The Rotofor® System As a Prefractionation Device Used Prior to 2-D Gel Electrophoresis

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Introduction

When researchers started working with two-dimensional (2-D) gel electrophoresis, they quickly understood the great complexity of the samples they were analyzing and began to search for ways to fractionate crude samples before analysis. Depending on the kind of material being analyzed — tissue, cells, or body fluids — many approaches have been used to reduce sample complexity.

One challenge remaining in today's research is to be able to detect low-abundance proteins in samples. This is particularly true when studying body fluids like serum, cerebrospinal fluid (CSF), and urine because they contain highly abundant proteins (for example, albumin and immunoglobulins), rendering the analysis of low-abundance proteins very difficult. Many efforts have been made to remove highly abundant proteins like albumin from a sample by affinity chromatography, and they have been more or less successful. Another approach is to divide the sample into different fractions and exclude those that contain highly abundant proteins. The low-abundance proteins will then be enriched in the remaining fractions. This can be done at a preparative scale by prefractionation of the sample in the Rotofor cell, as proteins are separated in liquid phase according to their isoelectric point (pI).

We compared the CSF proteome of Alzheimer's disease (AD) and frontotemporal dementia (FTD) patients with that of nondementia controls to identify proteins that play a role in these disease processes and to study their pathogenesis. Detection of new biomarkers by screening 2-D gels could further strengthen diagnosis and provide useful information in drug trials. Our strategy to increase the detection of less-abundant proteins in CSF was to use liquid-phase isoelectric focusing (IEF) in the Rotofor cell as a prefractionation step prior to 2-D gel electrophoresis (Davidsson et al. 2002a).

Methods

CSF samples from individual patients were prefractionated using a mini Rotofor cell. Each 3 ml CSF sample was mixed with 9 ml deionized water, 1% SERVALYT ampholytes, pH 3–10, (SERVA Electrophoresis, GmbH, Germany), 20 mM DTT, and 0.5 ml complete antiprotease solution (Roche Diagnostics, Mannheim, Germany). Focusing was performed at 4°C and at 12 W constant power for 2.5 hr. Twenty fractions were harvested (approximately 0.5 ml in each fraction). Selected fractions (2–5, 6–9, and 10–14) were pooled and concentrated to 300 µl in a vacuum centrifuge.

Prior to 2-D gel electrophoresis, the 300 µl protein fractions were precipitated with 900 µl 90% ice-cold ethanol and stored for 2 hr at –20°C. For unfractionated samples, which were used directly for 2-D electrophoresis, 300 µl CSF proteins were precipitated using 900 µl ice-cold acetone and stored for 2 hr at –20°C. The mixtures were centrifuged at 10,000 x g for 10 min at 4°C, and the resulting protein pellets were air-dried. Prefractionated samples were precipitated with ethanol instead of acetone because ethanol was more effective in removing ampholytes, which were present in the fractionated samples and may interfere with first-dimension separations (Folkesson Hansson et al. 2004). For unfractionated samples, acetone and ethanol precipitation had similar efficiencies.

First-dimension electrophoresis was carried out on 7 cm pH 3–6, pH 4–7, and pH 5–8 IPG strips for 20,000 V-hr on a PROTEAN® IEF cell. Second-dimension electrophoresis was performed using a 10% NuPAGE mini gel system (Invitrogen) run with MES buffer (50 mM MES, 50 mM Tris, 0.8 mM EDTA, 3.5 mM SDS) for 35 min. Gels were stained with SYPRO Ruby protein gel stain according to the supplier's protocol. Image acquisition and analysis were performed on a Fluor-S™ multiimager. Protein spots were detected, quantitated, and matched using PDQuest™ 2-D gel analysis software, v. 6.0.2.

Protein spots were excised, digested with trypsin, and analyzed by mass spectrometry (MS) as previously described (Folkesson Hansson et al. 2004).

Results and Discussion

Comparison of Direct and Prefractionated CSF Proteins Separated by 2-D Gel Electrophoresis

We first compared the 2-D separation of prefractionated CSF samples with that of unfractionated controls (direct 2-D). CSF samples from control (nondementia) patients were used. For unfractionated samples, 300 µl CSF was prepared for 2-D gel electrophoresis as described above and separated on pH 3–6, 4–7, and 5–8 IPG strips. For prefractionated samples,

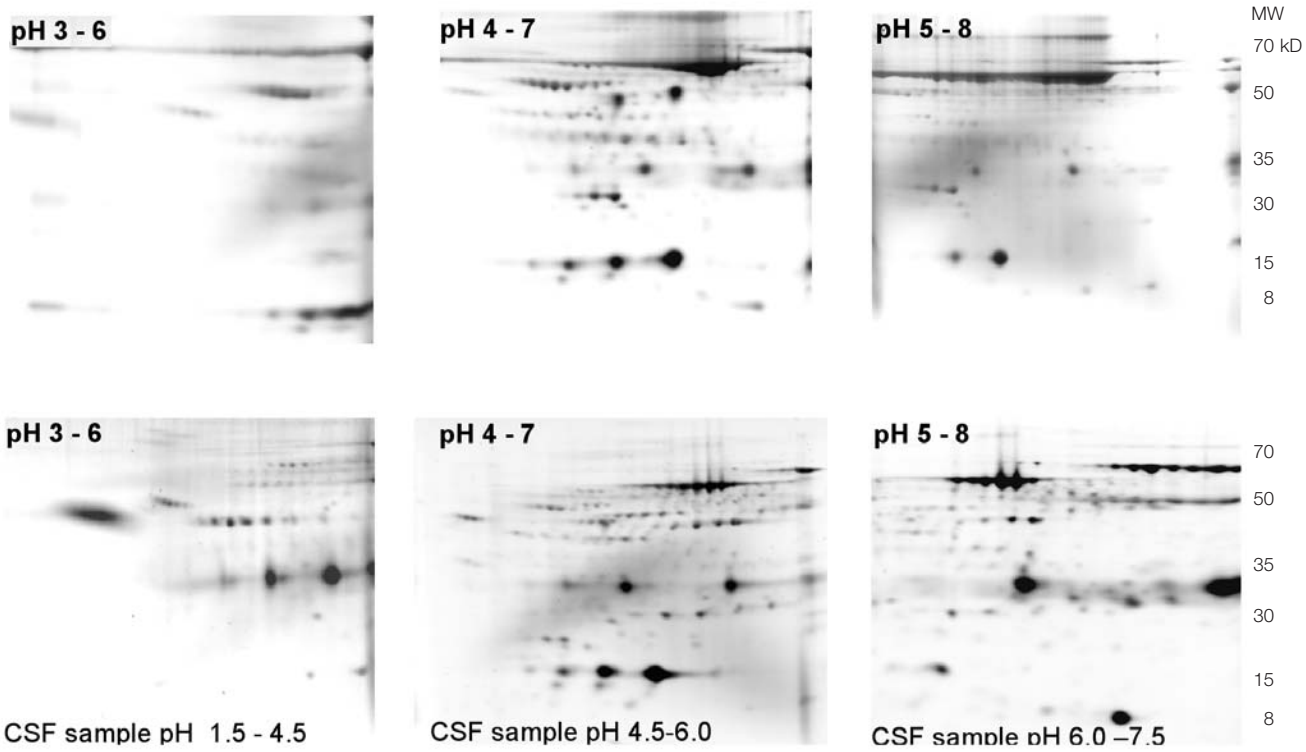

Fig. 1. Comparison of direct and prefractionated CSF on SYPRO Ruby-stained 2-D gels. The upper figures represent direct 2-D gel electrophoresis of unfractionated CSF samples, and the lower figures represent 2-D gel electrophoresis after prefractionation on the mini Rotofor cell. The pH interval of the IPG strips is denoted in the upper-left corner of the gels, and the pH range of the prefractionated CSF samples, at the bottom of the gel images.

fractions were pooled according to their pH, prepared for electrophoresis as described above, and loaded on the IPG strip with the corresponding pH range.

The results (Figure 1) showed that the gels of prefractionated samples contained more spots with a higher protein concentration. In particular, the spots in the pH 3–6 and pH 5–8 gels increased in both number and density.

Another advantage of using the Rotofor cell is its capacity for a high protein load (milligram amounts in the small fractionation cell, grams in the large fractionation cell) and the ability to concentrate the sample further after prefractionation, for example, by protein precipitation in ethanol or acetone.

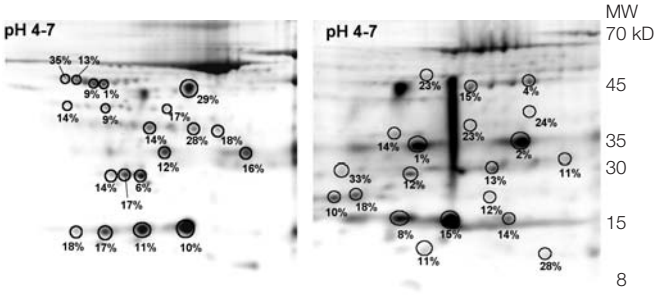

Fig. 2. Reproducibility of direct 2-D gel electrophoresis (left) and 2-D gel electrophoresis after prefractionation of CSF (right). Images are of SYPRO Ruby-stained 2-D gels. Numbers represent the CV values of circled protein spots from four replicate gels analyzed by PDQuest software.

Reproducibility of 2-D Gel Electrophoresis of CSF Samples

The effect of prefractionation on the reproducibility of 2-D gel electrophoresis was also assessed. Four aliquots of one CSF sample were individually prefractionated on the mini Rotofor cell, and for each aliquot, fractions 6–9 (having a pH of 4.5–6.0) were pooled and analyzed by 2-D gel electrophoresis using pH 4–7 IPG strips. The spots chosen for analysis included proteins of differing molecular weights, pIs, and abundance, as well as different isoforms of the same protein. In a selection of 20 proteins from the four replicate gels (Figure 2), the coefficient of variation (CV) (SD/mean x 100) of the spots was 1–33% (mean 15.4%). These values were comparable to those obtained for 2-D gels of unfractionated samples (CVs of 1–35%, mean 14.6%), and agreed with those described in another study (Nishihara and Champion 2002). These results show that the prefractionation step did not introduce additional variation and was reproducible from sample to sample.

A Proteomic Study Comparing Prefractionated CSF Proteins From AD and FTD Patients With Control Subjects

The prefractionated 2-D gel electrophoresis method was used to screen for changes in the CSF proteome of AD (Puchades et al. 2003) and FTD (Folkesson Hansson et al. 2004) patients compared to that of nondementia controls.

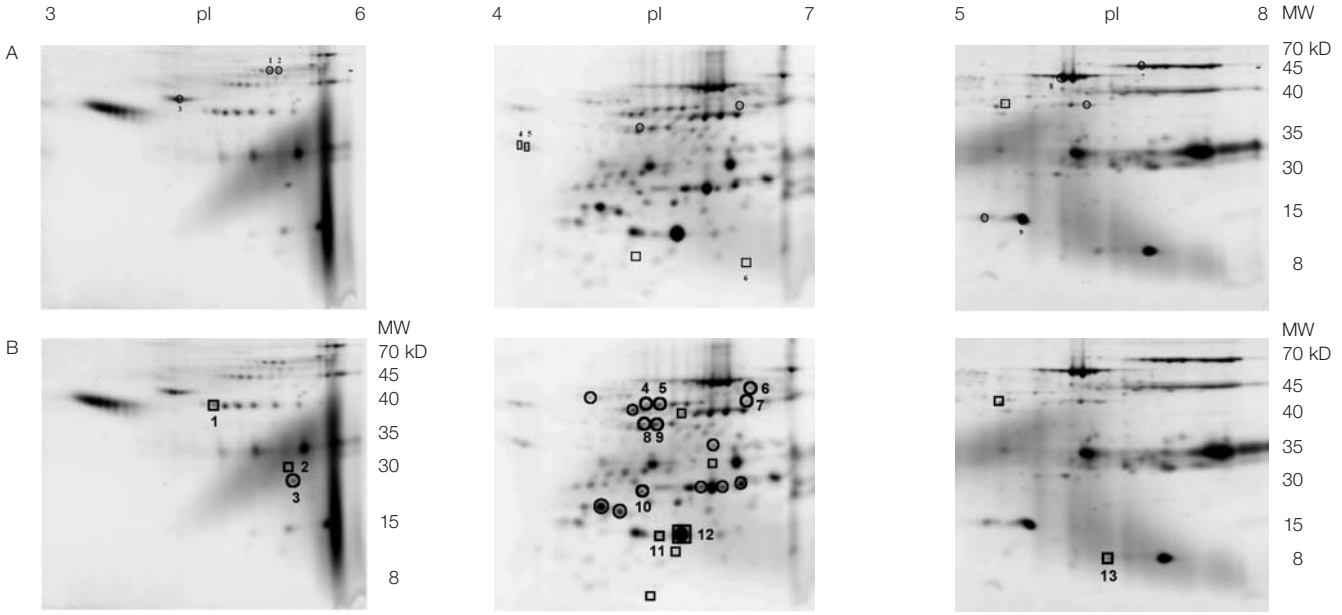

Fig. 3. SYPRO Ruby-stained 2-D gels of prefractionated CSF proteins from dementia patients. A, CSF from AD patients; B, CSF from FTD patients. Fractions 2–5, 6–9, and 10–14 were analyzed on pH 3–6, pH 4–7, and pH 5–8 IPG strips, respectively, followed by electrophoresis in 10% NuPAGE gels. Typical gels are shown. Circles, protein spots that decreased 2-fold in intensity; squares, protein spots that increased 2-fold in intensity in CSF of dementia patients compared to controls.

Table. Proteins up- or downregulated at least 2-fold in dementia patients compared to controls. CSF was prefractionated before 2-D gel electrophoresis, and gel spots identified by MS as described in Methods. Spot numbers refer to those in Figure 3A for AD patients, and Figure 3B for FTD patients.

Spot #	Protein Identity	NCBI Accession #	MW (kD)*	pI*	# of Peptides Matched	Sequence Coverage (%)	Levels Relative to Control	Normalized Spot Density (Mean ± SD) Dementia Patients	Controls
AD Patients									
1	α-1β glycoprotein	23503038	51.87	5.51	4	11	↓	3,460 ± 839	12,098 ± 6,889
2	α-1β glycoprotein	23503038	51.87	5.51	4	11	↓	3,604 ± 976	11,520 ± 8,433
3**	α-2-HS glycoprotein	2521983	40.20	5.4	4	9	↓	5,732 ± 4,255	20,548 ± 15,819
4	α-1 antitrypsin precursor	177836	44.32	5.37	7	27	↑	5,559 ± 2,967	2,310 ± 1,408
5	α-1 antitrypsin precursor	177836	44.32	5.37	–	–	↑	4,636 ± 1,299	969 ± 891
6	β-2 microglobulin	229995	11.58	6.5	4	46	↓	2,722 ± 1,550	951 ± 471
7	Transferrin precursor	4557871	77.03	6.9	21	31	↓	7,924 ± 3,586	19,159 ± 5,308
8	Albumin precursor	6013427	69.21	5.9	13	19	↓	10,831 ± 7,298	23,019 ± 11,727
9**	Transthyretin	339685	13.76	5.3	8	81	↓	21,425 ± 12,812	68,603 ± 27,957
FTD Patients									
1	Zn-α-2-glycoprotein	141596	31.6	5.70	4	22	↑	42,256 ± 7,227	4,189 ± 979
2	Proapolipoprotein A1	178775	28.9	5.45	14	43	↑	11,572 ± 10,432	4,575 ± 2,309
3	Retinol-binding protein	20141667	21.1	5.27	4	35	↓	3,680 ± 1,675	8,131 ± 4,857
4	Serum albumin	113576	52.0	5.69	11	22	↓	1,819 ± 1,657	7,272 ± 1,812
5	Serum albumin	113576	52.0	5.69	11	22	↓	2,415 ± 964	7,839 ± 1,906
6	Serum albumin	113576	66.0	5.69	12	20	↓	1,003 ± 309	3,098 ± 1,428
7	Serum albumin	113576	66.0	5.69	9	15	↓	1,528 ± 587	4,896 ± 2,551
8	Alloalbumin	178345	69.2	5.99	12	19	↓	1,361 ± 530	7,454 ± 1,748
9	Alloalbumin	178345	69.2	5.99	12	19	↓	2,463 ± 806	8,395 ± 1,475
10	Retinol-binding protein	20141667	20.9	5.27	4	35	↓	1,744 ± 551	5,871 ± 2,040
11	Transthyretin	339685	13.8	5.3	8	81	↑	45,198 ± 26,202	9,149 ± 2,685
12	Transthyretin	339685	13.8	5.3	8	81	↑	303,002 ± 72,750	14,7732 ± 30,928
13	β-2-microglobulin	4757826	12.9	5.77	5	46	↑	41,938 ± 43,510	12,287 ± 3,161

* Theoretical molecular weight.

** Mass spectrometric results from Davidsson et al. (2002a),

AD Patients — Five AD patients aged 81 ± 5 years (mean ± SD) and five nondementia controls aged 62 ± 12 years were studied. Three ml of CSF from each of the patients and controls was prefractionated in the mini Rotofor cell, and the fractions were pooled and analyzed by 2-D gel electrophoresis as follows: fractions 2–5 (pH 1.5–4.5) on pH 3–6 IPG strips, fractions 6–9 (pH 4.5–6.0) on pH 4–7 strips, and fractions 10–14 (pH 6.0–7.5) on pH 5–8 strips (Figure 3A). Comparing the intensities of the protein spots, we found 15 protein spots that were up- or downregulated at least 2-fold in AD patients compared to controls (five were upregulated and ten were downregulated). All altered proteins are marked in Figure 3A. Seven proteins, including α-1β glycoprotein, α-2-HS glycoprotein, transthyretin, α-1 antitrypsin precursor, β-2 microglobulin, transferrin precursor, and albumin precursor, were identified by MS and are listed in the table.

FTD Patients — In the same manner, 3 ml of CSF from each of five FTD patients aged 70.6 ± 5.6 years (mean ± SD) and five nondementia controls aged 59.2 ± 11.9 years was prefractionated in the mini Rotofor cell and compared by 2-D gel electrophoresis (Figure 3B). We found that 10 spots were upregulated and 16 spots downregulated at least 2-fold in FTD patients compared to nondementia controls. Thirteen of the protein spots, corresponding to seven different proteins, were identified by MS (see table). In CSF samples from FTD patients, levels of one isoform of Zn-α-2-glycoprotein, proapolipoprotein A1, and β-2-microglobulin as well as two isoforms of transthyretin were increased, while four isoforms of serum albumin, two isoforms of alloalbumin, and two isoforms of retinol-binding protein were reduced compared to controls (see table).

Levels of apolipoproteins have been shown to change in previous direct 2-D gel electrophoresis studies by our group (for example, Davidsson et al. 2002c). However, these proteins were poorly detected in gels from prefractionated CSF compared to direct 2-D gel electrophoresis of CSF. This might be explained by the fact that lipoproteins tend to adhere to plastic vials (Hesse et al. 2000) and could be lost in the additional sample transfer step from one test tube to another that is needed in the prefractionation procedure.

Summary

The results shown here describing altered amounts of specific proteins in the CSF of AD and FTD patients are consistent with previous results from direct 2-D gel electrophoresis experiments (Davidsson et al. 2002b, c). Therefore, these results support the implication of these proteins in AD and FTD pathology. By adding a prefractionation step to the 2-D gel electrophoresis workflow, new proteins were also identified, such as fetuin (α-2-HS glycoprotein), which showed reduced levels in AD patients and has never been previously associated with AD. Similarly, Zn-α-2-glycoprotein, which showed increased levels in FTD patients, has not been associated previously with dementia. Due to the small sample size, it must also be emphasized that the protein changes found in these studies are preliminary.

By using 2-D gel electrophoresis to compare proteins expressed in patients with different neurodegenerative diseases like AD and FTD, we found specific protein patterns for both diseases. This was also the case in the prefractionation studies; we could observe that different isoforms of the proteins were affected or that proteins were regulated differently. The significance of such changes is still unknown and necessitates further investigations.

In conclusion, this study shows that the prefractionation 2-D gel electrophoresis method is reproducible to the same extent as traditional 2-D gel electrophoresis and can enrich CSF proteins in the gel, which may offer new perspectives on the pathology of neurodegenerative diseases.

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