Two-dimensional Electrophoresis

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Introduction

Two-dimensional (2D) polyacrylamide gel electrophoresis is a classical technique for the separation of proteins. It first appeared in the mid-1970s but for a long time it only found limited applications. Recently it has enjoyed an impressive renaissance. The major reasons for this are the introduction of the immobilized pH gradient (IPG) strips and the development of analytical methods capable of identifying proteins present in very low quantities. 2D electrophoresis represents the core methodology of the new, technology-driven science **proteomics**. Proteomics finds a wide application, in both clinical diagnosis and in pharmaceutical research, for the detection of novel drug targets. **Figure 1** demonstrates the application of 2D electrophoresis for the detection of variable protein levels between diseased and healthy brain tissue. In a sample from the parietal lobe of the brain of a patient with Alzheimer's disease, a strong spot representing glial fibrillary acidic protein (GFAP), a marker for neuronal loss, is present, whereas in the control sample, the corresponding spot is very weak.

The aim of proteomics is the high throughput analysis of the proteome (protein complement expressed by a genome) of various organisms or tissues. It consists of two steps: (1) the separation of protein mixtures by 2D electrophoresis, and (2) the identification of the separated proteins by analytical techniques, such as mass spectrometry and amino acid composition analysis. The process is facilitated by the use of highly sophisticated software for advanced image analysis and the high reproducibility of images in intra- and inter-laboratory studies. The 2D electrophoresis itself involves: (1) separation of the proteins on the basis of differences in their net charge, called isoelectric focusing (IEF), and (2) separation of the focused proteins on the basis of differences in their molecular masses. **Table 1** gives a summary of the most significant chronological events in the development of 2D electrophoresis. The state-of-the-art technology will be discussed without entering into extensive technical details that can be found in the literature provided.

First-Dimensional Separation (IEF)

Proteins carry positively and negatively charged side groups and are, therefore, amphoteric molecules. The

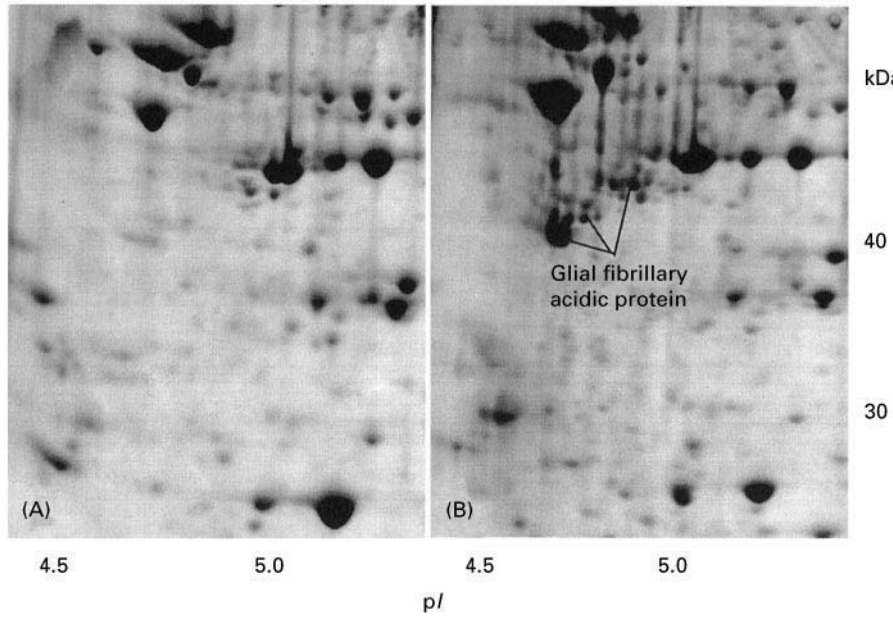

Figure 1 Partial 2D gel images showing human brain proteins from a control (A) and a patient with Alzheimer's disease (B). The proteins were separated on pH 3–10 nonlinear strips, followed by 9–16% SDS gels, stained with colloidal Coomassie blue. The spots representing glial fibrillary acidic protein (GFAP) in (B), a known marker of neuronal loss, are indicated.

Table 1 Important advances in 2D electrophoresis technology

1970	Introduction of sodium dodecyl sulfate in 1D gel electrophoresis to efficiently separate complex protein mixtures (Laemmli UK, *Nature* 227: 680)
1975	Separation of protein mixtures by 2D gel electrophoresis using tube gels and pH gradients formed with carrier ampholytes (O'Farrell PH, *Journal of Biological Chemistry* 250: 4007)
1980–1990	Pioneering work to improve pH gradient stability; synthesis of Immobilines and preparation of IPG strips (Bjellqvist B, *Journal of Biochemistry Biophysics Methods* 6: 317); electrotransfer of proteins from gels to PVDF membranes
1990–today	IPG strips became commercially available; introduction of sigmoidal strips, efficient separation of basic proteins, improvement of gel staining and protein solubilization techniques (Bjellqvist, Dunn, Goerg, Hochstrasser, Rabilloud, Righetti and others); development of high throughput protein analytical techniques (MALDI-MS, amino acid analysis); development of software for protein identification and image comparison; establishment of databases accessible via the WorldWideWeb; sequencing of the complete genome of microorganisms; preparation of 2D protein maps for organs, cell lines, organisms; new terms *Proteome*, *Proteomics* were introduced

protein charge depends on the pH value of the solution. IEF is an equilibrium process, during which, under the influence of a high voltage field the proteins move along a pH gradient, according to their net charge, to a position, where they have no net charge and consequently stop moving. This pH value is called the isoelectric point (p*I*). The resolving power of IEF is defined by the equation of Svensson:

$$\Delta pI = [D[d(pH)/dx]:E[-du/d(pH)]]^{1/2}$$

where ΔpI = resolution capacity (pI difference required to resolve neighbouring spots), D = diffusion coefficient of the protein, E = field strength (V cm^{-1}), $d(pH)/dx$ pH gradient, $du/d(pH)$ mobility slope at pI.

According to this equation, the resolution capacity is influenced by the pore size of the gel, which affects the diffusion of the protein, the slope of the pH gradient and the voltage value.

An efficient and reproducible protein separation during IEF requires a stable pH gradient. There are two pH gradient systems in use. In the first one, the pH gradient is created by an excess of carrier ampholytes during the IEF run. Ampholytes are amphoteric compounds of low molecular mass with closely related p*I* values. Upon application of an electric field, the ampholyte molecules move and align themselves between the electrodes, forming a pH gradient, which increases from anode to cathode. This type of IEF is usually performed in tube acrylamide gels.

In the second pH gradient system, the pH gradient is immobilized and has been formed prior to IEF run. IPGs are formed by acrylamide derivatives, called immobilines, which are weak acids and bases with a buffering capacity. Immobilines are copolymerized in a polyacrylamide gel, such that a pH gradient is formed between basic and acidic molecules. When an electric field is applied, the pH gradient does not move. Only the charged molecules of the protein sample move and are focused according to their p*I*s into narrow bands. This type of IEF is usually performed in strips of acrylamide gel fixed on a plastic sheet.

Carrier Ampholytes

The pH gradient formed by the carrier ampholytes during IEF can be affected by the amount of total protein loaded. Proteins when applied in large quantities, having themselves a buffering capacity, can affect the focusing position along the pH gradient and consequently the reproducibility. Therefore, factors such as protein quantity, temperature, voltage and chemicals can strongly affect performance. Only small quantities of protein (of the order of 0.1 mg) should be applied for IEF with the carrier ampholytes approach. The difficulties in controlling the various factors which affect reproducibility together with the difficulties of preparing and transferring the tube gels to the second dimensional separation, contribute to the reasons why carrier ampholyte-based IEF remained a scientific speciality of only a few laboratories. Nevertheless, these laboratories are able to control the conditions affecting reproducibility, so that IEF with carrier ampholytes is still used. This approach allows a very reliable protein quantification of complex mixtures. Because only a small amount of protein can be applied, 2D gels made following the carrier ampholyte approach are more suitable for analytical purposes. IEF based on carrier ampholytes can efficiently separate proteins with p*I*s within the pH range of 3–8. Proteins with higher pIs separate poorly due to cathodic drift during isofocusing. The cathodic drift is the result of a high electroosmotic flow, caused by the charged groups on the glass walls of the gel tubes.

IPG Strips

The increased application of two-dimensional gel electrophoresis today is to a large extent due to the introduction of IPG strips. The major advantage of using IPG strips is the ability to maintain high

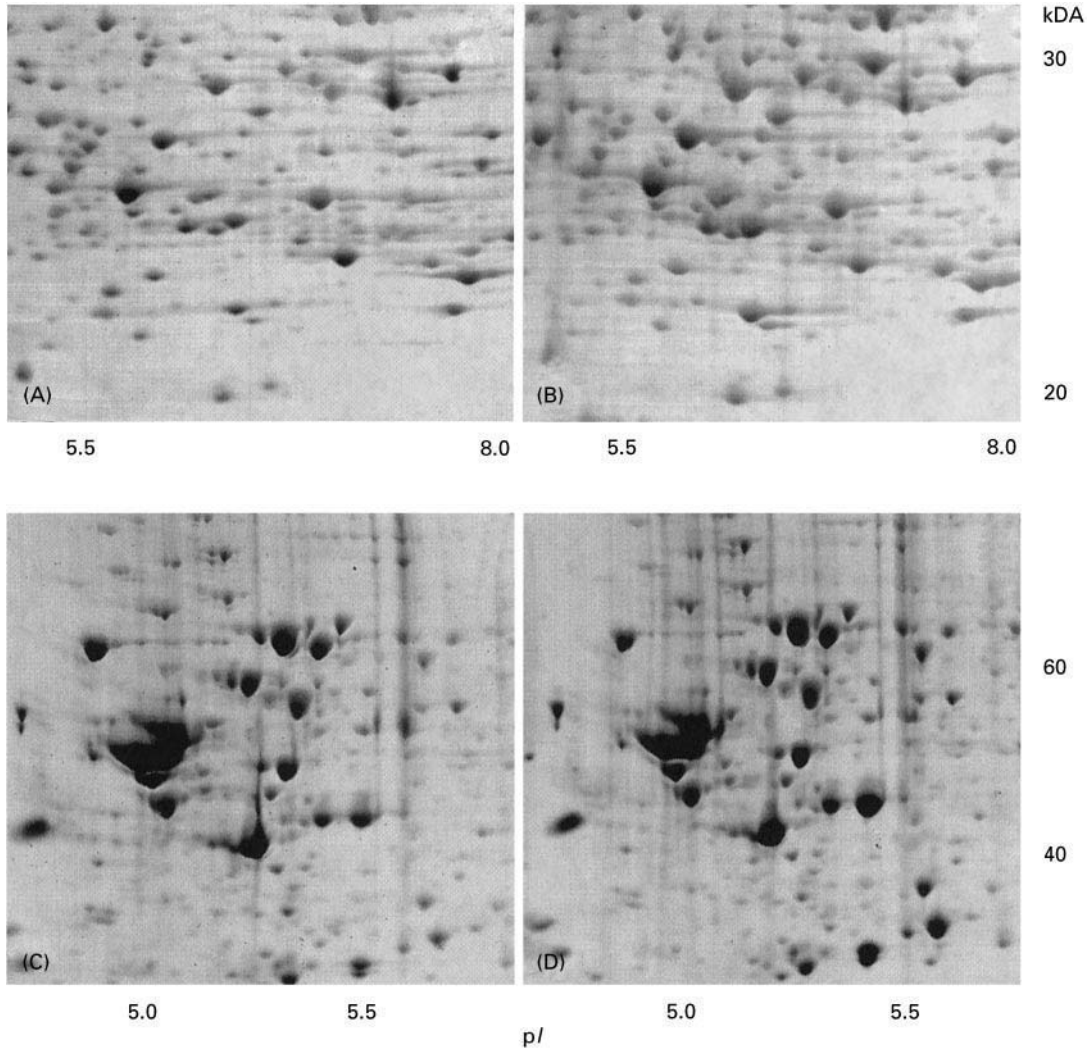

Figure 2 Partial 2D gel images showing the high fidelity in the reproducibility of separation of bacterial (A, B) and mammalian (C, D) proteins. The proteins were separated as stated in the legend to Figure 1. (A, B). Separation of proteins of *Haemophilus influenzae* were 1.5 and 3.0 mg, respectively. (C, D) Separation of rat brain proteins, 1.5 and 2.0 mg, respectively.

reproducibility. The increase in reproducibility has allowed a high throughput analysis of proteomes and the application of larger sample quantities, a requirement for protein spot analysis. **Figure 2** provides examples of the reproducibility of separation of bacterial and mammalian proteins, following IEF on pH 3–10 nonlinear IPG strips. Although minor differences can be detected, the reproducibility concerning both the position and the intensity of the protein spots can be considered as satisfactory.

As mentioned earlier, immobilines are polymerized in a gradient, in a polyacrylamide gel, and the gel is then dried on a plastic sheet. Before the IEF run, the dry strips are rehydrated in a specific rehydration solution, containing a reducing agent, ampholites and high concentrations of urea and a zwitterionic or nonionic detergent (usually CHAPS). Rehydration can also be performed in a solution containing the protein sample to be analysed (see sample application). Large numbers of strips can be rehydrated at a time and this represents an advantage of the method regarding high throughput analysis and performance. Today, IPG dry strips are commercially available from Amersham Pharmacia Biotechnology in two lengths – 11 and 18 cm and in three pH ranges – 3–10, 4–7 and 6–11. The pH 3–10 strips are available in a linear and nonlinear (sigmoidal) form. The latter allow a more efficient focusing of proteins with p*I*s between 4.5 and 7. A large percentage of proteins from various organisms have p*I* values within this range. The dry strips can be kept frozen at $-20^{\circ}C$ for a long time (the expiration date is indicated on the packaging).

The use of narrow pH range strips (i.e. of 1 pH unit) provides a higher resolution and allows the detection of protein isoforms; this is an additional advantage of IEF using IPG strips. Strips of more narrow pH ranges are not currently commercially available and have to be prepared by the user. IPG strips can be made in any biochemical laboratory using a gradient marker and Immobilines of various p*K* values which can be purchased. Recipes for the preparation of IPG strips can be found in handbooks, for example in *Electrophoresis in Practice* (see Further Reading). On the narrow pH range the spots appear stretched compared to the wide range strips. **Figure 3** shows an example of a protein which appears as one spot following IEF after focusing on a pH 3-10 strip and as five spots after IEF on a pH 4–7 strip. IEF on strips of an even narrower pH range would result in the detection of additional spots resulting from further isoforms of the protein. Following IEF, the IPG strips can be either immediately used for the second dimensional separation or stored frozen at $-20^{\circ}C$ for long periods (for example, in petri dishes sealed with parafilm). Strips stored for 4 months have been used at $-20^{\circ}C$ without any effect on the spot resolution. Longer storage times of up to 1 year have been reported.

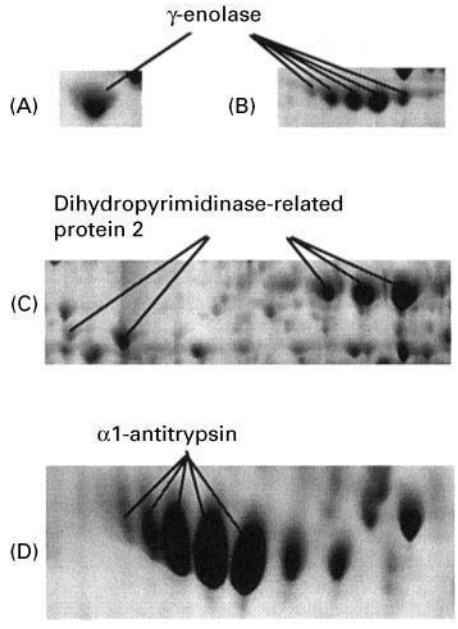

Figure 3 Partial 2D gel images showing examples of proteins represented by multiple spots. (A, B) γ-enolase from human brain. The protein is represented by one spot when IEF was performed on pH 3–10 nonlinear IPG strips (A), and by five spots when IEF was performed on pH 4–7 strips (B). (C) Dihydropyrimidinase-related protein 2 from human brain shows a high heterogeneity, represented by five spots, localized into two regions on the gel. (D) α1-antitrypsin from human cerebrospinal fluid is represented by many spots, most likely denoting different glycoforms of the protein.

Sample Preparation

Careful sample preparation is a prerequisite of a successful analysis. Most proteins are soluble and are easily recovered in the sample preparation solution, which includes urea, CHAPS, a reductant and, optionally, protease inhibitors. Recovery of the proteins that are insoluble in this solution is often a problem. A centrifugation step is necessary for removal of nondissolved material. The addition of thiourea and of a noncharged reducing agent, such as tributyl phosphine, to the sample buffer increases protein solubility during IEF. It would appear that hydrophobic interactions between proteins and the acrylamide gel of the IPG strips are responsible for protein losses during IEF. Nucleic acids present in the sample can also seriously affect spot resolution. Enzymatic digestion with an endonuclease prior to sample application is usually recommended.

Sample Application

The protein application mode can affect the amount of protein entering the IPG strip during IEF. There are several ways of applying the sample. In the system supplied by Amersham Pharmacia Biotechnology (Multiphor II), the sample is usually loaded into application cups (also supplied by Amersham Pharmacia Biotechnology). Up to 150 μL can be applied in one cup. The cups are fixed in special 'cup accommodating bridges' which are placed near the basic or acidic end of the strip. It seems that sample application at the basic end of the strip is more advantageous compared to the application at the acidic end. We have, however, found that simultaneous sample application at both the basic and the acidic ends of the strip can result in the detection of more and stronger protein spots compared to sample application at only one end. It also allows the simultaneous application of sample volumes larger than 150 μL. From a technical point of view, sample application using the cups is the most difficult operation to perform. The cups should touch the polyacrylamide gel on the strip, otherwise the sample will leak; they should also not damage the gel at the contact point, otherwise the proteins will not enter the strip.

An alternative method of sample application is the rehydration of the strip in a solution containing the protein sample. This approach is convenient to perform and theoretically it should result in the detection of all proteins present in the sample. However, more comparative studies are required to prove that this approach is more efficient than the loading of sample

into cups. Amersham Pharmacia Biotechnology has recently introduced a new IEF apparatus (IPGphor) in which sample application and IEF can be performed. The strips are placed in special ceramic strip holders and rehydrated for the desired time in a solution containing the protein sample. Each strip holder holds a single IPG strip throughout rehydration and IEF. IEF starts automatically after rehydration according to the conditions programmed. Whether the performance of IEF will be improved with the use of this instrument is not clear at present.

The quantity of protein to be applied on the strip naturally depends on the goal of the analysis. If the identification of protein spots is intended, the amount loaded should be in the order of 1 mg or higher, depending on the number of proteins in the mixture. A 1D gel analysis of the sample prior to 2D electrophoresis may provide helpful information as to defining the right protein quantity. If large amounts of protein are applied, a percentage of the proteins may not enter the strip. Presently, this constitutes a drawback of the IPG strip approach. Because certain proteins in the sample (mainly major components) only partially enter the strip, this can result in an unreliable quantification of a particular protein in a given mixture. While the application of 15 mg or more of protein sample has been reported, we consider that 2–4 mg is the limit for a productive separation, using the strips that are presently available.

IEF using IPG strips can separate basic proteins efficiently with pIs up to about 12. The introduction of low concentrations of isopropanol in the rehydration buffer improves focusing of basic proteins. Hydrophobic proteins probably precipitate at the point of application and efficient separation has not yet been reported. Hydrophobic proteins can be analysed in a different 2D electrophoresis system, which uses the interaction of the proteins with a cationic detergent in the first dimension rather than p*I*. The second dimension is, as described below, dependent on the molecular mass. The separated proteins form approximately a diagonal line. Relatively, only a small number of proteins can be successfully separated using this approach.

Second-Dimensional Separation (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, SDS-PAGE)

Following IEF, the proteins are separated according to their molecular masses. During this nonequilibrium step, the proteins are negatively charged by addition of the anionic detergent SDS. Upon application of an electric field, the charged proteins move along a porous polyacrylamide gel and are separated according to their size. A reducing agent is also included to disrupt disulfide bonds. In comparison with IEF, SDS-PAGE is relatively easy to control. The terms ISO-DALT and IPG-DALT are often used to mean 2D gel electrophoresis employing IEF with carrier ampholytes or IPG strips, respectively.

Horizontal or more usually vertical slab gels, running in a discontinuous buffer system are employed. A high throughput analysis is facilitated by the use of tanks accommodating 6–20 gels running in parallel. An efficient separation of thousands of proteins present in complex mixtures, can only be performed on gels of a large format (18×20 or 25×25 cm). Either gradient gels or gels of a constant acrylamide concentration can be used. Because of the complexity of the

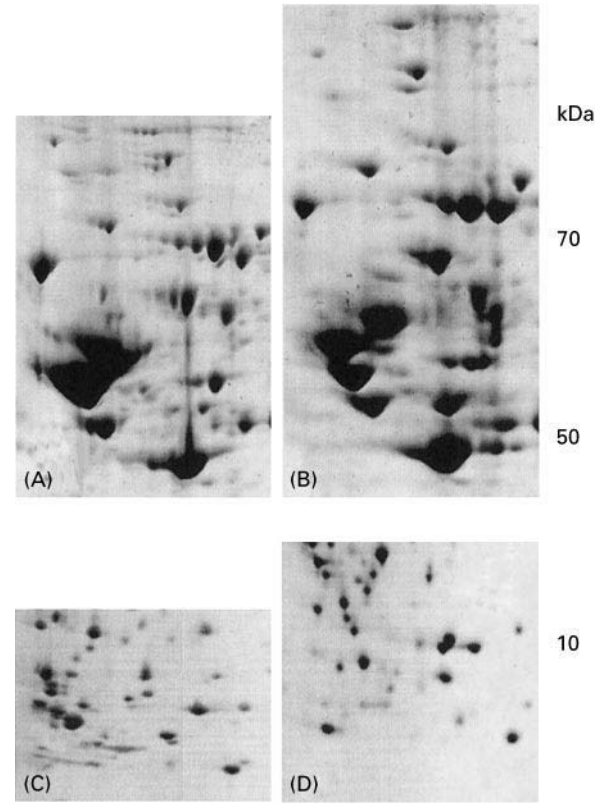

Figure 4 Partial 2D gel images showing an improved spot resolution by using different acrylamide concentrations. Separation of rat brain proteins on a 9–16% SDS gel (A) and on a 7.5–16% SDS gel (B). Separation of low molecular mass soluble proteins from *H. influenzae* on 9–16% SDS gel (C) and on a 10% SDS Tricine gel (D). (B, D) The gel parts comprising the corresponding proteins shown in A and B, respectively, are longer on account of the different acrylamide concentrations.

technology and the large diversity of the samples to be analysed, and in order for the data to be useful to a broad research community, 2D PAGE has been to a large extent standardized. In the second dimension, for example, we usually use 9–16% linear gradient gels. This gel system represents a good compromise, as it separates proteins between 5 and 200 kDa. However, efficient separation is limited to a range of approximately 15–40 kDa. Outside this range, in particular above 50 and below 10 kDa, the separation is often suboptimal. For more efficient separation, gels of a different acrylamide concentration should be tried. **Figure 4** gives examples of the improved separation of high molecular mass brain proteins using gels of lower acrylamide concentration and of low molecular mass proteins from *Haemophilus influenzae*, using gels with Tricine as the trailing ion instead of Tris.

For spot visualization, the gels can be stained with either silver or Coomassie blue (usually colloidal Coomassie blue), depending on the quantity of protein sample applied and the aim of analysis. Silver stain may be preferentially used for gel comparison studies, whereas staining with Coomassie is preferred when the spots are intended for protein identification. Colloidal Coomassie blue has the advantage that the stain is sensitive enough and the gels can be easily destained with water. The simultaneous staining of many gels in one tank substantially increases the throughput. Apart from silver and Coomassie blue, several other protein detection methods exist, such as staining with various metals, labelling with fluorescent agents or detection of radiolabelled compounds, after gel drying and exposure, for example to a film.

Proteome Analysis

An essential step of proteomics is the identification and mapping of the proteins separated by 2D electrophoresis. From a mammalian organism, comprising approximately 100 000 possible gene products,

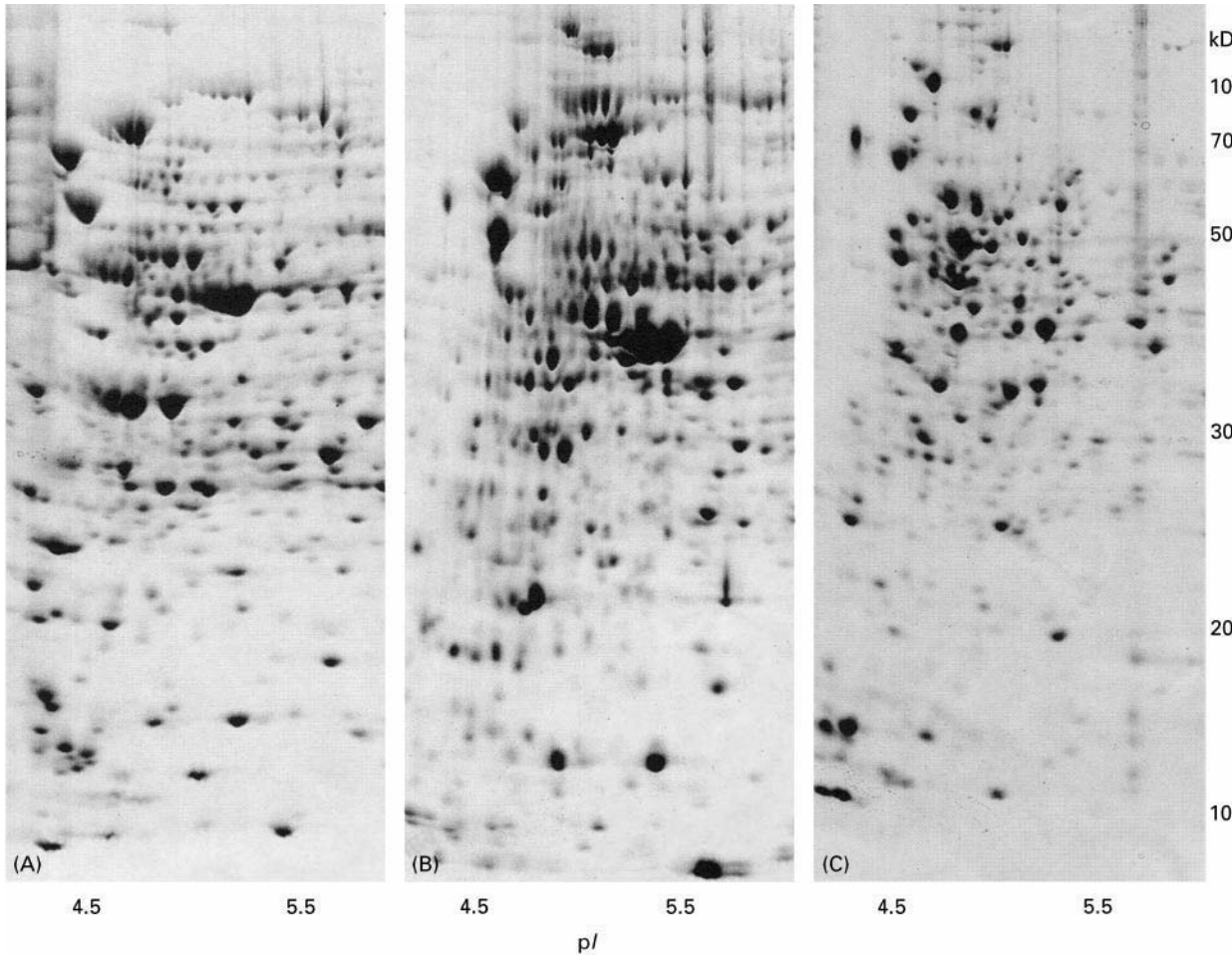

Figure 5 Partial 2D gel images showing soluble proteins of (A) *H. influenzae*, (B) *E. coli* and (C) *B. subtilis*. The proteins were separated as stated in the legend to Figure 1. Note the similarity in the distribution of the major proteins in the three bacterial organisms.

approximately 1000–2000 protein spots can be visualized on one 2D-gel, using Coomassie blue. Higher numbers can be detected, following staining with silver or after radiolabelling. Approximately one-half of the visible spots are available in sufficient quantities to be analysed for identification. **Figure 5** shows the analysis by 2D electrophoresis of the proteomes of three bacteria, *H. influenzae*, *Escherichia coli* and *Bacillus subtilis*. The genomes of the three microorganisms have been completely sequenced, so that theoretically all expressed proteins can be mapped. This has however not yet been accomplished. The largest 2D proteome maps, such as that of *H. influenzae* prepared at F. Hoffmann-La Roche, Basel, include approximately 500 mapped proteins. Many of the unidentified proteins are not expressed in sufficient amounts to be visualized.

For the mapping of proteomes of the various organisms, protein enrichment steps need to be introduced before analysis. We have used several chromatographic steps, such as heparin chromatography, hydrophobic interaction chromatography, chromatofocusing, hydroxyapatite chromatography and several other approaches, to enrich the low-abundance gene products of *H. influenzae* and *E. coli*. Additional enrichment steps are required for an efficient mapping of proteins present at low abundance, such as cytokines or transcription factors. **Figure 6** shows an example of protein enrichment by hydrophobic interaction chromatography. One protein (enolase), represented by a strong spot in the 2D map of the total protein extract, is highly enriched after chromatography. Another example of protein enrichment, this time using heparin chromatography is shown in **Figure 7**. In two fractions collected from the column, proteins which are not visible in the 2D gel image of the total extract can also be detected.

On a 2D map, proteins are often represented by more than one spot. Figure 3C shows an example of a brain protein represented by five spots, in two locations, with different p*I* and M_r values. Presently, we do not know the reasons and the biological significance for most of these cases of observed heterogeneity. It may be the consequence of post-translational modifications, such as deamidation, phosphorylation or glycosylation, which result in the alteration of the p*I* of the molecule and its focusing position. Another reason may be the carbamylation of the protein upon contact of the sample with urea. In Figure 3D an

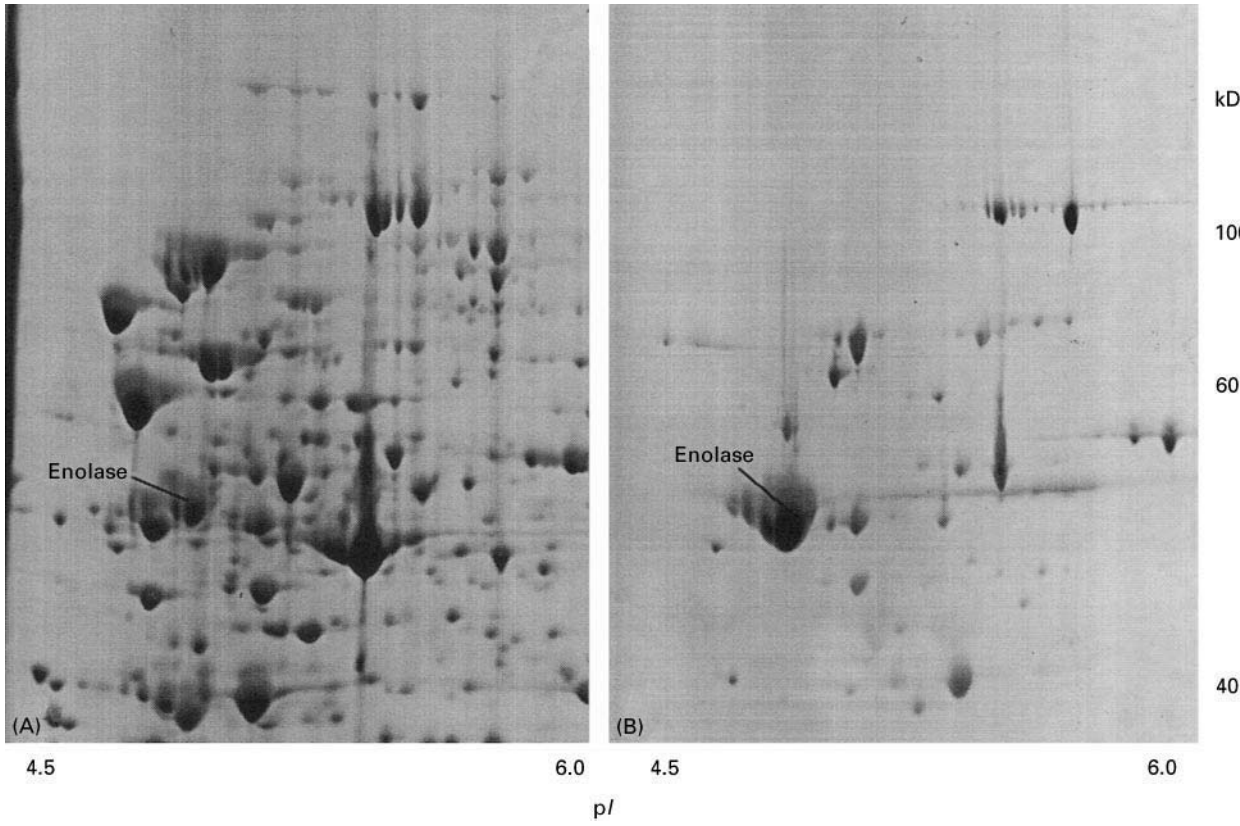

Figure 6 Partial 2D gel images showing the enrichment of enolase by hydrophobic interaction chromatography. (A) Total extract; (B) proteins from a fraction collected from the column.

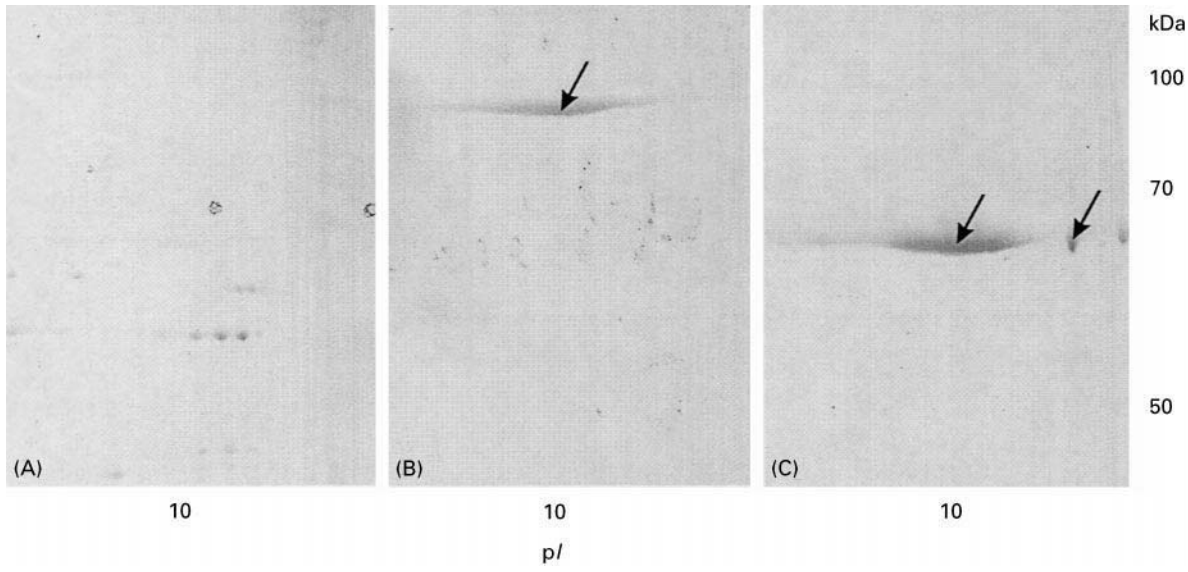

Figure 7 Partial 2D gel images showing the enrichment of low abundance proteins of *H. influenzae* by heparin chromatography. (A) Total extract; (B, C) proteins from fractions collected from the heparin column. The arrowheads indicate spots representing two proteins (B, topoisomerase I; C, ATP-dependent RNA helicase) which are not visible in total extract (A).

example of protein heterogeneity, most likely due to glycosylation, is presented.

Following 2D electrophoresis, proteins can be identified by mass spectrometric analysis of the peptides resulting from the in-gel digestion with a specific protease, such as trypsin. In another approach, the proteins can be electrotransferred onto membranes and identified by immunoreaction with specific antibodies, by N-terminal sequencing or amino acid composition analysis. For those proteins for which the

Table 2 Steps in the preparation of 2D electrophoresis

IPG strip rehydration and sample preparation Protein extraction, centrifugation, recovery in sample solution
Sample application Application in cups at either or at both ends of the strip or strip rehydration in a solution containing the protein sample
First dimensional separation (isoelectric focusing) Start at 200 V and increase gradually to 5000 V; keep 5000 V for 6–48 h, depending on sample, quantity and strip range; narrow pH range strips require longer focusing times
Reduction and alkylation of proteins on IPG strip Equilibration of strip with reducing and alkylating agents or freeze until use
Second dimensional separation (SDS-PAGE) Preparation of gel of the desired acrylamide concentration; gels should carry a label to identify them afterwards; establishment of contact between strip and gel with agarose solution; run at 40 mA/gel
Protein fixing and staining or blotting Fixation of proteins within the gel and staining with silver or Coomassie blue or drying of the gel and exposure to a film or phosphorimager for detection of radiolabelled proteins or electrotransfer of proteins to membranes for immunoblot, MS or amino acid analysis
Gel scanning Storage of image in a database
Gel comparison Gel comparison and protein quantification using specific software; comparison with database master gels via the WorldWideWeb
Identification of proteins Identification of protein spots from gels by mass spectrometry or from membranes by N-terminal sequencing, amino acid composition analysis, MS or immunoblots

genomic sequence is in a database, the most efficient identification method presently available is matrix-associated laser desorption ionization mass spectrometry (MALDI-MS) with which about 500 spots can be analysed daily by one person. The method tolerates small amounts of salt in the sample, so that no time-consuming desalting steps are required after digestion. Several approaches using a combination of protein digestion on membranes and MS have also been reported. **Table 2** summarizes the essential steps of 2D electrophoresis and protein analysis.

Future Developments

2D electrophoresis is still in a developmental stage. Several technical improvements, mainly concerning further simplification of the technology and possible automation, an increase in reproducibility and sensitivity, and expansion of the pH detection spectrum, have to be achieved in order for the method to become routine in any biochemical laboratory. Gel grinding techniques, together with sophisticated software using the mass spectroscopic data, may be developed to produce a gel image without previous staining of the gel. Such a development could be decisive as to whether the technology will reach its major goal, i.e. the investigation of biological problems by a faithful comparison of protein expression levels. The completion of the sequencing of more genomes together with improvements in the analytical techniques will also lead to a more widespread application of the technology.

See Colour Plate 43.

Further Reading

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Two-dimensional Polyacrylamide Gel Electrophoresis

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Introduction

The evolution of tools utilized in biology and medicine, together with the exponential progress accomplished recently in the area of bioinformation, enables analysis of whole organism constituents. Such analyses are best exemplified by complete genomic sequences of different microorganisms, and by the recent development in techniques permitting dissection of the whole protein repertoire of an individual, namely its proteome. Furthermore, the dramatic growth in the number of genome projects as well as the speed with which genome sequences are determined has generated huge amounts of information. This progress has boosted techniques, notably two-dimension polyacrylamide gel electrophoresis (2D-PAGE), enabling the analysis of a proteome consisting of all the proteins expressed by a genome. Such analyses give information on the effector molecule itself, namely the protein, and take into account highly sophisticated mechanisms regulating gene expression. 2D-PAGE is the most powerful tool to separate a multitude of polypeptides that are contained in a single biological sample. Various procedures have been described to separate proteins according to biophysical parameters. In 1975, O'Farrel, Klose and Scheele described optimized 2D procedures in which proteins were denatured and separated by electrophoresis on polyacrylamide gel. The first gel dimension comprised a separation according to the protein charge by isoelectric focusing,