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Extraction and proteomic analysis of proteins from normal and multiple sclerosis postmortem brain

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Abstract

In this study, a reproducible fractionation procedure was developed to reduce levels of the abundant cytoskeletal proteins that are present in normal and pathological central nervous system (CNS) tissues. The fractionation and proteomic analysis techniques employed greatly facilitated comparison of the spectrum of proteins in normal postmortem brain with proteins in samples from patients with multiple sclerosis, an inflammatory demyelinating disease in which complex changes in protein expression occur as lesions develop. This approach may be of value for the proteomic identification and quantitation of proteins which undergo disease-related changes in CNS disorders, and also for protein expression studies on normal adult and developing CNS tissues.

Keywords: Central nervous system; Human brain; Postmortem; Proteomics; Protein extraction; Multiple sclerosis; MS

1. Introduction

More than half of the 30,000 genes in the human genome may be expressed in the central nervous system (CNS), and 20% of them may be expressed only in the CNS. Many CNS disorders not only involve the interplay of many proteins but also the variation of specific gene products which are expressed in different facets of a disease [1]. The use of proteomic technologies offers the opportunity to identify CNS proteins and follow post-translational modifications indicative of complex pathophysiological events in CNS disorders [2]. Proteomic-based analyses are now starting to make progress on building databases for utilization in studies on the normal brain and spinal cord and on neurological and psychiatric disorders. The development of proteome databases of human CNS tissues may help avoid experimental model ambiguities, accelerate detection of specific diagnostic and prognostic disease markers, and develop more selective therapeutics [3]. However, there are many challenges in proteomic neuroscience, and to make more detailed analyses of the CNS proteome further improvements are needed in proteomic technologies to increase the sensitivity and efficiency of protein detection [4,5].

A number of proteomic studies have produced databases of proteins in normal and disease state cerebrospinal fluid (CSF) [6,7]. In a proteomic analysis of CSF from neurologically normal elderly people, an immunobased method enhanced detection of low-abundance proteins by greatly decreasing albumin and immunoglobulins levels, and considerable subject-to-subject variability was detected in the CSF proteome [8]. Using liquid chromatography tandem mass spectrometry on multiple sclerosis patient CSF samples, 65 proteins were identified from 300 spots, and a number of these proteins had not been reported previously in two-dimensional (2-D) electrophoresis studies on CSF [9]. As high immunoglobulin levels are present in multiple sclerosis CSF, a method has been developed for removing immunoglobulins to improve protein recovery and resolution which facilitates excision from gels for peptide mass

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fingerprinting. Using this method proteins not known to be present in normal CSF were identified in multiple sclerosis CSF [10,11].

Protein expression is dynamic and is affected by stress, disease, drug treatment and ageing [1]. Although proteins may be subject to some degradation immediately after death, the majority of proteins degrade only to a minor degree [12]. The correct interpretation of data should take into account age, gender, ethnicity, medicative history, ante-mortem status and postmortem intervals, and specific postmortem factors should be closely controlled. Identification of CNS marker proteins which show reproducible and quantitative changes as a function of the time between death and tissue preservation is of importance. In studies on the effects of postmortem time on protein expression patterns using proteomics on rodent brain, dihydropyrimidinase related protein-2 (DRP-2) was identified as a possible biomarker of postmortem time [13] and also temperature [14]. Another possible biomarker is *n-myc* downstream regulated gene 2 (NRDG-2), the levels of which appeared to change consistently as a function of time with postmortem interval in mouse brain [15].

In an early study on normal human CNS tissue using 2-D electrophoresis and matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry, about 400 spots corresponding to 180 parietal cortex proteins were identified on a 2-D map [16]. Preparative liquid phase 2-D electrophoresis has been used prior to mass spectrometry to examine proteins in detergent-solubilized normal frontal cortex and also in CSF [17]. Many human frontal cortex or CSF proteins identified had not been identified previously using proteomics, possibly because of low protein concentrations or low solubility. Advantages of this methodology were speed of analysis, high loadability in IEF separation, and non-discrimination of membrane or low abundance proteins. Using proteomic technologies to produce a human normal fetal brain database, about 1700 protein products of 437 different genes were identified and seven gene products were identified for the first time in fetal brain [18].

Proteomic analyses have also generated new data in studies on protein expression profiles in fetal Down's syndrome [19] and molecular mechanisms of human brain aging [20]. In a quantitative proteome analysis on Alzheimer's disease and age-matched control brain samples 37 differentially expressed proteins were identified [21]. Proteomics offer a rapid means of identifying modified proteins in aging and age-related neurodegenerative disorders without the limitations of immunochemical detection. Specifically oxidized proteins [22,23] and six targets of protein nitration have been identified in Alzheimer's disease brain [24], which provided evidence of the importance of oxidative stress in this disease.

In schizophrenia, bipolar disorder, and major depressive disorder no biological markers have been documented at the pathological, cellular or molecular level. However, using proteomics eight frontal cortex protein species that displayed disease-specific alterations in level were identified in these disorders [25]. In another study, almost half the altered proteins identified by proteomics in brain tissue from schizophrenia patients were associated with mitochondrial function and oxidative stress responses, and therefore oxidative stress and ensuing cellular adaptations may be linked to the schizophrenia disease process [26]. Human primary gliomas have been examined using a direct-tissue mass spectrometric technique to profile and map biomolecules. Mass spectral patterns reliably identified glial neoplasms of similar histological grade and differentiated them from tumors of different histological grades and from non-tumor brain tissues [27]. Another area of CNS research where proteomics has great potential to give new insights into complex alterations in protein spectra is the tissue damage and repair mechanisms occurring in neurotrauma [28].

Multiple sclerosis is an inflammatory disease of the human CNS with an unknown aetiology [29,30]. Neurological symptoms vary with the localization of multiple sclerosis plaques in the CNS. Lesion development is characterized by breakdown of the blood–brain barrier, loss of myelin from axons, and infiltration of lymphocytes and monocytes which release soluble inflammatory mediators. Myelin is removed from demyelinating lesions by macrophages, and limited remyelination may be carried out by oligodendrocytes which migrate into the lesion. However, these new oligodendrocytes and many axons are lost in subacute plaques. Astrocyte cells proliferate and form a dense glial scar as the plaque develops into a chronic lesion in which demyelination has ceased and inflammatory cells are no longer present.

The complex series of changes in protein expression which result from these events in plaques have been examined at low resolution by one-dimensional electrophoresis and immunoblotting [31,32]. Proteomic screening has been carried out on brain microsomes from mice with experimental allergic encephalomyelitis (EAE), the autoimmune disease model of multiple sclerosis [33]. This study showed that glucose regulated protein 94 (which plays roles in antigen presentation and pro-inflammatory cytokine production) and coagulation factor VIII (a marker of blood–brain barrier breakdown) were elevated in EAE. However, so far there has not been any published study on the proteomic evaluation of CNS tissue samples from multiple sclerosis patients.

Although very sensitive mass spectrometric techniques have been introduced recently, the high levels of intermediate filaments and tubulin present in CNS tissues make it difficult to examine less abundant proteins on gels. The primary aim of this study was to make proteins present at low levels more prominent for detailed proteomic image analyses by developing a fractionation procedure to reduce levels of abundant cytoskeletal proteins in normal and pathological CNS samples. The secondary aim was to determine conditions which would enhance resolution of proteins in gels for proteomic identification and quantitation of proteins which are differentially expressed in multiple sclerosis lesion areas.

These methodologies were used to identify landmark proteins in multiple sclerosis lesions and form a basis for building a platform for a database of proteins present in different types of multiple sclerosis lesions.

2. Experimental

2.1. Tissue samples

Normal control and multiple sclerosis brain samples from the NeuroResource tissue bank, London, UK were dissected from tissue blocks which had been snap-frozen in isopentane cooled on liquid nitrogen. Cryostat sections cut from each block were stained with oil red-O and haematoxylin for histological evaluation before blocks were thawed for dissection. For development of the fractionation and proteomic methodologies a work-up sample set of 16 normal control and eight plaque samples were used.

A detailed proteomic analysis on 22 additional samples was then carried out using the optimized fractionation and electrophoresis procedures. These samples were derived from three tissue blocks of brain white matter from two control subjects with no known CNS disorder, and three tissue blocks from the brain of a multiple sclerosis patient (Table 1). Each multiple sclerosis block was dissected into separate samples of plaque and macroscopically normal white matter immediately adjacent to each lesion (shown schematically in Fig. 1). Normal control or multiple sclerosis tissues were finely chopped to facilitate solubilization for fractionation, and then separated into either duplicate or triplicate homogenous 30–50 mg samples depending on amount of tissue available prior to rapid freezing in tubes for homogenization. As myelin loss, perivenular lymphocytic cuffing and large numbers of oil red-O positive macrophages containing myelin lipid breakdown products were present throughout all of these lesions they were classified as actively demyelinating [34].

2.2. Preparation of the total-extracts

A total-extract containing all the proteins soluble in the rehydration (RH) buffer was prepared initially from each sample in the work-up set, and was later prepared from selected samples in the proteomic analysis set. The RH buffer contained 2 M thiourea, 8 M urea (Perkin-Elmer MEN, Upplands Väsby, Sweden), 4% 3-(3-cholamidopropyl) dimethylammonio monohydrate (CHAPS; Sigma–Aldrich, Stockholm, Sweden), 0.5% immobilized pH gradient (IPG) buffer pH 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden), dithiothreitol (DTT) 0.3%, glycerol 7% and NP-40 (0.5%, Kebo Lab). Nineteen volumes of RH buffer were added to each frozen tissue sample in FastPrep tubes. After FastPrep beads were added to two-thirds of the mixture volume samples were homogenized in a FP120 FastPrep apparatus (Tamro, Mölndal, Sweden; speed 5, 5 s, ×2). Following vigorous vortexing for 3 h, liquid phases were transferred to new tubes and

Table 1
Data on samples examined in the detailed proteomic analysis

Sample codes	Sample type	WW (mg)	ORO score	Age (years)	DD (years)	M/F	DFT (h)	CSK-supernatant gel codes in Fig. 6
B504-33	NC W OVL	50	0.1	49	–	M	11	5NC
B504-33	NC W OVL	50	0.1	49	–	M	11	6NC
B501- 12	NC W PVR	50	0.0	65	–	M	15	8NC
B501- 12	NC W PVR	50	0.0	65	–	M	15	9NC
B501-11	NC W FVR	50	0.0	65	–	M	15	13NC
B501-11	NC W FVR	50	0.0	65	–	M	15	14NC
B488-107	XV OSvR	50	0.2	47	20	F	9	16XV
B488-107	XV OSvR	50	0.2	47	20	F	9	17XV
B488-107	XV OSvR	50	0.2	47	20	F	9	18XV
B488- 107	Plaque OSvR	50	4.4	47	20	F	9	21MS
B488- 107	Plaque OSvR	50	4.4	47	20	F	9	22MS
B488- 107	Plaque OSvR	50	4.4	47	20	F	9	23MS
B488-111	Plaque FVL	45	4.3	47	20	F	9	24MS
B488-111	Plaque FVL	32	4.3	47	20	F	9	25MS
B488-111	XV FVL	50	0.2	47	20	F	9	26XV
B488-111	XV FVL	50	0.2	47	20	F	9	27XV
B488-115	Plaque FSvL	50	3.2	47	20	F	9	28MS
B488-115	Plaque FSvL	43	3.2	47	20	F	9	30MS
B488-115	Plaque FSvL	30	3.2	47	20	F	9	Total-extract gel
B488-115	XV FSvL	50	0.2	47	20	F	9	31XV
B488- 115	XV FSvL	32	0.2	47	20	F	9	32XV
B488- 115	XV FSvL	30	0.2	47	20	F	9	Total-extract gel

Abbreviations: Sample type: tissue type and brain region; NCW: normal control brain white matter; XV: macroscopically normal-appearing white matter immediately adjacent to a MS plaque; O: occipital; P: parietal; F: frontal; V: ventricular; Sv: subventricular; R: right; L: left; WW (mg): mg wet weight of tissue; ORO: oil red-O and haematoxylin histological staining to assess lesion stage [34]; DD: disease duration; M/F: male or female; DFT: time between death and sample cryopreservation.

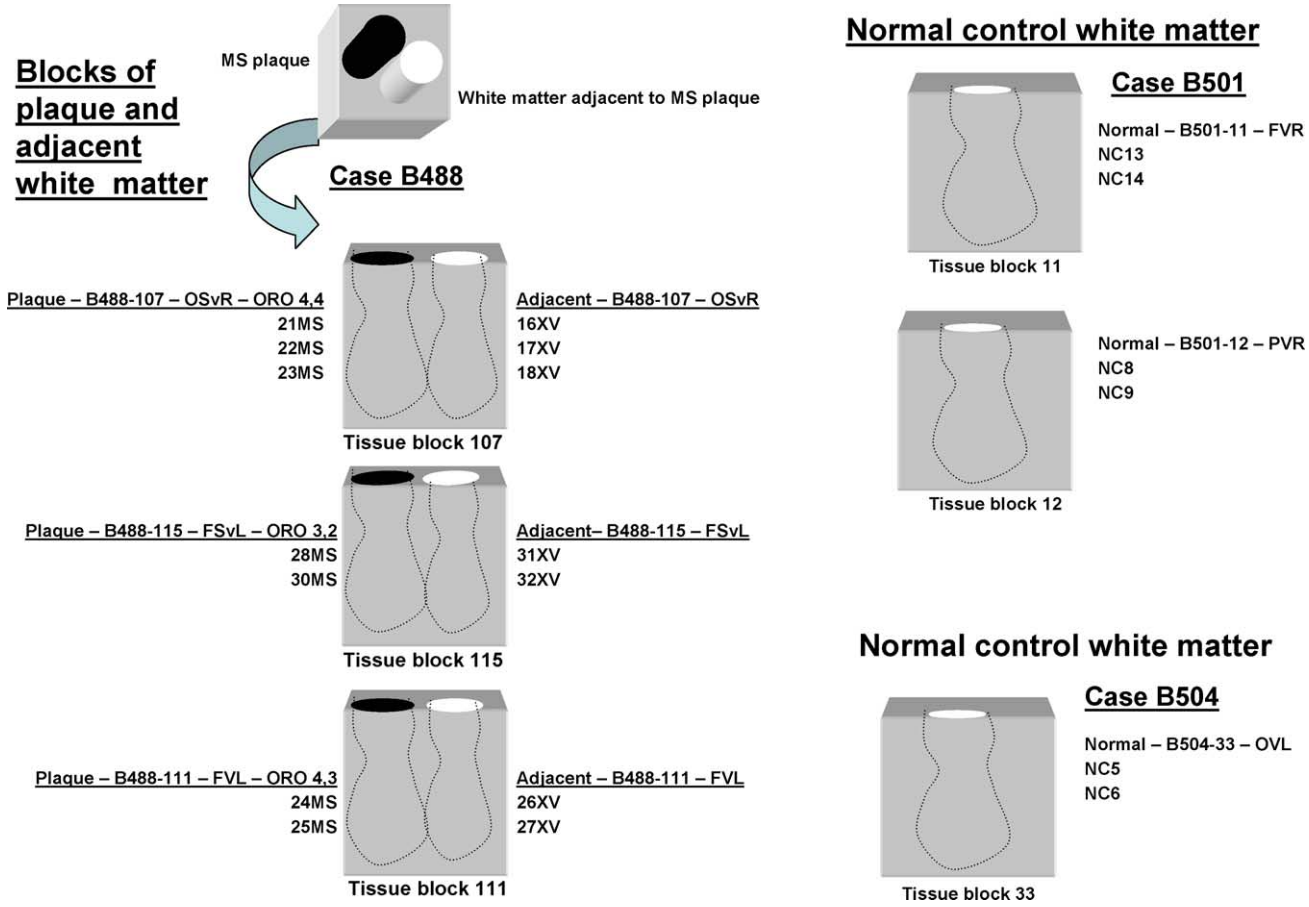

Fig. 1. Dissection of normal control and MS tissue blocks. Clinical information on these samples is shown in Table 1.

centrifuged (15,000 × *g*, 15 min, room temperature) and supernatants were retained as the total-extracts and pellets (very small amounts of insoluble material) were discarded.

2.3. Fractionation for reduction of cytoskeletal proteins

The cytoskeleton (CSK) buffer was used in order to visualize or enhance weak proteins on gels by extracting as much as possible of the intermediate filaments and tubulin seen in gels of proteins soluble in RH buffer. Experiments for optimizing the efficiency of the CSK buffer for removing cytoskeletal proteins were carried out on samples in the work-up set, and the optimized buffer was then used for preparation of the proteomic analysis sample set. The optimal composition, based on buffers used in several studies [35–39], was 1% Triton X-100 (Sigma–Aldrich), 0.6 M KCl, 10 mM $MgCl_2$, 1 mM EDTA (Merck, Spånga, Sweden), 10 mM hydroxyethylepiper-azinylethane sulfonic acid (HEPES), 1 mM GTP and protease inhibitors (complete protease inhibitor cocktail tablets, Roche Diagnostics, Bromma, Sweden).

CSK buffer (7 volumes) was added to frozen tissue samples in FastPrep tubes (Fig. 2). After beads to two-thirds of the mixture volume were added, tubes were shaken in the FastPrep apparatus (velocity 5, 5 s, ×2) with cooling between shakings. Samples were vortexed vigorously for 2 h, beads were removed and samples were centrifuged (1000 × *g*, 4 °C, 5 min). Pellets and fatty layers were washed in 1 ml CSK buffer without KCl, Triton X-100 or GTP, dissolved in 20 volumes of RH buffer with 0.5% IPG buffer pH 4–7, and the pellet-1s were stored at −70 °C. Supernatants transferred to new tubes were centrifuged (15,000 × *g*, 4 °C, 15 min) and resulting CSK-supernatants were stored at −70 °C for future desalting. Pellets were washed in 1 ml CSK-buffer without KCl, Triton X-100 or GTP and centrifuged (15,000 × *g*, 4 °C, 15 min), and supernatants were discarded. The resulting CSK-pellets were dissolved in 20 volumes of RH buffer with 0.5% IPG buffer pH 4–7and stored at –70 °C.

2.4. Desalting

After thawing the CSK-supernatants were transferred into Ultrafree-0.5 Centrifugal Filter Units (cut-off 5 kDa; Millipore AB, Sundbyberg, Sweden), and CSK-buffer without KCl, GTP or Triton X-100 was added up to 500 μl maximum volume. The maximum centrifugal force was 12,000 × *g* and the centrifugal filter unit was centrifuged until the level was low enough to refill with CSK-buffer

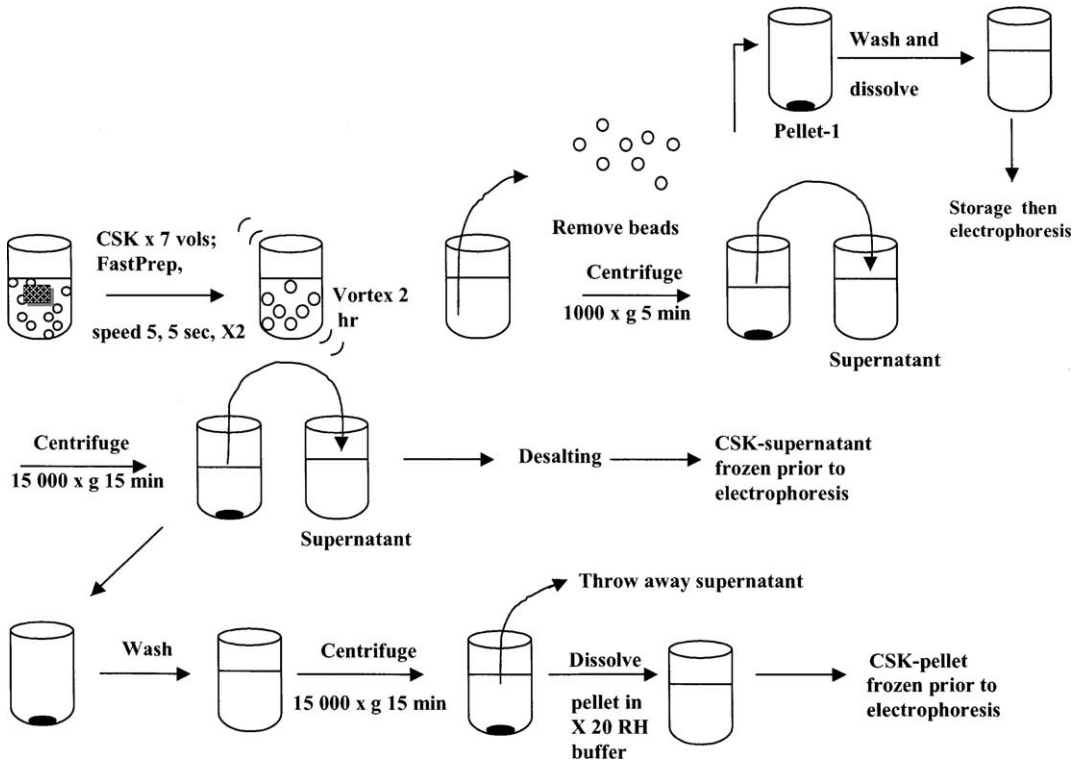

Fig. 2. Fractionation for reduction of cytoskeletal protein levels. Pellet-1 contained mainly insoluble material such as membranes and the CSK-pellet contained intermediate filaments and tubulins not required for analysis. The CSK-supernatants were desalted before electrophoresis.

up to 500 μl to gain a dilution of the salt concentration. This procedure was repeated several times with different samples requiring individual centrifugation times, and some samples had centrifugation times of up to 540 min. After diluting samples at least 2000 times, RH buffer with 0.5% IPG buffer pH 4–7 was added up to 500 μl to give salt concentrations of 0.3 mM or lower. Protein concentrations were determined at 590 nm using a Bradford assay [40] after addition of Bio-Rad Protein Assay solution (25 μl/100 μl sample; Bio-Rad, Sundbyberg, Sweden). Albumin (Pierce, Rockford, USA) standards were used with appropriate controls to minimize assay interference by detergents.

2.5. Electrophoresis conditions

Samples were solubilized in RH buffer containing 0.5% IPG buffer pH 4–7 after fractionation or desalting for first dimension electrophoresis using isoelectric focusing (IEF). For each sample 280 μg protein in 460 μl solution containing bromophenol blue for visualization was applied to IPG strips (Amersham Pharmacia Biotech). To determine whether to work with 18 or 24 cm length strips, total-extracts from the same brain area in the same patient which had been run on 18 cm strips were pooled and then run on 24 cm strips. As the 24 cm strips gave higher resolution additional pooled samples were then used to optimize focusing conditions for 24 cm pH 4–7 IPG strips. Samples were run in duplicate at 78, 129 and 140 kV h. The optimized electrophoretic conditions were then used for the detailed sample analysis. DryStrip cover fluid (Amersham Pharmacia Biotech) was added to 24 cm pH 4–7 linear IPG strips until the strip was completely covered and electrophoresis was carried out for 31 h at 120 kV h. Strips were equilibrated with DTT and iodoacetamide as previously described [14].

Polyacrylamide gels for second dimension electrophoresis using sodium dodecyl sulphate polyacrylamide electrophorosis (SDS-PAGE) were cast in 1 mm × 230 mm × 190 mm glass cassettes and allowed to polymerize for at least 3 h. The gel casting solution for 12.5% gels contained 686 g Duracryl (Kebo Lab., Stockholm, Sweden), 400 ml Tris slab gel buffer, 512 ml of Milli-Q water, 16 ml SDS 10%, 0.8 ml *N*,*N*,*N*′*N*′-tetra-methylethylenediamine (TEMED) and 4 ml of 10% (w/v) ammonium persulphate. Melted agarose (1%; Sea Plaque Agarose, FMC BioProducts, Rockland, USA) in running buffer was applied to the top of the gel cassette and the IPG strip was allowed to sink into the warm agarose, followed by electrophoresis at 100 V for about 17 h. The temperature holder for the running buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3) was 19 °C. Gels were soaked in fixation solution (20% ethanol, 7% acetic acid [41]), rinsed once with Milli-Q water and soaked overnight in Sypro Ruby (200 ml per gel; Molecular Probes Europe BV, Leiden, The Netherlands). Light was excluded during staining as Sypro Ruby, a metal chelate fluorescent dye containing ruthenium complexes which bind avidly to proteins [42], is light-sensitive. Excess Sypro Ruby was removed by gel in-

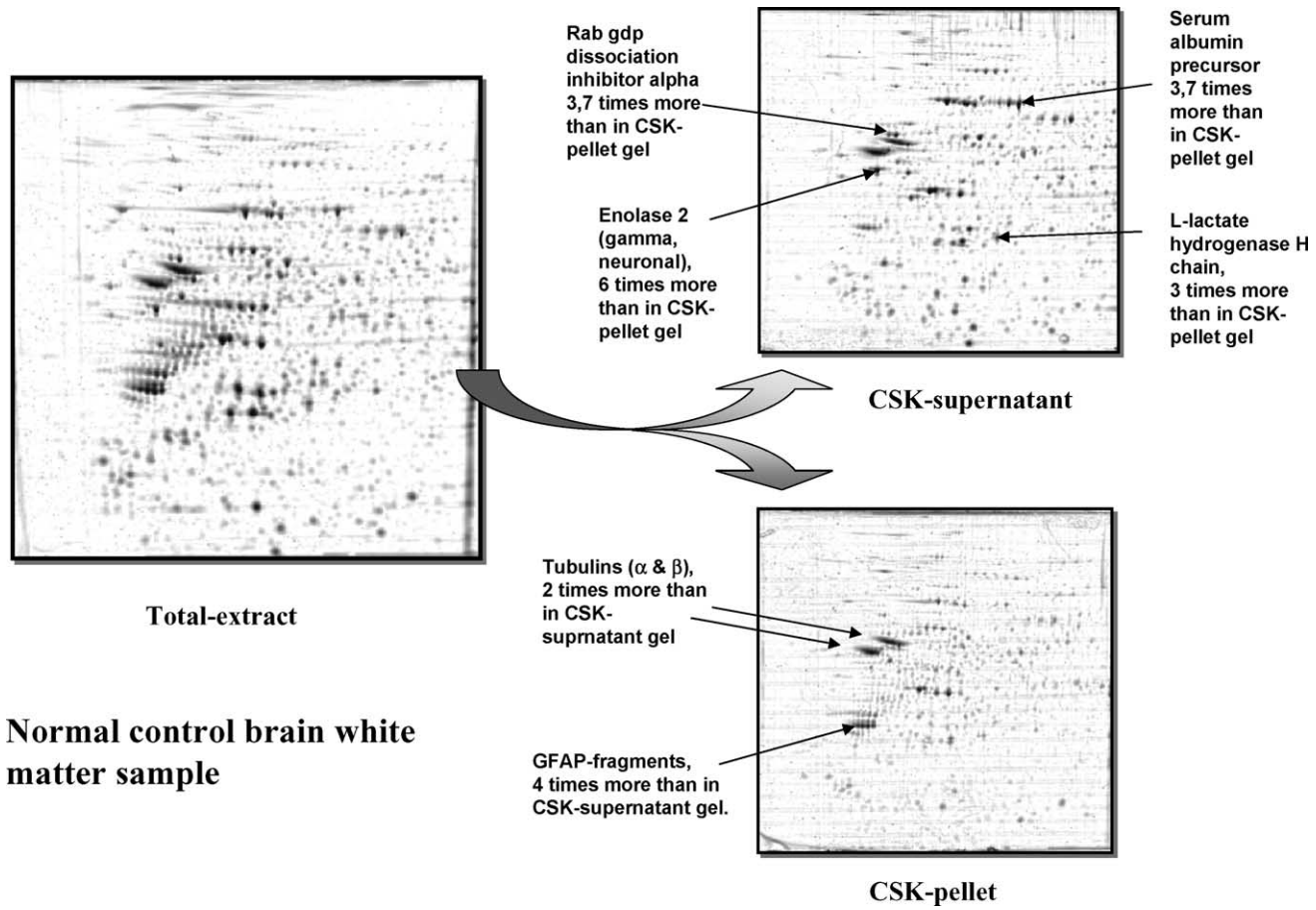

Fig. 3. Major proteins in normal control brain white matter. This figure shows the relative distribution of a number of identified protein spots in the total-supernatant, i.e. the total-extract, CSK-supernatant and CSK-pellet from normal control brain white matter.

cubation in fixation solution (2 × 30 min per gel) and rinsing with Milli-Q water (30 min).

2.6. Detection and analysis of spots

A Molecular Imager® FX (Bio-Rad) was used to scan the 20 gels in the created matchset at 100 μm resolution to detect protein spots. The detection limit for Sypro Ruby was 1–2 ng of protein per spot [43]. Digitized information was analyzed using PDQUEST software (Bio-Rad) and 2-D Gaussian fitting [44] was used to detect and quantify partially overlapping spots as well as isolated spots. To enable an accurate comparison to be made between gels, prior to analysis gels were normalized so that the volume of each protein peak was related in parts per million (ppm) to the total volume of all the protein peaks on each gel.

The first statistical analysis was a Student's *t*-test (0.05 significance level) between replicate groups consisting of closely related gels, for example, multiple gels from the same sample. In one analysis, plaque samples were in one replicate group and white matter samples adjacent to these lesions were in the other replicate group, both having originated from the same tissue block. The Student's *t*-test revealed the number of spots differing significantly between the replicate groups. These differences were compared again to find out how many of the significantly differing spots were present in two pairs of replicate groups using a Boolean test to combine information from different groups of data.

Data was exported to other systems for principal component analysis (PCA) using the SIMCAP 8.1 program (Umetri AB, Sweden) in combination with the Spotfire 6.2 program (Spotfire AB, Sweden). Spotfire was only used for visualization of data. After cutting out from gels with the Spot Cutter (Bio-Rad), gel spots were placed in 96-well microtitre plates and the cut gel was scanned to confirm cutting accuracy.

2.7. MassPREP and mass spectrometry

A MassPREP robot was used to extract proteins from gel spots by digesting proteins to give peptides for running on a MALDI time-of-flight (MALDI-ToF) mass spectrometer. Sypro Ruby-stained gel pieces were dehydrated using acetonitrile and trypsinated, followed by 1% formic acid/2% acetonitrile peptide extraction. After peptides were moved to their corresponding positions on another microtiter plate, ZipTips containing C18-gel were used for peptide concentration, desalting and elution together with the spot matrix onto a MALDI target plate, and crystals of matrix and pep-

tide were formed. After MALDI-ToF mass spectrometry was used to attain the mass of each peptide the resulting peaklist was imported into the search engine MASCOT, and several databases were selected as information sources. Protein modifications were oxidation and carbamidomethyl, and the upper limit for miscleaveage was set to 1 and the peptide charge to +1. The charge on peptides as a result of the MALDI-ToF mass spectroscopy process was always +1 and peptide tolerance was ±100 ppm. A more detailed description of these procedures is given in [14].

3. Results

3.1. Reduction of cytoskeletal protein levels by fractionation

The CSK-buffer was designed to extract as much as possible of intermediate filaments and tubulin, and as little as possible of other CNS proteins. A total-extract, CSK-supernatant and CSK-pellet were generated from samples in the work-up set, or from selected proteomic analysis samples for reference (Fig. 3). A number of proteins were stronger in CSK-supernatant gels than in total-extract gels. In normal control brain samples, on average a >4-fold increase in soluble proteins was seen.

Fig. 3 shows the relative distributions (i.e. ratios) of selected cytosolic and cytoskeletal proteins from normal control white matter that were identified using MALDI. In CSK-supernatant fraction gels in comparison to CSK-pellet gels, there were seven times more Rab gdp dissociation inhibitor alpha 3, six times more enolase 2 (gamma, neuronal), seven times more serum albumin precursor 3, and three times more L-lactate hydrogenase H chain. However, in CSK-pellets there were two times more α- and β-tubulins and four times more GFAP than in CSK-supernatants.

In the detailed proteomic analysis study, application of this protocol to white matter adjacent to the plaque (coded XV) and the plaque (coded MS) dissected from tissue block B488-115 gave a very reproducible and efficient extraction

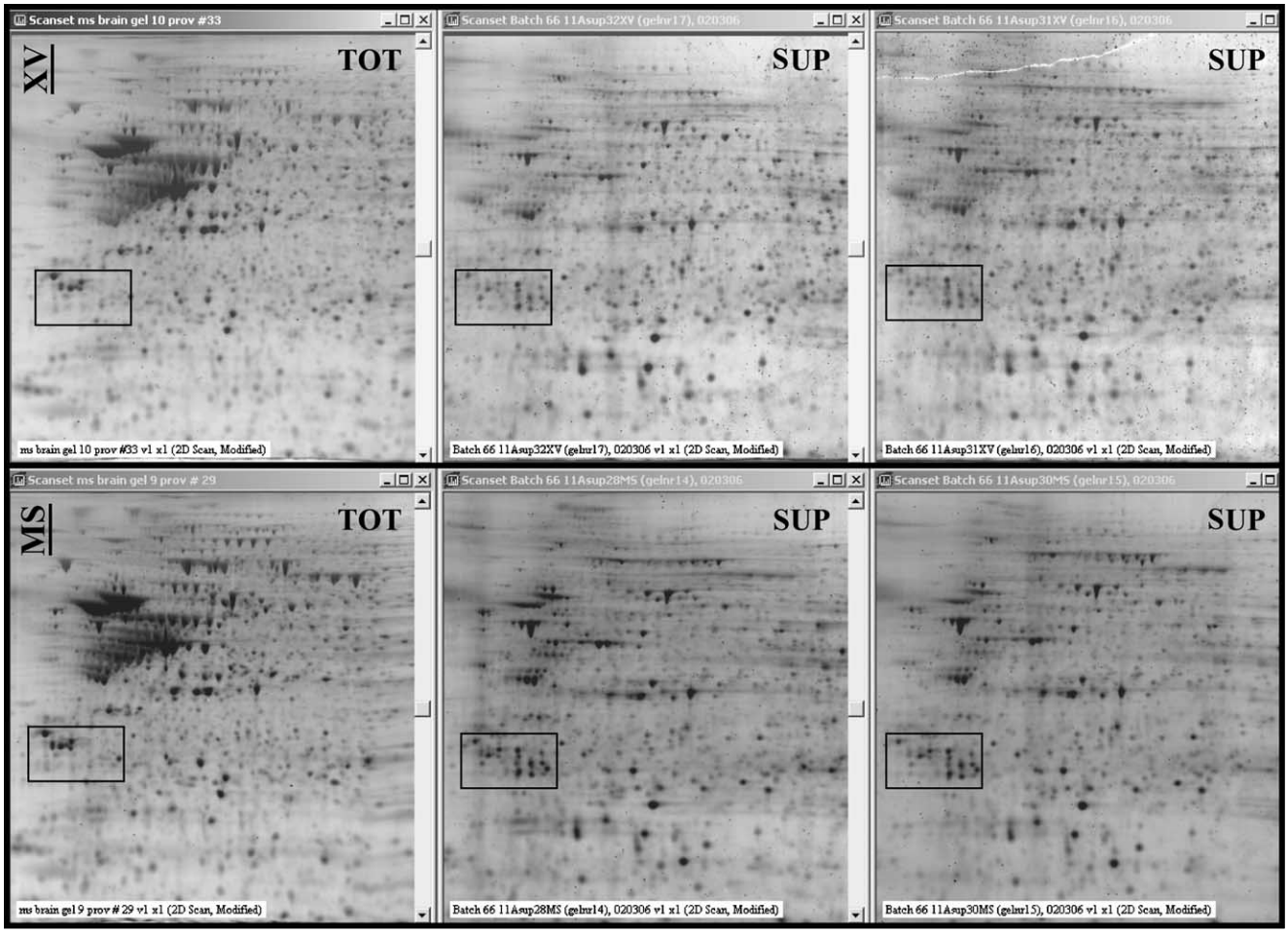

Fig. 4. Total-extracts (TOT) and CSK-supernatant (SUP) fraction gels for white matter adjacent to plaque (XV) and plaque (MS) from sample block B488-115. The XV and plaque samples were finely chopped and separated into three homogenous aliquots, one of which was used for preparing the TOT extract and two were used for SUP preparation. This figure shows the good extraction of cytoskeletal proteins that were present in the TOT gel but were absent or much weaker in SUP gels, and the visualization of new groups of proteins in SUP gels which were not seen or very weak in TOT gels, e.g. as in the boxes on the gels. The high levels of reproducibility attained in both the paired SUP gels can be seen.

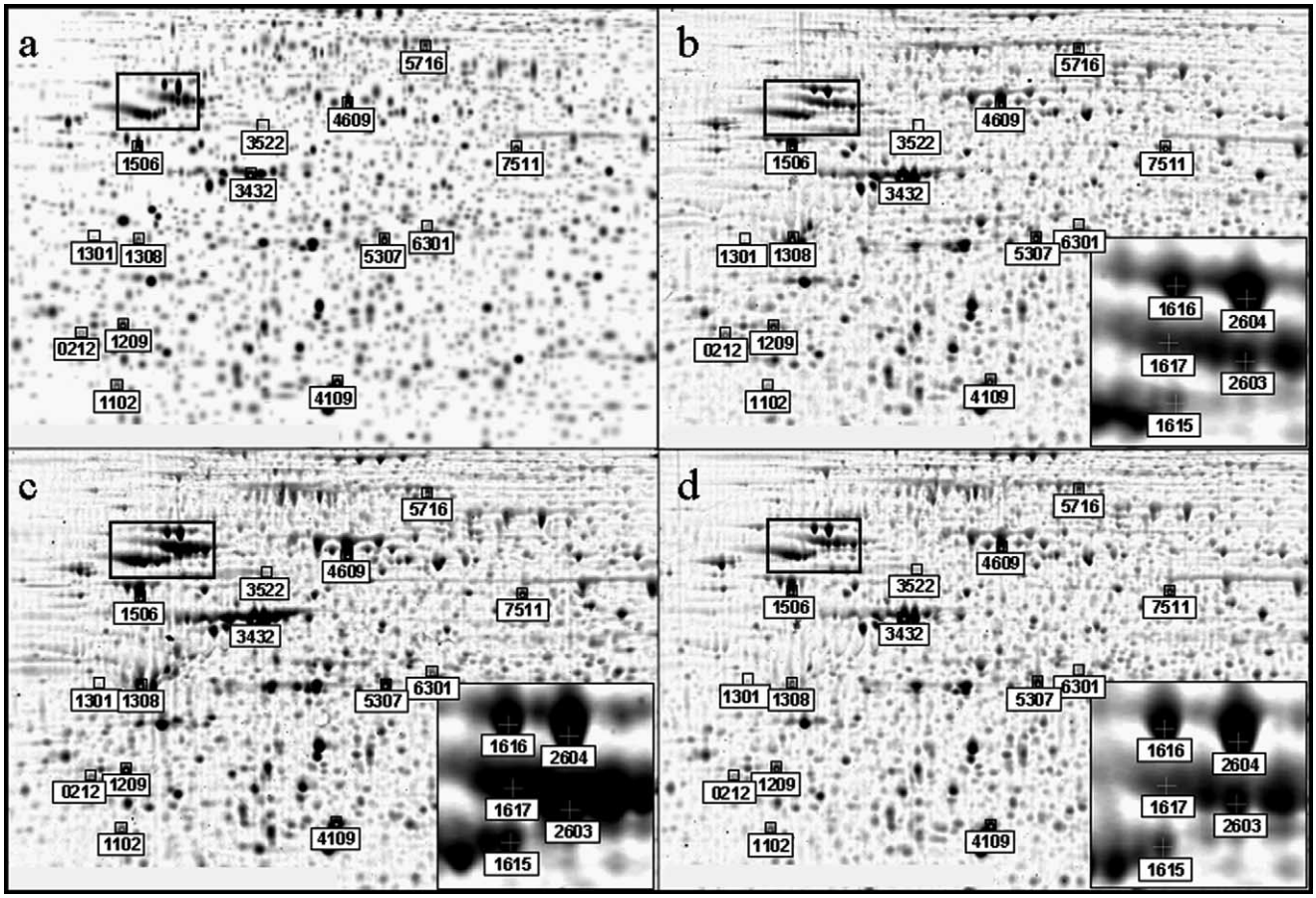

Fig. 5. Master image (a) and triplicate gels (16XV in b, 17XV in c and 18XV in d) of white matter immediately adjacent to the B488-107 plaque. The identity of landmark standard spot numbers on these gels are given in Table 2. The boxed areas at the top left of gels b–d are seen zoom-in, bottom right.

of cytoskeletal proteins including GFAP and tubulins. This extraction allowed undetectable or weaker protein spots to be visualized in CSK-supernatant (SUP) in comparison with total-extract (TOT) gels (Fig. 4). Figs. 5 and 6 also provide further evidence of the high reproducibility of the fractionation procedure (see Sections 3.2–3.4).

3.2. Resolution of gel spots

Optimization of electrophoresis conditions resulted in a protein spot resolution of an average of 1500 spots per gel for CSK-supernatants prepared from normal control or multiple sclerosis samples. Increased resolution of proteins was observed with the 24 cm pH 4–7 linear IPG strips in comparison to 18 cm strips. Only electrophoresis for 31 h at 120 kV h gave reproducible high resolution in the 6–7 p*I* range (not shown).

3.3. Expression profiles on proteins of interest

The Boolean test revealed 24 spots that were significantly different and present in each of the two analysis sets compared (A and B). These 24 spots were all shown to be present in analysis set B according to the Student's *t*-test. Six hundred spots were calculated to differ significantly in that analysis set, but many of these spots were below 500 ppm of protein and therefore difficult to quantify as the detection limit for Sypro Ruby was close to that value. Differences in total amount of protein on each gel results in qualitative differences as very faint spots in one gel may not be detected in another gel. Therefore all spots under 500 ppm were removed from the set, leaving only 116 spots. The 24 spots that differed between analysis sets A and B were present in the remaining 116 spots. All together there were 192 spots for the spot cutting procedure, and another 76 spots were selected by using the Image Stack Tool. The most interesting of the 192 spots were selected by examining the expression profile (manuscript in preparation).

As examples of identified landmark gel spots, Fig. 5 shows the master image and triplicate sample gels of white matter immediately adjacent to the B488-107 plaque. The identity of standard spot (SSP) numbers in this figure are shown in Table 2. The very close similarities between the three triplicate samples demonstrated the high level of reproducibility attained using the methodology described in this study.

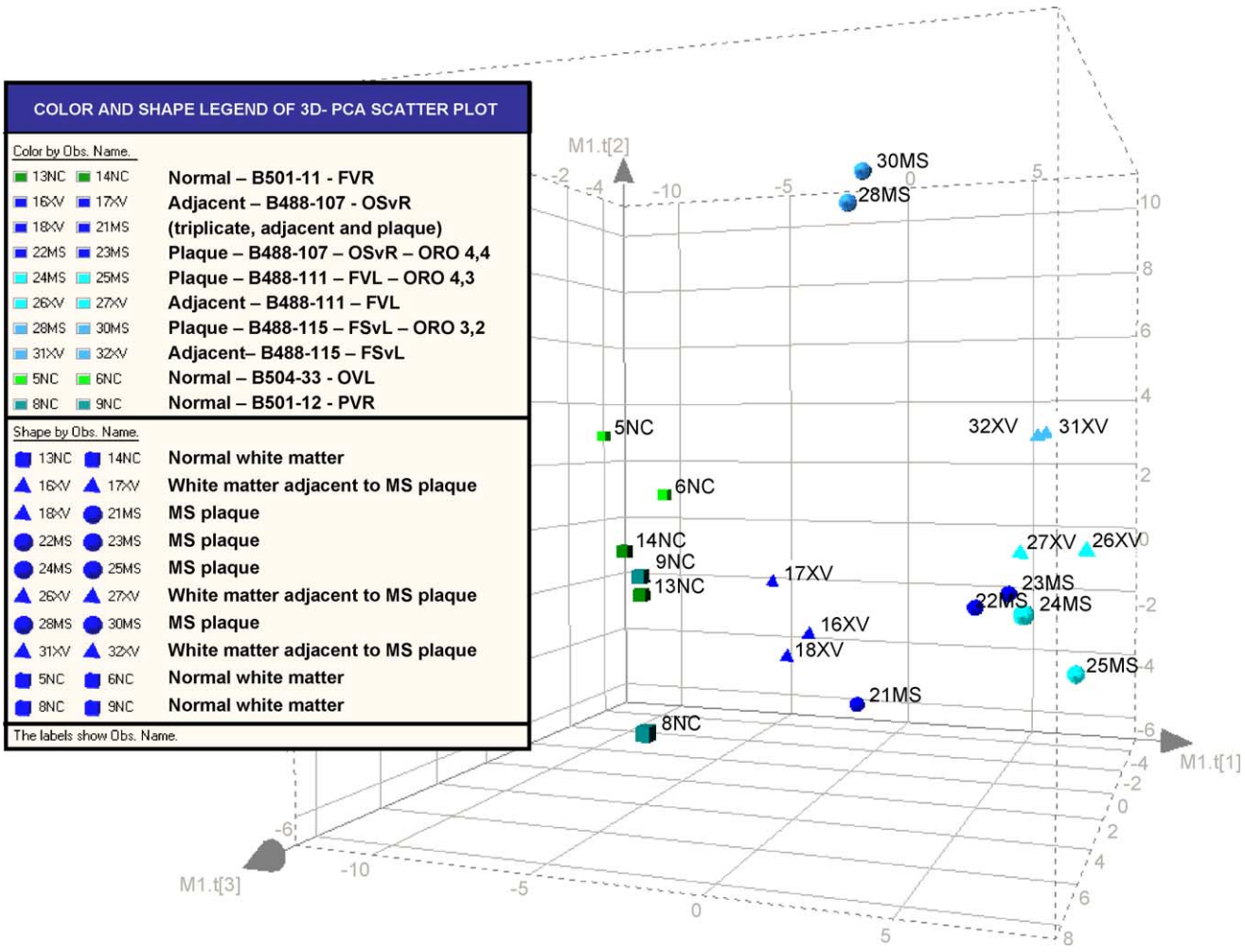

Fig. 6. 3-D view of a PCA-analysis. The CSK-supernatant gel codes are shown in Table 1. The color code shows that gels originate from the same tissue block, and the symbols show gels belong to the same sample type, i.e. normal control white matter (coded NC), plaque (coded MS) or white matter adjacent to a plaque (coded XV). Each figure represents a gel of NC white matter, XV white matter or MS plaque CSK-supernatant proteins, identified by shape. The NC gel group clusters together well whilst the XV and MS plaque gel groups are somewhat separated between and within groups. The separation between NC and XV/plaque results from differences in protein expression. The separation within the plaque and XV groups may result from plaques being at slightly different stages of development.

3.4. PCA-analysis

PCA is a test for determining how protein expression profiles differ between individual sample gels and groups of gels. A total of 109 proteins that were matched in all gels in the matchset were used. Groups of gels positioned close to each other in this test have a very similar expression profile of the 109 proteins, whilst groups positioned far from each other differ in the protein expression. Sample data for the 109 proteins were plotted in a 3-D plot, where the different axis or principal components were drawn through space as t_1–t_3. On the plot t_1 was drawn where the largest variation in the plot was situated, and t_2 was drawn where the next largest variation in the plot was situated, perpendicular to t_1. The t_3 was drawn where the next largest variation in the plot was situated, perpendicular to t_1 and t_2. Together, t_1–t_3 created a 3-D plot or diagram in which grouping of gels, equal to a similar protein expression profile, could be seen.

Four views from different directions of the same plot were compared (Fig. 6, a 3-D diagram of a PCA-analysis). This gives a further indication of the high reproducibility of the fractionation procedure and homogeneous expression of proteins in the tissue volume from which these samples originated. Examples are plaque sample gels 28MS and 30MS which are lying close together, as are gels 16XV, 17XV and 18XV. Gels 8NC and 21MS appear to be outsiders, not clearly belonging to any cluster.

4. Discussion

The aims of this study were to establish a fractionation procedure to reduce levels of the most abundant intermedi-

Table 2
Spots used in the evaluation of the sample preparation

SSP number	Mascot score	Accession number	Protein name
212	107	SWISSNEW:143Z_HUMAN	14-3-3 protein zeta/delta
1102	65	P13693	Translationally controlled tumor protein (tctp) (p|homo sapiens
1209	95	SWISSNEW:143E_HUMAN	14-3-3 protein epsilon
1301	110	P14136	GFAP (gfap)|homo sapiens
1308	83	P14136	GFAP (gfap)|homo sapiens
1506	120	AAH02745	Eenolase 2, (gamma, neuronal)|homo sapiens
1615	109	TREMBL:Q9BV28,	Tubulin beta fragment
1616	42	P31150	Rab gdp dissociation inhibitor alpha (rab gdi alph|homo sapiens
1617	80	SWISSNEW: TBA4_HUMAN	Tubulin alpha-4 chain
2603	66	SWISSNEW:TBA4_HUMAN	Tubulin alpha-4 chain
2604	114	P31150	Rab gdp dissociation inhibitor alpha (rab gdi alph|homo sapiens
3432	90	P02570	Actin, cytoplasmic 1 (beta-actin)|homo sapiens
3522	152	P14146	GFAP (gfap)|homo sapiens
4109	84	P09211	Glutathione s-transferase p (ec 25118) (gst class)|homo sapiens
4609	124	SWISSNEW:DPY2_HUMAN	Dihydropyrimidinase related protein-2 (DRP-2) (collapsin response mediator protein 2) (CRMP-2) (N2A)
5307	84	P07195	L-Lactate hydrogenase H chain (EC 11127) (LDH-B)|homo sapiens
5716	68	P02768	Serum albumin precursor|homo sapiens
6301	142	SWISSNEW:KPY2_HUMAN	Pyruvate kinase, M2 isozyme
7511	73	P50395	Rab gdp dissociation inhibitor beta (rab gdi beta)|homo sapiens

Abbreviations: SSP, standard spot. This is the unique number which maps the location on a gel where the spot is found. The first two digits refer to the gel *x* and *y* coordinates, and the second two digits refer to the individual box spots. These SSP numbers are shown on Fig. 5.

ate filament proteins and tubulins in normal and pathological CNS tissues, and to optimize electrophoresis conditions to facilitate proteomic analyses of complex changes in CNS protein expression. Our focus of interest was on proteins which are expressed at different levels between plaques and adjacent tissue as well as comparing normal control brain proteins with those in multiple sclerosis brain. The identification of landmark proteins in multiple sclerosis samples will be used to build a platform for the proteomic identification and quantitation of extracted proteins in future studies.

The multiple sclerosis lesions examined in the detailed proteomic analyses in this study were actively demyelinating plaques with a short death to freezing time to minimize proteolytic activity occurring prior to cryopreservation. There have been several studies on biomarkers which could be used as sensitive and reliable indicators of proteolytic activity as they are changed as a function of postmortem conditions. Levels of DRP-2 decreased within 6 h postmortem and two new spots were detected representing shorter forms of the protein in rat brain [13]. The ratio between the truncated form of DRP-2 (fDRP-2) and full length DRP-2 in mouse brain appeared to be an internal control which could be a biomarker of postmortem temperature as well as time between unrelated brain protein samples [14]. In the current study, DRP-2 was identified in human brain sample gels (SSP number 4609, Fig. 5 and Table 2), but so far we have not located the truncated form in gels. However, as oxidatively modified DRP-2 is present in Alzheimer's disease brain [45,21], there may be a disease-associated interference with the use of DRP-2 as a biomarker. Therefore, further studies are needed to characterize possible biomarkers of postmortem change.

Two important considerations in the development of the CNS protein fractionation used in this study were the prevention of proteolysis during preparation of samples for electrophoresis, and the removal of CNS lipids [4]. Inhibition of proteolytic enzymes is an important consideration as there are high levels of these enzymes in active multiple sclerosis lesions, and therefore a protease inhibitor cocktail was included in the CSK-buffer. Lipids were removed very effectively which is necessary because high levels of lipids are present in brain and spinal cord white matter and even traces of lipids can cause streaking of proteins in gels.

The optimized CSK-buffer was proven to effectively remove selected proteins by comparing the CSK-supernatant gels with CSK-pellet gels. It was shown that intermediate filaments and α- and β-tubulins were reduced in the CSK-supernatant gel and enriched in the CSK-pellet gel, as intended. Comparison of the ppm values for respective spots in CSK-supernatant and CSK-pellet gels showed a two-fold increase of α- and β-tubulins and a four-fold increase of GFAP in CSK-pellet gels compared to CSK-supernatant gels. Although there was not a complete absence of intermediate filaments in CSK-supernatant gels, when the fractionation protocol was applied to plaque samples which contain high GFAP levels as the result of astrogliosis, very effective reduction of GFAP and also α- and β-tubulins was observed.

Electrophoretic conditions were optimized to give enhanced resolution for the identification and quantitation of proteins present at low concentrations. A number of proteins seen in 24 cm IPG strips were not detected in 18 cm strips. The 24 cm IPG strips gave superior protein separation and more protein could be loaded, allowing detection of less abundant proteins and increased probability of success-

ful identification using MALDI. It was crucial that focusing time was optimized for first dimension electrophoresis. A too long focusing time can lead to water loss from the strip in the basic pH range causing protein streaking, whilst a too short focusing time results in both streaking and poorly separated proteins. When comparing different kV h more distinct protein spots were seen in gels run for 31 h at 120 kV h.

The PCA-analysis of normal control and multiple sclerosis gels in the proteomic analysis set showed several gels clustered together which is an indication of similarity in the expression of the selected 109 proteins in those gels. Gels 28MS and 30MS were clustered together, as were gels 16XV, 17XV and 18XV. Gels 31XV and 32XV and 22MS and 23MS stayed together, and gels 26XV and 27XV also formed a cluster. All the normal control gels formed a cluster on one side of the diagram with the exception of gel 8NC. This might be explained by a small tear in the gel which impaired quantitation in that area. The reason why gel 21MS was separated from the other plaque clusters and why gels 24 and 25MS were slightly displaced could result from relatively weak protein expression in those gels. As this phenomenon depends on the amount of protein applied to gels, the highest possible amount of protein was used. A weak protein expression makes matching of gels more difficult and sometimes impossible, leading to mismatches between gels.

There was a clear difference in protein expression between several gel groups as they differed in position in the PCA diagram. Care has to be taken when comparing gels of proteins from normal control with pathological tissues as they do not of course originate from the same tissue block. There was an obvious difference between normal control and multiple sclerosis clusters which will be investigated in future studies. In this study, samples from the same tissue block were compared, i.e. plaque samples and the adjacent white matter XV samples. Clearly, every adjacent white matter cluster (31 and 32; 26 and 27; 16, 17 and 18XV) was more or less separated from the plaque clusters (28 and 30; 22 and 23MS), indicating there were proteins which differ in expression between adjacent white matter and plaque gels.

These differences between the plaque clusters could be caused by variations in the disease activity of the lesions. Comparing the histopathology data for the plaque gels, the least active plaque was represented by gels 28 and 30MS which clustered together far away from other gels. The 24 and 25MS gels belonged to the plaque showing intermediate activity, but these gels did not tend to cluster together. The most active plaque was represented by the 21, 22 and 23MS gels, and the 22 and 23MS gels clustered together whilst 21MS was an outsider. Thus, some of the differences between the plaque clusters might be explained by small differences in levels of inflammation or the stage of lesion development.

In conclusion, the fractionation and proteomic methodologies developed in this study can be used for the identification and quantitation of disease-related proteins in neurological or psychiatric disorders. These methods can also be used for examining protein expression in normal adult or developing animal and human CNS tissues. We have used these techniques to examine the complex changes in protein expression which take place in multiple sclerosis lesions and to identify landmark protein spots. In future studies a database over the proteins in multiple sclerosis CNS samples will be developed which has the potential to provide new information on disease pathogenesis.

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References

[1] S.I. Kim, H. Voshol, J. van Oostrum, T.G. Hastings, M. Cascio, M.J. Glucksman, Neurochem. Res. 29 (2004) 1317.
[2] P. Davidsson, A. Brinkmalm, G. Karlsson, R. Persson, M. Lindbjer, M. Puchades, S. Folkesson, L. Paulson, A. Dahl, L. Rymo, J. Silberring, R. Ekman, K. Blennow, Cell. Mol. Biol. 49 (2003) 681.
[3] D.A. Lewis, Neuropsychopharmacology 26 (2002) 143.
[4] W.M. Freeman, S.E. Hemby, Neurochem. Res. 29 (2004) 1065.
[5] M. Fountoulakis, Mass Spectrom. Rev. 23 (2004) 231.
[6] M. Yun, W. Wu, L. Hood, M. Harrington, Electrophoresis 13 (1992) 1002.
[7] X.L. Yuan, T. Russell, G. Wood, D.M. Desiderio, Electrophoresis 23 (2002) 1185.
[8] B.R. Wenner, M.A. Lovell, B.C. Lynn, J. Proteome Res. 3 (2004) 97.
[9] D. Dumont, J.P. Noben, J. Raus, P. Stinissen, J. Robben, Proteomics 4 (2004) 2117.
[10] B.N. Hammack, B.P. Owens, M.P. Burgoon, D.H. Gilden, Mult. Scler. 9 (2003) 472.
[11] B.N. Hammack, K.Y.C. Fung, S.W. Hunsucker, M.W. Duncan, M.P. Burgoon, G.P. Owens, D.H. Gilden, Mult. Scler. 10 (2004) 245.
[12] M.R. Hynd, J.M. Lewohl, H.L. Scott, P.R. Dodd, J. Neurochem. 85 (2003) 543.
[13] M. Fountoulakis, R. Hardmeier, H. Hoger, G. Lubec, Exp. Neurol. 167 (2001) 86.
[14] B. Franzen, Y. Yang, D. Sunnemark, M. Wickman, J. Ottervald, M. Oppermann, K. Sandberg, Proteomics 3 (2003) 1920.
[15] H. Voshol, M.J. Glucksman, J. van Oostrum, Curr. Mol. Med. 3 (2003) 447.
[16] H. Langen, P. Berndt, D. Röder, N. Cairns, G. Lubec, M. Fountoulakis, Electrophoresis 20 (1999) 907.
[17] P. Davidsson, L. Paulson, C. Hesse, K. Blennow, C.L. Nilsson, Proteomics 1 (2001) 444.
[18] M. Fountoulakis, J.F. Juranville, M. Dierssen, G. Lubec, Proteomics 2 (2002) 1547.
[19] M.S. Cheon, M. Fountoulakis, M. Dierssen, J.C. Ferreres, G. Lubec, J. Neural Trans. (Suppl.) 61 (2001) 311.
[20] W. Chen, J.G. Ji, X.M. Xu, S.Z. He, B.G. Ru, Int. J. Dev. Neurosci. 21 (2003) 209.
[21] S.J. Schonberger, P.F. Edgar, R. Kydd, R.L.M. Faull, G.J.S. Cooper, Proteomics 1 (2001) 1519.
[22] A. Castegna, M. Aksenov, M. Aksenova, V. Thongboonkerd, J.B. Klein, W.M. Pierce, R. Booze, W.R. Markesbery, D.A. Butterfield, Free Radic. Biol. Med. 33 (2002) 562.

[23] A. Castegna, M. Aksenov, V. Thongboonkerd, J.B. Klein, W.M. Pierce, R. Booze, W.R. Markesbery, D.A. Butterfield, J. Neurochem. 82 (2002) 1524.
[24] A. Castegna, V. Thongboonkerd, J.B. Klein, B. Lynn, W.R. Markesbery, D.A. Butterfield, J. Neurochem. 85 (2003) 1394.
[25] N.L. Johnston-Wilson, C.D. Sims, J.P. Hofmann, L. Anderson, A.D. Shore, E.F. Torrey, R.H. Yolken, Mol. Psychiatr. 5 (2000) 142.
[26] S. Prabakaran, J.E. Swatton, M.M. Ryan, S.J. Huffaker, J.T.-J. Huang, J.L. Griffin, M. Wayland, T. Freeman, F. Dudbridge, K.S. Lilley, N.A. Karp, S. Hester, D. Tkachev, M.L. Mimmack, R.H. Yolken, M.J. Webster, E.F. Torrey, S. Bahn, Mol. Psychiatr. 9 (2004) 684.
[27] S.A. Schwartz, R.J. Weil, M.D. Johnson, S.A. Toms, R.M. Caprioli, Clin. Cancer Res. 10 (2004) 981.
[28] N. Denslow, M.E. Michel, M.D. Temple, C.Y. Hsu, K. Saatman, R.L. Hayes, J. Neurotrauma 20 (2003) 401.
[29] A. Compston, G. Ebers, H. Lassman, I. McDonald, B. Matthews, H. Wekerle, McAlpine's Multiple Sclerosis, 3rd ed., Churchill Livingstone, London, 1998.
[30] B.M. Keegan, J.H. Noseworthy, Ann. Rev. Med. 53 (2002) 285.
[31] J. Newcombe, M.L. Cuzner, M. Roytta, H. Frey, J. Neurochem. 34 (1980) 700.
[32] J. Newcombe, P. Glynn, M.L. Cuzner, J. Neurochem. 38 (1982) 267.
[33] T. Duzhak, M.R. Emerson, A. Chakrabarty, M.A. Alterman, S.M. Levine, Cell. Mol. Biol. 49 (2003) 723.
[34] H. Li, J. Newcombe, N. Groome, M.L. Cuzner, Neuropathol. Appl. Neurobiol. 19 (1993) 214.
[35] D. Gard, P. Bell, E. Lazarides, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 3894.
[36] P. Steinert, Isolation and characterization of intermediate filaments, in: L. Wilson (Ed.), Methods in Cell Biology, Academic Press, New York, 1982.
[37] E. Fey, K. Wan, S. Penman, J. Cell Biol. 98 (1984) 1.
[38] M. Paz, C. Tasca, A. Mattos, R. Puriur, J. Nutr. 121 (1991) 1349.
[39] M. Ramsby, G. Makowski, E. Khirallah, Electrophoresis 15 (1994) 2655.
[40] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[41] T. Rabilloud (Ed.), Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods, Springer Verlag, Berlin, 2000.
[42] W. Patton, Electrophoresis 21 (2000) 1123.
[43] A. Görg, C. Obermaier, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037.
[44] J. Garrels, J. Biol. Chem. 264 (1989) 5269.
[45] G. Lubec, M. Nonaka, K. Krapfenbauer, M. Gratzer, N. Cairns, M. Fountoulakis, J. Neural. Trans. (Suppl.) 57 (1999) 161.