Technique Review

The untiring search for the most complete proteome representation: reviewing the methods

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Advance Access publication date 29 May 2008

Abstract

Proteomic research has proved valuable for understanding the molecular mechanisms of biological processes, as well as in the search for biomarkers for a variety of diseases which lack a molecular diagnostic. While several new approaches are being developed, two-dimensional (2-DE) gel electrophoresis is still one of the most commonly used techniques, despite its many limitations. However, for biomarker research, 2-DE gel electrophoresis alone does not fulfill the necessary pre-requisites. If such a technique is utilized exclusively, a great part of a given proteome remains unseen. Therefore, very precise and sensitive techniques are needed. Here, we present a brief review of known methodologies that try to overcome the limitations of conventional proteome analysis as well as their respective advantages and limitations.

Keywords: two-dimensional gel electrophoresis; 2D; 2DE; 2-DE; proteome; proteomics; shotgun; narrow-range; NEPHGE; pre-fractionation; HPLC; depletion; DIGE; protein arrays; low-expressed proteins; high-expressed proteins

PROTEOMICS

The proteome, defined by Wilkins et al. [1] in the 1990s as ‘the total protein complement of a genome’, has contributed to the emergence of the discipline we now call proteomics, which includes protein-expression profiling, the comparison of protein

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profiles [2, 3], protein–protein interaction analysis [4, 5] and 3D structure determination [6, 7].

Proteomics, which continues to grow, uses different approaches to reveal a total protein profile expressed by a cell or tissue (called ‘proteome’), with two-dimensional electrophoresis (2-DE) being the main approach. Since 1999, shotgun peptide sequencing in combination with stable isotope methods has been considered a powerful technique.

The search for the best method for representing proteomes is however still on-going, with 2-DE and shotgun peptide sequencing being continually improved.

THE CONTINUOUS SEARCH FOR BETTER PROTEOME CHARACTERIZATION USING 2-DE

Despite all the new technologies, the most commonly used approach to reveal the proteome of cells, tissues or organisms remains 2-DE [2], a powerful technique that permits the separation of thousands of proteins (also called ‘spots’) in a single experiment followed by a mass spectrometry (MS) analysis to identify the proteins. While 2-DE has formed the basis of Proteomics, and despite all its powerful qualities, it still has major drawbacks; it excludes both the smallest and the largest proteins, those which are extremely acidic and those which are extremely basic [8]. Furthermore, proteins such as transcription factors and low-expressed proteins that could be disease-associated proteins or drug targets are not detected due to the low sensitivity of the system [9, 10]. The same is true for proteins with low values of codon-bias index or codon-adaptation index (Codon usage bias is the probability that only one of several possible codons will be used: this probability can be calculated as an index (codon-bias index or codon-adaptation index) considering the whole genome and can be a tool to predict the protein abundance inside the cell) [11]. It is estimated that the proteome of a cell contains at least 10 000–30 000 different types of proteins, but only 2000–10 000 can be visualized on a silver stained 2-DE gel, and not all of these are present at sufficient levels for MS identification [12].

The limits in the detection of extremely basic or acidic proteins can be partially counteracted by using narrow-range immobilized pH gradient (IPG) strips. The problems in finding proteins with a very high or very low molecular weight can be offset in part by using polyacrylamide gels of different concentrations. However, there are still unsolved problems concerning the low capacity for detecting low-abundance proteins as well as the resolution and identification of membrane proteins (MPs) by 2-DE.

Low-expressed proteins and MPs in 2-DE analysis

One way to better detect the low-expressed proteins, such as transcription factors, which are present at ∼10 copies per cell is to increase the quantity of protein loaded onto the gel, mainly after the introduction of IPG isoelectric focusing (IEF), which allows a greater loading capacity in a single gel [13]. However, higher amounts of protein loaded onto the gel can result in poorly resolved 2-DE profiles: spots of high-abundance proteins, such as proteins of the cellular structure that are present at ∼1 000 000 copies per cell, overlay the spots of less abundant proteins. Revealing the low-expressed proteins is one of the great proteomics challenges because many high-abundance proteins can be found in proteomic analysis of different organisms and tissues. As an example, consider the identification of the same set of proteins belonging to the family of Heat Shock Proteins (HSPs) in comparative proteomics studies of the bacterial proteome [14], in rice stress response against bacteria [15], in the fungus Penicillium expansum in the presence of borate [16], in lymph node metastasis in colorectal cancer [17] and in Alzheimer’s disease [18]. The presence of HSPs in these situations is obvious [19]. However, many low-expressed proteins considered as determinant protein targets in cell or tissue processes are part of the ‘unseen proteome’ [20]. Righetti et al. [21] have also interestingly described proteomes as ‘oligarchic’ because ‘a few proteins dominate the landscape and often obliterate the signal of the rare ones’. The above cited ‘rare proteins’ are the low-expressed proteins such as transcription factors and protein hormones, important players in the proteome. Using traditional 2-DE, the most abundant proteins hide the low-expressed ones, preventing their identification.

MPs are also an important protein class that plays a critical role in cellular processes and thus accounts for a large percentage of drug targets [22]. The poor solubility of MPs in aqueous solutions remains a considerable challenge. The use of different protein extraction buffers combining different kinds of detergents can improve the sensitivity and resolution of the 2-DE profile. Detergents initially employed in
2-DE to solubilize MPs in aqueous solution were Triton X-100 and Nonidet P-40 until the emergence of the sulfobetain CHAPS, which has become the most used detergent [reviewed in 23]. In 1998, another class of more polar detergents, the zwitterionic sulfobetaine known as ASB (amidosulfobetain), was synthesized specifically for protein extraction and 2-DE analysis [24] improving the 2-DE MPs resolutions considerably [25].

The poor detection of low-expressed proteins and MPs as well as their poor resolution by 2-DE called for improvements for the traditionally most popular proteomic technique.

**Improvements to overcome the main limitations of 2-DE**

In the 1980s, researchers realized that there was a need to introduce new strategies to overcome the main limitations of 2-DE and to obtain a better characterization of very complex mixtures of proteins expressed within biological systems. In recent years, several methodologies and strategies to overcome the cited limitations of 2-DE have been described.

**Narrow-range IPG strips**

The introduction of IPG strips in 1982 [13] provided reproducibility, higher resolution, higher loading capacity and simplicity to IEF of 2-DE. Since the IPG strip concept allowed the development of any narrow pH gradient, the narrow-range IPG strips (NR-IPG) were created with the aim of increasing the number of detected and identified proteins and, consequently, generate a more complete 2-DE profile. Multiple IEF gels covering many different narrow pH ranges (for example, 3.5–4.5 or 4.5–5.5 or 5.5–6.7 or 4–7 or 6–11 or 7–11) are run and the resulting images are concatenated by overlaying 2-DE profiles. The resulting image allows the visualization of a higher number of proteins since several different proteins can be resolved where previously a single spot was present in the gel [26, 27].

Using NR-IPG, Cordwell et al. [28] obtained a 30% increased resolution in microbial proteome analysis. Furthermore, this work showed that, in combination with MS, this method allows for a better quantitative as well as qualitative identification of proteins. Due to the increased resolution with the use of NR-IPG, Cordwell et al. [28] could load higher protein amounts onto the gel and visualize some low-expressed proteins.

Another advantage obtained using NR-IPG is a more reliable identification of proteins by MS since there are fewer overlapping proteins in the same spot, eliminating the doubles, triplets and multiples common in broad-range 2-DE profiles [29].

The disadvantages of this method are the great number of gels required for the analysis of one sample, and consequently, the amount of sample required, which might be limiting in some cases [27]. Rational exploitation of NR-IPG would involve their use alongside broader range IPG in a first-pass investigation in order to determine which pH region deserves a deeper analysis [30].

Although NR-IPG has overcome certain limitations of the conventional 2-DE with broad-range IPG by allowing the visualization of some low-expressed proteins, several others limitations still remain, such as the detection of very low-expressed proteins and resolution of the proteins present in the very basic or acidic regions of the 2-DE gels [29, 31].

**Non-equilibrium pH gel electrophoresis method**

Klose and Kobalz [32] refined the non-equilibrium pH gel electrophoresis method (NEPHGE), a high-resolution 2-DE-based technique introduced in the 1970s [33] that allows for better resolution of the protein spots compared to the immobiline IPG-strip technique [32].

The NEPHGE IEF gel is a non-equilibrium pH gradient electrophoresis that prevents protein precipitation and in which the pH range is built up by a mixture of carrier-ampholytes. After sample preparation, the first dimension is performed using rod-like gels placed in glass tubes with lengths of 24–48 cm. After IEF is completed, gels are extruded from the glass capillaries, equilibrated to the second dimension buffer conditions, transferred to the SDS-PAGE slab gels and polymerized by addition of agarose buffer solution. The method can separate up to 5000 proteins from large 2-DE gel (24 x 32 cm) and up to 10 000 protein spots from giant 2-DE gels (48 x 32 cm). The main advantages of this technique include good reproducibility of sample preparation, optimum solubilization of MPs and shorter electrophoresis. Moreover, in the large NEPHGE 2-DE gel technique, more protein spots become visible and most of the streaks and stripes, frequently visible in the IPG-strip method can be prevented. Some areas in the protein pattern can be specifically spread out depending on the pI of the proteins of interest in
order to investigate protein variants, protein modifications and proteins up to 250 kDa [34].

The application of NEPHGE in protein studies as an alternative method has diminished due to the difficulties in reproducing the method in different laboratories. Nevertheless, the method was recently successfully applied in proteome investigation of the alterations in the infiltrating ductal carcinomas (IDCA) of the male breast [35].

Nowalk et al. [36] showed some differences in the resolution of proteins between NEPHGE and IPG in their comparative proteome analysis of subcellular fractions of membrane associated and soluble proteins from Borelia burgdorferi. The NEPHGE technique allowed a better resolution and identification of extremely basic proteins compared to the IPG method, but the separation of proteins by IPG did allow a better resolution for protein isoforms.

Subcellular proteome

For eukaryotic cells, a single-step study is not a sufficient tool for the characterization of the complete proteome. An alternative to overcome this limitation lies in analyzing the ‘subcellular proteomes’, which consists primarily in a separation of all organelles and/or macromolecular structures of the cell, using methods such as differential purification, immunoprecipitation or sucrose density gradients, with a subsequent analysis of each organelle using 2-DE and MS or another proteomic technique.

Jung et al. [37] reviewed the application of subcellular proteomes in many different organelles and macromolecular structures including mitochondria, lysosomes, peroxisomes, endoplasmic reticulum, Golgi apparatus and nuclei. In some cases, the nucleus has been divided into multi-protein complexes such as the nuclear matrix, spliceosome, spindle pole and the nuclear pore complex [38–40]. The 2-DE profile of total cell proteins was different when compared with the protein profile of one specific organelle and the 2-DE profile of organelles allowed the identification of more proteins which could not be observed in total protein 2-DE profile. Moreover, the 2-DE profiles of organelles provide an overview of the proteins present specifically in each subcellular compartment.

The subcellular fractionation and purification of organelles reduces the complexity of the proteome, allows the identification and the localization of new proteins and may provide indications of possible roles for these proteins. The main drawback of this procedure is the difficulty to achieve a clean purification of specific subcellular compartments, such as organelles, which makes further experiments to confirm the localization of the protein necessary [41, 42].

Proteome pre-fractionation

Since one of the main purposes of the pre-fractionation of the proteome is to reduce the complexity of the analyzed samples by dissociating the abundant proteins, thus giving the low-expressed proteins a chance to appear, proteome pre-fractionation using a chromatographic method prior to 2-DE can be considered a good strategy to increase proteome coverage. High performance liquid chromatography (HPLC) is a traditional, well-trusted technique for biochemical separation of complex protein mixtures that has not yet received much attention, despite the fact that it offers the potential for further enhanced protein pre-separations that should lead to a corresponding increase in overall detection coverage [43]. Although HPLC’s potential has not been completely explored in proteome analysis, some previous studies have made use of this technique. Lysates of human breast epithelial cell line were pre-fractionated by reverse-phase HPLC (RP-HPLC), with a five-step gradient of increasing acetonitrile concentrations. Subsequently, the RP-HPLC fractions were distinctly separated by 2-DE with optimal reproducibility. The 2-DE fractions’ profiles revealed the detection and identification of many proteins, such as ‘nucleophosmin’, ‘hnRNP C1/C2’ and ‘Nuclear distribution gene C’, interesting components that were not found in the 2-DE total profile (non-fractionated). This method was also successful when applied to rat lung tissue [12].

Görg et al. [44] developed a proteome pre-fractionation with Sephadex IEF prior to 2-DE. Complex sample mixtures can be pre-fractionated in Sephadex gels and after IEF, up to 10 gel fractions alongside the pH gradient are removed with a spatula and directly applied onto the surface of the corresponding narrow pH range IPG strips as the first dimension of 2-DE.

The pre-fractionation method using ion exchange chromatography prior to 2-DE revealed that the denaturant condition associated with the specific fractionation of proteins increases the visualization of low-abundance tumor marker protein related with
prostate-specific antigen in serum of a patient with prostate cancer [45]. Martosella et al. [46] developed effective RP-HPLC conditions for protein fractionation of highly complex and hydrophobic protein mixtures, such as lipid raft proteins, identifying specific integral MPs.

Biofluids analysis

The biofluids proteome analysis approach to look for biomarkers can be an ideal way to improve future clinical medicine, since serum and plasma offer especially promising resources for biomarker discovery and these materials also can be obtained in a non-invasive way. The main challenge in this analysis is to recognize as many proteins as possible, including the very low-abundance proteins [47].

Due to the large dynamic range of the proteins, the proteome analysis of serum is already a task that needs improvement. In the human serum, proteins can be found with a dynamic range greater than 10 orders of magnitude, including the following six most abundant serum proteins: albumin, IgG, IgA, haptoglobin, alpha-1-antitrypsin and transferrin, which together represent ~85% of the total protein mass [48]. Several techniques, gel and non-gel based, have been applied to make the analysis of proteins with low orders of magnitude, which are also present in the biofluids, possible.

One applicable strategy to access the identity of the low-abundance proteins present in the biofluids is to decrease the complexity of the protein samples through the reduction of the amount of the most abundant proteins in the sample, which could mask the presence of the low-abundance proteins. Based on this concept, several methods were developed to decrease the total amount of the high-abundance proteins, including antibody-based affinity methods and centrifugal ultrafiltration [49].

Among these methods is the Multiple Affinity Removal System (Agilent Technologies, Santa Clara, CA, USA) based on the specificity of polyclonal antibodies against the six most abundant serum proteins described above, reaching an albumin depletion of around 99.4%. This system gives reliable performance in the depletion of highly expressed protein and the best performance in not binding the low-abundance proteins to the column [50]. Another method, the Cibacron Blue (CB) column, does not work well considering its specificity in retaining only high-abundance serum proteins. Granger et al. [51] showed that several low-abundance proteins, including the ATP-binding protein, gelsolin and cytokines, which can play important roles in inflammatory pathways, bind to the CB column, removing them from the analysis.

A disadvantage of serum high-abundance proteins depletion resides in the fact that albumin can interact with low-molecular weight proteins, protein fragments and peptides during the depletion method and act as a carrier, binding to molecules as cytokines, hormones and lipoproteins that are depleted as well [52].

Usually, depleted proteins are separated by 2-DE or multidimensional chromatography methods and identified by MS. It has been proven that a good depletion method for the high-abundant proteins from serum and plasma improves the protein resolution in gel-based techniques and permits the enrichment of the low-abundant proteins in the proteomic profile [47, 50, 53]. In addition to the technical strategy used for depletion, the most important step in biofluid analysis is the necessity to optimize the collection and handling of the samples and standardize the analytical methods in a manner that allows comparative data analyses to be performed by other researches of different groups [52].

Some new applications that have been proposed to analyse the low-abundant proteins in biofluids include combining centrifugal ultrafiltration and multidimensional chromatography [49], depletion processes in integrated microcolumn-based systems [54] and different immunoaffinity fractionations [55]. Results show an increased number of identified low-expressed proteins in serum samples and an increased confidence in the peptide identification from 73.6 to 99% [55]. The obtained data confirm that these methods seem to be showing the way towards a more complete alternative for the analysis of the proteome of complex protein samples.

Another available procedure for biofluid protein extraction without protein depletion is protein precipitation. Khan and Packer [56] have compared different protocols for protein precipitation with organic solvents in order to obtain the most representative one for urine samples using 2-DE. For this kind of sample, they have concluded that acetonitrile was the most effective solvent in the sample preparation, with the highest number of spots and best resolution.

For biofluids, single procedures will not be robust enough to analyse the whole pool of interesting biomarkers, but the combination of technologies can
provide the opportunity to look more closely into the complexity of the samples, and the challenge in this specific area is not the search for a single excellent method, but the search for a strategy that can be broadly applied to obtain the most reliable and reproducible data.

Fluorimetric detection methods: difference gel electrophoresis

In comparative proteome studies, images from different gels must be compared to check protein expression differences between samples. Due to the experimental variations between gels, numerous replicates must be generated to give confidence that an observed difference is significant and real, which can demand much time in the image analyses. To overcome this limitation and the limitations of protein quantification and related to protein load quantities, pre-electrophoretic fluorimetric methods have been developed, such as SYPRO [57] and fluorescent 2-DE difference gel electrophoresis (DIGE) [58]. These methods require only a single gel to detect differences between two samples reproducibly, use low quantities of sample, and linearly detect protein expression differences.

The introduction of DIGE has made it possible to detect and quantify minute differences between experimental pairs of samples. In DIGE-based proteomics, the experimental and control samples are labelled with different fluorophores and are run in the same gel, thereby minimizing technical variation, and using small amounts of sample (detection limit is between 150 and 500 pg) [59].

DIGE is based on the pre-electrophoretic labelling of the samples to be compared with distinct fluorescent dyes that are mass and charge matched. The cyanine-derived fluorophores commonly used interact with the amino group of lysine side chains and are named Cy2, Cy3 and Cy5. These three fluorophores allow the labelling of two comparable samples plus an internal standard that is composed of equal amounts of the samples that will be compared. Run gels are digitalized in a fluorescence scanner that reveals the volume of internal standard spots. Also, the spot volumes of each distinct sample are related to the internal standard spot volumes, providing a correspondence between sample and internal standard spots. The relative abundance of the spots in distinct samples is compared, revealing the differentially expressed proteins [60]. This experimental design should permit much more accurate statistical analysis of expression differences [61].

DIGE limitations include the cost of fluorophores, the necessity of special devices for gel analysis, and the inability to excise spots for MS analysis directly without post-staining techniques.

Other approaches

Many other approaches and strategies aiming to overcome the 2-DE limitations have been described. Since the usual 2-DE procedure combining IEF and SDS-PAGE leads to artifacts and irreproducible results due to aggregation and precipitation of proteins and protein-phospholipid complexes, the optimization of solubilization procedures for hydrophobic MPs and the use of continuous free flow electrophoresis (FFE) devices (to fractionate complex protein mixtures by solution-phase IEF) have been successfully implemented in proteome studies [62].

Aiming to obtain a better MPs representation in 2-DE, a method was developed based on the use of organic co-solvents like trifluoroethanol (TFE), which helps to increase the solubility of hydrophobic MPs [63], increasing the extraction efficiency of intrinsic MPs [64]. Additionally, the use of 100 mM sodium carbonate in cell lysis helps to dissociate many of the peripheral proteins from the membrane and results in a greater yield of integral MPs [65].

Many researchers looked for the best buffer for protein extraction, since the different buffers can favour different classes of proteins and improve the 2-DE resolution. Gazzana et al. [66] described the use of two different lysis buffers in sequence followed by a liquid-phase IEF pre-fractionation and separation of protein mixtures at different pH ranges, aiming for a better 2-DE resolution of liver proteins. The combination of CHAPS, the most used zwitterionic detergent in proteome studies, and the aminosulfobetain ASB-14, into a urea/thiourea-based buffer to extract proteins from human brain tissue appears the best option for hydrophobic proteins. Furthermore, 2-DE resolution is improved for acid proteins [25].

PROTEOMIC STUDIES SKIPPING 2-DE

Since 2-DE is not the most complete technique for proteome study, many attempts have been made to develop alternative methods and strategies apart from 2-DE and electrophoretic methods to obtain the best
proteome representation, while retaining the 2-DE’s grade of protein separation.

**Shotgun proteomics**

All the methods and strategies described above are gel based. Trying to overcome the limitations of gel-based proteome analysis, Link et al. [67] have described a revolutionary strategy, known nowadays as shotgun proteomics, that is based on the digestion of a whole proteome with specific enzymes. The resultant peptides are separated using multidimensional chromatographic systems and analysed using tandem-MS, aiming to overcome the limitations of conventional proteomic strategies and to be sensitive enough to identify the diverse classes of proteins [68]. Several algorithms allow automated assignment of MS/MS spectra by matching acquired data with spectra predicted on the basis of protein sequence databases [69]. Shotgun proteomics has been extensively used [70] and may be one of the most powerful tools available to a proteome characterization if combined with stable isotope labelling experiment (SILE) methods such as Isotope-Coded Affinity Tags (ICAT) [71], Global Internal Standard Technology (GIST) [72], Isobaric Tags for Relative and Absolute Quantification (iTRAQ) [73] and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) [74] in order to offer a precise comparison and quantification of peptide sequencing.

Despite the use of shotgun strategies as a powerful tool for global proteome analysis, these methods can be performed according to the research interest. Bledi et al. [75] described a shotgun strategy to detect proteins exposed on the surface of the plasma membrane which consists of applying proteases to whole intact living cells and analysis of the resulting peptide fragments by liquid chromatography followed by MS/MS, providing a satisfactory detailed profile of the MPs.

Chen et al. [76] analysed the distribution of molecular weight (MW), isoelectric point and cellular localization of the eluted proteins from mammalian cancer cell lysate in a shotgun experiment. They showed that 60% of the identified proteins have a pI higher than 7.0, 39% of the identified proteins have a MW higher than 100 kDa and 28% of the identified proteins were MPs. Nunn et al. [77] could identify 4530 proteins from *Salmonella typhimurium* using shotgun from which 46.5% of the identified proteins showed a pI higher than 7.0, overcoming the extremely basic proteins limitation. In that same work, they identified 7.9% of the proteins with a pI between 3 and 4.6, a satisfactory coverage of extremely acid proteins. In both cases, the shotgun data proved the advantages of this method in regard to the main limitations of 2-DE.

It is important to highlight that despite all the benefits of a shotgun method for proteome characterization, there are proteins which can be revealed better using 2-DE than shotgun methods. Thus, a powerful proteomic strategy consists of the combination of 2-DE and shotgun as complementary techniques [78, 79].

**Protein arrays**

The concept of DNA arrays introduced another alternative method to proteomics studies in the 2000s: protein arrays and antibody microarrays. Microarrays are printed with thousands of recombinant antibodies carrying the desired specificities. The generated protein array patterns can be converted into proteomic maps, or molecular fingerprints, revealing the proteome [80]. Despite the fact that protein arrays are a novel technology, there are many published articles proving their applicability in the study of cell surface membrane proteomics [81], the study of the Ankara virus [82] and the study of cytokine expression in human embryonic mesenchymal stem cells [83] as well as articles showing improvements of the protein array method [84], which will be used more in the coming years.

**CONCLUSION**

We have discussed above some of the several proteomics methods and strategies available that aim to reveal the most complete proteome possible. As all methods have their own limitations, it appears difficult to obtain comprehensive information about the whole proteome from a single experiment, even if we consider the powerful analytical methods now available, such as Shotgun Proteomics or stable isotopic applications. The combination of the best proteome solubilization procedure, reproducible techniques of protein separation, a very sensitive analysis of protein identification with trustable protein quantification and a precise statistical interpretation of the data promises to be an effective avenue for proteome research to pursue.
Methodologies for proteome representation

Key Points

- Proteome, the total protein complement of a genome, is valuable for understanding the molecular mechanisms of biological processes through the study of protein expression.
- Two-dimensional gel electrophoresis is the most traditional technique used to study proteomes, despite its limitations.
- We presented a review of known methodologies that aim to overcome these limitations as well as the respective advantages and limitations of the methodologies.

Acknowledgements

The authors would like to thank FAPESP (Fundação de Apoio a Pesquisa do Estado de São Paulo, Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for their support of this work. They thank Benjamin Gaede from the University of Potsdam by the help on the corrections of the manuscript.

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