electrophoresis

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Combination of 2-D Gel and Liquid-Phase Electrophoretic Separations as Proteomic Tools in Neuroscience

Pia Davidsson, Department of Clinical Neuroscience, Experimental Neuroscience Section, Göteborg University, Sahlgrenska University Hospital/Mölndal, Mölndal, Sweden

Introduction

The most widespread strategy for studying protein expression in biological systems employs analytical two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) followed by enzymatic degradation of isolated protein spots, peptide mapping, and bioinformatic database searches. However, certain types of proteins, many of which may have important cellular functions, are not easily analyzed using this general strategy. These primarily include membrane and low-abundance proteins. Proteomic approaches using combinations of analytical or preparative 2-D electrophoresis — liquid-phase isoelectric focusing (IEF) in the Rotofor® cell, SDS-PAGE in the Model 491 prep cell, or one-dimensional (1-D) SDS-PAGE and electroelution in the mini whole gel eluter — followed by immunoblotting, mass spectrometry (MS), and database searches will be presented and discussed. The electrophoretic strategies will be illustrated with examples from our work on human cerebrospinal fluid (CSF) and brain tissue to show the powerful combination of analytical and preparative 2-D electrophoresis.

Investigations of alteration of multiple protein expression patterns provide a powerful strategy for investigating complex pathophysiological processes in neurodegenerative disorders such as Alzheimer's disease (AD). Among the current methods for studying protein expression, 2-D electrophoresis in combination with MS is the principal approach for proteome analysis. To study neural proteins — markers for neuropathological processes or mechanisms — requires further developments in proteomic methodology. Neuron-specific proteins are often low-abundance or membrane-associated proteins. Therefore, alternative separation methods including combinations of analytical or preparative 2-D electrophoresis have to be developed to provide information complementary to that derived from analytical 2-D electrophoresis. Our study of neural proteins focuses on CSF. Analysis of proteins in CSF is of major diagnostic importance because of its close connection to the brain and the ease of clinical availability. Today, CSF proteins identifiable on a traditional 2-D gel are restricted to high-abundance proteins. To study low-abundance proteins like neuron-specific proteins, a prefractionation step prior to analytical or preparative 2-D electrophoresis has to be performed. Many of the limitations of 2-D electrophoresis arise from its use of a gel as the separation medium; a liquid medium for electrophoretic separation may offer significant advantages due to reduced loss of proteins. A variety of approaches for preparative-scale IEF have been developed. Bio-Rad's Rotofor cell was developed for preparative-scale, free-solution (liquid-phase) IEF applications.

Methods and Results

To study neural proteins, some alternative proteomic methods have been developed which provide important information complementary to that derived from 2-D gels. Analytical and preparative methods, including direct 2-D electrophoresis on mini gels, liquid-phase IEF in the Rotofor cell combined with 2-D gels, immunoblotting, SDS-PAGE in the Model 491 prep cell, and 1-D SDS-PAGE and electroelution in the mini gel eluter (Figure 1), each provide a different analytical "window" through which protein expression in a biological system can be followed.

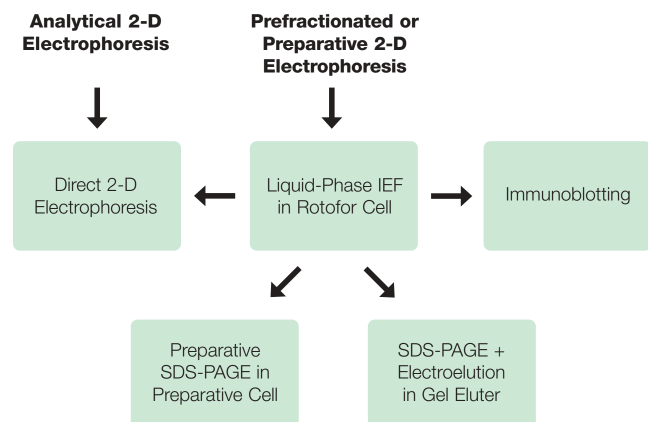

Fig. 1. Possible combinations of analytical and preparative 2-D electrophoretic methods.

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Direct Analytical 2-D Electrophoresis of Human CSF

We have used mini-gel 2-D electrophoresis, SYPRO Ruby protein staining, and MS for clinical screening of disease-influenced CSF proteins in AD patients (Davidsson et al. 2002). The first-dimension separation was carried out using ReadyStrip™ immobilized pH gradient (IPG) strips (pH 4–7) in the PROTEAN® IEF system. In the second dimension, the proteins were separated on small vertical gels (NOVEX NuPAGE system). Image acquisition and analysis were performed on a Fluor-S MAX™ multiimager system. The gels were analyzed digitally with PDQuest™ software. The criterion for a successful protein match was that the PDQuest software could detect and quantitate the protein in all CSF controls. The mean intensity of each matched protein spot in 15 AD samples was statistically compared with the mean intensity of the same protein spot in 12 controls. By comparing the intensity of spots between AD samples and normal controls, 15 spots were found to be significantly up- or down-regulated (Mann-Whitney U test, $p < 0.05$ in PDQuest software), and these proteins were identified by MS. Since multiple statistical comparisons were made, the Bonferroni correction was used for further comparisons of the selected protein spots between the groups, which were done using Student's t test. The levels of ten protein spots were still significantly altered in CSF of AD patients (Figure 2). The average concentration of the identified proteins on the SYPRO Ruby-stained mini 2-D gels was 1–20 mg/L. We have been able to identify ubiquitin, which is present in CSF at 0.050–0.200 mg/L, on 2-D gels of CSF samples.

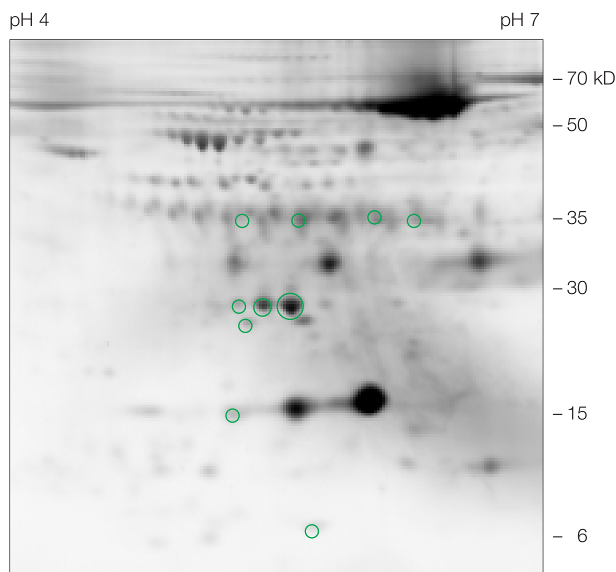

Fig. 2. Direct analytical 2-D electrophoresis of human CSF from AD patients. A typical image of a SYPRO Ruby-stained 2-D mini gel of CSF proteins. CSF proteins from 15 AD patients and 12 controls were analyzed on pH 4–7 ReadyStrip IPG strips, followed by separation in 10% NuPAGE gels. The gels were stained with SYPRO Ruby protein gel stain; proteins were detected and compared using PDQuest software. Selected spots, representing CSF proteins with different levels in AD patients compared to controls, are indicated.

Enrichment of Neuron-Specific Proteins in Human CSF Using Liquid-Phase IEF and Immunoblotting

Liquid-phase IEF in combination with immunoblotting has been used to enrich trace amounts of neuron-specific proteins that are involved in AD (Davidsson et al. 1999b, Sjögren et al. 2001). Biological fluids such as CSF and serum were prepared for prefractionation by solubilizing them in a neutral detergent (0.1% octylglucoside). Ampholytes were added to a concentration of 2.5%, and 18–55 ml samples were loaded into a Rotofor cell for fractionation over a broad pH range (3–10). After IEF, 20 separate fractions were rapidly harvested. The proteins in the IEF fractions were then separated by SDS-PAGE, stained with colloidal Coomassie Blue (Figure 3A), and detected by immunoblotting using specific monoclonal antibodies (Figures 3B and 3C). Tau protein, a marker for neurodegeneration, and the synaptic protein rab3a, a marker for synaptic function, were detected in nanogram per liter quantities in CSF. Tau protein in CSF was detected as four bands with molecular weights of 25–80 kD, using four different types of antibodies that recognize both phosphorylated and unphosphorylated forms. Our results (Figure 3B) show that tau protein is present in both phosphorylated and unphosphorylated isoforms, and in full-length and truncated forms in CSF (Sjögren et al. 2001). Five synaptic proteins (rab3a, synaptotagmin, GAP-43, SNAP-25, and neurogranin) have been demonstrated in CSF using this method (Figure 3C showing rab3a in CSF; Davidsson et al. 1999b).

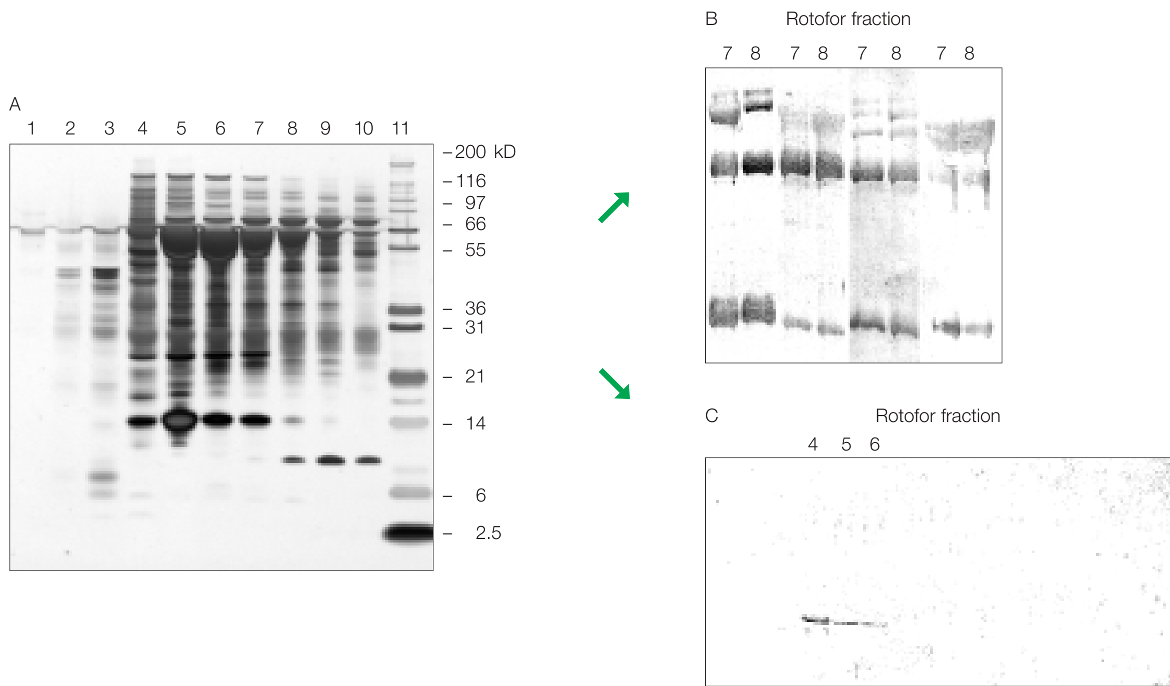

Fig. 3. Enrichment of neuron-specific proteins in human CSF using liquid-phase IEF in combination with immunoblotting. The 50 ml CSF sample was concentrated by precipitation in 10% TCA. The TCA precipitate was brought to a volume of 10 ml with 0.1% octylglucoside and 2.5% ampholytes. The CSF protein sample was loaded onto the Rotofor cell and run at 10 W constant power. Twenty fractions were harvested. A, SDS-PAGE (12%) followed by colloidal Coomassie Blue staining of the Rotofor fractions. Lanes 1–10 correspond to Rotofor fractions 1–10. Lane 11 contains molecular weight standards. A 300 µl aliquot of each Rotofor fraction was concentrated 5-fold by vacuum centrifugation. The proteins were separated through a 12% SDS mini gel followed by immunoblotting using antibodies to B, tau protein, and C, rab3a protein. Tau protein was detected in Rotofor fractions 7 and 8 by the antibodies AT120, BT2, AT270, and AT8, respectively (left to right). The protein rab3a was detected in Rotofor fractions 4–6 using MAbCl 42:2 from mouse.

Identification of Proteins in Human CSF Using Liquid-Phase IEF Prefractionation, 2-D Electrophoresis, and MS

We have developed a method for prefractionating proteins from individual CSF samples by liquid-phase IEF in the Rotofor cell and subsequent analysis of selected fractions by 2-D electrophoresis on mini gels. Human CSF (3 ml) was diluted with 9 ml of distilled water, and the proteins were separated using the Rotofor cell as described above. Selected IEF fractions were pooled prior to 2-D electrophoresis, and the proteins were precipitated with ice-cold acetone. The first-dimension separation was carried out using narrow-range ReadyStrip IPG strips, and the second-dimension on NuPAGE mini gels. When comparing 2-D gels of prefractionated and unfractionated CSF samples, more protein spots were detected in the 2-D gels of prefractionated CSF than in direct 2-D electrophoresis of unfractionated CSF (Figure 4). Furthermore, variation in glycosylation states of different proteins, for example, α1-acid glycoprotein, was easily visualized using this method (Figure 4B). We have also shown that 2-D electrophoresis with liquid-phase IEF prefractionation improved the sequence coverage of transthyretin compared to conventional 2-D electrophoresis alone (Westman-Brinkmalm and Davidsson 2002). Furthermore, tryptic peptides from posttranslationally modified forms of transthyretin were not detected in matrix-assisted laser desorption/ionization (MALDI) analysis from gel spots, but only in the gel spots from the combined

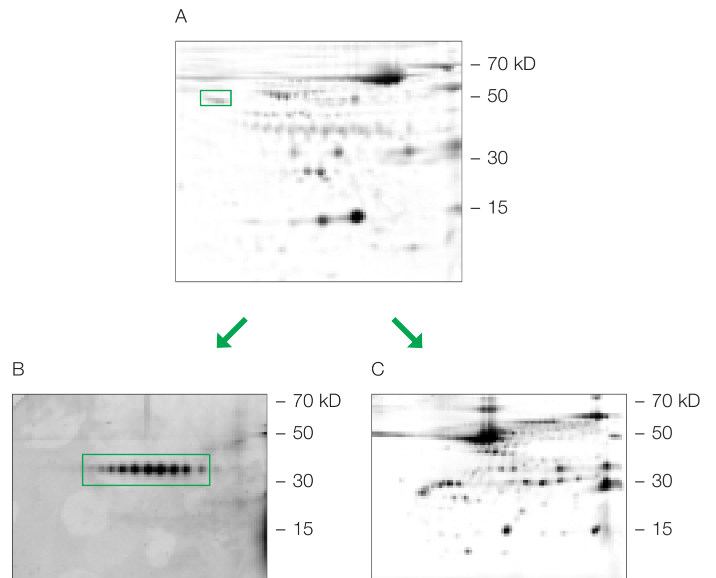

Fig. 4. Identification of proteins in human CSF using liquid-phase IEF as a prefractionation step followed by 2-D electrophoresis. A, typical image of a SYPRO Ruby-stained 2-D mini gel of CSF proteins. The CSF proteins were analyzed on pH 4–7 ReadyStrip IPG strips, followed by separation in 10% NuPAGE gels. B, CSF proteins were prefractionated in the Rotofor cell. Fractions corresponding to pH 3.0–3.5 were separated on pH 3–6 ReadyStrip IPG strips, then run on 2-D mini gels and stained with SYPRO Ruby. The glycosylated forms of α1-acid glycoprotein are indicated with boxes on both the unfractionated (A) and prefractionated (B) CSF 2-D gel. C, Rotofor fractions corresponding to pH 5.0–5.5 were separated on pH 4–7 ReadyStrip IPG strips.

liquid-phase IEF/2-D electrophoresis procedure (Westman-Brinkmalm and Davidsson 2002). The prefractionation step prior to 2-D electrophoresis thus enhanced the detection of less abundant proteins and the visualization of posttranslationally modified proteins.

Identification of Proteins Using Preparative 2-D Liquid-Phase Electrophoresis and MS

A strategy employing 2-D liquid-phase electrophoresis and MALDI time-of-flight MS was used to characterize tryptic digests of low-abundance proteins in human CSF (Davidsson et al. 1999a). 2-D liquid-phase electrophoresis is based on the same IEF and gel electrophoresis principles as the widely used analytical 2-D electrophoresis, except that analytes remain in solution throughout the separation process. In 2-D liquid-phase electrophoresis, the first step fractionates proteins by liquid-phase IEF into defined pH ranges in the Rotofor cell as described above. In the second purification step, the proteins from the Rotofor fractions were applied to the Model 491 prep cell for separation by SDS-PAGE on the basis of molecular weight. Individual fractions, each highly enriched for a single protein band and virtually free of other proteins, can be selected for MS analysis. Three CSF proteins, transthyretin, cystatin C, and β(2)-microglobulin, with concentrations of 3–20 mg/L, have been isolated and characterized (shown for transthyretin in Figure 5). Analysis of intact proteins using this method has also been performed (Puchades et al. 1999a).

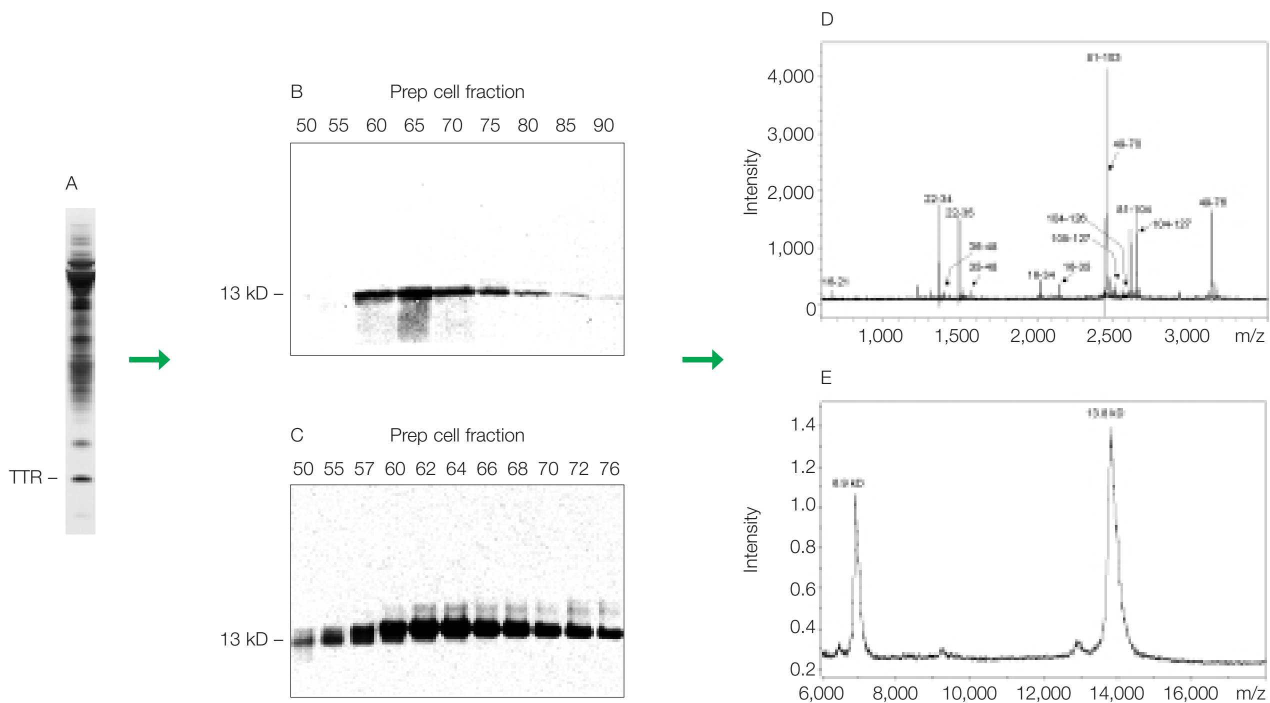

Fig. 5. Identification of proteins using preparative 2-D liquid-phase electrophoresis and MS. Undiluted CSF (15 ml) was fractionated in the first dimension into defined pH ranges using the Rotofor cell. A, Rotofor fraction 8, containing transthyretin (TTR), was further separated using the Model 491 prep cell. B, western blotting, and C, silver staining of transthyretin after SDS-PAGE using the Model 491 prep cell. Only the TTR band was detectable on the silver-stained gel. D, MALDI analysis of tryptic digest of TTR. E, direct MALDI MS analysis of TTR.

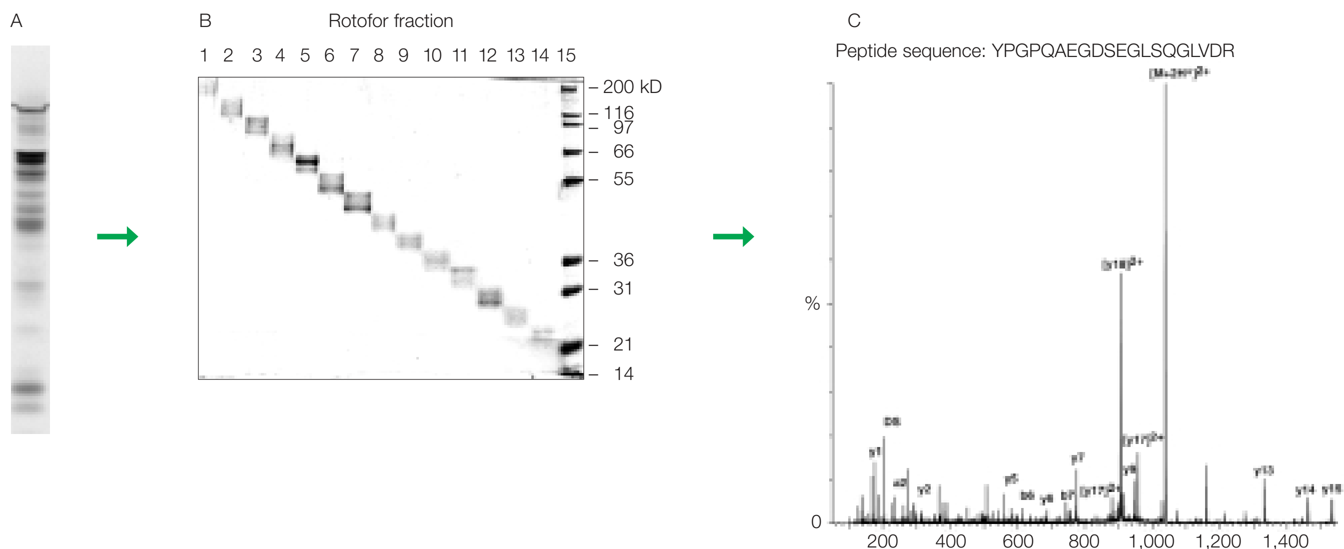

Fig. 6. Identification of proteins using preparative 2-D liquid-phase electrophoresis, NuPAGE system plus mini whole gel eluter, and MS. CSF (15 ml) was fractionated in the first dimension into defined pH ranges using the Rotofor cell. A, Rotofor fraction 2 was used for further purification by molecular weight using SDS-PAGE and electroelution in the second dimension. B, colloidal Coomassie Blue staining of whole gel eluter fraction 13, which was selected for MS analysis. C, fragment ion analysis of a doubly charged tryptic peptide from the human protein chromogranin A.

Alternatively, in the second dimension, the proteins from the Rotofor fractions were separated by 1-D SDS-PAGE with the NuPAGE system and electroeluted in Bio-Rad's mini whole gel eluter. This method has been used to purify proteins from human CSF and brain tissue (Davidsson and Nilsson 1999, Davidsson et al. 2001). Eluted proteins were precipitated with ice-cold acetone, trypsinized, and purified with ZipTip sample preparation tips prior to MS. We have identified several neuron-related proteins in CSF, such as amyloid precursor-like protein, chromogranin A (Figure 6), chromogranin B, glial fibrillary acidic protein, β-trace, transthyretin, ubiquitin, and cystatin C. Proteins from a detergent-solubilized human frontal cortex homogenate have also been characterized, including membrane proteins such as synaptophysin, syntaxin, and Na^+/K^+ ATPase. One-third of the identified proteins had not previously been identified in human CSF or human frontal cortex using proteomic techniques. Advantages of this method compared to 2-D liquid-phase electrophoresis are the speed of analysis and the easy handling. The whole process, from dialysis of CSF to MALDI analysis, can be completed in 18 hr.

Preparative 2-D liquid-phase electrophoresis allows high protein loads (up to 1 g) and larger volumes of sample (up to 55 ml), thus yielding sufficient amounts of low-abundance proteins for further characterization by MS. Removal of SDS with chloroform/methanol/water or ice-cold acetone easily interfaces electrophoresis with MALDI-MS (Puchades et al. 1999b).

Discussion

Given the complexity of protein expression patterns in living organisms, it is difficult to claim that a single type of separation is ideal for global protein analysis. Analytical 2-D electrophoresis will continue to be a cornerstone in the field of proteomics in the near future, because of the technique's ability to resolve and visualize several thousand proteins. But because standard 2-D electrophoresis fails to resolve some types of proteins, combinations of 2-D electrophoresis with other methods (preparative liquid-phase electrophoretic separations) will continue to be key approaches to studying global protein expression, especially in biomedically important areas such as neuroscience.

The advantages of using liquid IEF to separate and identify proteins from complex biological mixtures are the speed of analysis, high load capacity in the IEF separation, and retention of very large, small, highly basic, membrane-associated, or low-abundance proteins, yielding sufficient amounts for characterization by MS. In a proteomic approach, direct 2-D electrophoresis or a combination of liquid-phase IEF and 2-D electrophoresis will be used to study the expression and posttranslational modifications of disease-related proteins, while preparative 2-D electrophoresis will be used to identify and characterize low-abundance and membrane-associated proteins.

The use of complementary strategies in proteome studies of cerebrospinal fluid and brain tissue is expected to offer new perspectives on the pathology of neurodegenerative diseases, and reveal new potential markers for brain disorders.

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