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Applications and current challenges of proteomic approaches, focusing on two-dimensional electrophoresis

Review Article

F. G. G. Vercauteren[1,2], **L. Arckens**[2], and **R. Quirion**[1]

[1] Douglas Hospital Research Center, Department of Psychiatry, McGill University, Montréal, Québec, Canada
[2] Laboratory of Neuroplasticity and Neuroproteomics, Katholieke Universiteit Leuven, Leuven, Belgium

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Summary. Since the formulation of the concept of "proteomics" in 1995, a plethora of proteomic technologies have been developed in order to study proteomes of tissues, cells and organelles. The powerful new technologies enabled by proteomic approaches have lead to the application of these methods to an exponentially increasing variety of biological questions for highly complex protein mixtures. Continuous technical optimization allows for an ever-increasing sensitivity of proteomic techniques. In this review, a brief overview of currently available proteomic techniques and their applications is given, followed by a more detailed description of advantages and technical challenges of two-dimensional electrophoresis (2-DE). Some solutions to circumvent currently encountered technical difficulties for 2-DE analyses are proposed.

Keywords: Proteomics – Two-dimensional electrophoresis – Mass spectrometry – Applications – Shortcomings

Abbreviations: ASB-14, Amidosulfobetaine-14; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2-DE, two-dimensional electrophoresis; DIGE, difference gel electrophoresis; HUPO, human proteome organization; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1. New research opportunities offered by proteomics

The proteome of a cell or an organelle provides information about the ensemble of proteins and protein isoforms expressed in that cell or organelle under specific physiological conditions and at a specific time (Wasinger et al., 1995; Wilkins et al., 1996). Proteomic approaches provide several novel possibilities to address biological questions. In fact, the large-scale screening approach of proteomics enables protein expression studies that are impossible to perform using classical molecular biology techniques, in which the expression of only one or a few proteins is studied at a time. Globally, proteomic approaches have three major advantages over more traditional approaches. First, these technologies allow for the analysis of up to thousands of proteins simultaneously, in any tissue or organelle, under any given physiological condition.

Second, prior to proteomic applications, no limitations are set for the proteins analyzed on cellular functions or role in specific signal transduction pathways. Therefore, these studies enable the investigation of proteins and protein populations that are not a priori expected to be linked to any physiological conditions, allowing for the discovery of novel molecular mechanisms, opening novel research avenues. In addition, proteomic studies are not limited to proteins that have already been characterized. They allow for the study of links between physiological conditions and novel proteins, of which thus far only hypothetical amino acid sequences exist, deduced from the nucleic acid sequence of corresponding genes.

Third, the high sensitivity of proteomic technologies allows for these large-scale screening studies utilizing only a minimal amount of protein. For example, while for a typical Western blotting experiment, 40 μg of protein sample is loaded on a gel for the analysis of one or a few proteins, proteomic expression analysis of more than a thousand proteins in a single sample can be performed using as little as 50 μg of protein. The requirement of such a little amount of protein sample for these high-throughput studies is particularly advantageous when the availability of tissues is limited, for example when samples are

obtained from transgenic animals, small brain regions or human tissues.

Because of these unique properties, an overview of expression profiles of thousands of proteins can be established for each sample investigated (Washburn et al., 2001; Taylor et al., 2004; Kislinger et al., 2006). Comparative proteomic studies in which protein expression in diseased and control tissue is quantitatively compared are particularly suitable for the discovery of novel biomarkers of diseases (Fu and Van Eyk, 2006). For example, the application of gel-based and other proteomic approaches in research on complex protein mixtures of brain extracts is providing large data sets on protein expression under normal and pathological conditions (Vercauteren et al., 2004). In addition, by investigating tissues or cell cultures before and after drug treatment, comparative proteomic studies can be used for drug target identification, drug interaction studies and studies on mechanisms of action for a variety of compounds (Rothe et al., 2006). Finally, time point proteomic studies permit follow-up studies of changes in expression levels of hundreds of proteins at different time points before and after drug treatment, or after various physical conditions (Geho et al., 2005, Van den Bergh et al., 2006). Data obtained from proteomic analyses can subsequently be combined with more traditional approaches such as Western blotting and confocal microscopy for biological validation and functional studies.

2. A plethora of proteomic techniques and applications

A wide range of proteomic approaches is available. Gel-based applications include one-dimensional and two-dimensional polyacrylamide gel electrophoresis (2-DE; Vercauteren et al., 2004; Van den Bergh and Arckens, 2005). Several gel-free high-throughput screening technologies for protein analysis are equally available, including multidimensional protein identification technology (MudPIT; Florens and Washburn, 2006), isotope-coded affinity tag (ICAT; Gygi et al., 1999); isobaric tagging for relative and absolute quantitation (iTRAQ; Ross et al., 2004), yeast two-hybrid and reverse two-hybrid assays (Vidal and Legrain, 1999), protein microarrays (Cutler, 2003; Melton, 2004), phage-display antibody libraries (Sidhu et al., 2003) and HysTag reagent (Olsen et al., 2004).

The choice of a given proteomic approach should depend on the type of biological question asked, since each proteomic technology is characterized by specific applications, technical advantages and limitations. Proteomic technologies allow for a wide variety of applications. In general, proteomic approaches can be used a) for proteome profiling, b) for comparative expression analysis of two or more protein samples, c) for the localization and identification of posttranslational modifications, and d) for the study of protein–protein interactions. Shotgun proteomics (Washburn et al., 2001), 1-DE and 2-DE (Klose et al., 2002) as well as protein microarrays (Melton, 2004) are being applied to obtain overviews of protein expression in tissues, cells and organelles. For quantitative comparison studies of two or more protein samples, 2-DE (Unlu et al., 1997), protein microarrays (Ren et al., 2006), HysTag (Olsen et al., 2004) and stable isotope labelling (Gygi et al., 1999; Stewart et al., 2001; Krijgsveld et al., 2003; Sebastiano et al., 2003; Ross et al., 2004) are being used. Posttranslational modifications of hundreds of proteins can be screened simultaneously by means of 2-DE (Aksenov et al., 2001) as well as by mass spectrometry-based applications (Unlu et al., 1997; Mann and Jensen, 2003; Reinders and Sickmann, 2005; Wuhrer et al., 2005). Finally, yeast two-hybrid, reverse two-hybrid assays (Uetz et al., 2000), phage display (Sidhu et al., 2003) and mass spectrometry (Gavin et al., 2002; Ho et al., 2002) allow for large-scale screening of protein–protein interactions in complex mixtures. In combination with affinity purification techniques, 1-DE and 2-DE (Hedman et al., 2006), MudPIT and protein microarrays can equally be used to identify protein interaction partners.

3. Current challenges of proteomic analyses

Despite major advances in the development of proteomic technologies, various methodological challenges are still encountered in proteomic analyses. These difficulties are mainly due to the origin and complexity of the protein extract, physical and chemical properties of certain proteins, and instrumental limitations. For example, the number of proteins expressed in a cell at any given time point is estimated to largely exceed 100000, rendering protein extracts extremely complex. In addition to the presence of the high number of gene transcripts in a cell, most proteins are present in a number of isoforms, due to differential splicing and hundreds of possible posttranslational protein modifications to which a protein may be subject (http://prowl.rockefeller.edu/aainfo/deltamassv2.html). Moreover, various gene products, including microRNA (Taganov et al., 2006), as well as epigenetic factors influence the expression levels of genes and their transcripts (Strohman, 1994). Hence, the proteome and subproteomes of any living cell are highly dynamic and of unknown complexity, rendering the characterization of proteomes

a formidable challenge. Accordingly, the complete mapping of the proteome of any cell type and for any species is far more complex than of the corresponding genome, and is yet to be achieved.

While the concentration range of proteins in a given sample may exceed 10 orders of magnitude (Anderson and Anderson, 2002), currently available proteomic approaches are estimated to focus on the 30% most abundant proteins in an extract. Indeed, highly abundant proteins such as cytoskeletal and metabolic proteins tend to interfere with proteomic analyses by masking proteins with lower copy numbers. In order to increase the likelihood to detect the expression of less abundant proteins, a variety of protein separation techniques are being applied to reduce the complexity of a sample prior to proteome analysis. Because of their unique amino acid composition and sequence, as well as posttranslational modifications, all protein isoforms have a unique profile of physical and chemical properties, including molecular weight, size, shape, ionic charge, relative hydrophobicity, biological affinity as well as compartmentalization. These properties allow for the separation of proteins from a given mixture (Table 1). For example, proteins with an isoelectric point or hydrofobicity score within a specific range may be selected for proteomic analysis. For the study of serum proteins, abundant proteins such as albumin, IgG and transferrin may be removed prior to proteomic analysis using affinity chromatography (Fountoulakis et al., 2004; Bjorhall et al., 2005; Righetti et al., 2006). Due to compartmentalization of most proteins, organelle purification using differential centrifugation and/or sucrose gradient centrifugation may equally be used during sample preparation in order to remove abundant proteins from other organelles (Foster et al., 2006; Vercauteren et al., 2006). An advantage of selecting protein populations for proteomic analysis is that it substantially decreases the complexity of extracts and thus increases the likelihood of identifying proteins with a lower copy number (Fountoulakis and Juranville, 2003; Brunet et al., 2003; Yates et al., 2005; Kislinger et al., 2006).

Table 1. Commonly used techniques for protein separation and analysis, as well as the respective protein properties on which these techniques are based

Protein separation technique	Physical or chemical property
native gel electrophoresis	molecular weight, charge
SDS-PAGE	molecular weight
Isoelectric focusing	charge
chromatofocusing	charge
ion exchange chromatography	charge
gel filtration chromatography	molecular weight, size
ammonium sulfate precipitation	hydrophobicity
hydrophobic interaction chromatography	hydrophobicity
reverse phase chromatography	hydrophobicity
affinity chromatography	biological affinity
ultracentrifugation	compartmentalization

However, it should be noted that each additional protein purification step inadvertently leads to random loss of protein species (Yocum et al., 2005). Taking into account the high sensitivity of proteomic approaches, additional steps in sample preparation may hence be deleterious for quantitative comparative expression studies. Moreover, additional sample manipulation increases proteolysis and alters posttranslational modifications. For the establishment of global proteome maps, random protein loss during sample preparation is a significant limiting factor. For example, when shotgun analysis is repeated on the same protein extract, protein loss as well as limitations in mass spectrometric detection usually reduces the average of overlap of identified protein species between the shotgun experiments to 60%. Therefore, a compromise between improved sample purity and complete protein representation must be a target for each proteomic analysis.

4. Advantages and technical limitations of two-dimensional electrophoresis

Each proteomic application currently available presents characteristic technical limitations. Below, we focus on technical advantages and challenges currently encountered for two-dimensional electrophoresis (2-DE). During 2-DE, proteins in complex mixtures are electrophoretically separated according to their isoelectric point (pI) and subsequently according to their molecular weight by a sequential combination of isoelectric focusing (IEF) and sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 1). 2-DE has been used for large-scale protein separation since 1975 (Klose, 1975) and allows for purification, identification as well as quantification of proteins. 2-DE analysis provides several types of information about the hundreds of proteins investigated simultaneously, including molecular weight, pI and quantity, as well as of possible posttranslational modifications. There are two ways to study posttranslational modifications by means of 2-DE. First, posttranslational modifications that alter the molecular weight and/or pI of a protein are reflected in a shift in location of the corresponding proteinous spot on the proteomic pattern. Second, in combination with Western blotting, antibodies specific for posttranslational modifications can reveal spots on 2-DE patterns containing proteins with these modifications (Boyd-Kimball et al., 2006). As a consequence, in parallel to genomic microarray studies

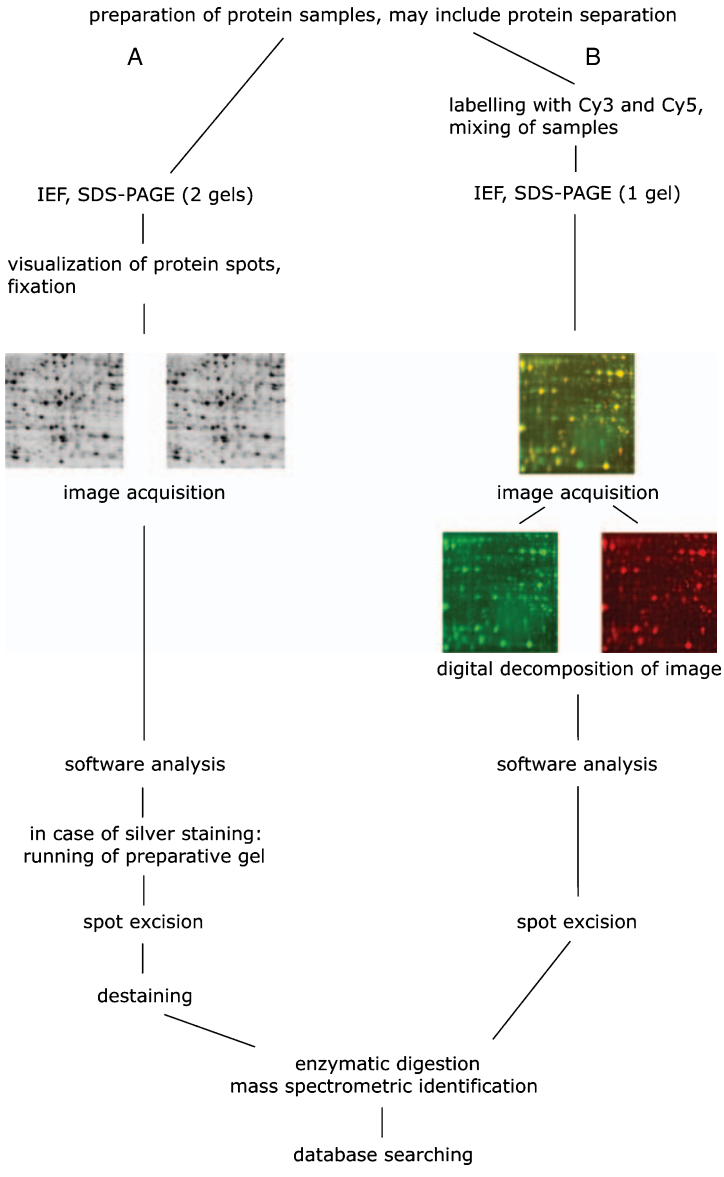

Fig. 1. Diagram of experimental steps in two-dimensional electrophoresis (2-DE) analysis (**A**) and 2-DE Difference Gel Electrophoresis (2-DE DIGE) analysis (**B**) for 2 protein samples. Proteins extracted from cells or tissues are separated by means of isoelectric focusing on immobilized pH gradient strips. In the second dimension of 2-DE, proteins with similar isoelectric points are separated according to their molecular weight on a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. Proteomic patterns visualized using Sypro ruby staining (**A**) or Cy-dyes (**B**) are compared by means of ProFinder (Perkin Elmer; **A**) or DeCyder Differential In-gel Analysis (Amersham Biosciences; **B**) software, in order to identify differentially expressed protein spots on the gels. Spots of interest are excised. Proteins in these spots are enzymatically degraded and subsequently identified by means of mass spectrometry and database searching

(Kozarova et al., 2006; Wang and Cheng, 2006) 2-DE approaches have proven to be particularly useful in screening for molecular changes in healthy and diseased tissues (Lubec et al., 2003; Vercauteren et al., 2004).

However, each of the different steps in 2-DE (Fig. 1) is characterized by specific technical challenges for proteins with a variety of chemical and physical profiles. Commonly encountered technical difficulties for 2-DE protein separation are discussed below and some ways to resolve these issues are suggested.

4.1 Protein solubilization

Protein extraction and solubilization are key steps for proteomic analysis using 2-DE. For example, highly hydrophobic proteins tend to precipitate during IEF. In combination with their low copy number, the insolubility of transmembrane and membrane-associated proteins renders quantitative analysis of these peptides and polypeptides via 2-DE proteomic approaches very challenging (Santoni et al., 2000a). In order to enhance protein solubilization, denaturation and reduction, and to minimize protein–protein interactions, different treatments and conditions are necessary to efficiently solubilise different types of protein extracts (Santoni et al., 2000b; Fountoulakis and Takacs, 2001; Tastet et al., 2003; Taylor and Pfeiffer, 2003; Twine et al., 2005; Zahedi et al., 2005). Mixtures of chaotropes, surfactants and reducing agents are applied during the first and second dimensions of 2-DE, creating denaturing conditions

Table 2. Solubilization compounds for unfolding and denaturation of proteins and breaking of protein–protein interactions during 2-DE experiments

Compound	Suggested concentration	Function, mode of action
urea	8–9.8 M	chaotropes: disrupt hydrogen bonds
thiourea	2 M	
DTE/DTT	40 mM	reducing agents: break intra- and intermolecular
iodoacetamide	2% w/v	disulfide bonds
tributylphosphine[a]	2 mM	
SDS	20%	anionic detergent: blocks hydrophobic interaction
16-BAC[b,c]	7.5% w/v	cationic detergent
CHAPS	4%	zwitterionic detergents
ASB-14, ASB-16, ASB-C8O[d,e]	$\geq$2%	
Zwittergent[f]	2%	
L-α-lysophosphatidyl-choline[f]	1%	
decanoyl-N-methylglucamide[f,g]	1%	nonionic detergents
Nonidet P-40[h]	0.5–2%	

DTE dithioerythritol; *DTT* dithiothreitol; *SDS* sodium dodecyl sulfate; *16-BAC* benzyldimethyl-n-hexadecylammonium chloride; *CHAPS* 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; *ASB* amidosulfobetaine

[a] Ferrari et al. (2006)
[b] Coughenour et al. (2004)
[c] Bierczynska-Krzysik et al. (2006)
[d] Molloy et al. (1999)
[e] Tastet et al. (2003)
[f] Churchward et al. (2005)
[g] Poole and Halestrap (1988)
[h] Collins et al. (1990)

(Table 2). During IEF, protein solubility is enhanced by carrier ampholytes or IPG buffers, which reduce charge–charge interactions. SDS used during the second dimension unfolds proteins further by disrupting hydrogen bonds and by blocking hydrophobic interactions. Although compatibility of highly hydrophobic protein species and proteomic analyses remains problematic and non-gel-based approaches such as ICAT (Gygi et al., 1999) and liquid chromatography-mass spectrometry (LC-MS; Gygi and Aebersold, 2000) may be more suitable for the analysis of these proteins, recent technical improvements render their study using 2-DE more feasible (Bierczynska-Krzysik et al., 2006, Helling et al., 2006). In general, ideal extraction and solubilization conditions need to be determined empirically for each type of sample investigated, and for each type of proteomic approach used.

4.2 Removal of contaminants

Contaminants such as ionic detergents, small ionic molecules, salts, nucleic acids, polysaccharides, phospholipids and phenolic compounds, present in protein extracts, often interfere with protein migration during IEF as well as during SDS-PAGE. The presence of these contaminants results in streaking on the 2-DE patterns and thus limits the number of proteins that can be analyzed. These contaminants can be removed by means of dialysis, solvent extraction, gel filtration and centrifugation.

4.3 Isoelectric focusing

During IEF, proteins are separated according to their isoelectric point. Under the influence of an electric field, each protein migrates through the pH gradient of a gel until it reaches its pI. Until recently, a mixture of carrier ampholytes was commonly used to create the pH gradient for IEF. However, these carrier ampholytes-created pH gradients show certain limitations. Problems occurring with these gradients are so-called *cathode drifting* as well as pH flattening near the anode. As a consequence, protein separation resolution is limited for highly basic or highly acidic proteins. In addition, some ampholyte molecules tend to bind certain proteins, and hence interfere with the focusing of these proteins. Finally, reproducibility of IEF using ampholytes is often reduced because of batch-to-batch variability.

In order to circumvent the technical difficulties in using ampholytes, commercially available immobilized pI gradients are being used more often. In immobilized pI gradient gels (immobilized pH gradient or IPG strips), the pH gradient is created by acryl amido buffers containing acryl amide polymers linked to acidic or basic buffering groups. Covalent immobilization of the buffering groups inside the polyacrylamide gel prevents drifting of the pH gradient under the influence of an electric current, increasing the resolution as well as the reproducibility of 2-DE patterns.

Interference with IEF results in horizontal streaking on 2-DE patterns. The resolution of the protein separation during IEF can be increased by applying a stronger electric field, up to 5000 V, during an extended period of time (e.g. 10 h). In general, the main difficulties encountered during IEF occur for the separation of highly hydrophobic proteins.

Finally, a large number of protein spots on 2-DE patterns contain several proteins with a similar pI. However, reducing the slope of the pH gradient can significantly increase the resolution of protein separation. A pH gradi-

Table 3. Commonly used protein staining methods for 2-DE analysis

Staining method	Sensitivity	Reversible	Ms compatibility	Straightforward protocol	Highly reproducible	Linearity: order of magnitude
silver	1 ng	−	−	−	±	1
coomassie	100 ng	+	+	+	+	2
coomassie R250	50 ng	+	+	+	+	2
colloidal coomassie	10 ng	+	+	+	+	2
sypro ruby	1 ng	+	+	+	+	>3
Cy-dyes	125 pg	+	+	+	+	4
deep purple	125 pg	+	+	+	+	>4

ent with a narrower range allows zooming into different proteins with the same molecular weight. This principle is used for so-called zoom-gels, allowing for high-resolution separation of spots containing proteins with the same molecular weight but slightly different pI.

4.4 SDS-PAGE

During the second dimension of 2-DE, proteins with similar pI are separated according to their molecular weight by means of SDS-PAGE. Factors that influence protein migration during SDS-PAGE include the strength of the electric field, relative hydrophobicity of the samples, size and shape of the molecules, and ionic strength and temperature of the electrophoresis buffer. For spots on 2-DE patterns containing more than one protein with similar molecular weight, protein separation may be improved by changing the percentage of polyacrylamide and by using gradient gels (Fountoulakis et al., 1998). However, SDS-PAGE separation of high molecular weight proteins as well as very low molecular weight ones is still rather challenging. The applicability of 2-DE analysis generally remains limited to proteins with molecular weight between approximately 10 and 120 kDa. This limitation excludes most neuropeptides, which are of great interest in brain function and neuropharmacology, and non-gel-based methods may prove more appropriate for the analysis of small proteins (Baggerman et al., 2004).

4.5 Visualisation of 2-DE proteomic patterns

Protein spots on 2-DE patterns can be visualized by a variety of protein staining techniques, each with specific technical aspects, sensitivity, linear range for quantitation, reproducibility and compatibility with mass spectrometric analysis (Table 3). The major challenge for protein visualization in 2-DE is the compatibility of sensitive protein staining methods with mass spectrometric analysis. Among the most commonly used protein staining methods, Coomassie staining (which is characterized by a straightforward and reversible staining protocol, a sensitivity of up to 30 ng and compatibility with mass spectrometry) is mainly used for preparative gels for mass spectrometric protein identification. For a higher sensitivity of protein spot staining, the originally used silver staining is incompatible with mass spectrometric analysis, due to fixation steps in the staining protocol.

Therefore, several fluorescent staining methods have been developed for the visualization of 2-DE patterns, including sypro stainings and Cy-dyes. Although sypro ruby (Berggren et al., 2000) and silver staining have a comparable sensitivity, sypro ruby staining allows for a much higher reproducibility, a significantly wider dynamic range, less false-positive staining, and is compatible with mass spectrometric analysis. In addition, sypro ruby allows for the detection of lipoproteins, glycoproteins, metalloproteins, calcium-binding proteins, fibrillar proteins and low molecular weight proteins that are less ''stainable'' using other methods. The recently developed Flamingo stain (Bio-Rad) may prove even more sensitive.

Proteins can also be fluorescently labelled with Cy2™, Cy3 or Cy5 prior to 2-DE (Fig. 1; Unlu et al., 1997). Cy-dyes are cyanine dyes carrying an N-hydroxysuccinimidyl ester reactive group that covalently binds the ε-amino group of lysine residues in proteins. Staining with these dyes is more sensitive than silver staining (Table 3), giving a linear response to protein concentrations of up to four orders of magnitude. In addition, no fixation or destaining steps are required during DIGE analysis (Fig. 1), reducing protein loss from the gels. Cy-dyes have the same charge, a very similar mass and are relatively pH-insensitive between pH 3 and 10. Therefore, they do not influence significantly the relative migration of the labelled proteins through the gel (Unlu et al., 1997; Gharbi et al., 2002).

During Difference Gel Electrophoresis (2-DE DIGE; Van den Bergh and Arckens, 2004, 2005), proteins in each

of up to 3 samples can be labelled with one of these fluorescent dyes, and the differentially labelled samples can be mixed and loaded together on one single gel, allowing the quantitative comparative analysis of 3 samples using a single gel. Hence, a major advantage of this technique is a significant reduction in inter-gel variability, facilitating spot identification and matching during software analysis, thus increasing the number of analyzable spots.

The binding of Cy-dyes with lysine residues has an implication for the compatibility of Cy-dyes with subsequent mass spectrometric protein identification. Typical lysine content of a protein is about 7%. Since enzymatic cleavage by trypsin requires unlabelled lysine residues, a minimal labelling approach must be applied to ensure mass spectrometry compatibility (Unlu et al., 1997; Tonge et al., 2001). During minimal labelling, about 1 in 5 protein molecules are labelled, thereby giving a statistical probability that each labelled protein has only one dye molecule attached. Additional advantages of minimal labelling, in comparison with labelling all lysines, include a reduced weight as well as a reduced hydrophobicity of the protein-dye complex, resulting in increased solubility and reduced streaking during the second dimension.

Overall, the major advantages of 2-D DIGE are the high sensitivity and linearity of its dyes, its straightforward protocol (Fig. 1) as well as its significant reduction of inter-gel variability, increasing the possibility to unambiguously identify biological variability, and reducing bias from experimental variation. Moreover, the use of a pooled internal standard, loaded together with the control and experimental samples, increases quantification accuracy and statistical confidence (Alban et al., 2003).

4.6 Software analysis of 2-DE patterns

After visualization of proteomic spot patterns, 2-DE gels are scanned and analyzed by means of specialized software. Commonly used commercial softwares include DeCyder Differential In-gel Analysis (http://www.amershambiosciences.com), ProFinder (http://www.perkinelmer.com), Phoretix 2D Advanced (http://www.phoretix.com), Melanie 4 (http://ca.expasy.org/melanie), PDQuest (http://www.proteomeworks.bio-rad.com/html/pdquest.html), and ImageMaster 2D Elite (http://www.imsupport.com). These softwares are used for spot detection, spot matching on the different gels of a gel set, aligning spot patterns and comparing spot intensities and spot surfaces on several gels, as well as automated spot picking. Despite continuous optimization of algorithms used by these software, a time-consuming visual verification remains necessary for all steps of software analyses of 2-DE patterns.

4.7 Protein identification

Great progress has recently been made in the sensitivity of mass spectrometry analysis (Domon and Aebersold, 2006), increasing significantly the applicability of proteomic technologies (Lahm and Langen, 2000). Thanks to the development of electrospray ionization (Henzel et al., 1993) and new mass analyzers, and to their continuously increasing sensitivity, mass spectrometry-based strategies have replaced Edman degradation for the identification of the amino acid sequences. Peptide mass fingerprinting (PMF; Henzel et al., 1993) and LC-MS/MS (Mann and Wilm, 1994; Yates et al., 1995) are commonly used for protein identification on two-dimensional proteomic patterns. Mass spectrometry-based methods are equally being used for determining the type and location of posttranslational modifications (Mann and Jensen, 2003).

Some major challenges in mass spectrometric analysis remain the identification of proteins and peptides with labile posttranslational modifications such as glycosylation and sulfatation, as well as low molecular weight proteins and peptides. Enzymatic digestion of low molecular weight proteins and peptides yields only few peptides that are masked during mass spectrometric analysis by fragments originating from large and abundant proteins, in the case of 1D and non-gel-based proteomic applications. In the case of neuropeptides, mixtures of ion types are generated during tryptic digestion, in contrast to normal tryptic peptides which lead to y-type ions in mass spectrometric analysis. The generation of ion type mixtures hampers mass spectrometric identification of these peptides (Baggerman et al., 2004).

Although mass analyzers can be used to identify proteins in femtomolar range, many protein spots that can be visualized on 2-DE patterns thanks to the increasing sensitivity of staining methods, contain protein quantities that are too small for identification using presently available mass analyzers. Moreover, the concentration range spanned by proteins expressed in a cell largely exceeds the dynamic range of any analytical method or instrument. However, mass accuracy, resolving power, sensitivity, and dynamic range of mass analyzers are improving continuously, and tandem MS analysis now offers a solution for mass spectrometric identification of many of these protein species.

Finally, mass spectrometric protein identification depends on the availability of the corresponding nucleic acid and/or amino acid sequences in databases. Despite the

incompleteness of presently existing gene and protein databases, the recent completion of sequence databases for several species by genomic studies (Goffeau et al., 1996; The *C. elegans* Sequencing Consortium, 1998; Venter et al., 2001; Waterston et al., 2002; Gibbs et al., 2004) has greatly increased the likelihood of identifying proteins using mass spectrometry. The success rate of mass spectrometric protein identification has now increased to about 80%.

4.8 Database searching

Recent advances in database searching (Pandey and Mann, 2000; Aebersold and Mann, 2003) as well as the development of bioinformatics tools allow for accurate protein identification and analysis of complex data sets from proteomic studies (reviewed in Vercauteren et al., 2004). While the amount of data generated by proteomic and other molecular biology analyses increases exponentially, bioinformatics and data mining tools for storage and processing of these data (Buckingham, 2003; Orchard et al., 2004; Quackenbush, 2004) still are being developed.

Taken together, the technical difficulty of 2-DE experiments, the limited possibilities of automation and the highly time-consuming data analysis, are currently limiting factors for the use of 2-DE proteomic approaches, and limit the number of biological replicates analyzable by 2-DE. Further research to enhance the compatibility of 2-DE with highly hydrophobic and highly basic or acidic proteins is also needed.

5. Concluding remarks

In conclusion, despite persisting technical challenges, 2-DE remains a very useful method for display and quantification of a majority of proteins in biological samples. Its technologies are robust and increasingly reproducible. The sensitivity and applicability of presently available proteomic technologies are improving continuously, and for many types of biological questions, specific proteomic approaches are currently available. International initiatives by the Human Proteome Organization (HUPO), including the Proteome Standard Initiative (PSI; Hermjakob et al., 2004), are working towards standardization of methodology, data storage and processing for 2-DE and other proteomic technologies. Finally, standardization of experimental design and data representation (Hunt et al., 2005; Wilkins et al., 2006) will greatly facilitate interpretation of results obtained from different proteomic experiments and different research groups.

Proteomic studies complemented by genomics and more traditional molecular biology techniques are contributing significantly to build complete proteome maps for cells and organelles, under both normal and pathological conditions. The valuable information provided in qualitative and quantitative proteome maps will enable further identification of mechanisms of diseases, as well as those underlying drug actions, and may contribute to the development of more effective drug treatments.

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Authors' address: Rémi Quirion PhD, FRSC, CQ, Scientific Director, Douglas Hospital Research Center, Institute of Neuroscience, McGill University, Mental Health and Addiction, 6875 LaSalle Boulevard, Montréal, Que., Canada H4H 1R3,

Fax: +1-514-762-3034, E-mail: remi.quirion@douglas.mcgill.ca