

Gel-based Proteomic Characterization of Soluble and Insoluble Fraction Proteins in Rat Spinal Cord

Shuguang Yang^{1*}, Qinxue Ding^{1,2*}, Yaojun Guo³, Congjian Zhao¹, Yufeng Jia¹, Haiping Que¹, Hongxia Wang⁴, Kaihua Wei⁴, Dacheng He², Shuqian Jing^{1,5}, Shaojun Liu^{1*}

¹State Key Laboratory of Proteomics and Department of Neurobiology, Department of Neurobiology, Institute of Basic Medical Sciences, Beijing 100850, P.R. China

²Institute of Cell Biology, Beijing Normal University, Beijing 100875, P.R. China

³Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, P.R. China

⁴China National Center of Biomedical Analysis, Beijing 100850, P.R. China

⁵Department of Clinical Immunology, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA, USA

*These authors contributed equally to this work

Abstract

Fractionation efficiency and protein characterization of neural soluble and insoluble proteins by sequential extraction was scrutinized by gel-based proteomic analysis. Spinal cord proteins of adult rats were first extracted with aqueous buffer (fraction A), followed by standard (fraction B) or modified (fraction C) enhanced solutions. Of the top 30 most abundant proteins in fractions A, B and C, the percentage of cytoplasmic proteins was 74% (28/38), 37% (14/38) and 42% (15/36), respectively; membrane organellar proteins accounted for 8% (3/38), 45% (17/38), and 44% (16/36); membrane proteins accounted for 13% (5/38), 18% (7/38) and 14% (5/36). The number of hydrophobic domains was 5, 15 and 9. Shared proteins in three fractions were only 13%. When additional less abundant 30 spots enriched the insoluble fraction C were characterized, membrane proteins accounted for 31%, among which 83% were peripheral membrane proteins and 17% were integral membrane proteins. Functional analysis also revealed some difference between different fractions although all fractionated proteins are involved in energy metabolism, redox regulation, signal transduction and cellular architecture.

Keywords: Fractionation efficiency; Membrane protein; Quantitative analysis; Sequential extraction; Spinal cord

Abbreviations: CNS: Central Nervous System; 2-DE: Two-Dimensional Electrophoresis; GRAVY: Grand Average of Hydropathicity; HR: Hydrophobic Region; NOG: N-octyl-D-glucopyranoside; PMF: Peptide Mass Fingerprint; SB3-10: N-decyl-N, N-dimethyl-3-ammonio-1-propanesulfonate; SP: Signal Peptide; TMH: Transmembrane Helix

Introduction

Membrane proteins play crucial roles in biology from cell interaction and recognition to ion transport and signal transduction. In the central nervous system (CNS), membrane proteins are of particular relevance to their pathophysiological disciplines involved with specific functions, such as neural degeneration and regeneration. Due to high hydrophobicity and low abundance, membrane proteins remain a great challenge in proteomic study (Ahram and Springer, 2004; Tan et al., 2008). Considering the complexity and extensive protein-protein and protein-lipid interactions in the CNS, membrane proteins are extraordi-

narily stable and become more resistant to disruption and isolation, leaving many unknown yet potentially very interesting proteins to be characterized.

Sequential extraction has been applied in many studies as a successful approach to membrane protein enrichment in *Escherichia coli* (Molloy et al., 1998), eukaryotic cells (Lehner et al., 2003; Abdolzade-Bavil et al., 2004) and animal tissues (Yao and Li, 2003; Cheng et al., 2005; Mangum et al., 2005). Given its important contribution to proteomics, it is worthy to systematically evaluate the fractionation efficiency of the technique itself. In fact, some basic questions have no satisfactory answers yet. For example, what's the percentage of cytoplasmic/secreted proteins in the soluble fraction? what's the percentage of membrane/membrane organellar proteins in the insoluble fraction? How many proteins are overlapped in both fractions? Of those membrane proteins, how many are peripheral and how many are transmembrane proteins? Functional categorization of proteins in each fraction is also interesting to address.

In the present study, we used sequential extraction coupled with solubility enhanced solution to fractionate soluble and insoluble proteins from rat spinal cord. Protein profiling was accomplished with 2-DE and mass spectrometry. Fractionation efficiency was evaluated by two strategies. The top 30 most abundant protein spots in each fraction were firstly characterized and compared; as an indispensable strategic complement, additional less abundant 30 spots enriched in the insoluble fraction were characterized. Our quantitative data confirmed that sequential extraction combined with optimized lysis system is an efficient approach to fractionating soluble and insoluble proteins. When integrated with such strategies, 2-DE-based platform remains an effective technique to resolve insoluble proteins, including hydrophobic neural integral membrane proteins.

***Corresponding author:** Professor Shaojun Liu, State Key Laboratory of Proteomics and Department of Neurobiology, Neurobiology Department, Institute of Basic Medical Sciences, Beijing, 100850, P.R.China, Tel: +86-10-66931304; Fax: 86-10-68213039; E-mail: liusj@nic.bmi.ac.cn

Received January 22, 2010; **Accepted** February 23, 2010; **Published** February 23, 2010

Citation: Yang S, Ding Q, Guo Y, Zhao C, Jia Y, et al. (2010) Gel-based Proteomic Characterization of Soluble and Insoluble Fraction Proteins in Rat Spinal Cord. *J Proteomics Bioinform* 3: 074-081. doi:10.4172/jpb.1000124

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Materials and Methods

Animal

Three adult male Wistar rats (200-250g, 2.5 months of age) were killed and fresh spinal cord tissues were collected for proteome analysis immediately. Every effort was made to minimize the number of animals used and their suffering. The animal subjects review board of our institute approved all the experiment procedures, which were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals revised 1996.

Sample preparation

Sequential extraction of soluble and insoluble proteins was performed as described by Molloy et al. (1998) with moderate modifications. Briefly, 3 fresh spinal cords (from the medulla oblongata through the horsetail, about 0.35g each) were quickly taken out after decapitation and homogenized with Heidolph DIAX900 polytron (Heidolph, Germany) (speed 3, 10 strokes) in 5 volume of ice-cold buffer, which contained 40 mmol/L Tris (Sigma, St. Louis, MO, USA), 1% w/v dithiothreitol (DTT; Promega, Madison, WI, USA) and 0.5% w/v carrier ampholytes (pH3-10; Amersham Biosciences, San Francisco, CA, USA). The homogenate was sonicated in UP200s sonicator (Dr. hielscher, Germany) to facilitate protein solubility (30 seconds, under ice water bath) then centrifuged at 40,000 g for 30 min at 15°C. Supernatant (fraction A) was recovered for later use. Insoluble pellets were rinsed with 1mL Tris (Sigma) buffer four times to maximally avoid soluble protein contamination. Rinsed pellets were reconstituted and sonicated in standard enhanced solution, which consisted of 5 mol/L urea (Sigma), 2 mol/L thiourea (Sigma), 2% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS; Sigma), 2% w/v n-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-10; Sigma), 1% w/v dithiothreitol (DTT; Promega), 0.5% w/v carrier ampholyte (pH3-10; Amersham Biosciences) and 40 mM Tris (Sigma) (fraction B). In parallel, same amount of pellets were suspended and sonicated in same volume of modified enhanced solution (fraction C, whose recipe was the same as fraction B except addition of 40 mmol/L N-Octyl -D-glucopyranoside (NOG; Sigma)). The final pellets (less than 1% w/w of total tissue) were boiled in about 100 µL of SDS buffer (1% w/v SDS (Sigma), 0.375 mol/L Tris (Sigma), 1% w/v dithiothreitol (DTT; Promega), and 25% v/v glycerol (fraction D). A cocktail of protease inhibitors (0.7 µg/mL pepstatin A (Sigma), 0.5 µg/mL leupeptin (Sigma), 0.3 mg/mL EDTANa2 (Sigma) and 100 µg/mL PMSF (Sigma)) was added in all sample solutions except fraction D. Protein concentration in each fraction was determined by the Bradford method on a Unicam UV300 spectrometer (Unicam, Cambridge, UK) except fraction D, for which A280 method was used to estimate the protein concentration.

2-DE and image analysis

Each fraction proteins were separated in duplicate by 2-DE as described elsewhere (Ding et al., 2006). Briefly, 100 or 1000 microgram protein mixture from fractions A, B or C were loaded on a ceramic immobilized pH gradient (IPG) gel strip holder with in-gel rehydration mode for analytical and micropreparative purposes respectively. Using the IPGphor Isoelectric focusing

(IEF) system (Pharmacia Biotech, San Francisco, CA, USA), the IPG gels (pH3-10L, 18 cm; Amersham Biosciences) were rehydrated for 12 h under low voltage of 30 V at 20°C. IEF was performed with the following presetting: 200 V for 1h, 500 V for 1h, 1000 V for 1h, 8000 V (gradient) for 0.5 h, and finally 8000 V for a total of 50 kWh. Then vertical SDS-PAGE was performed using the Ettan DALT II system (Amersham Pharmacia Biotech AB, San Francisco, CA, USA) with laboratory-made homogeneous acrylamide gel (13%T, 3%C, 245X180X1mm³). About 25 microgram D fraction proteins were loaded for 2-DE analysis, which had to be diluted enough before loading for IEF with rehydration buffer (8M urea (Sigma), 2%w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS; Sigma), 0.5% v/v IGP buffer (pH3-10L; Amersham Biosciences), 1% w/v dithiothreitol (DTT; Promega)) with final sodium dodecyl sulfate (SDS; Sigma) concentration below 0.25%. Gels were stained with Coomassie Brilliant Blue R250 (higher loading amount; Sigma) or silver nitrate (lower loading amount; Sigma). Image analysis was performed by the ImageMaster® Platinum 5.0 software (Amersham Biosciences), from which not only the relative abundance of each protein spot can be obtained (based on staining intensity), also obtained are matched spots in different gels (based on spot location). According to imaging data, the top 30 most abundant proteins were selected for validating fractionation efficiency. Additional 30 enriched spots in fraction C were further characterized. The volume increase of all spots enrolled in this group should reach statistical significance during image analysis when compared to Fraction A.

Mass spectrometric analysis

Protein spots were excised from gels and digested with in-gel digestion mode (Scheler et al., 1998; Gharahdaghi et al., 1999; Ding et al., 2006). Peptide mass spectra were recorded in reflect mode with delayed extraction on a Bruker Daltonics autoflex MALDI-TOF-MS instrument (Bruker Daltonics, Bremen, Germany). Positively charged ions were analyzed and 100 single-shot spectra were accumulated to improve the signal-to-noise ratio. Internal calibration was performed with trypsin autolysis peptide masses at m/z 842.51 (M⁺) and m/z 2211.10 (M⁺). Nano ES tandem mass spectrometry was performed on a Micromass Q-TOF2 mass spectrometer (Manchester, Britain) as described in reference (Kristensen et al., 2000).

Protein identification (Database search)

Protein identification by peptide mass fingerprinting (PMF) was completed using the ProFound search algorithm at <http://prowl.rockefeller.edu/prowl-cgi/profound.exe>. Searching parameters were preset as follows: NCBI nr database (2007/11); maximum of one missed cleavage; partial oxidation with methionine residue and carbamidomethylation with cysteine residue; maximum mass tolerance of 0.3 Da; taxonomic category *rattus norvegicus* unless otherwise indicated. Contaminated masses from matrix, keratins and trypsin were manually deleted before database searching (Ding et al., 2003). Relative mass intensity of peptide fragments was taken into consideration during database searching. Minimum of 4 matched peptide masses was required for unambiguous protein identification when PMF was used alone. For tandem mass spectrometric analysis, raw MS/MS data were directly transferred to the Mascot engine and

searched against NCBI nr database. In both cases, the quality of the search result, as indicated by Z score for PMF and ion score for MS/MS, reached statistical significance ($P < 0.05$).

Western blot

Thirty microgram of protein mixture in different fractions was separated by 10% polyacrylamide gel and electrotransferred to nitrocellulose membrane in a Trans-Blot® Semi-dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Non-specific binding sites were blocked in TBS-T (25mM Tris (Sigma), 150mM NaCl, 0.05 w/w Tween20, pH7.5) containing 5% non-fat milk overnight at 4°C; membranes were incubated with the primary antibody of vimentin (Santa Cruz Biotechnology, USA)(1:300) for 1hr at room temperature and followed by anti-rabbit IgG horseradish peroxidase conjugate (1:2000). The immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech AB). The film signals were digitally scanned and then quantified using the ImageMaster® Platinum 5.0 software (Amersham Biosciences).

Grand average of hydropathicity (GRAVY) calculation and signal peptide (SP), transmembrane helix (TMH) and hydrophobic region (HR) prediction

Protein GRAVY (Kyte and Doolittle, 1982) was calculated using the ProtParam tool at <http://us.expasy.org/tools/protparam.html>. SP was predicted with SignalP 3.0 server at <http://www.cbs.dtu.dk/services/SignalP/>. TMH was predicted with TMHMM server at <http://www.cbs.dtu.dk/services/TMHMM-2.0>. The number of predicted hydrophobic regions inside protein sequences was based on the combination analysis of SP, TMH and HMMTOP (<http://www.enzim.hu/hmmtop/>).

Functional and subcellular localization sorting

Function and component sorting of proteins were primarily based on the annotations in Swiss-Prot/UniProt knowledge database (<http://www.expasy.uniprot.org>). In case of "one protein multiple locations" (for example, HSP8 can be translocated between cytoplasm and nucleus), the denominator used for component percentage calculation is the counts of all locations rather than the number of proteins.

Results

Spinal cord proteins were sequentially extracted by aqueous Tris buffer (fraction A), standard (fraction B) or modified (fraction C) enhanced solution and finally by SDS solution (fraction D). Concentration determination showed that more proteins were harvested in the soluble fraction than in each insoluble fraction (Table 1). Between the two insoluble fractions, it seems that more proteins were extracted in fraction C than in fraction B ($P < 0.05$, t-test, Table 1). In consistence to this observation, the staining intensity of many proteins in Figure 1C was higher than that of matched spots in Figure 1B (Table 2). Representative 2-DE maps of the four fractions are shown in Figure 1. Approximately 1400, 680, 700 and 50 protein spots were visualized by silver staining from fractions A, B, C, and D, respectively. More basic proteins seem to be collected in fractions B and C than in fraction A, which are located on the right region in the 2-DE map. The percentage of proteins with $pI > 7$ in fractions A, B and C was 43.3%, 46.7 and 50% ($p < 0.05$, compared to Fraction A, t-test, Table 3). Since membrane proteins are more

Sequential Fractions	Lysis Buffer (ml)	Protein Con. (mg/ml)	Total Protein (mg)	Percentage ^{a)}
Fraction A	7.8	4.05	31.59*	38%*
Fraction B	5.2	4.13	21.48	26%
Fraction C	5.2	4.93*	25.64*	31%*

^{a)}This percentage was calculated with no consideration of fraction D since there were only trace amounts of proteins in that final fraction. * $P < 0.05$, compared to fraction B, t-test.

Table 1: Protein yields in different extraction fractions.

Proteins	Fraction B	Fraction C
4-aminobutyrate aminotransferase	185	335*
Fumarate hydratase	402	713*
Mitochondrial aconitase	404	697*
Pyruvate dehydrogenase, beta chain	110	246*
Voltage-dependent anion channel 1	1215	2114*

* $P < 0.05$, compared to Fraction B, t-test.

Table 2: Intensity volume (relative abundance) of enriched proteins in insoluble fractions B and C.

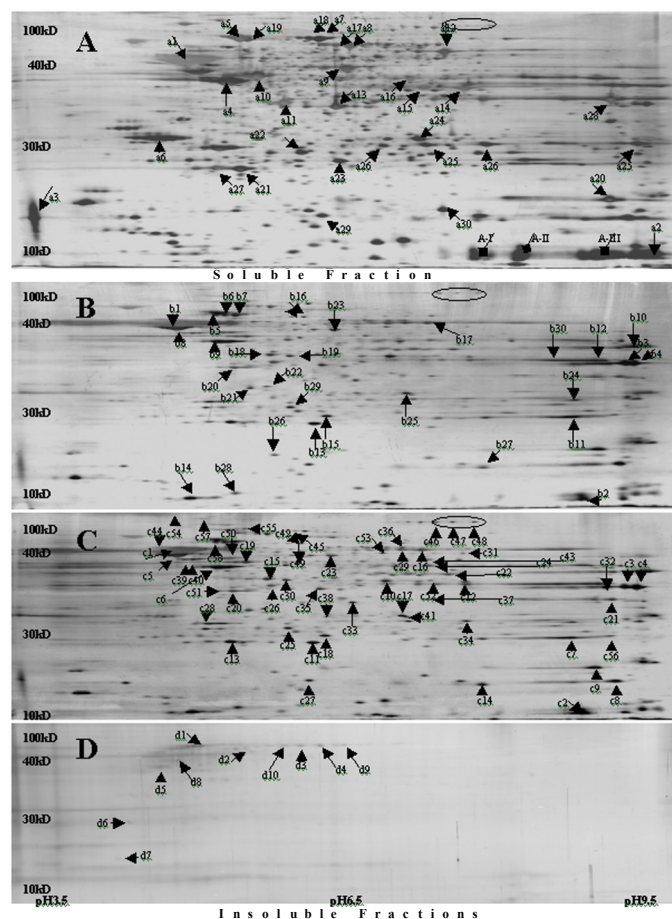


Figure 1: Representative 2-DE maps of sequential protein extraction fractions from rat spinal cords. Top 30 most highly abundant proteins spots are arbitrarily marked with arrows by the order of spot staining intensity (scanned volume) in each gel. Figure 1A, soluble fraction; Figures B and C, insoluble fractions with different enhanced extraction systems. Spots A-I, II, III (marked with solid squares) in Figure 1A are contaminated hemoglobins and excluded in our analysis. In Figure 1C, another 30 enriched spots (two are overlapped with the top 30 most highly abundant proteins) are marked, which are highly expressed in the insoluble fractions. Elliptical areas show representative insoluble-protein-enrichment effect in enhanced fractions B and C. Gel size: 245 x 180 x 1 mm³ (13% T, 3% C). Silver staining.

likely basic proteins (Wilkins et al., 1998; Schwartz et al., 2001), this fact encouraged us that more membrane proteins may have been harvested in the enhanced solutions. Due to very low abundance, proteins from fraction D had very poor mass spectrometric signals, making their identification rather uncertain (data not shown). Further analysis with this fraction was not included in current study.

To quantitatively evaluate the fractionation efficiency, the top 30 most strongly stained spots in each fraction were firstly analyzed (protein identification see Supplementary Table 4). These proteins were listed in the descending order of abundance (Tables 4, Supplementary Table 1 and 2). Venn diagram analysis showed that 22% (20/90) were exclusively displayed in the soluble fraction A while 39% (35/90) were exclusively displayed in the enhanced fractions B and C. Proteins overlapped in all three fractions were only 13% (12/90) (Figure 2).

The average GRAVY of abundant proteins in fractions A, B and C is -0.317, -0.255 and -0.253, respectively. One protein

Sequential Fractions	pI<7	pI>7
Fraction A	56.7%	43.3%
Fraction B	53.3%	46.7%
Fraction C	50.0%	50.0%*

*P<0.05, compared to fraction A. t-test.

Table 3: Isoelectric point distribution of the top 30 most abundant proteins from soluble fraction A and insoluble fractions B and C.

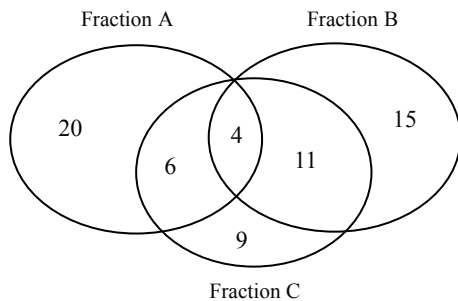


Figure 2: Venn diagram analysis of the top 30 most abundant proteins in the sequential extraction fractions A (soluble), B (insoluble) and C (insoluble).

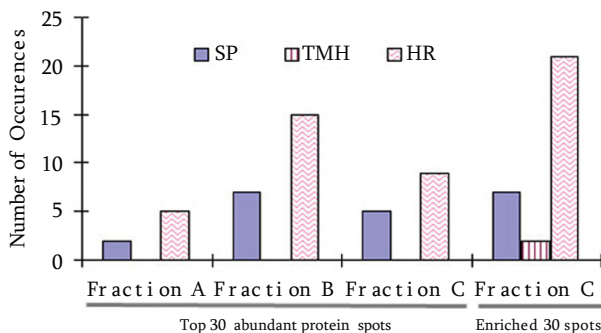


Figure 3: Hydrophobic analysis of the top 30 most abundant protein spots in soluble fraction A and insoluble fractions B and C. Value is subject to change if all proteins are analyzed. 30 less abundant spots enriched in the insoluble fraction C were analyzed as an independent group. SP, signal peptide; TMH, transmembrane helix; HR, hydrophobic region.

(3%) in fraction A has a positive GRAVY value while six (20%) and five (17%) was found to be such proteins in fractions B and C (Tables 4, Supplementary table 1 and 2). As for the number of hydrophobic regions in each fraction, there were five in fraction A and fifteen and nine in fractions B and C. Among those hydrophobic regions, two were signal peptides in fraction A and seven and five in fractions B and C. No transmembrane helix was found in most abundant proteins either from soluble or insoluble fractions (Tables 4, Supplementary table 1 and 2; Figure 3).

Protein localization sorting in different fractions is demonstrated in Figure 4. Cytoplasmic proteins accounted for 74% (28/38), 37% (14/38) and 42% (15/36) in fractions A, B and C, respectively. Membrane organellar proteins were 8% (3/38), 45% (17/38) and 44% (16/36). Membrane proteins were 13% (5/38), 18% (7/38) and 14% (5/36). Secreted proteins only appeared in the soluble fraction A which accounted for 5% (2/38). Pooled analysis of most abundant proteins in three fractions revealed that cytoplasmic, membrane organellar, membrane and secreted proteins accounted for 51% (57/112), 32% (36/112), 15% (17/112) and 2%(2/112). Among the organellar proteins, mitochondrial, nuclear and endoplasmic reticulum proteins accounted for 72% (26/36), 20% (7/36), 8% (3/36), respectively (Figure 5).

As a critical complement to most abundant protein analysis, we characterized another 30 less abundant protein spots which were significantly enriched in the enhanced solutions (Supplementary table 3). The average GRAVY of proteins in this group was -0.266, three proteins (10%) possessed positive GRAVYs.

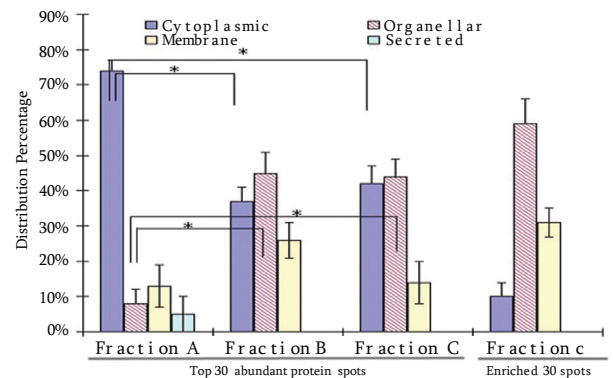


Figure 4: Protein localization analysis of top 30 most abundant protein spots in soluble fraction A and insoluble fractions B and C. Value is subject to change if all proteins are analyzed. 30 less abundant spots enriched in fraction C were analyzed as an independent group. *P<0.05, compared to Fraction A, t-test.

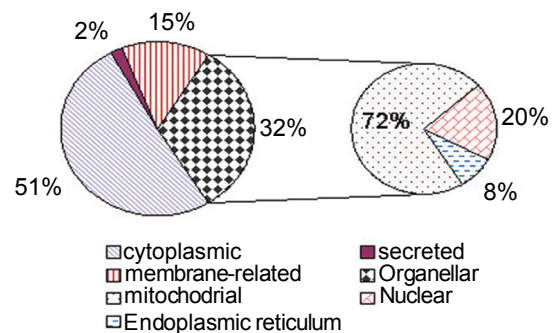


Figure 5: Pooled analysis of protein localization of most abundant protein spots in soluble and insoluble fractions. Value is subject to change if all proteins are analyzed.

There were twenty one hydrophobic domains in total, among which seven were signal peptides and particularly, two were transmembrane helixes (Figure 3). Component analysis demonstrated that cytoplasmic, membrane organellar and membrane proteins accounted for 10% (4/39), 59% (23/39) and 31% (12/39), respectively (Figure 4). Among various membrane organellar proteins, mitochondrial, endoplasmic reticulum and nuclear proteins accounted for 56% (13/23), 22% (5/23) and 22% (5/23), respectively. Among those membrane proteins, peripheral-related proteins accounted for 83% (10/12) and integral mem-

brane proteins accounted for 17% (2/12). Besides, two unknown proteins with novel amino acid sequences (7%) were also found in this enriched group.

Functional categorization of identified proteins in soluble and insoluble fractions was shown in Figure 6. Involved proteins were mainly clustered into energy metabolism, signal transduction, redox regulation, and cytoarchitecture. For energy metabolism-involved proteins/enzymes, it is noted that glycolytic enzymes dominated in the soluble fraction (Table 4) whereas tri-carboxylic acid cycle-related enzymes were major in the insoluble

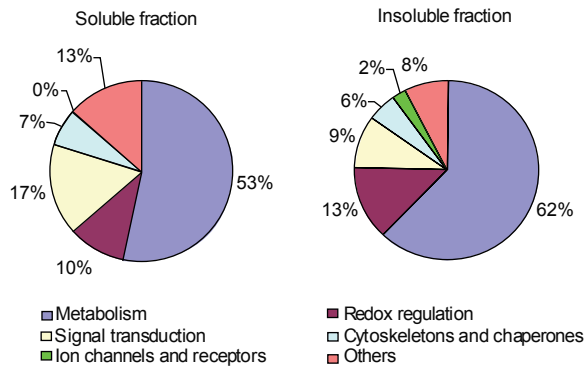


Figure 6: Functional sorting of identified spinal cord proteins in soluble and insoluble fractions. Value is subject to change if all proteins are analyzed.

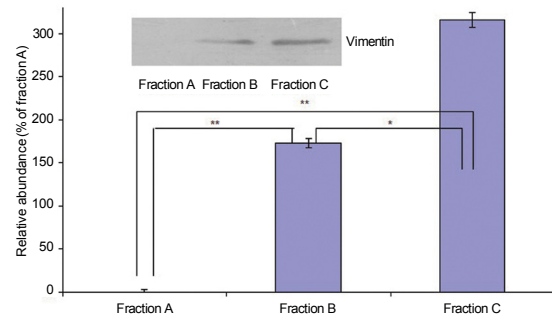


Figure 7: Western blot of nuclear protein vimentin in fractions A, B and C. *P<0.05; **P<0.01, t-test.

Intensity order No.	Protein name	Swiss_Prot entry	GRAVY a)	SP b)	TMH c)	HR d)	Localization (Component)	Function
a1	Tubulin, alpha	TBA1A_RAT	-0.229	0	0	0	Cytoplasmic	Skeleton protein
a2	Peptidylprolyl isomerase A	PPIA_RAT	-0.326	0	0	0	Cytoplasmic	Protein folding
a3	Calmodulin	CALM_RAT	-0.654	0	0	0	Cytoplasmic	Signal transduction
a4	Actin, beta	ACTB_RAT	-0.2	0	0	0	Cytoplasmic	Skeleton protein
a5	Heat shock protein 8	HSP7C_RAT	-0.452	0	0	0	Cytoplasmic/nuclear	Chaperone
a6	Calretinin	CALB2_RAT	-0.664	0	0	0	Cytoplasmic	Signal transduction
a7	Serum albumin	ALBU_RAT	-0.389	1	0	1	Secreted	Transporter
a8	Toad-64	DPYL2_RAT	-0.267	0	0	0	Cytoplasmic or membrane-bound	Axon elaboration
a9	Enolase 1	ENOA_RAT	-0.198	0	0	0	Cytoplasmic or membrane-bound	Glycolytic enzyme
a10	Creatine kinase-b	KCRB_RAT	-0.466	0	0	0	Cytoplasmic	Energy transporation
a11	Lactate dehydrogenase	LDHB_RAT	0.033	0	0	1	Cytoplasmic	Glycolytic enzyme
a12	Pyruvate kinase	KPYM_RAT	-0.096	0	0	0	Cytoplasmic	Glycolytic enzyme
a13	Malate dehydrogenase	Q6PCV2_RAT	-0.059	0	0	1	Cytoplasmic	Energy metabolism
a14	Aldolase C	ALDOC_RAT	-0.21	0	0	0	Cytoplasmic	Glycolytic enzyme

a15	Aldolase C	ALDOC_RAT	-0.21	0	0	0	Cytoplasmic	Glycolytic enzyme
a16	Glutamine synthetase 1	GLNA_RAT	-0.601	0	0	0	Cytoplasmic	Amino acid metabolism
a17	Toad-64	DPYL2_RAT	-0.267	0	0	0	Cytoplasmic or membrane-bound	Axon elaboration
a18	Serum albumin	ALBU_RAT	-0.389	1	0	1	Secreted	Transporter
a19	Heat shock protein 8	HSP7C_RAT	-0.452	0	0	0	Cytoplasmic or nuclear	Chaperone
a20	Cofilin 1	COF1_RAT	-0.388	0	0	0	Cytoplasmic or nuclear	Structural protein
a21	Phosphatidylethanolamine binding protein	PEBP1_RAT	-0.582	0	0	0	Cytoplasmic or membrane-bound	Signal transduction
a22	Peroxiredoxin 6	PRDX6_RAT	-0.225	0	0	0	Cytoplasmic	Redox regulation
a23	Neuronal rhoa gef protein	Q6RFZ7_RAT	-0.537	0	0	0	Cytoplasmic	Signal transduction
a24	Phosphoglycerate mutase 1	PGAM1_RAT	-0.506	0	0	0	Cytoplasmic	Glycolytic enzyme
a25	Triosephosphate isomerase 1	TPIS_RAT	-0.121	0	0	0	Cytoplasmic	Glycolytic enzyme
a26	Triosephosphate isomerase 1	TPIS_RAT	-0.121	0	0	0	Cytoplasmic	Glycolytic enzyme
a27	Peroxiredoxin 2	PRDX2_RAT	-0.175	0	0	1	Cytoplasmic	Redox regulation
a28	Glyceraldehyde 3-phosphate-dehydrogenase	G3P_RAT	-0.084	0	0	0	Cytoplasmic	Glycolytic enzyme
a29	Superoxide dismutase [cu, zn]	SODC_RAT	-0.419	0	0	0	Cytoplasmic	Redox regulation
a30	Nucleoside diphosphate kinase b	NDKB_RAT	-0.27	0	0	0	Cytoplasmic or membrane-bound	Neuclear acid metabolism

^a)GRAVY, Grand average of hydropathicity; b) SP, signal peptide; c) TMH, transmembrane helix; d) HR, hydrophobic region.

Table 4: The top 30 most abundant protein spots in soluble fraction A.

fractions (Supplementary table 1 and 2). It is also noted that, signal transduction and redox regulation-related proteins were more likely seen in the soluble fraction whereas ion channels and receptors were only found in the insoluble fractions.

Finally, we used western blot to validate our 2-DE/mass spectrometry-based results. Figure 7 is a quantitative result of western blot of vimentin, a nuclear protein enriched in the insoluble fractions. In consistence with 2-DE-based analysis, this protein was virtually invisible in soluble fraction A, but showed medium intensity in fraction B and was most intensive in fraction C.

Discussion

Gel-based (2-dimensional gel electrophoresis and mass spectrometry) and gel-free (liquid chromatography and mass spectrometry) approaches currently are the two most popular strategies for profiling protein expression. It is fair to say that gel-free strategy seems more powerful when very hydrophobic mem-

brane proteins are to be addressed. Integral membrane proteins bearing more than 4 transmembrane helices are hard to resolve by gel-based technique (Bunai and Yamane, 2005). However, 2-DE-based technique possesses its own attractive merits, such as high-throughput, high resolution, providing pI and molecular weight as well as other valuable information. Particularly, when integrated with fractionation, lysis buffer optimization and new electrophoretic system, quite a few membrane proteins, including integral membrane proteins, have been successfully characterized by this technique (Molloy et al., 1998; Lehner et al., 2003; Yao and Li, 2003; Abdolzade-Bavil et al., 2004; Cheng et al., 2005; Mangum et al., 2005).

In this gel-based proteomic study, we quantified the sequential fractionation efficiency of soluble and insoluble neural proteins dissolved in different buffers. Our study, for the first time to our knowledge, provides key data which definitely help to answer some basic questions raised in the introduction. In order to simplify research approach and avoid characterizing all hun-

dred of thousands of spots on the gels (which is ideal but really a daunting work), we took two complementary strategies to achieve our goal. Firstly, same number of most abundant proteins were analyzed and compared in different fractions; secondly, additional couples of enriched yet less abundant proteins were characterized. Our data unambiguously confirmed that sequential fractionation combined with optimized lysis buffer definitely facilitates 2-DE-based approach to addressing membrane proteomics.

High fractionation efficiency of soluble and insoluble proteins achieved in this study depends on the combination of two key factors: sequential extraction and optimized lysis buffer. We developed here a modified enhanced solution based on the recipe of Molloy et al. (1998), in which we introduced an additional key component of NOG, expecting to better solubilize membrane related proteins. NOG is a nonionic surfactant which was first used to solubilize membrane protein of insulin receptors (Gould et al., 1981). Compared with other surfactants such as CHAPS, NOG can solubilize more protein than lipids, making it more suitable for neural protein extraction. As expected, the staining intensity of many proteins in Figure 1C (standard enhanced solution) was higher than that of matched spots in Figure 1B (modified enhanced solution) (Table 2), confirming that NOG did improve membrane/membrane organelle-related protein solubility. Western blot provided additional evidence to this conclusion.

In an earlier 2-DE-based analysis, only 4% or 15% of identified proteins were membrane proteins when sequential extraction or detergent-based strategy was used alone (Lehner et al., 2003). In another one-step-for-all extraction study, 30% of identified proteins were membrane and membrane organelle-related proteins (Fountoulakis et al., 2005). Compared to the fact that as high as 90% of proteins (enriched in the insoluble fraction C) belonged to this category in our study, the advantage of current strategy is really impressive. In fact, when focusing on proteins enriched in the insoluble fraction, one can find that membrane proteins alone accounted for 31%, which seems to be even competitive to gel-free (LC-based) strategy (28% membrane proteins) (Chen et al., 2006).

It is also necessary to compare our experimental data with the theoretical component distribution. Gene ontology classification predicts 43% membrane, 14% extracellular and 43% intracellular proteins in entire mouse proteome (Hood et al., 2005). By contrast, the component distribution was 19%, 2% and 79% in our study (based on all identified proteins). Obviously, membrane and extracellular proteins are underestimated but intracellular proteins are overvalued. Similar phenomenon also appeared in a gel-free based technique (28%, 4% and 68%, (Chen et al., 2006)). The discrepancy between theoretical and experimental data may be explained from the following aspects. Firstly, membrane proteins are inherently hydrophobic and usually lowly abundant. Secondly, a great part of secreted proteins are also very low abundant (i.g., cytokines and hormones), which tend to be ignored (Anderson et al., 2004). Thirdly, a lot of intracellular proteins are highly abundant and have multiple isoforms (glycolytic and mitochondrial enzymes are typical examples), likely leading to overvalue bias of this fraction. Last but not least, our current analysis is based on partial proteins. Relevant data are subject to change if all proteins are analyzed.

GRAVY is a widely-used parameter for evaluating protein hydrophobicity (hydrophilic or hydrophobic). A protein with a positive GRAVY is highly hydrophobic while one with a negative value is more hydrophilic. However, membrane proteins are not necessarily GRAVY positive, even transmembrane proteins. Fountoulakis and Gasser, (2003) reported that only 10% of the proteins in the membrane fraction of E coli envelopes had positive values (Fountoulakis and Gasser, 2003). In our study, two integral membrane proteins of endoplasmic reticulum protein 29 and TNF-alpha-converting enzyme are both GRAVY negative. It seems that the GRAVY value of protein domain rather than overall sequence may be more meaningful because all signal peptides, transmembrane helices as well as other hydrophobic regions (predicted by HMMTOP) involved in this study bear positive GRAVYs with no exception (data not shown).

In summary, we successfully fractionated, quantified and characterized soluble and insoluble spinal cord proteins. Our quantification data strengthen gel-based proteomic application potentials in membrane proteomics. Additionally, a complete protein filing of enhanced solution fractions should reveal more interesting neural proteins which may play important functional roles in the CNS.

Acknowledgments

The authors are grateful to Ms. Janice Willson for editing this manuscript. This work was supported by grants from the China National Key Basic Research and Development Projects Foundation (Grant No.2001CB510206) and the Beijing Municipal Nature Foundation (Grant No. 7012032).

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