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Enrichment of integral membrane proteins from small amounts of brain tissue

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Summary. Subcellular fractionation represents an essential technique for functional proteome analysis. Recently, we provided a subcellular fractionation protocol for minute amounts of tissue that yielded a nuclear fraction, a membrane and organelle fraction, and a cytosolic fraction. In the current study, we attempted to improve the protocol for the isolation of integral membrane proteins, as these are particularly important for brain function. In the membrane and organelle fraction, we increased the yield of membranes and organelles by about 50% by introducing a single re-extraction step. We then tested two protocols towards their capacity to enrich membrane proteins present in the membrane and organelle fraction. One protocol is based on sequential solubilization using subsequent increases of chaotropic conditions, thereby partitioning hydrophobic proteins from hydrophilic ones. The alternative protocol applies high-salt and high-pH washes to remove non-membrane proteins. The enrichment of membrane proteins by these procedures, as compared to the original membrane and organelle fraction, was evaluated by 16-BAC-SDS-PAGE followed by mass spectrometry of randomly selected spots. In the original membrane and organelle fraction, 7 of 50 (14%) identified proteins represented integral membrane proteins, and 15 (30%) were peripheral membrane proteins. In the urea-soluble fraction, 4 of 33 (12%) identified proteins represented integral membrane proteins, and 10 (30%) were peripheral membrane proteins. In the high-salt/high-pH resistant sediment, 12 of 45 (27%) identified proteins were integral membrane proteins and 13 (29%) represented peripheral membrane proteins. During the analysis, several proteins involved in neuroexocytosis were detected, including syntaxin, NSF, and Rab3-interaction protein 2. Taken together, differential centrifugation in combination with high-salt and high-pH washes resulted in the highest enrichment of integral membrane proteins and, therefore, represents an adequate technique for region-specific profiling of membrane proteins in the brain.

* Both authors contributed equally to the paper

Keywords: Subcellular prefractionation, membrane proteins, 16-BAC-SDS-PAGE, high-salt/high-pH extraction, sequential solubilization, mass spectrometry, proteomics.

Abbreviations

CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; *16-BAC* benzyldimethyl-n-hexadecylammonium chloride; *SDS* sodium dodecylsulfate; *PAGE* polyacrylamide gel electrophoresis; *EDTA* ethylenediaminetetraacetic acid

Introduction

The identification of most genes in many model organisms, in combination with progress in protein and peptide separation techniques and mass spectrometry, offers the potential to perform extensive protein profiling analyses (Takahashi et al., 2003; Stasyk and Huber, 2004). Ultimately, the comprehensive identification and quantification of proteins will lead to an improved understanding of the molecular repertoire underlying tissue-specific functions. A major difficulty with proteomic approaches is the extraordinarily high protein complexity of biological samples. Likely more than 100,000 different protein isoforms exist in a cell and display a dynamic range of 7–10 orders of magnitude in concentration (Anderson and Anderson, 2002; Stasyk and Huber, 2004). This complexity requires the prefractionation of samples prior to protein analysis in order to make low-to-medium abundant proteins detectable (Dreger, 2003; Stasyk and Huber, 2004). One prominent class of less abundant proteins is formed by membrane proteins. They are assumed to constitute about 20–30% of cellular proteins (Lehnert et al., 2004; Yu et al., 2004) and fall into two groups. Integral membrane proteins contain transmembrane domains, whereas peripheral membrane proteins are associated with the membrane, e.g., via GPI-anchors or via non-covalent binding to integral membrane proteins. Membrane proteins are involved in many important cellular processes, for instance active transport, ion flow, energy transduction, or signal transduction. In the nervous system, membrane proteins are essential for such fundamental processes as neuronal circuit formation and neurotransmission. Examples include neurotransmitter receptors, ion channels, transporters, and proteins of the neuroexocytosis machinery.

Several protocols have been established to enrich membrane proteins. One such protocol applies high-salt and high-pH washes to the protein sample, which removes cytosolic and luminal proteins (Fujiki et al., 1982; Pasquali et al., 1997). In addition, this handling lowers the amount of peripheral membrane proteins by reducing non-covalent protein–protein interactions (Taylor et al., 2000). Finally, the high-pH causes the removal of actin bundles by their depolymerization (Galkin et al., 2001). Another approach is based on the sequential solubilization of proteins (Molloy et al., 1998; Lehner et al., 2003). It includes four steps of solubilization with subsequent increases of chaotropic conditions, thereby separating the hydrophobic proteins from the hydrophilic ones.

We recently established a protocol for the subcellular fractionation of minute amounts of biopsy samples, e.g., 500 mg of brain tissue (Guillemin et al., 2005). This protocol yields three fractions: a nuclear fraction, a cytosolic fraction, and a composite membrane and organelle fraction (M/O-fraction). The purpose of the current study was to enrich integral membrane proteins from the M/O-fraction. To pursue our goal, we first increased the yield of membranes and organelles by adding a re-extraction step to the initial differential centrifugation protocol. We then applied the M/O-fraction either to the sequential solubilization protocol or to high-salt and high-pH washes. To evaluate the enrichment of peripheral and integral

membrane proteins by the two procedures, protein samples were separated by 16-BAC-SDS-PAGE, and randomly selected spots were identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The data show that differential centrifugation, followed by high-salt and high pH washes of the M/O-fraction, is best suited for accumulating integral membrane proteins from small brain areas.

Materials and method

Animals

Sprague-Dawley rats (8–9 weeks old of both genders) were deeply anesthetized by a peritoneal injection of 700 mg/kg chloral hydrate and sacrificed by decapitation. Isolated brainstems were immediately frozen in liquid nitrogen and then stored at −80°C. All protocols complied with the current German Animal Protection Law and were approved by the local animal care and use committee (Landesuntersuchungsamt Koblenz).

Chemicals

Acetonitrile and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany) and α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany). All other chemicals were purchased from Sigma-Aldrich (Munich, Germany).

Subcellular prefractionation

Subcellular prefractionation of brain tissue was performed according to our previously reported protocol (Guillemin et al., 2005; Fig. 1). Frozen tissue (2 g) was transferred to 4 ml CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) and prehomogenized by applying 2 strokes in a glass/teflon homogenizer. The suspension was incubated on ice for 10 min, followed by 6 strokes of a motorized homogenizer at 250 rpm. After restoration with 0.1 volume 2.5 M sucrose, differential centrifugation was performed at 6,300 × *g* for 10 min. The supernatant was collected and stored on ice. The sediment was resuspended in 4 ml isotonic CLB buffer, containing 0.1 volume 2.5 M sucrose, and centrifuged at 6,300 × *g* for 10 min. The resulting supernatant was combined with the first supernatant and sedimented at 107,000 × *g* for 30 min in a Beckman SW40 rotor (Beckman Coulter, Krefeld, Germany). The resulting sediment represented the M/O-fraction and was stored at −80°C until further use.

Marker enzyme assays

The protein amount was determined using the method of Bradford (1976) with bovine serum albumine as standard. Marker enzymes for the various cellular compartments were as follows: alkaline phosphatase [EC 3.1.3.1] was used as a plasma membrane marker (Graham, 1993), and succinate dehydrogenase [EC 1.3.5.1] as a marker for mitochondria (Graham, 1993). All results represent mean values ± standard deviation of three independent experiments.

Sequential protein solubilization

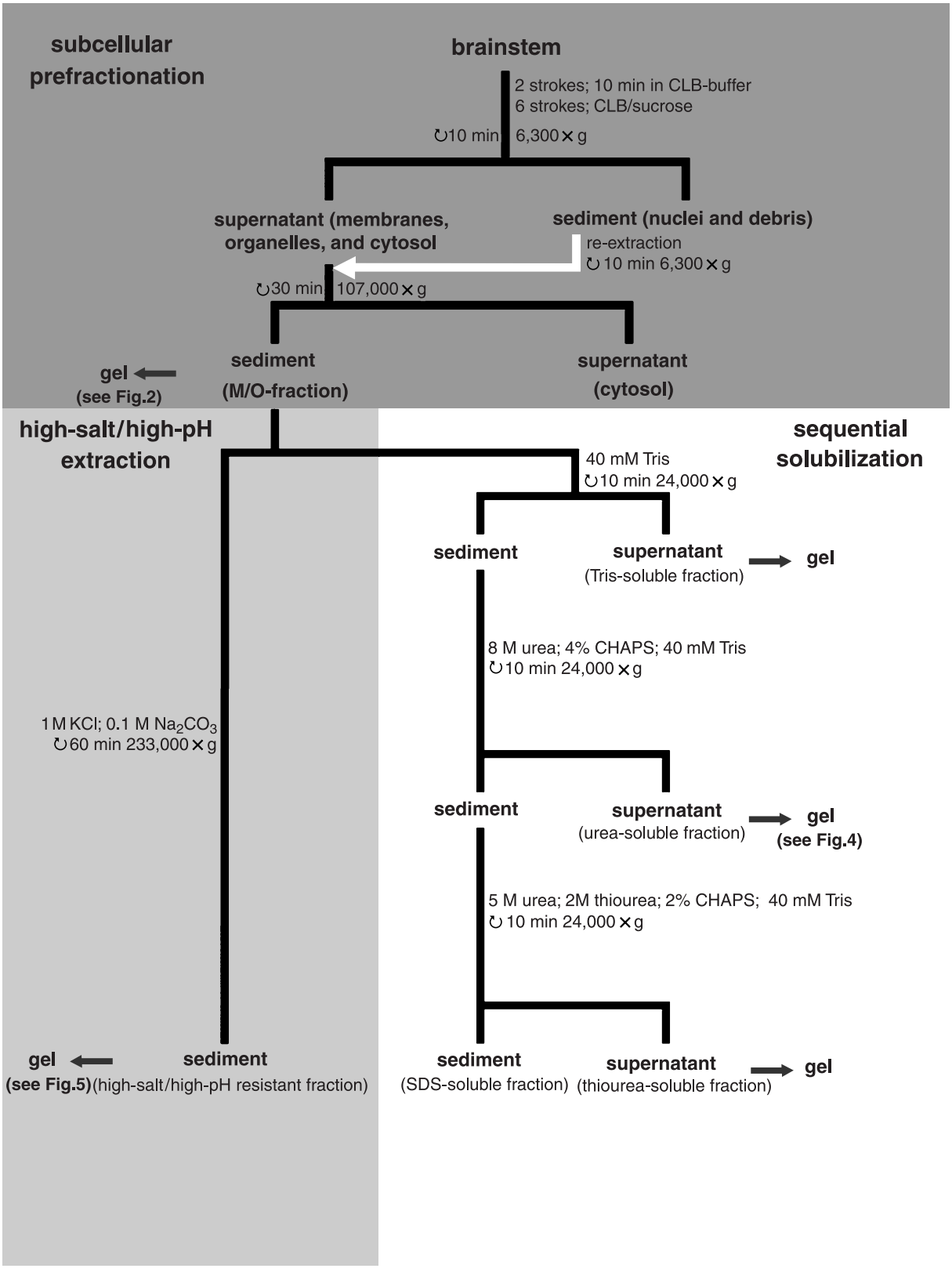
Sequential protein solubilization was performed as described previously (Molloy et al., 1998) with minor modifications. The M/O-fraction was resuspended in a buffer containing 40 mM Tris, pH 9.5 (Tris-buffer) and then centrifuged at 24,000 × *g* for 10 min in a tabletop centrifuge. The supernatant represented the Tris-soluble proteins. The sediment was resuspended in 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 40 mM Tris, pH 9.5. After centrifugation under the same conditions as described above, the supernatant was stored as the urea-soluble fraction. The sediment was extracted in 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, and 40 mM Tris pH 9.5. The supernatant, obtained after another round of centrifugation, represented the thiourea-soluble fraction. The final sediment was resuspended in 1% SDS, 40 mM Tris, pH 9.5 and represented the SDS-soluble fraction.

High-salt/high-pH extraction

The M/O-fraction was resuspended in an ice-cold solution of 1 M KCl and 15 mM Tris, pH 7.4 (high-salt extraction). After 15 minutes on ice, the solution was centrifuged at 233,000 × *g* for one hour in a SW40 rotor. The sediment was re-extracted twice with the high-salt solution. The resulting sediment was washed three times with 0.1 M Na_2CO_3, pH 11.5 (high-pH extraction) as described earlier (Fujiki et al., 1982).

16-BAC-SDS-PAGE

Proteins from various fractions were separated by two-dimensional 16-BAC-SDS-PAGE as this gel sys-


subcellular prefractionation
brainstem
2 strokes; 10 min in CLB-buffer
6 strokes; CLB/sucrose
↻10 min 6,300 × g
supernatant (membranes, organelles, and cytosol
sediment (nuclei and debris)
re-extraction
↻10 min 6,300 × g
↻30 min 107,000 × g
gel
(see Fig.2)
sediment
(M/O-fraction)
supernatant
(cytosol)
high-salt/high-pH extraction
sequential solubilization
40 mM Tris
↻10 min 24,000 × g
sediment
supernatant
(Tris-soluble fraction)
gel
8 M urea; 4% CHAPS; 40 mM Tris
↻10 min 24,000 × g
1 M KCl; 0.1 M Na2CO3
↻60 min 233,000 × g
sediment
supernatant
(urea-soluble fraction)
gel
(see Fig.4)
5 M urea; 2M thiourea; 2% CHAPS; 40 mM Tris
↻10 min 24,000 × g
gel
(see Fig.5)
sediment
(high-salt/high-pH resistant fraction)
sediment
(SDS-soluble fraction)
supernatant
(thiourea-soluble fraction)
gel

tem does not discriminate between hydrophobic proteins and hydrophilic proteins (Hartinger et al., 1996). In brief, proteins were separated twice by molecular mass using different detergents. In the first dimension, benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) was used as a detergent, and the separation was performed in a 7.5% polyacrylamide gel under acidic conditions at 10 mA until the dye had migrated to the lower end of the gel. In the second dimension, separation under alkaline conditions was performed with sodium dodecylsulfate (SDS) as the detergent in an 8% polyacrylamide gel (25 mA for about 6 hours). Due to the different effects of the two detergents on proteins with similar or equal mass, this method allows the separation of such proteins which is impossible in one-dimensional gels. Spots that were visible after colloidal Coomassie staining were excised using a Spot Cutter (Bio-Rad, Munich, Germany) and prepared for mass spectrometry as follows.

Protein identification by mass spectrometry

Excised spots were alternately washed twice with 50 mM NH_4HCO_3 and 25 mM NH_4HCO_3/50% acetonitrile. Protein disulfides were reduced with 10 mM dithiothreitol at 57°C for 30 minutes, followed by carbamidomethylation with 5 mM iodacetamide for 30 minutes. Finally, the spots were washed twice as described above and dried. Proteins were in-gel digested by adding 3.5 µl of a 25 µg/ml solution of trypsin (Promega, Madison, MA, USA) in 50 mM NH_4HCO_3 over night at 37°C. Peptides were extracted by incubating them with 0.1% trifluoroacetic acid for 45 min. Extracted peptides were concentrated using Perfect Pure C-18 tips (Eppendorf, Germany) and eluted onto a MALDI anchor target plate using α-cyano-4-hydroxycinnamic acid as matrix. Spectra were acquired using an Ultraflex MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany). One-thousand spectra per sample were summed up and processed with FlexAnalysis 2.2 (Bruker Daltonics, Bremen, Germany) to generate a mass list. Peptide mass fingerprints were analyzed with Biotools 2.2 (Bruker Daltonics, Bremen, Germany). MASCOT searches were performed considering carbamidomethylation as fixed modification and oxidation of methionine as variable modification. Peptide mass tolerance was set at 0.25 Da. The final subcellular assignment of proteins was achieved using the Protein Knowledgebase Swiss-Prot (http://www.expasy.org/sprot) or GeneCards (http://bioinformatics.weizmann.ac.il/cards/index.shtml).

Results

Increased yield in the M/O-fraction by introducing a single re-extraction step

To evaluate the recovery of membranes and organelles in the original protocol, we determined the enzymatic activity of the plasma membrane marker alkaline phosphatase and the mitochondrial marker enzyme succinate dehydrogenase. When applying the initial protocol for differential centrifugation (Guillemin et al., 2005), we noticed $21.3 \pm 3.8\%$ of plasma membrane marker activity and $3.4 \pm 0.4\%$ of mitochondrial marker activity in the supernatant (membranes, organelles, and cytosol), whereas $78.7 \pm 3.8\%$ of the plasma membrane marker activity and $96.6 \pm 0.4\%$ of the mitochondrial marker activity were found in the sediment (nuclei and debris). To increase the recovery of proteins in the supernatant, we re-extracted the nuclei and debris-containing sediment by resuspension in isotonic CLB and determined the enzyme marker activities in the supernatants (membranes, organelles, and cytosol; Fig. 1). Through the introduction of the re-extraction step, the yield of plasma membrane proteins and mitochondrial proteins was increased by $49.9 \pm 14.5\%$ and $56.6 \pm 13.5\%$, respectively. Further re-extraction steps

Fig. 1. Schematic illustration of the subcellular prefractionation and the enrichment protocols. Differential centrifugation was performed mainly as described previously (Guillemin et al., 2005). The only difference was the re-extraction of sediment (nuclei and debris), yielding a further supernatant (membranes, organelles, and cytosol). The combined supernatants were sedimented to obtain the M/O-fraction and the cytosolic supernatant. The M/O-fraction was subjected to either sequential solubilization or high-salt/high-pH extraction. Proteins in the resulting fractions were subsequently separated by 16-BAC-SDS-PAGE and identified by mass spectrometry

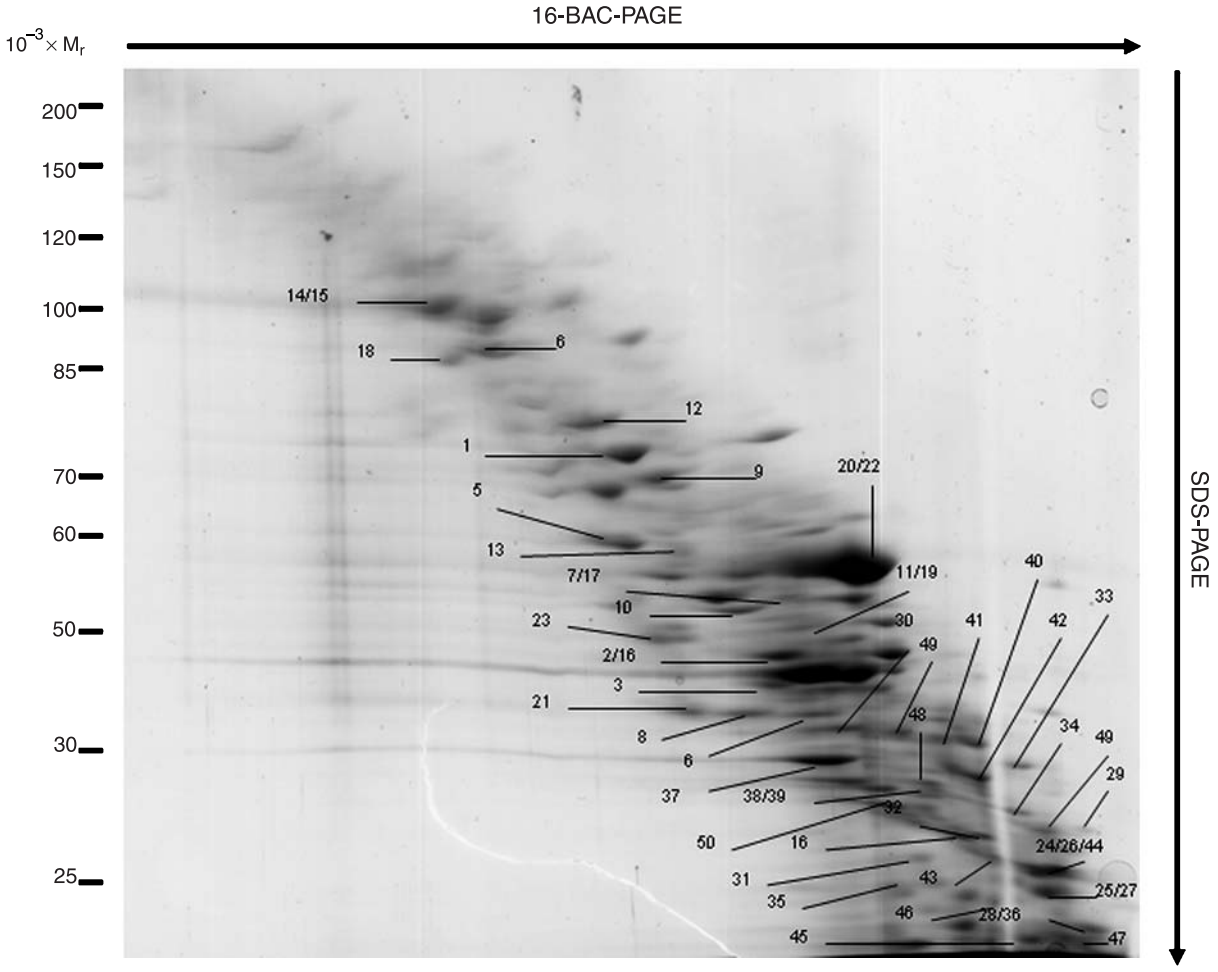


Fig. 2. 16-BAC-SDS-PAGE of the M/O-fraction from rat brainstem. 300 µg protein were separated by 16-BAC-SDS-PAGE. Numbered spots were excised from the gel, in-gel digested with trypsin, and identified by mass spectrometry. The results are listed in Table 2

of the nuclei and debris-containing sediment did not considerably increase the yield. Therefore, we conclude that the addition of a single re-extraction step is sufficient to improve the protein output of the subcellular prefractionation.

Table 1. Subcellular allocation of proteins in different fractions

Allocation	M/O-fraction	Urea-soluble proteins	High-salt/ high-pH resistant proteins
integral membrane	14% (7)	12% (4)	26% (12)
peripheral membrane	30% (15)	30% (10)	28% (13)
cytoskeleton	12% (6)	27% (9)	11% (5)
cytosol	16% (8)	18% (6)	20% (9)
endoplasmic reticulum	4% (2)	0	2% (1)
mitochondria	6% (3)	0	2% (1)
multiple	18% (9)	12% (4)	8% (4)
Total number of proteins	50	33	45

The number of proteins assigned to various allocations is given in parentheses

Table 2. Proteins of the M/O-fraction

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)
1	Heat shock cognate 71 kDa protein	P63018	HSP7C	mu	114	31	38
2	Creatine kinase, brain isoform	P07335	KCRB	mu	72	13	41
3	Glutamine synthetase	P09606	GLNA	c	121	22	46
4	Pyruvate kinase M1/M2	P11980	KPYM	c	63	20	13
5	Cytoplasmic aspartate aminotransferase	P13221	AATC	c	108	18	39
6	Heat shock protein HSP 90-beta	P34058	HS90B	mu	114	31	38
7	Protein kinase C and casein kinase substrate in neurons protein 1	Q9Z0W5	PACN1	pm	66	16	44
8	Phosphoglycerate kinase 1	P16617	PGK1	c	54	11	42
9	Syntaxin binding protein 1 splice isoform 2	P61765	STXB1	pm	79	15	19
10	Alpha enolase	P04764	ENOA	mu	70	16	28
11	Gamma enolase	P07323	ENOG	mu	83	18	44
12	78 kDa glucose-regulated protein	P06761	GRP78	er	130	29	46
13	Protein disulfide-isomerase A3	P11598	PDIA3	er	94	19	34
14	Na/K-ATPase alpha-2	P06686	AT1A2	im	102	30	31
15	Na/K-ATPase alpha-3	P06687	AT1A3	im	135	35	36
16	2′,3′-cyclic-nucleotide 3′-phosphodiesterase	P13233	CN37	pm	117	24	48
17	ATP synthase beta, mitochondrial precursor	P10719	ATPB	pm	109	21	53
18	Mitochondrial aconitate hydratase	Q9ER34	ACON	mito	76	19	22
19	Mitochondrial glutamate dehydrogenase 1	P10860	DHE3	mito	58	15	31
20	Tubulin beta-1	P04691	TBB1	cs	63	17	35
21	Fructose-bisphosphate aldolase A	P05065	ALDOA	c	73	17	49
22	Tubulin alpha-1	P68370	TBA1	cs	101	20	45
23	Septin-7	Q6Q137	SEPT7	cs	52	18	43
24	14-3-3 protein gamma	P61983	1433G	mu	50	17	43
25	14-3-3 protein zeta/delta	P63102	1433Z	mu	60	14	54
26	Actin, cytoplasmic 2	P63259	ACTG	cs	60	14	30
27	ADP/ATP translocase 1	Q05962	ADT1	im	68	18	52
28	ADP/ATP translocase 2	Q09073	ADT2	im	52	11	40
29	Alpha-soluble NSF attachment protein	P54921	SNAA	pm	80	17	63
30	Aspartate aminotransferase, mitochondrial	P00507	AATM	mito	55	18	37
31	ATP synthase alpha, mitochondrial	P15999	ATPA	pm	74	23	34

(*continued*)

Table 2 (*continued*)

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)
32	ATP synthase gamma, mitochondrial	P35435	ATPG	pm	54	15	37
33	ATPase, H+ transporting, V0 subunit D isoform 1	Gi|62665162	ATPase	pm	77	20	28
34	Beta-soluble NSF attachment protein	P28663	SNAB	pm	157	26	74
35	Carbonic anhydrase II	P27139	CAH2	mu	65	12	45
36	Glutathione S-transferase Mu 1	P04905	GSTM1	c	68	16	58
37	Glyceraldehyde-3-phosphate dehydrogenase	P04797	G3P	mu	74	16	52
38	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	P54311	GBB1	pm	71	16	48
39	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2	P54313	GBB2	pm	77	17	49
40	Guanine nucleotide-binding protein G(o), alpha subunit 1	P59215	GNAO1	pm	76	22	49
41	Guanine nucleotide-binding protein G(o), alpha subunit 2	P30033	GNAO2	pm	69	18	45
42	L-lactate dehydrogenase B	P42123	LDHB	c	69	14	45
43	Myelin-oligodendrocyte glycoprotein	Q63345	MOG	im	52	10	33
44	Phosphate carrier protein, mitochondrial	P16036	MPCP	im	50	14	35
45	Phosphatidylethanolamine-binding protein	P31044	PEBP	pm	55	11	60
46	Phosphoglycerate mutase 1	P25113	PGAM1	c	70	14	42
47	Ras-related protein Rab-14	P61107	RAB14	pm	100	14	66
48	sirtuin 2	NP_001008369	Sirt2	cs	79	17	48
49	Tubulin beta-5	P69897	TBB5	cs	97	23	51
50	Voltage-dependent anion-selective channel 1	Q9Z2L0	VDAC1	im	92	15	60

The spot number (spot no.) corresponds to the position marked on the gel (Fig. 2). Protein names and accession numbers were derived from the Protein Knowledgebase Swiss-Prot. Information on the subcellular location was obtained from Swiss-Prot or GeneCards. MASCOT score, no. of matching peptides, and sequence coverage (seq. coverage) for the identified proteins are indicated. Membrane proteins were classified as integral membrane proteins (im) or peripheral membrane proteins (pm). Non-membrane proteins were assigned to subcellular compartments: *c* cytosol; *cs* cytoskeleton; *er* endoplasmic reticular lumen; *mito* mitochondrial matrix; *mu* multiple localizations

Characterization of the M/O-fraction

In order to evaluate the percentage of integral membrane proteins in the M/O-fraction, we separated the proteins by 16-BAC-SDS-PAGE (Fig. 2). Fifty different proteins were identified by mass fingerprint analysis using a MALDI-TOF-TOF instrument. Seven (14%) integral membrane proteins and 15 (30%)

peripheral membrane proteins were detected (Table 1). Their identity and further details concerning the mass spectrometry data are provided in Table 2. The other 28 proteins represented cytosolic proteins (8 proteins), luminal proteins (5 proteins), cytoskeleton (-associated) proteins (6 proteins), or had no defined subcellular allocation (9 proteins; Table 2). A total of 42 (84%) proteins were assigned to a membrane or organelle locali-

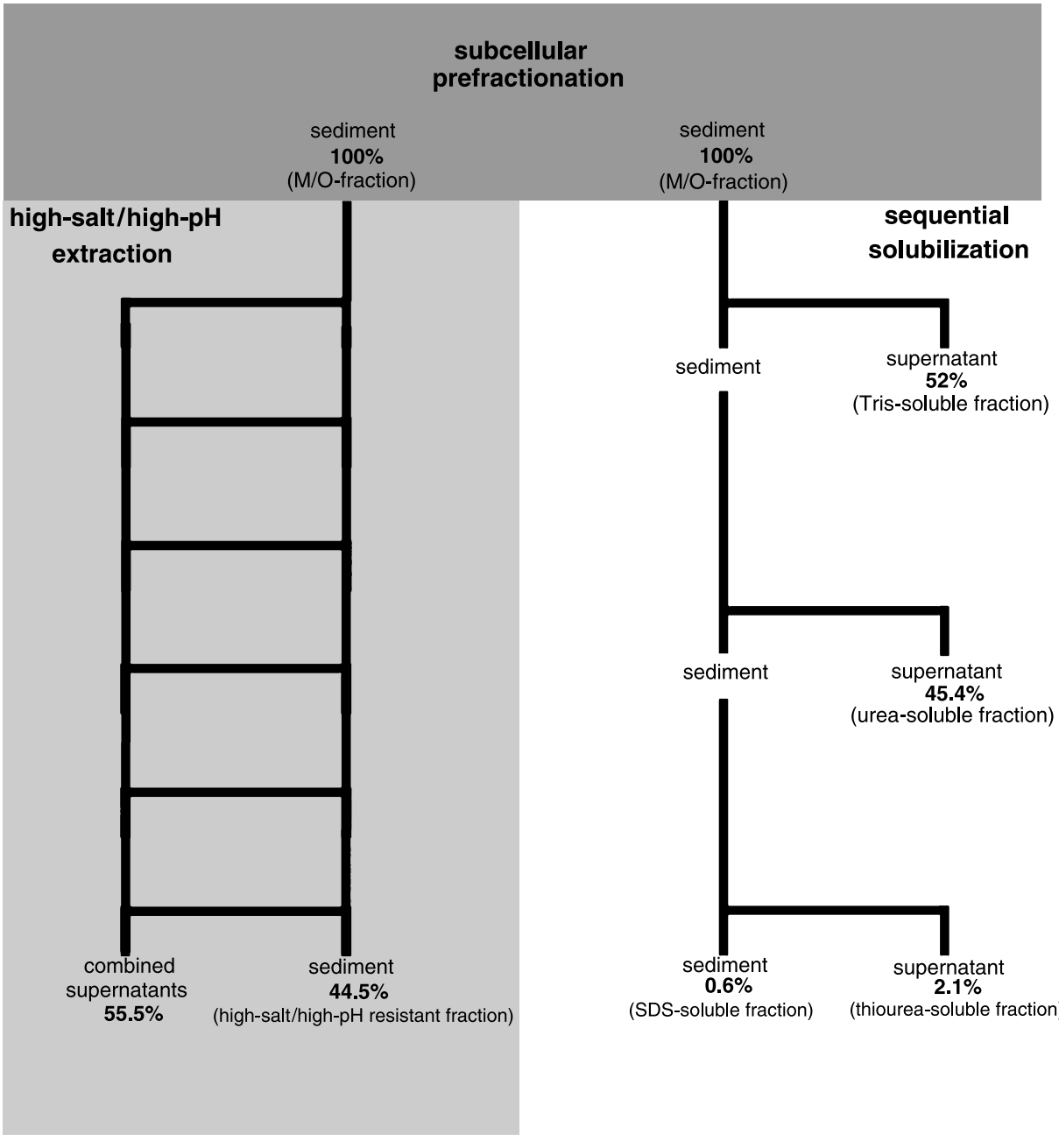


Fig. 3. Quantitiative analysis of the protein amount present in various fractions. The protein amount is displayed as the percentage of the protein amount determined in the M/O-fraction. Data represent mean values of three independent experiments except for high salt/high pH washes, which were performed twice

zation, thus confirming their previously reported enrichment in this fraction (Guillemin et al., 2005).

Enrichment of membrane proteins in the M/O-fraction by sequential solubilization

To determine the enrichment of integral membrane proteins obtained by the sequential solubilization protocol, the M/O-fraction was first solubilized in Tris-buffer. Based on Bradford assays, $52 \pm 17\%$ of the protein amount in the M/O-fraction was recovered in the Tris-soluble fraction, $45.4 \pm 18.3\%$ in the urea-soluble fraction, $2.1 \pm 1.2\%$ in the thiourea-soluble fraction, and only $0.6 \pm 0.2\%$ in the SDS-soluble fraction (Fig. 3).

Protein detection by 16-BAC-SDS-PAGE of the final two sediments (thiourea-soluble fraction and SDS-soluble fraction) was unfeasible as the combined protein amount of these two fractions was less than 3% of the total protein amount seen in the starting material (i.e., the M/O-fraction). We therefore focused our further analysis on the urea-soluble fraction and separated its proteins by 16-BAC-SDS-PAGE (Fig. 4). Thirty-three different proteins

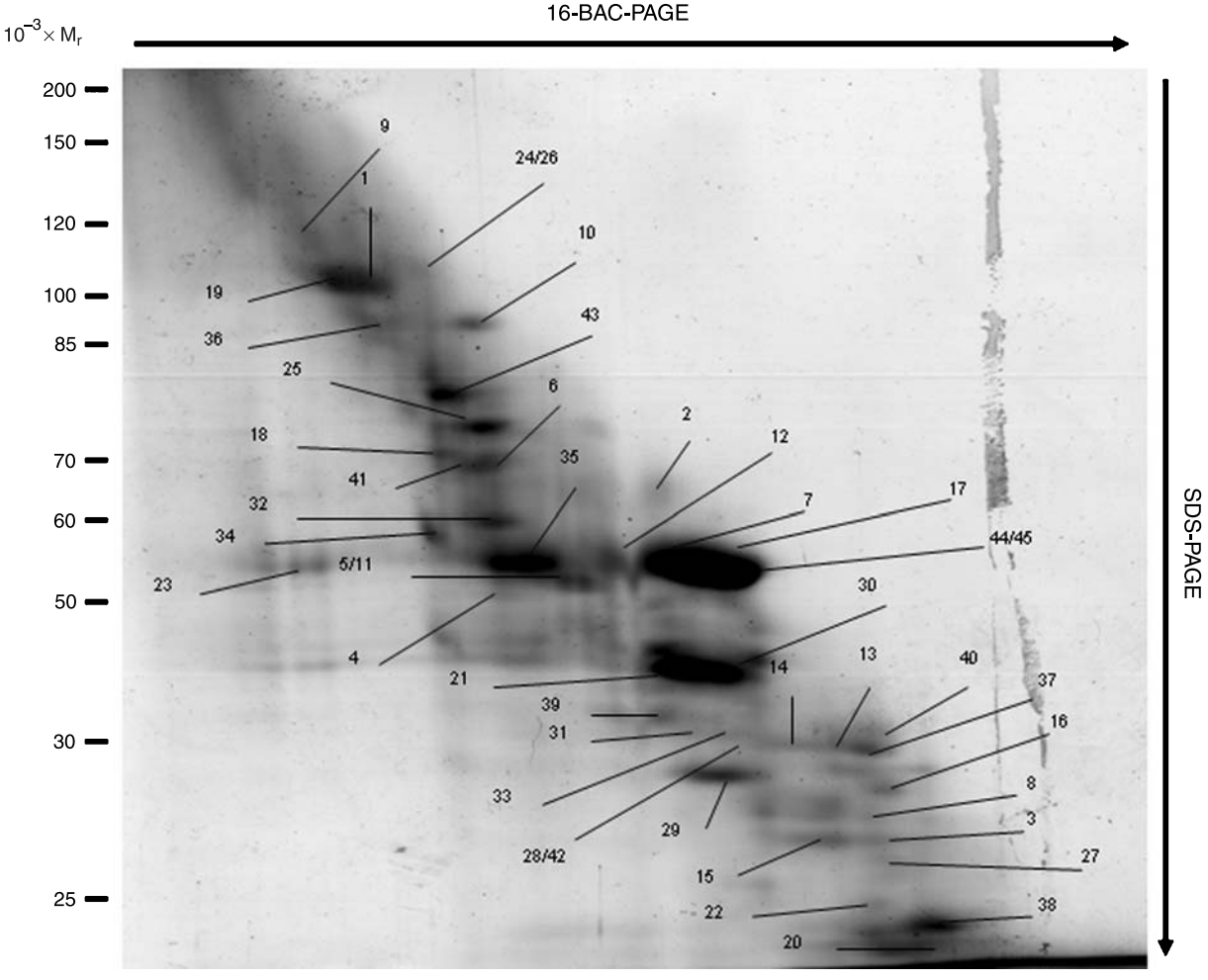


Fig. 4. 16-BAC-SDS-PAGE of urea-soluble proteins of the M/O-fraction from rat brainstem. 300 µg protein were separated by 16-BAC-SDS-PAGE. Numbered spots were excised from the gel, in-gel digested with trypsin, and identified by mass spectrometry. The results are listed in Table 3

Table 3. Proteins of the urea-soluble fraction

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)	Also detected in
1	Lamin A	P48679	LAMA	cs	53	19	26	–
2	Mitochondrial aspartate aminotransferase	P00507	AATM	pm	53	15	32	–
3	Mitochondrial ATP synthase beta	P10719	ATPB	pm	74	21	33	–
4	Mitochondrial ATP synthase gamma	P35435	ATPG	pm	58	16	39	–
5	Mitochondrial ubiquinol-cytochrome-c reductase protein 2	P32551	UQCR2	im	70	15	31	–
6	Voltage-dependent anion-selective channel 1	Q9Z2L0	VDAC1	im	95	14	57	HS
7	2′,3′-cyclic-nucleotide 3′-phosphodiesterase	P13233	CN37	pm	298	42	68	HS
8	Dihydropyrimidinase related protein-2	P47942	DPYL2	pm	197	32	63	HS
9	Myelin P0 protein	P06907	MYP0	im	63	12	33	–
10	Pyruvate kinase M1/M2	P11980	KPYM	c	221	38	55	HS
11	Syntaxin-1B2	P61265	STX1C	im	53	12	41	HS
13	3 beta-hydroxysteroid dehydrogenase type II	P22072	3BHS2	pm	52	11	33	–
14	Malate dehydrogenase 1	AAH59124	MDH1	c	72	17	46	–
15	citrate synthase	NP_570111	CS	c	88	19	34	–
16	Kinesin-like protein	O35787	KIF1D	cs	51	12	20	–
17	Myosin heavy chain	P02563	MYH6	cs	58	27	15	–
18	Glyceraldehyde-3-phosphate dehydrogenase	P04797	G3PDH	mu	83	18	55	HS
19	Gamma enolase	P07323	ENOG	mu	100	19	42	–
20	Glutamine synthetase	P09606	GLNA	c	81	16	32	–
21	Clathrin heavy chain	P11442	CLH	pm	124	35	27	–
22	Cytoplasmic aspartate aminotransferase	P13221	AATC	c	88	17	43	HS
23	Dynamin-1	P21575	DYN1	pm	56	19	20	HS
24	14-3-3 protein gamma	P61983	1433G	mu	85	22	43	–
25	Heat shock cognate 71 kDa protein	P63018	HSP7C	mu	162	34	43	HS

(*continued*)

Table 3 (*continued*)

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)	Also detected in
26	ACF7 neural isoform 1	AAC52988	ACF7	cs	69	29	18	–
27	sirtuin 2	NP_001008369	sirtuin 2	cs	120	20	54	–
28	Tubulin beta-1	P04691	TBB1	cs	85	26	26	–
29	Glycogen phosphorylase, brain	P53534	PHS3	c	111	26	35	–
30	Tubulin alpha-1	P68370	TBA1	cs	83	19	45	HS
30	Tubulin alpha-6	Q6AYZ1	TBA6	cs	73	17	44	–
30	Tubulin alpha-8	Q6AY56	TBA8	cs	52	15	43	–
31	Tubulin alpha-2	Q6P9V9	TBA2	cs	81	35	42	–
31	Tubulin alpha-3	Q68FR8	TBA3	cs	66	16	39	–
31	Tubulin beta-5	P69897	TBB5	cs	85	19	41	–
32	Rab3-interacting molecule 2	Q9JIS1	RIMS2	pm	52	27	20	–
33	Septin-7	Q9WVC0	SEPT7	cs	79	22	49	–
34	H+ transporting ATPase V1 subunit A, isoform 1	XP_340988	Atp6v1a1	pm	127	30	39	HS

Spot no. corresponds to the position marked on the gels (Fig. 2). Protein name and acc. no. were derived from the Protein Knowledgebase Swiss-Prot. Information on the subcellular location was obtained from Swiss-Prot or GeneCards. MASCOT score, no. of matching peptides, and sequence coverage for the identified proteins are indicated. Membrane proteins were classified as integral membrane proteins (im) or peripheral membrane proteins (pm). Non-membrane proteins were assigned to subcellular compartments: *c* cytosol; *cs* cytoskeleton; *mu* multiple localizations. Proteins that were also detected in the high-salt/high-pH resistant fraction are marked with HS

were identified (Table 1). Four of them (12%) represented integral membrane proteins and 10 (30%) were peripheral membrane proteins (Table 3). Furthermore, 9 cytoskeleton (-associated) proteins, 6 cytosolic proteins, and 4 proteins with multiple subcellular localizations were identified (Table 3). Therefore, neither peripheral membrane proteins nor integral membrane proteins could be enriched by urea solubilization.

Since the sequential solubilization resulted in insufficiently low protein amounts in both the thiourea-soluble fraction ($2.1 \pm 1.2\%$) and the SDS-soluble fraction ($0.6 \pm 0.2\%$), and since no enrichment of membrane proteins was seen in the urea-soluble fraction, we wondered whether membrane proteins were lost at earlier stages of the protocol. We therefore analyzed the Tris-soluble fraction and identified a surprisingly high percentage of membrane proteins [8 (16%) peripheral membrane proteins and 4 (8%) integral membrane proteins out of 50 proteins].

Enrichment of membrane proteins in the M/O-fraction by high-salt/high-pH extraction

In a next series of experiments, we analyzed the enrichment of membrane proteins by high-salt and high-pH washes. To do so, the M/O-fraction was solubilized 3 times in 1 M KCl, followed by 3 washing steps in 0.1 M Na_2CO_3. After these 6 steps, the insoluble protein fraction (= high-salt/high-pH resistant fraction) contained $44.5 \pm 2.1\%$ of the initial protein amount of the M/O-fraction, whereas the 6 combined supernatants in total

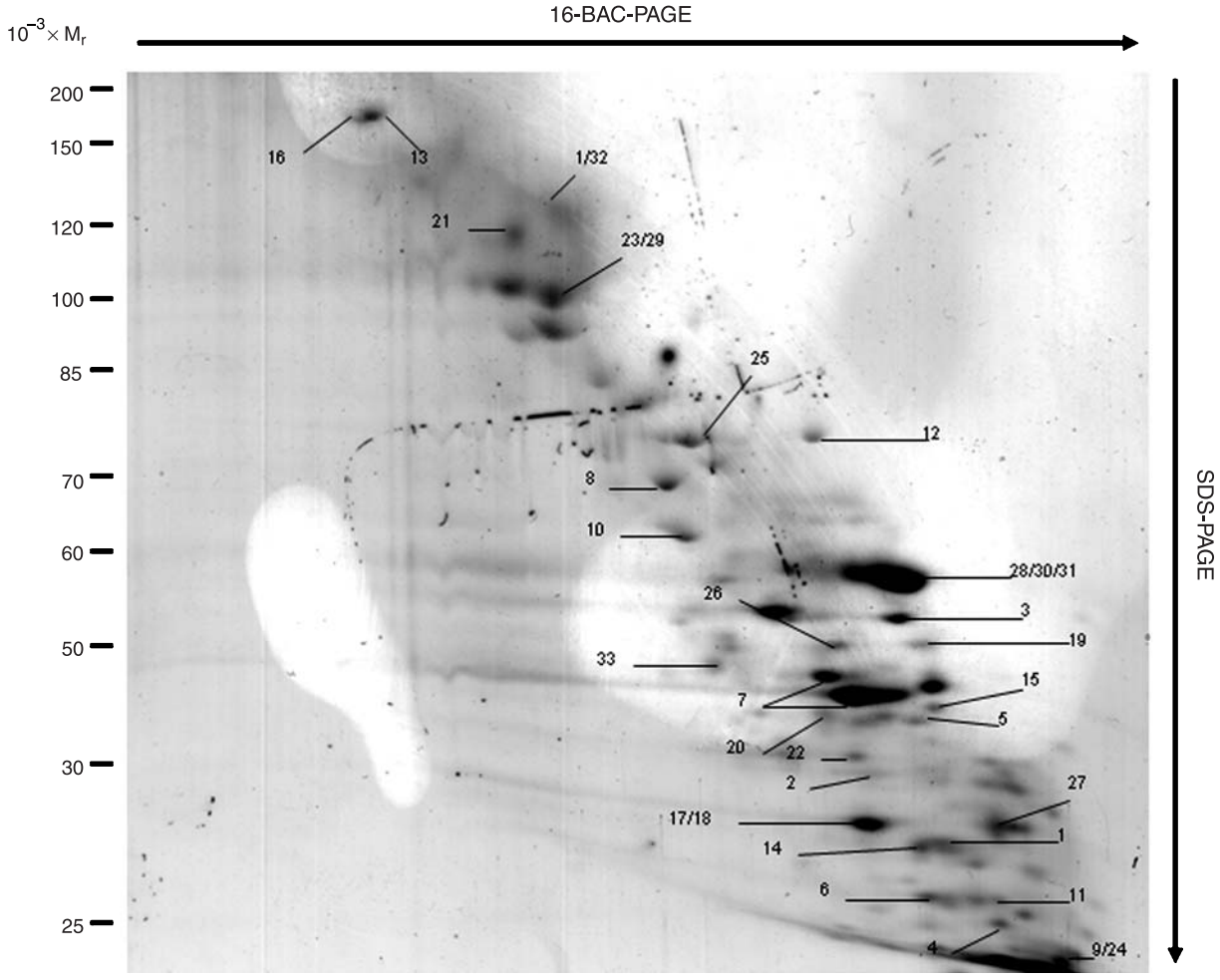


Fig. 5. 16-BAC-SDS-PAGE of the high-salt/high-pH resistant fraction from rat brainstem. 300 µg protein were separated by 16-BAC-SDS-PAGE. Numbered spots were excised from the gel, in-gel digested with trypsin, and identified by mass spectrometry. The results are listed in Table 4

contained the remaining $55.5 \pm 2.1\%$ (Fig. 3). The high-salt/high-pH resistant fraction was separated by 16-BAC-SDS-PAGE (Fig. 5). Forty-five different proteins were identified (Tables 1 and 4). The largest class represented 13 (29%) peripheral membrane proteins. The second largest class comprised integral membrane proteins with 12 (27%) members. Among the remaining proteins, 9 were cytosolic, 5 cytoskeleton (-associated), one from the lumen of the endoplasmic reticulum, and one from the mitochondrial matrix. Four proteins had multiple subcellular assignments (Table 4). Taken together, high-salt/high-pH extraction resulted in a 2-fold enrichment of integral membrane proteins in the final sediment.

Analysis of proteins relevant to neural processing

A final aspect important to us for evaluating the two enrichment techniques was the analysis whether each protocol was able to detect proteins which are relevant to neural processing. We identified 13 proteins (29%) of this kind after high-salt/high-pH extraction, yet only 7 (21%) in the urea-soluble fraction. Four of

Table 4. Proteins of the high-salt/high-pH resistant fraction

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)	Also present in
1	Dynamin-1	P39053	DYN1	pm	55	21	28	U
2	Synaptotagmin I	P21707	SYT1	im	53	14	39	–
3	Synaptotagmin II	P29101	SYT2	im	67	15	31	–
4	Clathrin-associated adaptor complex AP-2 mu2	JC6563	AP2MU2	pm	66	19	45	–
5	Mitochondrial ATP synthase alpha	P15999	ATPA	pm	90	20	43	–
6	Mitochondrial aspartate glutamate carrier 1	Q8BH59	CMC1	im	67	18	26	–
7	ADP/ATP translocase 1	Q05962	ADT1	im	56	15	42	–
8	Voltage-dependent anion-selective channel 1	Q9Z2L0	VDAC1	im	67	11	53	U
9	Contactin 1	Q63198	CNTN1	pm	114	29	33	–
10	Calnexin	P35565	CALX	im	100	22	30	–
11	Intracellular chloride channel protein 4	Q9Z0W7	CLIC4	im	50	9	29	–
12	Brain vacuolar ATP synthase subunit B	P62815	VATB2	pm	60	16	32	–
13	Adenylate cyclase-inhibiting G alpha	P04897	GNAI2	pm	72	15	50	–
14	Guanine nucleotide-binding protein G(o), alpha 2	P30033	GNAO2	pm	58	15	41	–
15	Dihydropyrimidinase related protein-2	P47942	DPYL2	pm	88	17	45	U
16	Transducin beta 1	P54311	GBB1	pm	68	13	46	–
17	GTP-binding regulatory protein Go alpha 2	GNAO2	GNAO2	pm	54	15	32	–
18	Ribophorin I	P07153	RIB1	im	54	14	29	–
19	Na/K- ATPase alpha 2	P06686	AT1A2	im	111.5	29	29	–
20	Na/K- ATPase alpha 3	P06687	AT1A3	im	84	26	29	–
21	2′,3′-cyclic-nucleotide 3′-phosphodiesterase	P13233	CN37	pm	157	27	44	U
22	Syntaxin-1B2	P61265	STX1C	im	64	17	47	U
23	H+ transporting ATPase V1 subunit A, isoform 1	XP_340988	ATP6V1A1	pm	67	12	24	U
24	V-ATPase 116-kDa isoform a1	P25286	VPP1	im	76	21	24	–

(*continued*)

Table 4 (*continued*)

Spot no.	Protein name	Accession no.	Gene name	Subcellular location	MASCOT score	No. of matching peptides	Seq. coverage (%)	Also present in
25	78 kDa glucose-regulated protein	P06761	GRP78	er	159	31	45	–
26	Myosin heavy beta isoform	P02564	MYH7	cs	56	30	18	–
27	Triosephosphate isomerase	P48500	TPIS	c	53	10	37	–
28	Aspartate transaminase	JT0439	no name	c	71	14	35	–
29	Glyceraldehyde-3-phosphate dehydrogenas3	P04797	G3PDH	mu	56	13	41	U
30	Creatine kinase, brain isoform	P07335	KCRB	mu	121	21	58	–
31	Fructose-bisphosphate aldolase C	P09117	ALDOC	c	82	15	49	–
32	Pyruvate kinase M1/M2	P11980	KPYM	c	192	32	60	U
33	Cytoplasmic aspartate aminotransferase	P13221	AATC	c	86	15	36	U
34	Aspartyl-tRNA synthetase	P15178	SYD	c	51	11	30	–
35	Calpain-3	P16259	CAN3	c	50	14	19	–
36	Heat shock protein HSP 90-beta	P34058	HS9B	mu	106	26	38	–
37	L-lactate dehydrogenase B	P42123	LDHB	c	88	18	41	–
38	Vesicular-fusion protein NSF	P46460	NSF	pm	87	21	43	–
39	Beta-actin	P60711	ACTB	cs	53	11	46	–
40	Microtubule-actin crosslinking factor 1	Q9QXZ0	MACF1	cs	54	51	11	–
41	Heat shock cognate 71 kDa protein	P63018	HSP7C	mu	65	16	29	U
42	Glutamate oxaloacetate transaminase 2	AAH61792	AATM	mito	71	14	35	–
43	ATPase 3	AAS89307	ATPase 3	c	56	21	20	–
44	Tubulin beta-1	P04691	TBB1	cs	80	19	45	U
45	Tubulin alpha-1	P68370	TBA1	cs	65	15	43	U

Spot no. corresponds to the position marked on the gels (Fig. 4). Protein name and acc. no. were derived from the Protein Knowledgebase Swiss-Prot. Information on the subcellular location was obtained from Swiss-Prot or GeneCards. MASCOT score, no. of matching peptides, and sequence coverage for the identified proteins are indicated. Membrane proteins were classified as integral membrane proteins (im) or peripheral membrane proteins (pm). Non-membrane proteins were assigned to subcellular compartments: *c* cytosol; *cs* cytoskeleton; *er* endoplasmic reticular lumen; *mito* mitochondrial matrix; *mu* multiple localizations. Proteins that were also detected in the urea-soluble fraction are marked with U

them were found with both protocols: their gene names were DYN1, VDAC1, STX1C, and ATP6V1A1 (see Tables 3 and 4 for further information). The gene names of the 9 proteins which were uniquely found by applying the high-salt/high-pH extraction protocol were SYT1, SYT2, AP2MU2, CLIC4, VATB2, AT1A2, AT1A3, VPP1, and NSF. Finally, the gene names of the 3 proteins uniquely identified in the urea-soluble fraction were MYP0, RIMS2, and CLH.

Discussion

The aim of the present study was the enrichment of membrane proteins of the membrane and organelle (M/O) fraction obtained from brain tissue. The procedure by which such a fraction is generated was described in a previous paper (Guillemin et al., 2005). Here, we assessed the quality of two different enrichment techniques, namely sequential solubilization and high-salt/high-pH extraction. As a first criterion, we analyzed the protein amount in the various fractions with Bradford assays. The finding that the protein amount in the final fraction obtained through sequential solubilization was much lower than that obtained via high-salt/high-pH extraction (0.6% versus 44.5%; cf. Fig. 3) suggests a significant disadvantage of the former protocol. Quantifying the protein content in the thiourea-soluble fraction also revealed a disappointingly low value (2.1%). Indeed, in both fractions, the protein amount was too low to enable spot detection by colloidal Coomassie staining in 16-BAC-SDS-PAGE gels of standard size and the identification by mass spectrometry. This demonstrates that the sequential solubilization protocol provides in the most chaotropic fractions only protein amounts which are insufficient for analyzing membrane proteins. In the paper by Molloy et al. (1998), who introduced the sequential solubilization technique, a protein amount of 11% was reported in the thiourea-soluble fraction and the SDS-soluble fraction of *E. coli* cell lysates. Using the same technique, a value of 7% was obtained in a human lung carcinoma cell line (Lehner et al., 2003). Although these values are higher than our value of 3%, they are still too low to detect membrane proteins from small brain samples.

As the protein amount *in general* is not the only criterion by which the quality of the two protocols can be assessed, we also compared their performance concerning the enrichment of *membrane* proteins. Sequential solubilization did not increase the yield of integral membrane proteins (12%, as assessed in the urea-soluble fraction which yielded a sufficiently high protein amount of 25%). In contrast, high-salt/high-pH extraction led to an approximately 2-fold enrichment (from 14 to 27%). Peripheral membrane proteins were not enriched in the analyzed fractions by both techniques. These data provide additional evidence that the high-salt/high-pH extraction protocol is superior to the sequential solubilization protocol when aiming at the analysis of integral membrane proteins obtained from small amounts of brain tissue.

In the M/O-fraction obtained by differential centrifugation, we found 14% integral membrane proteins and 30% peripheral membrane proteins among the 50 identified proteins. These results represent a considerable increase compared to our previous report which had found 6% integral and 22% peripheral membrane proteins among 18 identified proteins (Guillemin et al., 2005). The difference can be explained by the fact that all protein spots were taken from a 16-BAC-SDS-PAGE gel in the present study, whereas half of the protein spots had been selected from conventional two-dimensional gels in our previous report. These conventional gels are not suited for separating hydrophobic, integral membrane proteins (Wu et al., 2003; Yu et al., 2004). Hence, the separation of membrane proteins should not be performed with a conventional two-dimensional gel sys-

tem, although this was done in several recent studies (Molloy et al., 1998; Lehner et al., 2003; Abdolzade-Bavil et al., 2004).

In contrast to the approximately 2-fold enrichment of *integral* membrane proteins, *peripheral* membrane proteins were not enriched by high-salt/high-pH extraction applied to the M/O-fraction (initially 30%, after extraction 28%). Harsh washing conditions in the high-salt and high-pH buffers are used to remove cytosolic, luminal, and non-covalently associated proteins from the fraction (Taylor et al., 2000; Zhao et al., 2004). Although these conditions do not appear to affect integral membrane proteins, they are likely to remove some peripheral membrane proteins.

The sequential solubilization resulted in insufficiently low protein amounts in both the thiourea-soluble fraction (2.1%) and the SDS-soluble fraction (0.6%), and no enrichment of membrane proteins was found in the urea-soluble fraction. Therefore, we wondered whether membrane proteins were lost at earlier stages of the procedure. To address this issue, we analyzed the Tris-soluble fraction and identified a surprisingly high percentage of membrane proteins. This can be attributed to an incomplete separation of hydrophilic and hydrophobic proteins due to low centrifugal forces. Centrifugation was performed at $12{,}000 \times g$ by Molloy et al. (1998), and although the centrifugal force was increased to $24{,}000 \times g$ in the present study, this is likely still insufficient to quantitatively sediment the membranous vesicles in the M/O-fraction. Furthermore, our data (12 membrane proteins out of 50 proteins in the Tris-soluble fraction) are in contrast to those reported in human carcinoma cells (0 out of 12; Lehner et al., 2003). We assume that the discrepancy is due to the fact that Lehner and coworkers did not perform a subcellular prefractionation of their material, thus feeding not only the M/O-fraction into the sequential solubilization procedure, but also the proteins from nuclei, debris and cytosol. The use of conventional two-dimensional gel electrophoresis is another argument that their results were biased towards an under-representation of hydrophobic proteins in the Tris-soluble fraction.

Concerning proteins that are relevant to neurotransmission, the higher ratio of such proteins identified by high-salt/high-pH extraction than seen in the urea-soluble fraction obtained by sequential solubilization (29% versus 21%) provides further evidence in support of the superiority of the former extraction protocol. In the following, functional aspects of eight of these proteins will be discussed; 5 were solely found after high-salt/high-pH extraction, one in the urea-soluble fraction, and two were common to both. Five proteins are directly involved in synaptic transmission. Synaptotagmin 1 and 2 (SYT1 and SYT2) are Ca^{2+}-binding proteins of the synaptic vesicles and as such involved in vesicle docking at the plasma membrane (Murthy and De Camilli, 2003; Sorensen, 2005). Syntaxin 1B (STX1C) is a major component of the SNARE complex at the plasma membrane which is essential for fusion (Sollner, 2003). The vesicular fusion protein NSF is an ATPase which disassembles SNARE complexes after exocytosis (Hanson et al., 1997). Finally, dynamin-1 (DYN1) is part of clathrin coats which are involved in the endocytosis of synaptic vesicles (Murthy and De Camilli, 2003). Two identified proteins participate in the maintenance of the resting membrane potential (subunits of the Na^+/K^+-ATPase; AT1A2 and AT1A3). AT1A2 is also important for functional inhibitory neural activity as evidenced by the findings that ATP1A2 knockout mice display a high intracellular Cl^- concentration and depolarizing actions of inhibitory neurotransmitters (Ikeda et al., 2004). Another identified protein was the Rab3-interacting protein 2 (RIMS2) which is involved in synaptic release (Wang et al., 2000; Graham et al., 2004; Sudhof, 2004).

Although the subcellular prefractionation and high-salt/high-pH extraction protocol has several strengths, as outlined above, we do not want to conceal that there is also a limit imma-

nent to this approach. This is the low value (21.3%) of plasma membrane marker activity present in the M/O-fraction obtained with our initial differential centrifugation protocol (Guillemin et al., 2005). Even if this value is increased to about 32% by the introduction of a re-extraction step (higher yield of 49.9%), it still indicates that about 2/3 of the protein amount of the plasma membrane is lost and ends up in the sediment containing nuclei and debris. Ways to improve the yield of plasma membranes in the M/O-fraction can be modifications in the centrifugation and re-extraction conditions. Improvements in this direction are particularly desirable if one considers that approximately 20–30% of the total protein content is formed by membrane proteins (Lehnert et al., 2004; Yu et al., 2004) and that plasma membrane proteins comprise only about 2–5% of all proteins (Olsen et al., 2004), thus putting them into the category of low-abundant proteins.

In summary, our data demonstrate that the combination of subcellular prefractionation by differential centrifugation with high-salt/high-pH extraction provides a valuable and efficient enrichment protocol for integral membrane proteins from small brain areas.

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