from a control animal (**A**), and animals treated with 300 mg/kg of APAP (**B**) and 300 mg/kg of AMAP (**C**). The spots representing proteins whose levels changed following APAP or AMAP administration and the corresponding level changes are shown. A 2-D image of an animal treated with 100 mg/kg of APAP is not shown. *P13444*, S-adenosylmethionine synthetase; *P17563*, selenium-binding protein; *P19226*, mitochondrial matrix protein P1; *P27773*, protein disulfide isomerase

Acknowledgements

I thank Dr. H. Langen for his support, J.-F. Juranville for excellent assistance and the colleagues at F. Hoffmann-La Roche Ltd., Basel who provided me with materials and other help.

References

Abbott A (1999) A post-genomic challeng: learning to read patterns of protein synthesis. Nature 402: 715–720

Alaiya AA, Franzén B, Auer G, Linder S (2000) Cancer proteomics: from identification of novel markers to creation of artifical learning models for tumor classification. Electrophoresis 21: 1210–1217

Banks RE, Dunn MJ, Hochstrasser DF, Sanchez J-C, Blackstock W, Pappin DJ, Selby PJ (2000) Proteomics: new perspectives, new biomedical opportunities. Lancet 356: 1749–1756

Berndt P, Hobohm U, Langen H (1999) Reliable automatic protein identification from matrix-assisted laser desorption/ionization mass spectrometric peptide fingerprints. Electrophoresis 20: 3521–3526

Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, Postel W (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 6: 317–339

Borman S (2000) Proteomics; taking over where genomics leaves off. Chem Engin News issue July 31: 31–37

Cash P (2000) Proteomics in medical microbiology. Electrophoresis 21: 1187–1201

Chevallet M, Santoni V, Poinas A, Rouquié D, Fuchs A, Kiefer S, Rossignol M, Lunardi M, Garin J, Rabilloud T (1998) New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19: 1901–1909

Cohen SD, Khairalaah EA (1997) Acetaminophen. In: Sipes G, McQueen CA, Gandolfi AJ (eds) Comprehensive toxicology, vol 9. Hepatic and gastrointestinal toxicology. Pergamon, Cambridge, UK, pp 329–343

Corthals GL, Wasinger VC, Hochstrasser DF, Sanchez J-C (2000) The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 21: 1104–1115

Dove A (1999) Proteomics: translating genomics into products? Nat Biotechn 17: 233–236

Eisenberg D, Marcotte EM, Xenarios I, Yeates T (2000) Protein function in the post-genomic era. Nature 405: 823–826

Fountoulakis M (2000) Two-dimensional electrophoresis. Encyclopedia of separation science II/Electrophoresis. Academic Press, London, pp 1356–1363

Fountoulakis M, Langen H (1997) Identification of proteins by matrix-assisted laser desorption ionization-mass spectrometry following in-gel digestion in low-salt, non-volatile buffer and simplified peptide recovery. Anal Biochem 250: 153–156

Fountoulakis M, Lahm H-W (1998) Hydrolysis and amino acid composition analysis of proteins. J Chromatogr 826: 109–134

Fountoulakis M, Takács B (1998) Design of protein purification pathways. Application to the proteome of *Haemophilus influenzae* using Heparin chromatography. Protein Expression Purification 14: 113–119

Fountoulakis M, Takács B (2001) Effect of strong detergents and chaotropes on the protein detection in two-dimensional gels. Electrophoresis 22: 1593–1602

Fountoulakis M, Juranville J-F, Berndt P (1997a) Large-scale identification of proteins of *Haemophilus influenzae* by amino acid composition analysis. Electrophoresis 18: 2968–2977

Fountoulakis M, Langen H, Evers S, Gray C, Takács B (1997b) Two-dimensional map of *Haemophilus influenzae* following protein enrichment by Heparin chromatography. Electrophoresis 18: 1193–1202

Fountoulakis M, Juranville J-F, Röder D, Evers S, Berndt P, Langen H (1998a) Reference map of the low-molecular-mass proteins of *Haemophilus influenzae*. Electrophoresis 19: 1819–1827

Fountoulakis M, Langen H, Gray C, Takács B (1998b) Enrichment and purification of proteins of *Haemophilus influenzae* by chromatofocusing. J Chromatogr 806: 279–291

Fountoulakis M, Takács B, Langen H (1998c) Two-dimensional map of basic proteins of *Haemophilus influenzae*. Electrophoresis 19: 761–766

Fountoulakis M, Cairns N, Lubec G (1999a) Increased levels of 14-3-3 protein forms gamma and epsilon in the brain of patients with Down syndrome and Alzheimer's disease. J Neural Transm [Suppl 57]: 323–335

Fountoulakis M, Schuller E, Hardmeier R, Berndt P, Lubec G (1999b) Rat brain proteins: Two-dimensional protein database and variations in the expression level. Electrophoresis 20: 3572–3579

Fountoulakis M, Takács M-F Berndt P, Langen H, Takács B (1999c) Enrichment of low-abundance proteins of *Escherichia coli* by hydroxyapatite chromatography. Electrophoresis 20: 2181–2195

Fountoulakis M, Takács M-F, Takács B (1999d) Enrichment of low-abundance proteins of *Haemophilus influenzae* by hydrophobic interaction chromatography. J Chromatogr 833: 157–168

Fountoulakis M, Berndt P, Boelsterli UA, Crameri F, Winter M, Albertini S, Suter L (2000a) Two-dimensional database of mouse liver proteins. Changes in hepatic protein levels following treatment with acetaminophen or its non-toxic regioisomer 3-acetamidophenol. Electrophoresis 21: 2148–2161

Fountoulakis M, Hardmeier R, Höger H, Lubec G (2000b) Post mortem changes in the level of brain proteins. Exp Neurology 167: 86–94

Fountoulakis M, Hardmeier R, Schuller E, Lubec G (2000c) Differences in the protein level between neonatal and adult brain. Electrophoresis 21: 673–678

Fountoulakis M, Juranville J-F, Berndt P, Langen H, Suter L (2001) Two-dimensional database of mouse liver proteins. An update. Electrophoresis 22: 1747–1763

Fountoulakis M, Berndt P, Langen H, Suter L (2002) The rat liver mitochondrial proteins. Electrophoresis (in press)

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037–1053

Greber S, Lubec G, Cairns N, Fountoulakis M (1999) Decreased synaptosomal associated protein 25 levels in the brain of patients with Down syndrome and Alzheimer's disease. Electrophoresis 20: 928–934

Gyri SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc Natl Acad Sci USA 97: 9390–9395

Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C (1993) Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci USA 90: 5011–5015

Herbert BR, Molloy MP, Gooley AA, Walsh BJ, Bryson WG, Williams KL (1998) Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis 19: 845–851

Jungblut PR, Zimny-Arndt U, Zeindl-Eberhart E, Stulik J, Koupilova K, Pleissner K-P, Otto A, Müller E-C, Sokolowska-Köhler W, Grabher G, Stöffler G (1999) Proteomics in human disease: cancer, heart and infectious diseases. Electrophoresis 20: 2100–2110

Karlsson K, Cairns N, Lubec G, Fountoulakis M (1999) Enrichment of human brain proteins by Heparin chromatography. Electrophoresis 20: 2970–2976

Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis in mouse tissue. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231–243

Krapfenbauer K, Berger M, Lubec G, Fountoulakis M (2001a) Changes in the brain protein levels following administration of kainic acid. Electrophoresis 22: 2086–2091

Krapfenbauer K, Berger M, Lubec G, Fountoulakis M (2001b) Kainic acid-induced changes in the level of low-abundance brain proteins. Eur J Biochem 268: 3532–3537

Lahm HW, Langen H (2000) Mass spectrometry: a tool for the identification of proteins separated by gels. Electrophoresis 21: 2105–2114

Langen H, Gray C, Röder D, Juranville J-F, Takács B, Fountoulakis M (1997a) From genome to proteome: protein map of *Haemophilus influenzae*. Electrophoresis 18: 1184–1192

Langen H, Röder D, Juranville J-F, Fountoulakis M (1997b) Effect of the protein application mode and the acrylamide concentration on the resolution of protein spots separated by two-dimensional gel electrophoresis. Electrophoresis 18: 2085–2090

Langen H, Berndt P, Röder D, Cairns N, Lubec G, Fountoulakis M (1999) Two-dimensional map of human brain proteins. Electrophoresis 20: 907–916

Langen H, Takács B, Evers S, Berndt P, Lahm HW, Wipf B, Gray C, Fountoulakis M (2000) Two-dimensional map of the proteome of *Haemophilus influenzae.* Electrophoresis 21: 411–429

Lubec G, Labudova O, Cairns N, Berndt P, Langen H, Fountoulakis M (1999a) Reduced aldehyde dehydrogenase levels in the brain of patients with Down syndrome. J Neural Transm [Suppl 57]: 21–40

Lubec G, Labudova O, Cairns N, Fountoulakis M (1999b) Increased glyceraldehyde 3-phosphate dehydrogenase levels in the brain of patients with Down syndrome. Neuroscience Lett 260: 141–145

Lubec G, Nonaka M, Krapfenbauer K, Gratzer M, Cairns N, Fountoulakis M (1999c) Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level. J Neural Transm [Suppl 57]: 161–177

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007–4021

Pandey A, Mann M (2000) Proteomics to study genes and genomes. Nature 405: 837–846

Qiu Y, Benet LZ, Burlingame AL (1998) Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J Biol Chem 273: 17940–17953

Rabilloud T, Blisnick T, Heller M, Lunche S, Aebersold R, Lunardi J, Braun-Breton C (1999) Analysis of membrane proteins by two-dimensional electrophoresis: comparison of the proteins extracted from normal or Plasmodium falciparum-infected erythrocyte ghosts. Electrophoresis 20: 3603–3610

Righetti PG, Castagna A, Herbert B (2001) Prefractionation techniques in proteome analysis. Anal Chem 73: 321–326

Rohlff C (2000) Proteomics in molecular medicine: Applications in central nervous systems disorders. Electrophoresis 21: 1227–1234

Steiner S, Anderson NL (2000) Expression profiling in toxicology-potentials and limitations. Toxicol Lett 112–113: 467–471

Wang JH, Hewick RM (1999) Proteomics in drug discovery. Drug Discovery Today 4: 129–133

Weiss M, Manneberg M, Juranville J-F, Lahm HW, Fountoulakis M (1998) Effect of the hydrolysis method on the determination of amino acid composition of proteins. J Chromatogr 795: 263–275

Yoo BC, Cairns N, Fountoulakis M, Lubec G (2001a) Synaptsomal-proteins beta-soluble N-ethylmaleimide-sensitive factor attachment protein (beta-SNAP), gamma (gamma-SNAP) and synaptotagmin I in the brain of patients with Down syndrome and Alzheimer's disease. Dement Geriatr Cogn Disord 12: 219–225

Yoo BC, Fountoulakis M, Cairns N, Lubec G (2001b) Changes of voltage-dependent anion channel proteins VDAC1 and VDAC2 brain levels in patients with Alzheimer's disease and Down syndrome. Electrophoresis 22: 172–179

Author's address: Dr. Michael Fountoulakis, F. Hoffmann-La Roche Ltd., Pharmaceutical Research, Genomics Technologies, Buiding 93-444, CH-4070 Basel, Switzerland, E-mail: michael.fountoulakis@roche.com

Received March 5, 2001