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Journal of Chromatography B, 849 (2007) 190–202
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Two-dimensional electrophoresis in proteome expression analysis☆

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Received 29 June 2006; accepted 27 November 2006
Available online 22 December 2006

Abstract

Cell proteomes are complex, given they consist of several thousand proteins. Two-dimensional electrophoresis (2DE) is unique not only for its ability to simultaneously separate thousands of proteins but also for detecting post- and co-translational modifications, which cannot be predicted from genome sequences.

This review will describe the protocols applied to prepare 2D gels properly, and analyse and summarise the major challenges for successful proteome analysis using 2DE, i.e. the ability to analyse very alkaline, hydrophobic and/or low or high M_r proteins with high resolution and the ability to detect minor components. Challenges involving sample preparation and solubilisation prior to the first dimension IEF/IPG step will be studied in depth. Sample preparation is crucial in 2DE studies and greatly influences other stages of the technique.

It is the aim of this review not only to describe the challenges and limitations of 2DE but also to suggest the avenues, the evolution, the potential and the future of 2DE in proteomics.

Keywords: Two-dimensional electrophoresis; Sample preparation; Expression proteomics

Contents

1. Introduction

Most activities in living cells are performed by proteins. To study all the proteins expressed by the genome of an organism, it is necessary to analyse the proteome. The proteome is defined as the complete protein complement of a genome [1]. The proteome is like a photographic snapshot of the protein expression at a particular moment and under specific conditions. One can say that there is one particular genome for every given organism or cell, but there is an infinite number of proteomes when referring to protein expression. A comprehensive description of the proteome of an organism not only provides a catalogue of all proteins encoded by the genome but also data on protein expression under defined conditions. Proteomics allows to obtain a quantitative description of protein expression and its changes

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

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1570-0232/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2006.11.049

under the influence of biological perturbations, the occurrence of post-translational modifications and the distribution of specific proteins within the cell [2]. Thus, the complexity of a biological system can be approached in its entirety if proteomics allows a multiplicity of proteins to be studied simultaneously [3].

The proteomes of cells are extremely complex, consisting of several thousand proteins. Because of this complexity, two-dimensional polyacrylamide gel electrophoresis (2DE) has been widely used as the standard protein separation and display method. Usually multiple samples are produced at different stages after stimulation, gene deletion or over-expression, or drug treatment experiments, and separated in a number of 2D gels. The scope of applications extends from drug discovery to diagnostics, therapy, microbiology, biochemistry, etc.

The technology of high-resolution 2DE has been considerably improved, making the method more reliable and reproducible. Image analysis software of these complex spot patterns has been developed to such a degree that non-computer experts can also use it and get reliable results. The potential of the method for a systematic approach to create a protein database of human proteins – human protein index – has been recognised early [4].

Two-dimensional gel electrophoresis separates proteins according to two independent parameters, isoelectric point (p*I*) in the first dimension and molecular mass (M_r) in the second dimension by coupling isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [5]. Proteins separated on 2D gels are visualised by either staining with Coomassie blue dye, silver stains, fluorescent dyes, immunological detection or by radiolabelling and quantifying using densitometers, fluoro- and/or phosphor-imagers. Theoretically, 2DE is capable of resolving upto 10,000 proteins simultaneously, with approximately 2000 proteins being routine, and detecting and quantifying protein amounts of less than 1 ng per spot.

The position of a spot in the 2D map is not the enough information for an exact identification of a protein. For routine analysis, protein spots of interest (e.g. up- or down-regulated proteins) are excised from the 2D gel, digested into fragments by specific proteases and then identified using mass spectrometry (MS) and database mining [6].

Although the limitations of the 2DE approach are well known, i.e. poor solubility of membrane proteins, limited dynamic range and difficulties in displaying and identifying low-abundance proteins, 2DE will remain a powerful and versatile tool, and at least in the immediate future, it is the most commonly used technique in proteome analysis [7–9].

Compared with the 2DE, the amount of sample that can be used with multi-dimensional chromatography (LC/LC–MS/MS) is less restricted, the process is easier to automate and specific classes of proteins, such as very acidic, very basic and membrane proteins, which are difficult to detect by 2DE, may be more readily detected. However, many protein changes that are often readily detected on 2D gels may be difficult or impossible to detect using, for example, the ICAT method, including proteolytic processing and changes in post-translation modifications.

Just as an example of the incredible growth of the field, specific and non-specific journals over the years have published many special issues collecting several hundreds of articles dealing with 2DE and its application in different areas of biological research. New journals, such as *Proteomics*, *Journal of Proteome Research* and *Molecular and Cellular Proteomics*, have appeared on this topic.

Two-dimensional gel electrophoresis will remain as one of the major separation techniques for the next years because its resolution and the advantage of storing the isolated proteins in the gel matrix until further analysis is unrivalled by any other alternative technique.

2. Brief history and evolution of two-dimensional maps

Any single-dimension method cannot resolve more than 80–100 different protein components. Two-dimensional gel electrophoresis exploit a combination of two different single dimension runs. Two-dimensional maps could be prepared by using virtually any combination of 1D methods, but the one that has won universal recognition is that combining a charge (IEF protocol) to a size (SDS-PAGE) fractionation, since this results in a more even distribution of components over the surface of the map. While this combination of separation methods was used at quite an early stage in the development of 2D macro-molecular mapping, it was the elegant work of O'Farrell [5] that really demonstrated the full capabilities of this approach. He was able to resolve and detect about 1100 different proteins from lysed *Escherichia coli* cells on a single 2D map. Apart from the meticulous attention to detail, major reasons for the advancement in the resolution obtained by O'Farrell, compared to early workers, included the use of samples labelled with ^{14}C or ^{35}S to the high specific activity and the use of thin gel slabs for the second dimension, which could dry easily for autoradiography. For the first dimension, O'Farrell adopted gel rods measuring 13 cm in length and 2.5 mm in diameter. The idea was to run samples that were fully denatured in what became known as the "O'Farrell lysis buffer". For the second SDS-PAGE dimension, O'Farrell [5] used the discontinuous buffer system of Läemmli [10]. He went as far as to recognise that some protein losses could occur during the equilibration of the IEF gel prior to running the SDS-PAGE.

Some minor modifications were adopted and they certainly helped in further improving the IEF. By the same token, to overcome the difficulties of separating basic proteins in the first IEF dimension, O'Farrell et al. [11] introduced the technique of non-equilibrium pH gradient isoelectric focusing (NEPHGE), in which the sample was applied at the acidic end of the gel and IEF terminated prior to reaching steady-state conditions.

At the opposite end of the scale, instead of miniaturisation, Anderson and Anderson [12] started thinking of large-scale biology and building instrumentation (called ISO-DALT system) for preparing and running several gels together. This approach greatly enhanced reproducibility and comparison between the resulting protein maps while enabling a large number of samples to be handled in a short time.

Although the power of 2DE as a biochemical separation technique has been well recognised since its introduction, its application, nevertheless, has become particularly significant in the past few years as a result of a number of developments. This new technique replaces the tube gels with gel strips supported by a plastic film backing [13]. Immobilised pH gradients are so precise that they allow excellent correlation between experimentally found and theoretically predicted p*I* values of both proteins and peptides. Methods for the rapid analysis of proteins have been improved. More powerful, less expensive computers and software are now available, allowing routine computerised evaluations of the highly complex 2D patterns. In addition, data on entire genomes for a number of organisms are now available, allowing rapid identification of the genes encoding a protein separated by 2D.

Other applications of 2DE include identification, the study of genetic variation and relationships, the detection of stages in cellular differentiation and studies of growth cycles, the examination of pathological states and diagnosis of disease, cancer research, monitoring of drug action and other studies.

Perhaps a key point for the success of the present 2DE was the introduction of the IPG technique. The main event that made IPGs so powerful was the recognition that a much wider portion of the pH scale could be explored; thus, very acidic pH intervals, as well as very alkaline intervals were described.

3. Basic methodology in 2DE

Today, the first dimension is preferably performed in individual IPG strips laid side by side on a cooling platform, with the sample often adsorbed into the entire strip during rehydration. At the end of the IEF step, the strips have to be interfaced with the second dimension, almost exclusively performed by mass discrimination via saturation with the anionic surfactant SDS. After the equilibration step, the strip is embedded on top of an SDS-PAGE slab, where the 2D run is carried out perpendicular to the 1D migration. The 2D map displayed at the end of these steps is the stained SDS-PAGE slab, where polypeptides are seen, after staining, as spots, each characterised by an individual set of pI/M_r coordinates.

Although the protocols for properly performing 2D maps will be described later, it is worth recalling some important steps, especially those involving sample solubilisation and preparation prior to the first dimension IEF/IPG step.

3.1. Sample preparation

The sample treatment is absolutely essential for good 2D results. The protein composition of a sample must be reflected in the 2D gel pattern; hence, the sample must not be contaminated with foreign proteins. So, the method of sample collection is crucial. It is relatively easy to collect body fluids and lysates from cultured cells because the proteins are evenly distributed. However, when tissue material is studied, the cells to be analysed must be well defined. To avoid protein losses and modifications, sample treatment must be kept to a minimum. The proteins have to be denatured to display them in single conformations. Under native conditions, the proteins may be in several different conformations. This leads to an even more complex 2D pattern, which could not be evaluated.

Due to the great diversity of protein sample types and origins, only general guidelines for sample preparation are provided in this review. The optimal procedure must be determined empirically for each sample type. Ideally, the process will result in the complete solubilisation, disaggregation, denaturation and reduction of the proteins in the sample.

The proteins are extracted with a so-called "lysis buffer". The components of the lysis buffer have to convert all the proteins into a single conformation, prevent different oxidation steps and protein aggregates, get hydrophobic proteins into solution, deactivate proteases and cleave disulfide and hydrogen bonds. A standard lysis buffer contains: high urea concentration to convert proteins into single conformation, to get and keep hydrophobic proteins in solution and avoid protein–protein interactions. With very hydrophobic proteins, such as membrane proteins, another, stronger denaturating chaotrope like thiourea has to be added. Zwitterionic or non-ionic detergent like CHAPS, Triton X-100 or NP-40 must be added to increase the solubility of hydrophobic proteins. Reducing agents like DTT or DTE prevent different oxidation steps. Dedicated pH intervals, called IPG buffers and carrier ampholytes, improve the solubility of proteins. In practice, the use of carrier ampholyte mixtures for wide gradients instead of IPG buffers has shown better results for narrow gradients. The composition of the ampholytes or IPG buffers used must always be the same if they will influence the results.

Proteases may be liberated upon cell disruption. Proteolysis greatly complicates analysis of the 2DE result; thus, the protein sample should be protected from proteolysis during cell disruption and subsequent preparation. Proteases can be inhibited by disrupting the sample directly in strong denaturants such as urea, TCA or SDS [14–16]. Individual protease inhibitors are active only against specific classes of proteases, so it is usually advisable to use a combination of protease inhibitors. Broad-range protease inhibitor "cocktails" are available from a number of commercial sources. For more comprehensive discussions on the inhibition of proteases see refs. [17–20]. One must be aware that some of these agents may interfere with proteomic analysis. For example, phenylmethylsulfonyl fluoride (PMSF) is frequently used to prevent protein degradation during tissue processing. However, residual PMSF in some protein samples may inhibit tryptic digestion needed for further proteome analysis. Likewise, detergents may interfere both with some analytical protein separations and with proteolytic digestions.

Protocols on sample preparation can be obtained from papers describing specific 2DE applications. There are excellent reviews containing detailed protocols for gel preparation and electrophoresis conditions for prospective users of 2DE [21–23]. As an example, the standard protocol for sample preparation without the use of protease inhibitors describes that good solubilisation is achieved by first lyophilising the samples and then pulverising them. Proteins were extracted by suspending the lyophilised tissue immediately in lysis buffer to avoid proteolysis. Proteins were solubilised for 3 h at 30 °C with vigorous shaking. Sonication can be useful for solubilisation; however,

the procedure should be performed on ice. The mixture was centrifuged at 12,000 × *g* for 15 min. Supernatants were either used immediately for electrophoresis or stored at −70 °C.

Special care should be taken in preparing the samples to avoid producing chemical alterations in the proteins that could lead to changes in charges and doubling of spots. Hence, modifications to the protocol should be avoided. Temperatures above 37 °C for the lysis buffer are not recommended, if urea breaks down and the resulting isocyanate would carbamylate the proteins, causing significant changes in their charges [5,24]. Temperatures below 18–16 °C should not be reached either, if the crystallization of urea would result.

Too much salt in the sample disturbs the isoelectric focusing and produces streaky patterns [25,26]. The use of concentrators or dialysis gives good resolution patterns. However, the method involving protein precipitation with acetone and/or TCA and the later resuspension in the same lysis buffer gave the best patterns.

A crude extract can be contaminated with phospholipids and nucleic acids. Nucleic acids are visualised as horizontal streaks in the acidic part of the gel. They can precipitate with the proteins and be removed with DNAse and RNAse treatment. The easiest technique is sonication and/or precipitation. Precipitation is also useful to remove lipids. Precipitation followed by resolubilisation in sample solution is typically used for selective separation of proteins from other contaminants, such as salts and detergents, but rarely gives 100% recoveries. Thus, application of a precipitation step prior to a 2D map may alter the protein profile in the final 2D image. Therefore, such protocols should not be adopted if one aims at obtaining a complete and accurate profiling of all the proteins in the sample analysed. Precipitation followed by resuspension can also be used as a sample concentration step from dilute sources, although in this latter case Centricon tubings might be preferred, since during the centrifugation step both concentration and desalting occur simultaneously.

The precipitation methods have been recently evaluated by Jiang et al. [27], who have reported that TCA and acetone precipitation, as well as ultrafiltration delivered a higher protein recovery compared to ammonium sulfate and chloroform/methanol steps.

Additional sample preparation steps can improve the quality of the final result, but each additional step can result in the selective loss of protein species. The trade-off between improved sample quality and complete protein representation must be carefully considered.

Qualitative as well as quantitative studies of the separated proteins in the 2D maps can be, on occasions, problematic if similar amounts of proteins are not used when loading the gel in the first dimension [1,28]. This effect is particularly important when using a sensitive detection method like silver staining that allows the detection of amounts up to 0.05–0.1 ng protein/mm^2 of gel [29]. Knowing the concentration of the sample is fundamental to control the amount of protein to be loaded onto the gel of the first dimension. The concentration of protein dissolved in lysis buffer cannot be determined directly by the Bradford method, nor by any other protein determination method (Biuret or Lowry), the reasoning being that the components of the lysis buffer (urea, detergents, ampholytes, DTT) alter the spectrophotometric reading. To avoid this problem, Ramagli and Rodríguez [28] have developed an assay (modified Bradford) consisting in acidifying the lysis buffer (0.1 mol/L HCl), before dilution of the sample proteins. Thus, reliable recordings of the protein concentration can be taken in the range 0.5–50 μg.

Sample loads are often described as analytical or preparative. In practice, it is not easy to clearly differentiate between these terms. A strong protein spot in an analytical gel can contain enough material for further analysis whereas a weak spot in a preparative gel can be insufficient. Roughly, when 50–150 μg of total protein is applied on a gel and silver or sensitive fluorescent staining methods need to be used, usually we are dealing with an analytical gel. Preparative gels are loaded with 1 mg total protein and more. These gels are usually stained with Coomassie Brilliant Blue (CBB).

Analysis of human body fluids is a special case. They are important sources for the detection and monitoring of disease markers. A plasma protein map produced with IPGs has been published by Hughes et al. [30]. Unfortunately, most body fluids, like plasma, serum or cerebrospinal fluid, have abundant amounts of proteins or salt ions, which interfere in the first dimension. An abundance of albumin and globulins limits the capacity for the rest of proteins in serum and plasma. Today there are several methods to remove these proteins and the sensitivity of detection for other proteins is considerably improved. But currently there is no procedure available to get rid of albumin without losses of other proteins. More tips on sample preparation can be found in the review by Shaw and Riederer [31].

3.2. First dimension: isoelectric focusing

Proteins are amphoteric molecules containing acidic and basic groups. These become protonated or deprotonated, depending on the pH environment. In basic environment, the acidic groups become negatively charged; in acidic environment the basic groups become positively charged. The net charge of a protein is the sum of all negative or positive charges of the amino acid side chains. When an electric field is applied, it will start to migrate towards the electrode of the opposite sign of its net charge. At the p*I*, the protein has no net charge and stops migrating.

In the original procedure the first dimension, the isoelectric focusing (IEF) [5,32], is run in thin polyacrylamide gel rods in glass or plastic tubes. The gel rods contain urea, detergent, reducing agent and carrier ampholytes to form the pH gradient in the electric field. Usually, the sample is loaded onto the cathodal side of the gel rod, which becomes the basic end of the gradient. This O'Farrell technique [5] has been used for about two decades. Originally, the pH gradients for 2DE IEF were created by carrier ampholytes. These are mixtures of a few hundred different homologues of amphoteric buffers. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with p*I*s spanning a specific pH range and with a high buffering capacity near their p*I*. The mixtures contain buffers with p*I* distributed over a wide spectrum from 3 to 10. When an electric field is applied, the ampholytes start

to migrate according to their charges, towards the anode or the cathode, and automatically form stable pH gradients between the electrodes. Under denaturing conditions, long migration times are needed, which lead to a destabilisation of the gradient. Although this basic method has been used in hundreds of 2DE electrophoresis studies, it has several limitations that have prevented its more widespread application. The soft, thin and long gel rods demand great experimental skill, and the patterns were not reproducible enough. Thus, carrier ampholytes are mixed polymers and undergo batch-to-batch manufacturing variations. Obviously, these variations reduce the reproducibility of the first-dimension separation. Ampholyte pH gradients are unstable and have the tendency to drift toward the cathode. Gradient-drift affects reproducibility by introducing time variability and leads to the loss of almost all basic proteins rendering the 2DE technique less useful at basic pH. O'Farrell introduced a modification of the first dimension [11]. The sample is loaded onto the acidic end of the gel, and the proteins are not focused, but rather stacked between the different carrier ampholyte homologues. Due to the time factor, it is hard to achieve reproducibility, and the resolution is limited by the number of different carrier ampholyte homologues. Carrier ampholytes have other drawbacks, including the inability to load the large amounts of protein required for micro-sequencing of minor abundant proteins, theoretically more variable. The gel rods may stretch or break, affecting reproducibility.

To overcome this problem, immobilised pH gradient (IPGs) had been developed as an alternative to ampholytes by Bjellqvist et al. [13]. The introduction of commercial IPG strips offer greater reproducibility and allow to establish comparisons among laboratories [33,34]. The pH gradients in these gels are prepared by co-polymerising acrylamide monomers with acrylamide derivates containing carboxylic groups. In this case, the gradient cannot drift and it is not influenced by the sample composition. The use of immobilised pH gradients in the first dimension has allowed many methodical innovations for 2D electrophoresis [35]. Immobilised pH gradients are stable and capable of simultaneously focusing both acidic and basic proteins on a single gel prepared with a broad pH gradient. With IPGs, several wide gradients have been used (3–10, 4–7). New types of gel strips with narrow and basic gradients are being developed. Another important advantage of these strips is the possibility of loading greater amounts of sample, making the running of preparative gels possible for later characterisation analyses. On the other hand, the stability of some proteins at their isoelectric point can become a problem, particularly under alkaline conditions.

The complete voltage load during IEF is defined in volt–hour integrals (Vh). When the applied Vh are insufficient, not all spots are round and horizontal streaks are produced, and, in this way, higher Vh loads are needed for samples containing high molecular weight proteins, more hydrophobic proteins and preparative runs. On the other hand, when the proteins are focused too long, cysteins become oxidised and the p*I* of the proteins changes. Some proteins become unstable at their isoelectric point. The modified proteins have different p*I* and start to migrate again with the horizontal streaks radiating from the spots. The best results are obtained with the shortest possible focusing phase at the highest possible voltage.

When the optimal conditions have been found, samples have to be run in at least doublets or triplets, to check whether the pattern differences observed are caused by the noise of the system or by variations between different samples.

The sample application is also important to obtain good 2D results. There are different modes of sample application to the first dimension IPG strip. In-gel rehydration, loading is currently the preferred method, since it facilitates higher loads and reduces focusing times. With this technique, the dehydrated IPG strip is directly reswollen with the protein sample dissolved in the rehydration solution. After suitable rehydration time (12 h), the IPG strip is ready for the first dimension, with the proteins already uniformly distributed within the gel matrix. The clear advantage of in-gel rehydration is the large volume of sample that can be applied compared to conventional cup loading. The other major advantage is the minimisation of sample aggregation and precipitation since the sample is diluted through the entire gel strip.

The in-gel rehydration, as compared to sample cup loading, has been recently quantitatively evaluated by Zuo and Speicher [36], who have reported that, at high sample loads, cup loading can result in as much as 50% of sample loss; whereas in the case of in-gel rehydration, even when applying up to 0.5 mg protein, sample losses of about 15% are rarely observed. In turn, at least in the pH 6–11 range, cup loading is by far superior than the in-gel rehydration method. Other improvements in the basic ranges are described by López and Humphery-Smith [37] (Fig. 1).

A slight modification of the previous technique is the active sample loading, always called in-gel rehydration loading under low voltage. This technique is distinguished from the previous one (called passive), in which during IPG strip rehydration, a gentle voltage (typically 50 V) is applied. It is believed that this procedure would further facilitate sample entry of high-M_r proteins.

Either the strips containing the focused proteins are equilibrated in SDS buffer to transform the focused proteins into SDS-protein complexes, which are completely unfolded and carry negative charges only, and run on the second dimension right away, or they are stored at −60 to −80 °C in a deep-freezer.

Equilibration is performed twice on a shaker. SDS at 2% is also sufficient for preparative protein loads. Recently, it has been demonstrated that by increasing the SDS concentration (from 2% to 10%), the solubilisation of hydrophobic proteins is improved [38]. DTT is necessary in a first step because after the proteins have been focused they have to be treated with the reductant again. When very high protein loads are analysed, the concentration of DTT needs to be increased. In a second step, the iodoacetamide functions as scavenger of the excess reductant, increases spot sharpness and improves protein identification using mass spectrometry.

3.3. Second dimension: SDS-PAGE

The second dimension of 2DE separates proteins on the basis of their apparent molecular weights in polyacrylamide gels in the presence of SDS. The methodology for the second dimen-

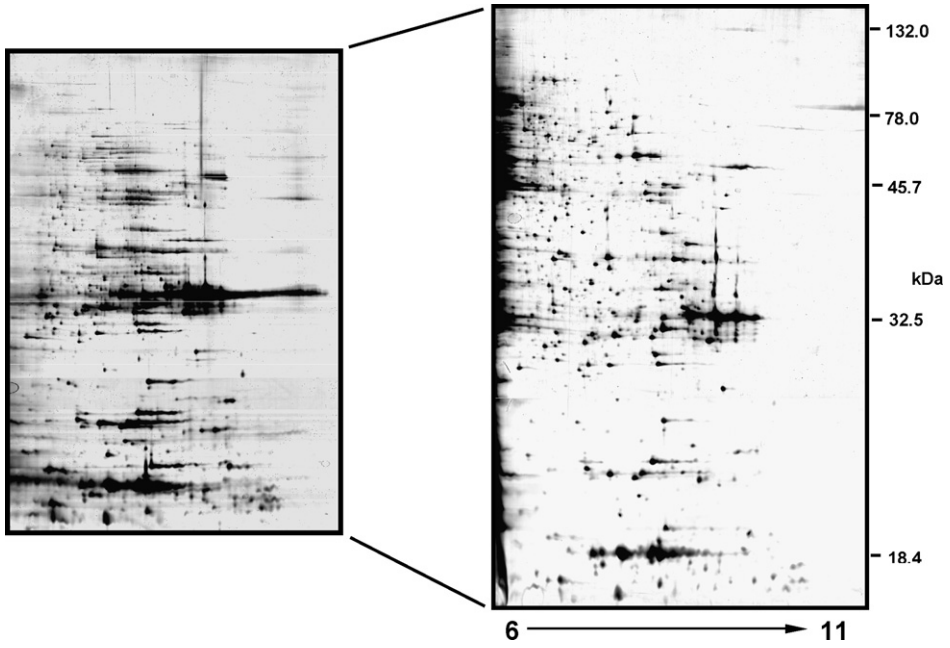

Fig. 1. Comparative 2D maps showing the improvements in the basic ranges.

sion did not change as much as for IEF. However, for SDS-PAGE some developments in the chemistry and instrumentation also contributed to improved handling and reproducibility of the spot positions. In this step, large amounts of SDS are incorporated into the SDS-protein complex in a ratio of approximately 1.4 g SDS/g protein. So, SDS masks the charge of the proteins themselves and the anionic complexes formed have a more or less constant net negative charge per unit mass. Thus, the electrophoretic mobility of proteins treated with SDS depends on the molecular weight of the protein. At a certain polyacrylamide percentage, there is an approximately linear relationship between the logarithm of the molecular weight and the relative migration distance of the SDS-polypeptide complexes of a certain molecular range. The molecular weights of the sample proteins can be estimated with the help of co-migrated standards of known molecular weights.

The standard buffer system for second dimension SDS-PAGE is based on the discontinuous Tris–chloride/Tris–glycine system described by Läemmli [10]. In the classical procedure the Läemmli system was used, including the stacking gel. For 2DE, the stacking gel is not needed because the proteins are already pre-separated by IEF and migrate from a gel into another gel and not from a liquid phase into a gel.

The gels, in the form of slab gels, are prepared as either single-concentration or gradient-polyacrylamide gels, which can be optimised to separate proteins over a specific M_r range.

The size of the gel clearly influences protein resolution. Typically, high-resolution 2DE systems use 1–1.5 mm thick slab gels measuring 20 cm × 20 cm and are capable of resolving over 1500 proteins (Fig. 2). Large gels provide a three- to four-fold increase in the number of proteins detected. However, increasing the gel size leads to enhanced complexity in profile analysis, and practical problems arise in handling these larger gels [39]. At the opposite end scale, 2DE can be carried out on smaller gel systems where relatively simple 2DE protein profiles are obtained [40]. An alternative approach is the production of 2DE "contigs" in which a series of several gels are prepared, each of which resolves proteins within a limited range of p*I* and M_r. The protein profiles produced by these gels are then combined by computer analysis to form a single large "virtual" gel covering broad p*I* and M_r ranges.

On the *y*-axis of the gel, the distribution of spots depends on the length, density and pH of the second dimension gel [5,39]. An appropriate acrylamide concentration is 12.5%. Lower per-

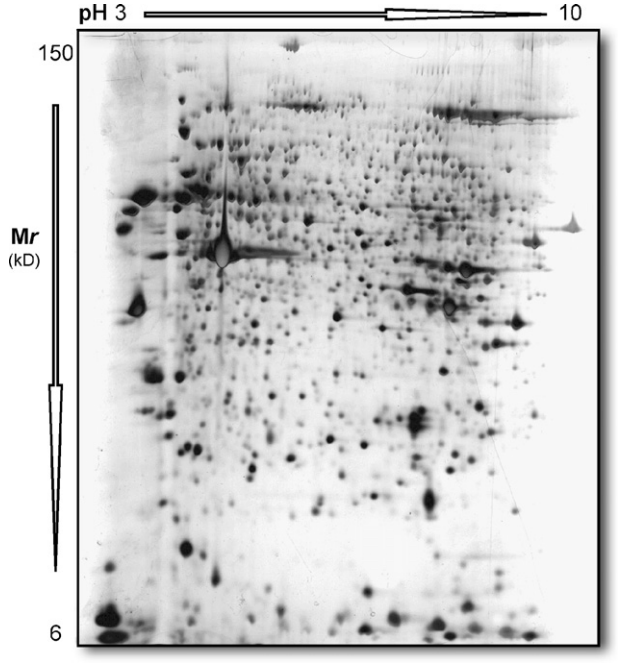

Fig. 2. Representative two-dimensional electrophoresis analytical gels of silver stained alkaline proteins.

centages worsen the resolution of two-dimensional maps, and higher percentages (15%) make the extraction of proteins from the gel more difficult for later studies. Generally, in gradient gels the overall separation interval is wider, also the linear relation interval between the logarithm of M_r and the migration distance is wider than that of homogeneous gels. Furthermore, the spots are sharper because the pore sizes are continuously decreasing. The preparation of gradient gels entails much more work, if it is more difficult to obtain reproducible gel properties in homogeneous gels. Usually, when homogeneous gels are run under optimal conditions, resolution and spot definition are high enough and a gradient gel is not necessary.

The resolution of proteins/peptides below 10–14 kDa is not sufficient in conventional Tris–chloride/Tris–glycine systems. Peptides smaller than 10 kDa co-migrate with the SDS front. Several modifications to the standard gel and buffer system have been proposed. The most efficient technique has been developed by Schägger and von Jagow [41]. In this modification, an additional spacer gel is introduced between the stacking and resolving gels. The gel buffer concentration is increased to 1 mol/L Tris–chloride, and the pH lowered to 8.4. Tricine is used as terminating ion instead of glycine. This method yields linear resolution for 100–1 kDa polypeptides.

The two-dimensional protein maps that allow the visualisation of more spots are called "analytical gels". These gels present the best patterns for a comparative analysis. To increase the concentration of several specific proteins for posterior characterisation, either by mass spectrometry or by any other analytical method, a greater sample load is applied in the first dimension, thereby obtaining protein maps containing greater amounts of protein. These gels are referred to as "preparative gels".

The absence of overlapping and the separation between spots make the analysis and comparison of gels easier, also facilitating the isolation of proteins from the 2D gels for later analysis. Additionally, the free spaces between spots can be covered by new spots, corresponding to small amounts of sample proteins. These spots could be detected by increasing the load or the sensitivity of the staining method. The distribution of the proteins throughout the surface of the gel is not usually problematic [39]. The focusing of proteins during the isoelectric focusing depends on the length of the strip of the first dimension, the voltage applied and the temperature used.

3.4. Detection of protein spots

Once the electrophoresis is finished, the proteins are detected using a variety of staining methods of different sensitivities.

There are several properties required for the ideal spot detection technique in 2D gels in proteomics. It should be sensitive enough for low copy number proteins, and allow quantitative analysis. It should have wide linearity and dynamic range, and be compatible with mass spectrometry. Moreover, the technique should be environmentally friendly. Unfortunately, there is no method that affords all these features together, and several different protocols exist for most of these methods.

Radiolabelling proteins with radioactive amino acid precursors can be used as a highly sensitivity detection method for studies in which prokaryotic and eukaryotic cells can grow in vitro to incorporate amino acid tracers during protein synthesis [42–44]. Obviously, for quantitative studies, radiolabelling is limited by the incorporation of the amino acid tracer. On numerous occasions and when working with tissues, biopsies, etc., it is difficult to radiolabel proteins. Hence, one must resort to using other staining methods.

In practice, the techniques most applied in proteomics laboratories are Coomassie Brilliant Blue, silver and fluorescence stainings.

It is important to know that different staining techniques stain proteins differently. There are proteins that do not stain with CBB, but that do with silver staining, and vice versa. The "classical" CBB recipe has a problem because during destaining, the spots are partly destained as well and because no steady state is reached, quantification is not reliable and not reproducible.

Colloidal Coomassie Blue staining contains ammonium sulfate, which increases the strength of hydrophobic interactions between the proteins and the dye. Perhaps the procedure takes a very long time and requires several steps, but it gives a sensitivity similar to that of silver staining.

Silver staining often produces a pattern different from the pattern achieved with Coomassie Blue and other procedures using Sypro Ruby [26]. Fig. 3 shows two separations of mussel foot extracts with the same protein load, one stained with CBB and the other one stained with silver. CBB detects approximately 0.1 μg of protein, and silver staining improves protein detection up to five-fold. The CBB procedure shows linear responses over

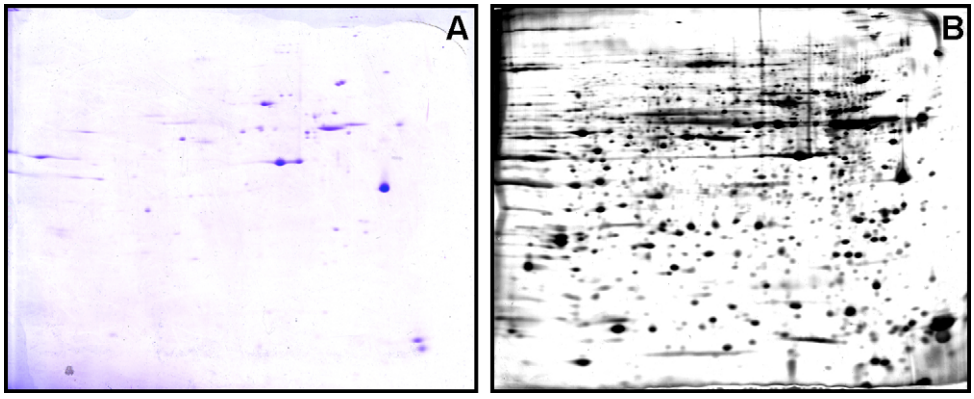

Fig. 3. Comparative figures of two gels having the same protein load, one with CBB and the other with silver staining.

a range of protein concentrations of 0.5–20 μg, whereas silver staining is linear at protein concentrations of 0.02–0.8 ng/mm^2 [45]. A limitation in the quantitative analysis using either CBB or silver staining is that the response slopes differ between proteins [46,47]. Silver staining is useful when searching for qualitative variations (presence/absence of spots). Therefore, what is preferred should be determined previously: number of spots, good definition or relative isolation of these. However, when the detection of quantitative variations is the aim, Coomassie staining with the assistance of computer programs was more reliable [48]. Incrementing the protein load in first dimension gels, staining the gels with CBB, destaining following the standard protocols and finally leaving the gels immersed in abundant distilled water gave practically the same number of spots as did a silver-stained analytical gel [49].

When the gel is first stained with Coomassie Blue and subsequently with silver staining, the pattern is the same as that with Coomassie Blue alone, just that this combination detects more spots because of the higher sensitivity.

Coomassie Blue stained gels are usually compatible with mass spectrometry analysis because the dye can be completely removed from the proteins. Moreover, CBB stained gels contain enough protein for the identification and characterisation using mass spectrometry. Nevertheless, silver staining can be modified for mass spectrometry compatibility by omitting the glutaraldehyde from the sensitising solution and formaldehyde from the silver solution [50].

An important recent advance was the development of high-sensitivity non-covalent fluorescent stains for general proteins, glycoproteins and phosphoproteins [51]. A major advantage of fluorescent stains with conventional chromogenic stains is a wider linear detection range, and they are therefore very well suited for quantification of proteins. Most of them show sensitivity similar or below that of the Coomassie Blue dyes. Fluorescence staining is mass spectrometry compatible. Sypro Ruby from Molecular Probes, Flamingo from Bio-Rad and Deep Purple from GE Healthcare are very sensitive, as sensitive as mass spectrometry compatible silver staining. A fluorescence scanner is required for visualisation and detection. A recent study showed that the Deep Purple stain can result in increased peptide recovery compared to the Sypro Ruby stain and can improve MS-based identification of lower intensity proteins [52].

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) method [53] is a variation of 2DE that labels complex protein mixtures using fluorescent dyes prior to conventional 2D electrophoretic separation. Up to three different samples can be labelled and mixed together and then separated on a single 2D gel. Cyanine dyes are used to label the proteins from different samples with dyes of different excitation and emission wavelengths. One advantage of this method over conventional methods is that since the samples are exposed to the same chemical environments and electrophoretic conditions, co-migration is guaranteed for identical proteins from separate samples; hence, simplifying the analysis of sample differences. The ratio of protein expression is always obtained from a single gel and an internal standard can be used in each gel, significantly reducing gel-to-gel variation of protein ratio measurements. Matching proteins between gels allows ratio measurements to be compared for several different samples. A specific software package (DeCyder from GE) has been developed to exploit the unique advantages of this technology allowing an automated approach in the analysis of differences found within and between gels. DeCyder uses ratio measurements obtained within gels rather than between gels to derive its statistical data. The labelling method used labels the proteins via amino group of lysine.

One limitation of this method is that excision of spots of interest for identification by MS can be problematic. Due to the incomplete labelling of lysines, a portion of most proteins may not be labelled and will migrate slightly more rapidly than the larger, fluorescently labelled form of the protein that is used for detection and quantitative comparisons. Even if a spot cutting robot is used that can detect fluorescent spots, the amount of the protein that is fluorescently labelled may be too low for facile protein identification using LC–MS after in-gel digestion. The position of the bulk amount of unlabelled protein may be estimated as being shifted about one spot diameter down, but this strategy can lead to excision of a protein other than one of interest [53]. Alternatively, one can attempt to correlate the 2D DIGE gel pattern with a colloidal Coomassie pattern from a parallel preparative 2D gel. But this method is not entirely reliable because different stains show protein-to-protein variations in staining intensity. A second limitation of the 2D DIGE method is that many low abundance proteins are not detected because the partial lysine labelling method is usually less sensitive than a good silver stain.

3.5. Image analysis

Once the gels are stained, the images are then analysed. For few gels, and especially when the gels present simple patterns, the 2DE protein profiles can be compared simply by overlaying the gels and manually inspecting the profiles for proteins with any electrophoretic mobilities. As the number of proteins being screened increases, the use of computer programs for profile matching becomes necessary. In addition, the detection of quantitative changes in protein expression between different states, different species or any other condition is an absolute requirement for computer-assisted programs. Specialised computer programs have been developed for the analysis of 2DE protein profiles [54–58]. However, for the detection of qualitative changes visual analysis is essential.

Therefore, the gel images have to be converted into digital data using a scanner or camera and analysed with a computer. For proper evaluation using the image analysis software, it is important to acquire the image as a grey-scale TIFF file with adequate resolution and preferably as much as 16 bit intensity.

To study quantitative changes, intensity levels of the spots must be normalised by expressing the intensity of each protein spot in a gel as a proportion of the total protein intensity detected for the entire gel (relative volume, %vol) [48,49,59–61].

The resolution of the gel must be maintained for proper analysis. At present, there are CCD cameras with sufficient resolution

to compete with the resolution of a scanner. Gels with visible spots have to be scanned in transmission mode and blot membranes are scanned in reflectance mode. It is necessary to calibrate the scanner using a grey step tablet.

Lasers with different wavelengths are combined with different filters for storage phosphor screens and fluorescent dyes. Radiolabelling provides the most sensitive signals. Storage phosphor screen scanners have a much wider dynamic range than X-ray films.

Most 2D programs follow these steps for the evaluation of 2D gels: spot detection, spot filtering, spot editing, background correction, gel matching, normalisation, comparison, quantification and reporting and exporting of data. One of the crucial steps is normalisation. Before the gels are compared for differences in the spot pattern, the spot volumes of the different gels have to be adjusted by normalisation. This step corrects for different protein loads and staining effectiveness. Gels are normalised according to the total spot volume or the volume of a single prominent reference protein.

4. Challenges of two-dimensional electrophoresis

Proteomes are very complex. Although the human genome is now estimated to contain only about 30,000 genes, the total number of unique protein components encoded by this genome is of the order of several million. There are currently no solid estimate of the numbers of unique protein components present in an "average" single human cell type, but it seems likely that at least 20,000–50,000 or more protein forms will be present in most types of human cells.

A second important attribute of most proteomes is the wide range in protein abundance levels that are usually encountered. Single cell organisms or individual cell types often contain many low abundance proteins that are present at about 100 copies per cell, while the most abundant proteins may be present at 100,000,000 copies per cell for a dynamic range of about 10^6 [62]. Current gels as well as non-gel protein profiling methods typically have detection dynamic ranges that are at best about 10^3–10^4. Furthermore, biological fluids usually have much wider dynamic ranges than cells and tissues.

Two-dimensional electrophoresis still experiences several shortcomings due to, in part, its capacity to resolve thousands of proteins simultaneously. Today, there is no standard 2DE system that separates all kinds of proteins equally well, i.e. hydrophilic and hydrophobic proteins, low and high M_r proteins and highly basic proteins. It is impossible to display all proteins in a single gel. So, several gels are most probably required for one sample. Unfortunately, 2DE cannot fully meet all these challenges.

Compared with alternative protein profiling methods, 2DE has several major strengths and weaknesses. Two-dimensional gels can detect many but not all post-translational modifications. In addition, 2DE is not limited to simultaneous comparisons of only two or three samples, and new samples can be compared to previously analysed samples that may no longer be available. However, 2DE also has several major weaknesses, including a limited capacity for the total amount of extract that can be analysed directly, which limits detection of the most abundant proteins in the sample. Very large, very small and membrane proteins are poorly recovered, and substantial gel-to-gel variation still persists.

The major challenges for successful proteome analysis are summarised in the following points: (i) the ability to analyse very alkaline, hydrophobic and/or low or high M_r proteins with high resolution; (ii) the ability to detect minor components in the presence of large quantities of housekeeping proteins; (iii) methods for protein quantitation and protein comparisons (quantitative comparisons) that are sensitive, rapid, simple, reliable and reproducible; (iv) simplification and automation of protein separation procedures.

Sample preparation is crucial in 2DE studies and has a great influence on several limitations of the technique. Although, the methodology for the protein extraction has become easier, reliable and reproducible, there is still a substantial list of improvements to be made.

Proteomic approaches have to select a good experimental system, reproducible sample preparation and any optional fractionation method such that the introduction of experimentally induced losses of specific proteins will be minimised. This is critical because experimentally induced protein changes, such as proteolysis, aggregation, precipitation and poor sample recoveries are frequently variable and can produce protein changes that can be difficult or impossible to distinguish from authentic changes associated with the biological question being investigated. It is therefore essential that all experimental parameters be carefully controlled. For good reproducibility, samples between the same individual as well as among different individuals should be run.

The reproducibility of the patterns should even reflect characteristics such as the size or intensity of the spots in-gels run with similar amounts of protein. This, along with the wide distribution of relative amounts of proteins of the sample, allows to recognise the spots even when complex patterns are obtained [5].

In the analysis with 2D gels, it is necessary to fine-tune and optimise the resolution of the protein maps as a function of the nature and characteristics of the samples studied. Testing in the first dimension with "Immobiline Dry Strips" of different pH ranges and lengths is recommended. Then, if most of the proteins focus at intermediate pH ranges, IPG strips in the range 4–7 should be used. This allows for a uniform distribution of the protein spots on the gel. To analyse other types of samples in which basic proteins are abundant, strips of pH 4–7 could not be used. Many proteins would be unresolved and concentrated at the basic end of the gel. In this case, broader pH ranges (i.e. 3–10) or basic narrow ranges such as 6–9 should be used.

A good 2D protein map should show spots that are not overlapping, well distributed throughout the gel, dark, having well defined borders, free of streaks and background staining. In general, all these characteristics should be present when comparing gels. The absence of overlapping and the separation between spots is particularly important for the detection of qualitative and quantitative variations of specific proteins between two conditions (Fig. 4). Moreover, this makes the analysis by den-

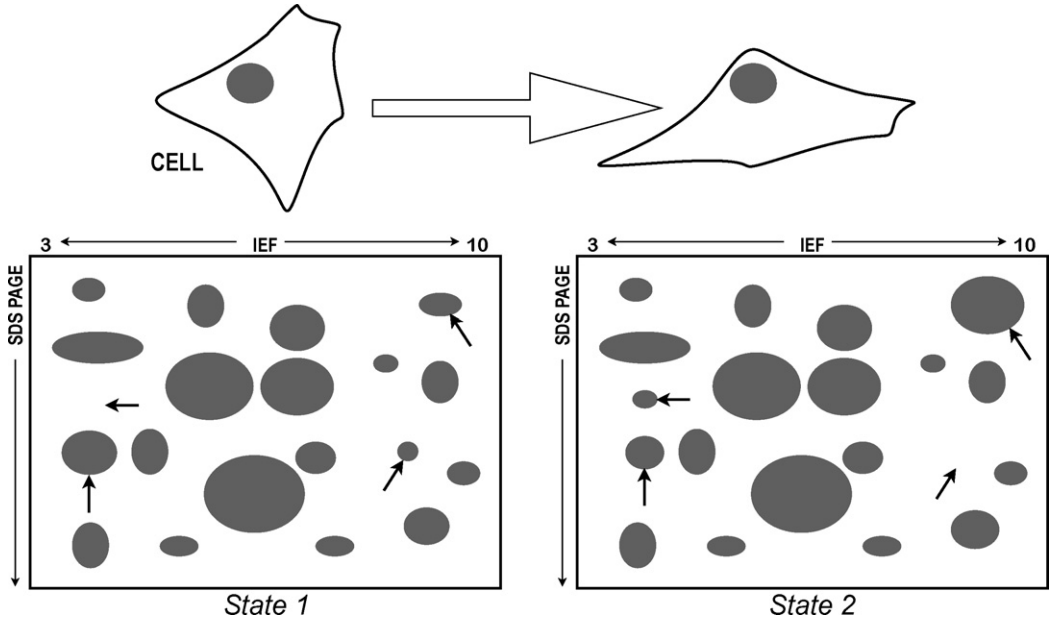

Fig. 4. Schematic representation of 2D gels showing different stages of a proteome. Up and down regulated genes (quantitative and qualitative changes) are marked with arrows.

sitometric imaging and the isolation of proteins for 2D gels for posterior structural analyses easier.

The protein extraction from the raw material is not total. Hydrophobic proteins, such as membrane proteins, are the most affected. The process of solubilisation continues to be, even today, a critical step in 2DE [63,64]. The protocols have been improved, incorporating the use of new reagents in the solubilisation and/or carrying out sequential protein extractions [65]. The absence in the gel of some proteins present in the sample can also be due to losses during the processing and limits in sensitivity. Some hydrophobic proteins do not go into solution, and others are lost during sample preparation and IEF.

Hydrophobic membrane proteins, which are one of the most interesting targets for drug discovery, are not readily solubilised in solvents, particularly due to the fact that the presence of SDS – an excellent solubilising agent – is not compatible with IEF. Nevertheless, new zwitterionic detergents such as sulfobetaines (e. g. SB 3–10 or ASB 14) increase the solubilisation of hydrophobic proteins [26,65].

The resolution of basic proteins has been, and continues to be, problematic. Notable examples include ribosomal proteins, histones and non-histone proteins, while the role of small, highly basic proteins as regulators of cellular physiology due to their ability to interact with nucleic acids remains poorly detailed. In *Bacillus subtilis*, approximately one third of the proteome is predicted to be alkaline [66]. Similar predictions have been made for a number of other bacterial species [67–69]. In addition, difficulties have been experienced in obtaining sufficiently large sample-loadings necessary to enhance the chances of successful protein characterisation of more than just the most abundant basic proteins. Unfortunately, as the sample load is increased, resolution decreases concomitantly. The developments in the IEF with the use of IPG strips allowed the analysis of very alkaline proteins or the introduction of overlapping narrow IPGs for higher resolution and the analysis of minor components [35].

High-quality 2D patterns of very alkaline proteins can be obtained with relative ease when wide pH gradients, short separation distances and running times and analytical sample loads are applied. Several approaches have been proposed to avoid streaking. These remedies include the application of an extra paper strip soaked with dithiothreitol (DTT) near the cathode to compensate for the loss of reducing agent DTT during IEF [70], alkilation of thiol groups with iodoacetamide prior to IEF [71] or oxidation of thiol groups with disulfides [72]. Herbert et al. [73] have proposed tributylphosphine (TBP) as an alternative to DTT. However, this reagent has also several disadvantages; the major among them are its low solubility in water and its short half-life. Moreover, TBP is toxic and volatile. Alternatively, tris(2-carboxyethyl)phosphine (TCEP) is used in the saturation labelling procedure in DIGE [26].

Narrow-range IPG gels in the acidic and neutral pH range work equally well with both in-gel rehydration and cup-loading. These gels are ideal for micropreparative separations where protein loads of several milligrams are applied. In contrast, alkaline narrow-ranges, especially in conjunction with micropreparative sample loads, are more difficult to handle and require additional optimisation steps. Additional improvements were made when the strip was transferred to a new strip holder after rehydration. As part of this transfer step, the strip was gently blotted against dry filter paper prior to focusing for 60 kVh (Fig. 1) [37].

Low-abundance proteins, which often are crucial to understand some biological changes and do the most important regulatory functions in a cell, may be present in only a few copies per cell. At present, there is not an analogous PCR system for amplifying proteins. On the other hand, these low abundant proteins cannot be detected without ultra-sensitive protein detection methods or without enrichment by sample pre-fractionation.

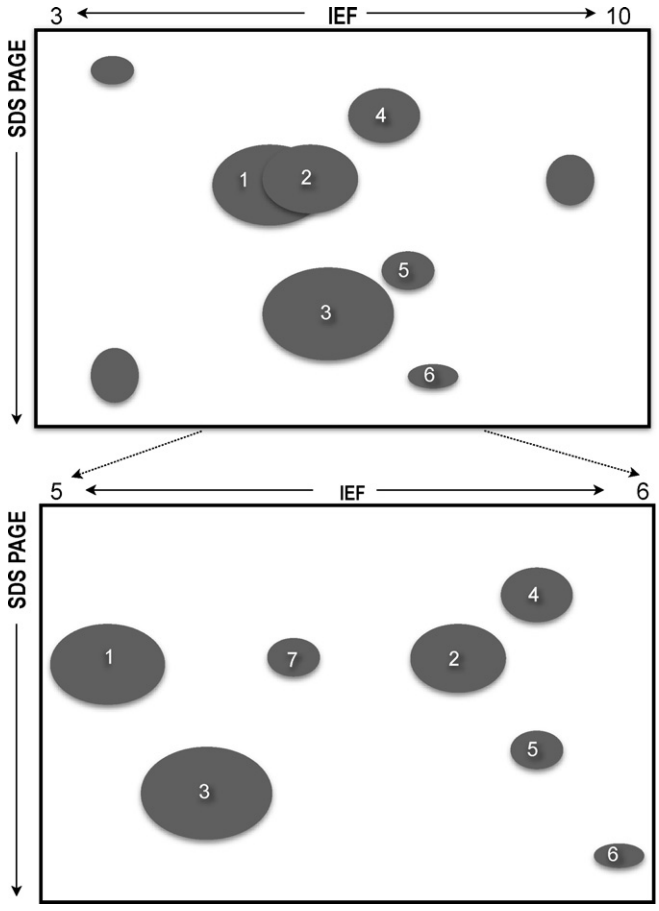

Fig. 5. Increase in resolving power in the first dimension with narrow pH range IPG strips (zoom gels). Note the improved resolution of proteins with numbers.

There are currently three major approaches to solve this problem: (i) ultrazoom gels, i.e. IPG strips which cover a series of narrow, overlapping ranges of p*I*; (ii) ultrasensitive protein stains; (iii) pre-fractionation steps to reduce the complexity of the sample and enrich low copy number proteins.

Current approaches to overcome the limitation when detecting low copy number proteins in the presence of highly abundant "housekeeping" proteins are sample pre-fractionation procedures and multiple overlapping narrow-range IPGs. Wide-range, linear or non-linear pH 3–10 (Fig. 2) gradients are often the method of choice for the initial analysis of a new type of sample and provide an overview of the total protein expression. However, more resolving power is needed for the separation of complex proteomes.

This problem can be overcome with the use of zoom gels that extended separation distances for higher resolution and improved detection of low copy number proteins. The major advantage of the overlapping narrow-range IPGs (e.g. IPG 4–5, 4.5–5.5, 5–6) is the gain in resolution by stretching the protein pattern in the first dimension (Fig. 5). Furthermore, computer-aided image analysis and protein identification by mass spectrometry are simplified due to the smaller number of co-migrating protein species and the more reliable database search results [74]. Moreover, it has been reported that ultrazoom gels allow the detection of proteins down to 300 copies [75] due to their higher sample loading capacity.

When the most sensitive method – radiolabelling – cannot be applied, more amount of protein must be loaded. Most staining techniques, like silver staining, have a rather limited dynamic range, and therefore, the results are not reproducible. Moreover, they are not absolutely quantitative. Today with the development of high-sensitivity fluorescent stains, several limitations are overcome. A major advantage of fluorescent stains is a wider linear detection range and the reproducibility [51].

In addition to electrophoretic methods, different chromatographic procedures have been successfully applied to enrich low-abundance proteins, e.g. hydrophobic interaction chromatography. Other, alternative methods are based on selective precipitation of proteins, e.g. with trichloroacetic acid/acetone for enrichment of alkaline proteins, or sequential extraction of proteins from a cell or tissue on the basis of their solubility properties.

No single current technology platform can reproducibly separate and quantitatively compare more than about a few thousand proteins, and therefore, only a modest portion of complex proteomes such as cell extracts, tissue extracts or biological fluids from higher eukaryotes can typically be analysed. One strategy for increasing the comprehensiveness of analysis of complex proteomes is to subdivide the proteome either by targeting a specific sub-proteome or by using a high-resolution pre-fractionation method.

Over the past several years, multiple research groups have attempted to expand the resolving power of 2D gels using various pre-fractionation methods to increase the number of proteins separated and to detect less abundant proteins.

The latter may be achieved by methods, such as sub-fractionation of cell components, e.g. organelles or centrifugation in a sucrose density gradient prior to 2DE analysis [30]. Pre-separation of cells into organelles by centrifugation is very useful not only to reduce the number of proteins, but also for the situation of the proteins in the cell. This procedure is complicated for most micro-organisms because in this case a lysis method is required, which is both efficient in disrupting the cell wall and gentle enough to guarantee that the organelles remain intact.

An alternative approach for pre-fractionation is electrophoresis in the liquid phase, e.g. microscale solution IEF [76,77] or multicompartment electrolysers with isoelectric membranes [78] or IEF in granulated gels [79]. It has been reported that this type of pre-fractionation allows higher protein load on narrow IPG gels without protein precipitation and allows detection of low-abundance proteins because major interfering proteins such as albumin have been removed [31].

The major disadvantage of most pre-fractionation procedures lies in the fact that they are either time consuming, complicated to handle and/or do not allow to process more than a few samples in parallel.

Due to the complexity of 2D patterns, only with informatics tools it is possible to find expressed changes in a series of gels, like up- and down-regulated proteins. Image analysis has still

been the bottleneck in the proteomics procedure because the spot detection parameters had to be adjusted and optimised manually.

The development of software for 2D electrophoresis gel image analysis is a continuously ongoing process. The functions become more reliable, reproducible and automated from year to year. With the latest developed program, it is already possible to compare gels of different sizes, shapes and even damaged gels. However, it seems that irreproducible results cannot be fixed, even not by the most sophisticated software. Bad separation results cannot be turned into good results using the software.

5. Future prospects

Proteomics has both rejuvenated an older technology, 2DE, and stimulated rapid progress in newer methodologies, particularly mass spectrometry, protein arrays, automation and nanotechnology.

High-resolution two-dimensional gel electrophoresis has been difficult to automate, but progress is being made, including programmable IEF units for automated overnight IPG strip rehydration and focusing, and even partially or fully automated 2D electrophoresis units. Even greater progress is being made in post-gel handling steps, including the use of robots for spot excision, in-gel trypsin digestion, post-digestion cleanup/concentration and sample spotting onto MALDI mass spectrometer targets or into injection vials for LC–MS analysis.

The great complexity of higher eukaryotic proteomes, their constantly changing nature, and most importantly, the wide diversity of protein properties and behaviour suggest that no single proteome analysis method will be able to effectively address all proteome analysis problems within the near future. Hence, it seems most likely that all the major current analytical technologies including 1D and 2D gels, multi-dimensional chromatography coupled with MS instruments, and antibody arrays will continue to play important and complementary roles in proteome analysis studies in the near future.

Despite some limitations, 2D gels remain as the gold standard to which any competing method should be compared. A viable alternative separation method should provide clear advantages over 2D gels, such as the following: detect more proteins, require less analysis time, detect lower abundance proteins or have other advantages. At present, it is not apparent that any non-2D gel protein separation method provides major advantages over 2D gels in terms of the number of proteins detected, analysis time or sensitivity. However, future advances in automation and chromatographic capacities may produce a clearly superior protein profile analysis technology.

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