Djuro Josic[1]
Mari Kino Brown[1]
Feilei Huang[1]
Helen Callanan[2]
Marijana Ručević[2]
Alison Nicoletti[1]
James Clifton[1]
Douglas C. Hixson[1, 2]

[1]Proteomics Core,
COBRE Center for Cancer
Research Development,
Rhode Island Hospital,
The CORO Center,
Providence, RI, USA
[2]Division of Medical Oncology,
Department of Medicine,
Rhode Island Hospital,
Providence, RI, USA

Use of selective extraction and fast chromatographic separation combined with electrophoretic methods for mapping of membrane proteins

A model system for selective solubilization and fast separation of proteins from the rat liver membrane fraction and purified rat liver plasma membranes for their further proteomic analysis is presented. For selective solubilization, high-pH solutions and a concentrated urea solution, combined with different detergents, are used. After extraction, proteins are separated by anion-exchange chromatography or a combination of anion- and cation-exchange chromatography with convective interaction monolithic supports. This separation method enables fast and effective prefractionation of membrane proteins based on their hydrophobicity and charge prior to one-dimensional (1-D) and 2-D electrophoresis and mass spectrometry. By use of this sample preparation method, the less-abundant proteins can be detected and identified.

Keywords: Convective interaction media monoliths / Plasma membranes / Protein mapping / Selective extraction
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1 Introduction

The first strategies for proteomic analyses of cells or tissues and organs started with protein extraction out of crude homogenates or body fluids, such as plasma or serum. The next step was usually tryptic digestion, followed by chromatographic separation and mass spectrometric analysis of digested peptides [1–4]. Alternatively, a protein mixture was separated by use of 1- or 2-DE, followed by excision of polypeptide bands or spots, tryptic digestion, and again by MS analysis [4–8]. However, without fractionation and enrichment of biological samples, the highly abundant proteins often masked less-abundant ones, and made their detection difficult or even impossible [9–11].

In recent years, the tendency has been to focus on subcellular proteomes, either organelles or macromolecular structures of the cell [8, 10, 12–15]. Separation of cell homogenates into organelles or other multiprotein fractions substantially increases the probability of detecting low abundance proteins. In this context, terms such as "subcellular proteomics" [15] or "organelle proteomics" [12, 13] have been established. The purity of organelles and other protein complexes is crucial to subcellular proteome research [13–17]. For the isolation of subcellular structures and organelles several methods, such as differential- and density-gradient centrifugation [18–20], immunoisolation [8], affinity purification, [21], and free-flow electrophoresis [22], have been applied. It has been shown that such fractionations improved identification of proteins from targeted subcellular structures [8, 10, 12–15]. However, the copurification and identification of possible contaminants from other structures and compartments was still one of the major unresolved questions. It was often difficult, and sometimes impossible, to conclude whether proteins, typically localized in other organelles but now identified in a targeted sample, represent true endogenous partners or were products of artificial association induced by cell disruption or incomplete purification [8, 17].

2-DE combined with mass spectrometric methods is one of the most widely used methods to perform protein expression mapping and to identify and characterize proteins of different cells and tissues in proteomic-based research. After initial enthusiasm [1–3], it has become more and more obvious that MS/MS analysis of peptides after tryptic digestion of whole cell/tissue lysates has its limitations [10]. There are thousands of peptides from hundreds of proteins to identify, even after chromatographic fractionation of the lysate. Additionally, the probability of identifying peptides from less-abundant proteins

Correspondence: Prof. Djuro Josic, Proteomics Core, COBRE Center for Cancer Research Development, Rhode Island Hospital, The CORO Center, Suite 4.208, One Hoppin Street, Providence, RI 02903, USA
E-mail: Djuro_Josic@brown.edu
Fax: +401-793-8908

Abbreviations: CAX, cation-exchange; **CEACAM**, carcinoembrionic antigen cell adhesion molecule; **CIM**, convective interaction media; **DEAE**, diethyl aminoethyl; **SELDI**, surface-enhanced laser desorption/ionization

is very low [9, 10, 23]. After initial criticism [10], fractionation of proteins before their tryptic digestion is gaining importance in laboratory protocols [4]. The group of Lubman [23, 24] developed a method for the fractionation of complex protein samples by a combination of IEF or chromatofocusing and RP-LC. Other groups have used alternative methods for prefractionation, such as CE, affinity chromatography, and ion-exchange chromatography [4, 21, 22].

More and more researchers in the field of proteomics have started using methods for subcellular fractionation before proteomic analysis of the sample [12–15]. Using methods, such as differential centrifugation, electrophoresis, or affinity fractionation, cell or tissue lysates are preseparated into fractions containing particular organelles [8, 12, 14]. After this prefractionation, the "organelle proteome" can be analyzed by use of established proteomics techniques, such as 1- and 2-DE and chromatographic methods followed by MS [8, 12–15, 25–28].

After isolation of organelles or subcellular structures, and before electrophoretic separation and final identification by MS, fractionation by selective extraction with different agents and chromatographic separation with different resins can further facilitate proteome analysis [4, 12, 23–30].

Membrane proteins, especially plasma membrane proteins, have a special position in proteome research due to their physiological roles, diversity, and behavior during the purification process [30–34]. For the analysis of membrane proteins several methods, including chromatography and different electrophoretical methods, have been used [12, 29, 33–36]. In the early eighties, methods for selective solubilization of membrane proteins by using salts, chaotropic reagents, and different detergents were developed. In the first step, membrane-associated, and peripheral proteins were removed, mainly by use of several different salt solutions or high-pH (pH 11) reagents, such as sodium carbonate or dilute NaOH solutions [29, 34, 35]. In the next step, more hydrophobic, integral membrane proteins were solubilized by use of different detergents [10, 29, 34, 36]. The remaining pellet, containing detergent insoluble proteins, was extracted by use of chaotropic reagents or "strong" detergents, such as SDS. Some detergent insoluble proteins were extracted by simple calcium chelation with EDTA or EGTA in the presence of "milder" detergents, such as octyl glucose or CHAPS [29, 36]. Bordier [37] developed a method for membrane protein solubilization with the nonionic detergent Triton X-114. After extraction at 4°C, the protein solution is warmed to induce phase partitioning. Less hydrophobic, mostly membrane-associated proteins are separated into the water-rich phase, away from more hydrophobic, integral membrane proteins in the Triton X-114 phase. Recently, this method was successfully used for prefractionation of proteins from Golgi membranes and the enrichment of less-abundant proteins from this organelle [27, 28]. Methods for selective capture of plasma membrane proteins from the cell surface following biotinylation of whole cells have also been developed. Proteins from the cell surface are isolated from detergent extracts by affinity chromatography with immobilized avidin and identified with MS [21]. Bledi *et al*. [38] have applied proteases to intact cells. The resulting peptide fragments were further analyzed by LC followed by MS/MS. However, neither of these methods is applicable to the fractionation of membrane proteins from whole tissues.

In the present paper, selective solubilization and fast chromatographic fractionation of liver plasma membranes with so-called convective interaction media (CIM) monolithic supports were used for sample preparation for 1- and 2-DE, followed by identification of separated proteins by MS. Protein fractionation based on their solubility and behavior during separation by anion- and cation-exchange chromatography enables further simplification of patterns in 1- and 2-DE and identification of less-abundant proteins by mass spectrometry.

2 Materials and methods

2.1 Liver plasma membrane preparation

Adult Fisher rat livers (three livers, wet weight 18–20 g each) were perfused with ice-cold PBS, excised, minced, and dissociated using a Dounce homogenizer, in a buffer of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.4 (lysis buffer), and protease inhibitor cocktail (CalBiochem, San Diego, CA, USA). Membranes were isolated using a two-phase polymer system, as previously described [39]. Using this method, about 33–40 mg membrane protein/rat liver can be isolated. For isolation of the so-called crude membrane fraction, the homogenate was spun at 8500 rpm (7000 $\times$ *g*, Beckman Centrifuge J2–21, Beckman Instruments, Palo Alto, CA, USA) for 30 min. The supernatant was discarded, the pellet containing the crude membrane fraction was resuspended in lysis buffer and pelleted again at 9500 $\times$ *g* (10 000 rpm).

2.2 Solubilization of plasma membranes

Rat liver plasma membranes were solubilized either according to Lin and Fain [35], using two different detergents: sodium cholate (Sigma, St. Louis, MO, USA) and monoethylene glycol monododecyl ether (Polydocanol, Sigma), or by the method of Josic and Zeilinger [29], which utilizes different solubilization agents combined

with the detergent Triton X-100 (Sigma). After solubilization with detergents, the membrane pellet was extracted with 25 mM EGTA, pH 7.4, containing 1% w/v octyl-glucopyranoside (Sigma). After centrifugation, the lipids were removed by stepwise extraction with ethanol/acetone. After lipid removal, the pellet containing detergent-resistant proteins was solubilized with 8 M urea containing 4% w/v CHAPS (Sigma).

2.3 Chromatographic separation

Solubilized plasma membrane proteins were separated on a system containing one anion diethyl aminoethyl (DEAE) and one cation (SO_3) exchange monolithic CIM disk or tube-shaped column (BIA Separations d.o.o., Ljubljana, Slovenia). The column volumes were either 50 or about 400 μL for the disks and 8 mL for the monolithic tube. Flow rates were 0.5, 4, and 8 mL/min for disks and tubes, depending on column size. For elution, the tandem columns were disconnected and for each column a step gradient with different concentrated buffered salt solutions was used. Chromatographic separation on DEAE CIM disk was compared to HiTrap Q FF 1 mL columns (Amersham Biosciences, Uppsala, Sweden). Buffer A was 5 mM Tris-HCl, pH 6.8. For elution, different amounts of NaCl were added to buffer A. The flow rate during separation with these columns was 1 mL/min. All separations were performed at 4°C. Proteins were detected optically at 280 nm. For separation, a BioLogic Duo Flow chromatographic system (BioRad, Hercules, CA, USA) was used.

2.4 SDS-PAGE

Protein samples were solubilized in NuPAGE LDS sample buffer and heated at 100°C for 5 min. SDS-PAGE was performed with precast NuPAGE 4–12% Bis-Tris gels in an XCell Sure Lock Mini-Cell (Invitrogen, Carlsbad, CA, USA), according to the manufacturer. The gels were stained with GelCode Blue (Pierce, Rockford, IL, USA) or SYPRO Ruby and visualized by a VersaDoc Imaging System (BioRad) before excising the bands of interest for in-gel digestion.

2.5 2-DE

Two different IPGs, either 3–10 or 7–10, were used. The dry IPG strips (BioRad) were passively rehydrated with proteins dissolved in IPG rehydration/sample buffer (8 M urea, 2 mM tributylphosphine (TBP), 4% w/v CHAPS, 0.2% w/v carrier ampholytes, and 0.0002% w/w bromophenol blue) overnight. The rehydrated strips were focused on the Protean IEF Cell (BioRad) at about 30 000 Vh. After focusing, the strips were equilibrated in equilibration buffers I and II for 20 min each. Both buffers contain 6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8, and 20% v/v glycerol. Buffer I contains 2 mM TBP as reducing agent; Buffer II has 2.5% iodoacetamide as alkylating reagent instead. In the second dimension, proteins were separated by size on 4–12% gradient Bis-Tris gels using the Criterion System (BioRad). The gels were then stained with SYPRO Ruby (BioRad) or SilverQuest silver staining kit (Invitrogen). Gels stained with SYPRO Ruby were visualized by the VersaDoc Imaging System and silver stained prior to excision of the spots of interest for in-gel digestion and subsequent analysis with MS.

2.6 In-gel digestion

The bands or spots of interest and a blank area of gel were excised by extracting six to ten gel plugs with clean glass Pasteur pipettes. The gel plugs were washed two times with 1:1 v/v of 0.1 M NH_4HCO_3/ACN (Sigma) for 15 min with agitation. The wash solution was then removed and enough ACN was added to cover the gel pieces. After few moments the gel pieces shrank and stuck together. ACN was removed and the gel pieces were rehydrated in 0.1 M NH_4HCO_3 for 5 min. An equal volume of ACN was then added to create a 1:1 v/v of 0.1 M NH_4HCO_3/ACN and the gel pieces were rehydrated for an additional 10 min. This procedure was performed at room temperature. After removal of all liquid, the gel pieces were completely dried in an Eppendorf Vacufuge (Eppendorf, Hamburg, Germany). After drying, the gel plugs were swelled with a solution of 10 mM DTT/0.1 M NH_4HCO_3 (Sigma) at 56°C for 45 min to reduce the protein. The tubes were then cooled to room temperature and the reducing solution removed. An alkylating solution of 55 mM iodoacetamide in 0.1 M NH_4HCO_3 was added and the gel plugs were incubated for 30 min at room temperature in the dark. After alkylation, the solution was removed and the gel pieces washed and dried. Gel pieces were rehydrated for 15 min at 4°C in a digestion buffer consisting of 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 μg/mL trypsin (porcine, sequence grade, Promega, Madison, WI, USA), and the protein was digested. After allowing the gel plugs to swell for 15 min, an additional volume of digestion buffer was added to cover the gel plugs that had completely absorbed all initially added buffer. The gel plugs were then placed in an incubator set at 37°C for 16 h. Subsequently, the peptides were recovered from the mixture by centrifugation. Peptides remaining in the gel were extracted with a solution of 50% v/v ACN containing 1% v/v TFA (Pierce) in 25 mM NH_4HCO_3 for 10 min with shaking, and subsequently pooled with the first fraction. The tryptic digest was held on ice until ready to spot on the surface enhanced laser desorption/ionization (SELDI)-MS chip (Ciphergen Biosystems, Fremont, CA, USA).

2.7 Protein chip array preparation and analysis

Twenty microliters of the tryptic digest was analyzed on a non-preactivated hydrophobic chip array (H4; Ciphergen Biosystems) and SEND-ID chip array (Ciphergen Biosystems). The array was spotted with repeated applications of 2 μL tryptic digest and allowed to dry between applications onto the H4 and SEND-ID chip arrays. H4 chip also received two applications of 0.5 μL of 20% saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Ciphergen Biosystems) dissolved in 10 mM ammonium acetate, 25% v/v ACN, and 1.25% v/v TFA. When the matrix was applied, the dispensed volume was pipetted up and down three times to mix the matrix with the spotted sample to allow even distribution of the peptides and matrix. The array was allowed to dry between applications. Mass analysis was performed in a ProteinChip Reader (PBS-II; Ciphergen Biosystems). The data were collected by hand to ensure capture of the low-mass peptide peaks (between 500 and 5000 Da) and to reduce background noise. An average of 200 laser shots to each spot, with a laser intensity of 180 for the H4 chip array and 175 for the SEND-ID chip array, were fired and the detector was run at a sensitivity of 9 and 7, respectively. Ciphergen's ProteinChip Software auto-identified peaks according to the following parameters: minimum valley depth of four times the noise, peak height at four times the noise, and real centroiding using the top 10% of peak-broadening of 3. Trypsin autolysis peaks were used for internal calibration, and were removed from the masses used to identify the proteins. MS-Fit (University of California San Francisco Mass Spec Facility) and ProFound (Rockefeller University, New York, NY) were used for protein identification using the databases SWISSProt.10.30.2003 and CNBInr.10.21.2003.

2.8 Immunochemical methods

Western blots were performed using the mAb 9.2. against the integral plasma membrane protein carcinoembrionic antigen cell adhesion molecule (CEACAM 1) [40, 41].

3 Results

3.1 Separation of the crude membrane fraction

Out of one rat liver (about 18–20 g wet weight), 150–200 mg protein in the so-called crude membrane fraction (*cf*. Section 2) can be isolated. The crude membranes from rat liver were extracted by use of two different solubilization schemes, which are shown in Fig. 1.

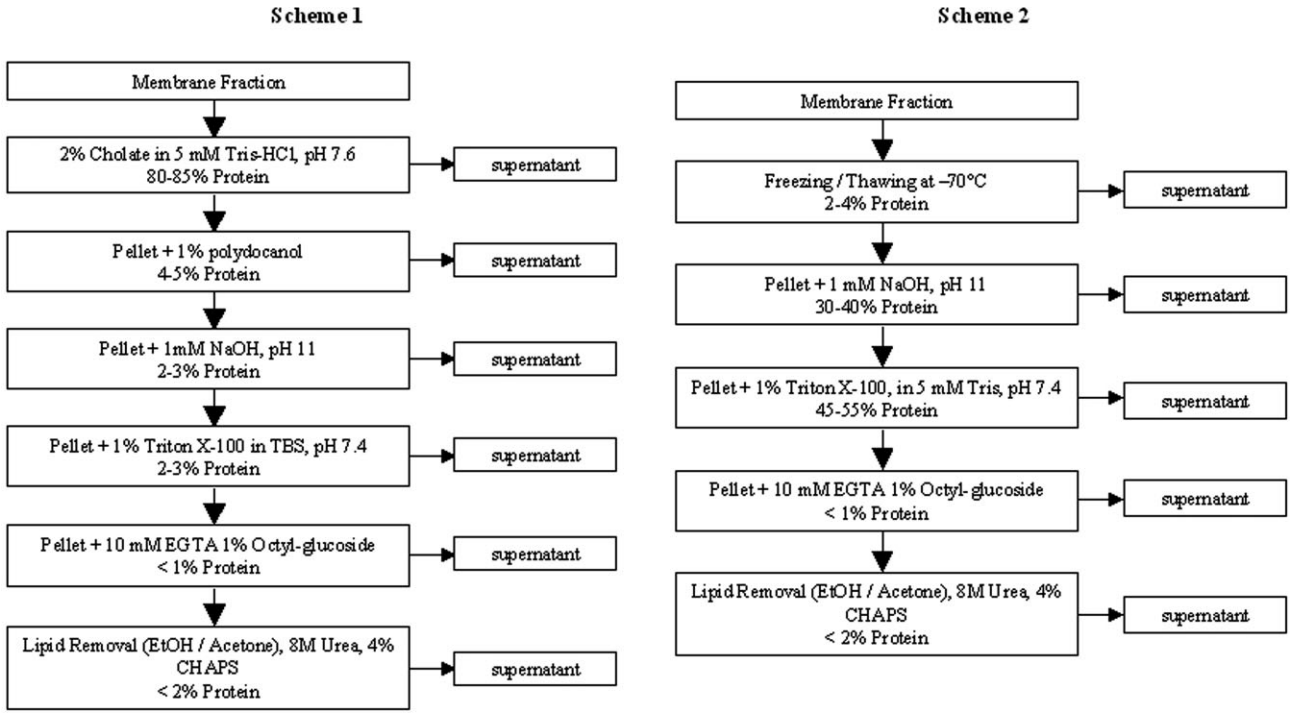

Figure 1. Schemes for extraction of membrane proteins according to their solubility. Left, by use of different detergents, according to Lin and Fain [35], Scheme 1; right, by use of different solubilization agents, according to Josic and Zeilinger [29], Scheme 2.

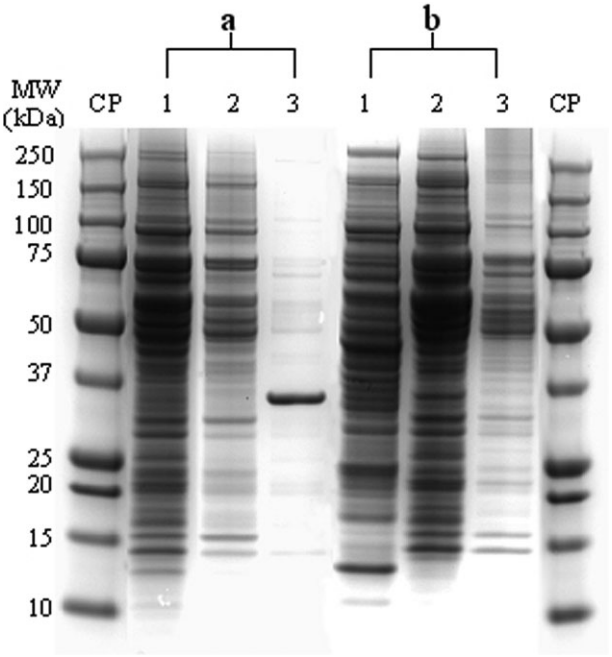

Figure 2. SDS-PAGE of solubilized proteins from the crude membrane fraction. (a) According to Scheme 1 (*cf.* left part of Fig. 1). Lane 1, proteins solubilized with sodium cholate; lane 2, proteins solubilized with polydocanol; lane 3, proteins solubilized with dilute NaOH, pH 11 after two detergents. (b) According to Scheme 2 (*cf.* right part of Fig. 2). Lane 1, proteins solubilized after freeze/thaw; lane 2, proteins solubilized after treatment with dilute NaOH solution, pH 11; lane 3, proteins solubilized with Triton X-100; CP, calibration proteins.

In the first scheme (*cf.* left side of Fig. 1), two different detergents were used sequentially. An SDS-PAGE of proteins extracted following this scheme is shown in Fig. 2. This scheme was developed by Lin and Fain [35] for selective solubilization of the integral membrane protein CEACAM 1. By use of the first detergent, sodium cholate, most contaminating cytosolic, membrane-associated, and peripheral membrane proteins are extracted [35]. In the next step, extraction with 1% polydocanol, more hydrophobic, integral membrane proteins are solubilized (lane 2 in the left part of Fig. 2). However, in our experiments, when crude membrane fractions were used, the majority of the cell adhesion molecule CEACAM 1 was already extracted by sodium cholate (data not shown). If the pellet remaining after extraction with both detergents is subsequently treated with dilute sodium hydroxide at pH 11 only one main polypeptide, with apparent molecular weight in SDS-PAGE between 30 and 40 kDa, was extracted (*cf.* left part of Fig. 2, lane 3). After tryptic digestion and peptide mapping on a SELDI-TOF mass spectrometer, this protein was identified as uricase, a protein found in liver peroxisomes [8, 52].

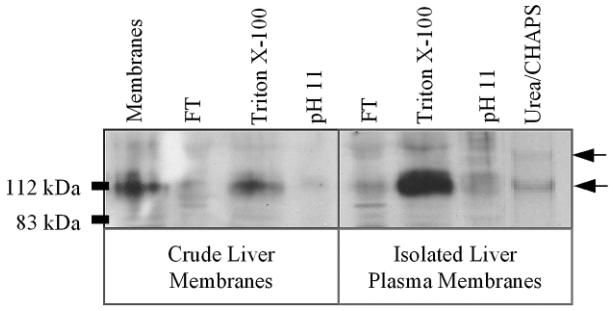

Figure 3. Immunoblot with monoclonal anti-CEACAM 1 antibodies. Left, crude membrane fraction; right, purified plasma membranes. FT, fraction solubilized after freeze/thaw.

Using Scheme 2 (*cf.* right side of Fig. 1), contaminating cytosolic proteins together with some membrane-associated proteins were removed by centrifugation after freezing twice at −80°C and thawing the membrane suspension. Residual membrane-associated and contaminating cytoskeletal proteins were extracted at high pH (pH 11) with dilute sodium hydroxide (see SDS-PAGE in Fig. 2, lane 2). In the next step, extraction with a 1% solution of the nonionic detergent Triton X-100, hydrophobic integral membrane proteins were solubilized. By use of this solubilization scheme, the cell adhesion molecule CEACAM 1 could be highly enriched in the Triton X-100 fraction (*cf.* right side of Fig. 3). The concentration of this protein is about 0.4 mg/rat liver (18–20 g wet weight, *cf.* in [40]). As shown in Fig. 3, the majority of this protein can be extracted with this non-ionic detergent. The residual pellet, containing detergent-insoluble proteins, was used for further solubilization with 8 M urea solution containing 4% zwitterionic detergent CHAPS. As shown in Fig. 3, traces of CEACAM 1, two bands with apparent molecular masses in SDS-PAGE of about 100 and 150 kDa, could still be detected in this fraction.

As shown in Fig. 2, the polypeptide patterns from membranes solubilized either by use of Scheme 1 or according to Scheme 2 were still very complex. To reduce the complexity before 2-DE, chromatographic separation of the extracts with anion-exchange monolithic disks was performed. Depending on the amount of the applied material, 50 or 400 μL monolithic CIM disks or 8 mL monolithic CIM tubes were used, having capacities for hydrophobic membrane proteins of about 500 μg, 5 mg, and 100 mg, respectively. The concentration of the protein solution applied was approximately 1 mg/mL. For the separation shown in Figs. 4a-c, a 400 μL DEAE monolithic disk was loaded with about 4 mg of sodium cholate extracted protein. As shown in Fig. 4a, further fractionation yielded a much less-complex protein pattern, at least in SDS-

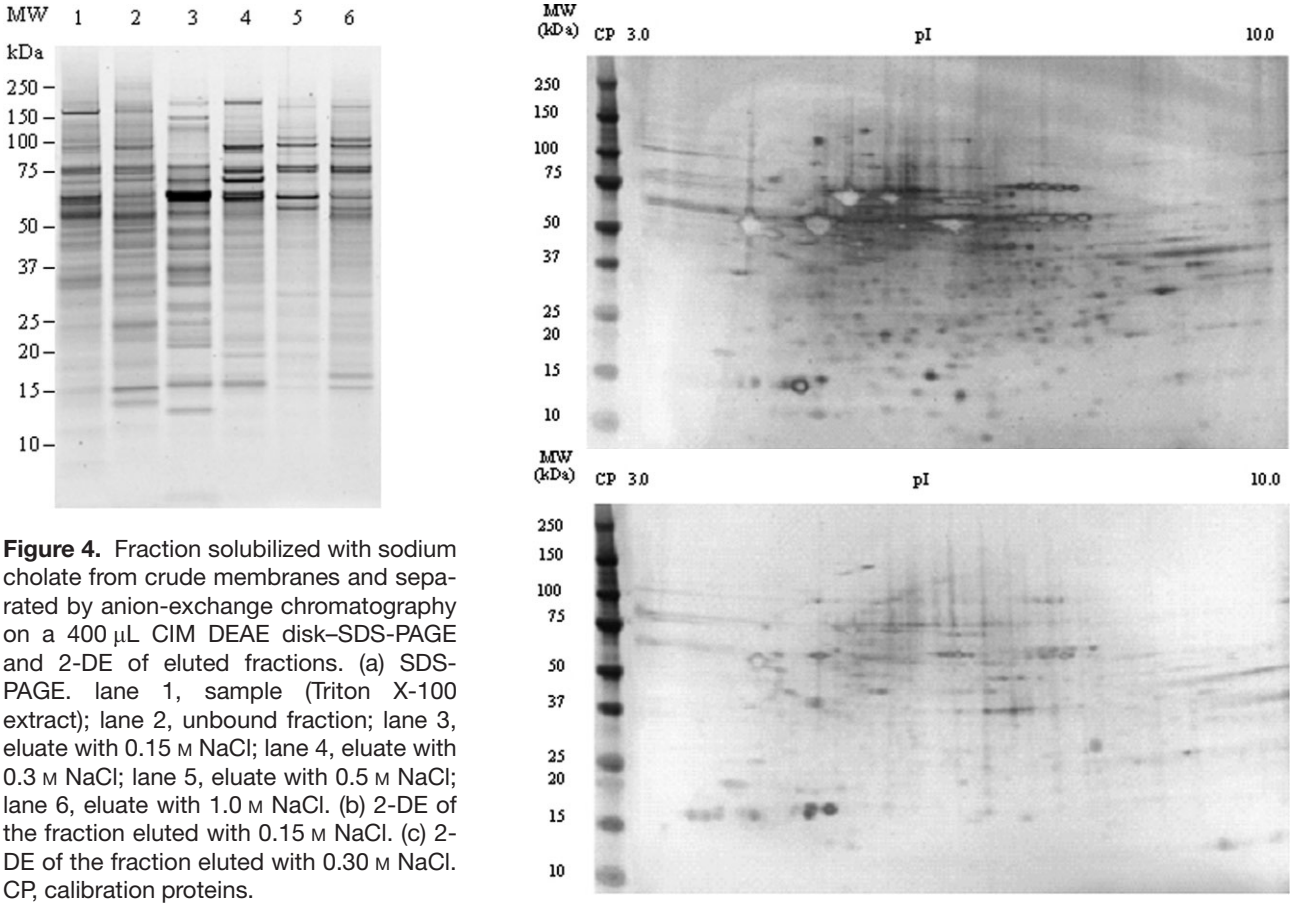

Figure 4. Fraction solubilized with sodium cholate from crude membranes and separated by anion-exchange chromatography on a 400 μL CIM DEAE disk–SDS-PAGE and 2-DE of eluted fractions. (a) SDS-PAGE. lane 1, sample (Triton X-100 extract); lane 2, unbound fraction; lane 3, eluate with 0.15 M NaCl; lane 4, eluate with 0.3 M NaCl; lane 5, eluate with 0.5 M NaCl; lane 6, eluate with 1.0 M NaCl. (b) 2-DE of the fraction eluted with 0.15 M NaCl. (c) 2-DE of the fraction eluted with 0.30 M NaCl. CP, calibration proteins.

PAGE. Further separations of two fractions from anion-exchange chromatography by 2-DE are shown in Figs. 4b and c.

3.2 Fractionation of purified liver plasma membranes

Further experiments were performed using plasma membranes purified with a two-phase polymer system [39]. Using this method, about 100–120 mg protein in highly purified plasma membranes, out of three rat livers (50–60 g wet weight) could be isolated. Figure 5 shows the SDS-PAGE analysis of fractions obtained by use of solubilization (Scheme 2). Again, after removal of cytoskeletal and peripheral membrane proteins at pH 11, integral membrane proteins were solubilized with Triton X-100 (*cf.* lane 3 in Fig. 5). This is demonstrated by an immunoblot analysis showing the presence of the membrane protein CEACAM 1 in the Triton X-100 extract. Figure 3 also shows a comparison between the crude membrane fraction and highly purified liver plasma membranes with regard to the content of CEACAM 1. If the Triton X-100 insoluble pellet is further treated with suitable reagents such as EGTA, urea/4% CHAPS mixture, or SDS, so-called detergent-insoluble proteins can be extracted [44, 45]. In previous experiments, we showed that membrane-associated annexin A6 can be solubilized by simple calcium-complexing with EDTA or EGTA (not shown here, *cf.* [29, 36, 45]). After this extraction step, residual proteins are solubilized with 8 M urea, containing 4% zwitterionic detergent CHAPS. This reagent is usually used for protein solubilization before IEF, the first dimension in 2-DE (*cf.* Section 2). To get good resolution in SDS-PAGE (*cf.* Fig. 5) and 2-DE (Fig. 6), a thorough delipidation of the sample by stepwise extraction with ethanol and acetone is necessary. Remaining lipids in the membrane pellet impair the solubilization in sample buffer and without their removal no proper separation, neither in SDS-PAGE nor in IEF can be achieved. The 2-DE pattern of proteins, extracted with 8 M urea/4% CHAPS after removal of detergent-soluble and membrane-associated proteins, is shown in Fig. 6. The proteins identified by SELDI-TOF-MS are listed in Table 1.

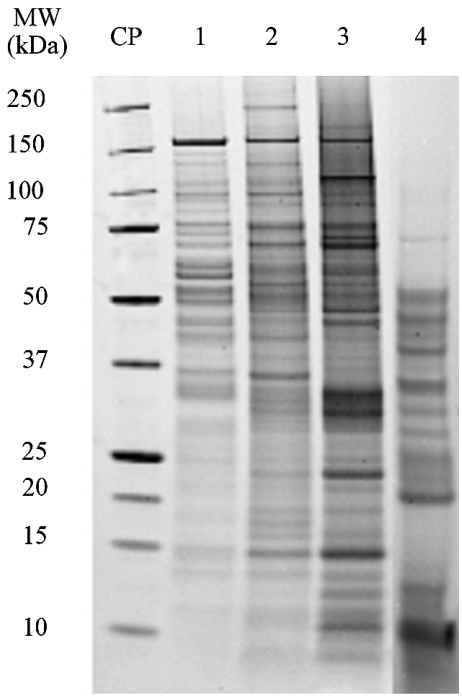

Figure 5. SDS-PAGE of solubilized proteins from the purified plasma membrane fraction. CP, calibration proteins; lane 1, proteins solubilized after freezing and thawing; lane 2, proteins solubilized after treatment at pH 11; lane 3, proteins solubilized with Triton X-100; lane 4, proteins solubilized with 8 M urea/4% CHAPS after lipid extraction.

As above, for further fractionation, fast ion-exchange chromatography with monolithic supports was used. In this case, a tandem containing both anion (DEAE) and cation (SO_3) CIM monolithic disks, so-called "conjoint LC" [46], was used. For separation, different extracts from purified liver plasma membranes were applied to this column tandem. In Fig. 7, separation of a Triton X-100 extract, containing integral membrane proteins, is shown. Using this fractionation method, further separation of these hydrophobic proteins was achieved (see SDS-PAGE in Fig. 7). As shown in Fig. 8, CEACAM 1, a model integral membrane protein from the rat liver, could be enriched in fraction 4, eluted with 0.3 M sodium chloride. The main form of this protein in rat liver, a band with apparent molecular mass in SDS-PAGE of about 100 kDa, could be separated from another band with lower molecular mass, which also reacts with the anti-CEACAM 1 mAb (*cf.* fraction 3 in Fig. 8a). If DEAE-Sepharose Fast Flow was used as the anion exchanger, the separation between the two forms of this protein could not be achieved (*cf.* Fig. 8b). By use of a "conjoint system", containing a monolithic DEAE CIM disk as the first and an SO_3 CIM disk as the second column, only three main bands, with apparent molecular mass in SDS-PAGE of 55, 53, and about 14 kDa, could be eluted with 1 M NaCl from the cation-exchange column (*cf.* Fig. 7). After 1- and 2-DE (*cf.* Figs. 7, 9), tryptic digestion of excised protein bands or spots, and peptide mapping with SELDI-TOF-MS, the upper two bands could be identified as two forms of cytochrome p450 (*cf.* Table 2 and Fig. 10). In 2-DE, the low-molecular-weight band could be separated into at least three spots with p*I* higher than 9 (*cf.* Fig. 9). Low-molecular-weight proteins identified in this subfraction are listed in Table 2. Hydrophobic proteins eluted from the anion-exchange column were also further fractionated by 2-DE. In Fig. 11, the 2-D pattern of the fraction eluted with 0.15 M NaCl is shown. Compared to the whole Triton extract, this pattern was simplified, and further analysis of

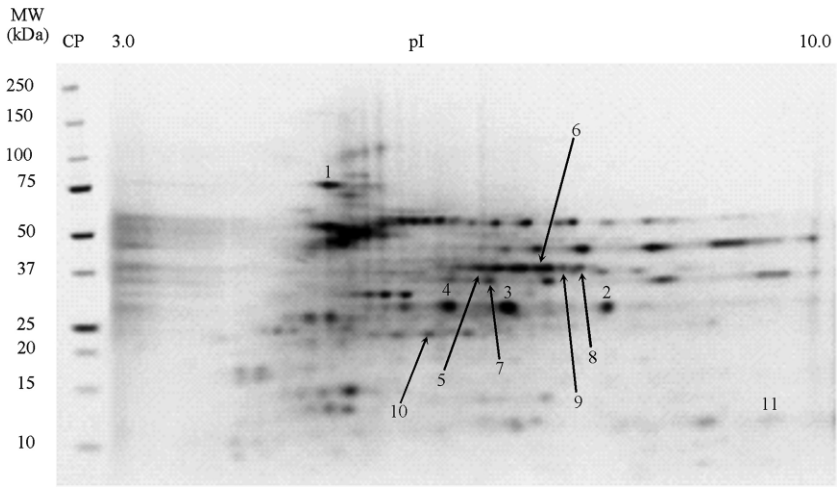

Figure 6. 2-DE of liver plasma membrane proteins solubilized with 8 m urea/4% CHAPS after lipid extraction. Identified proteins (*cf.* spot nos.) are listed in Table 1.

Table 1. Proteins identified in the fraction solubilized with 8 M urea/4% CHAPS after pH 11 treatment and Triton X-100 solubilization (see Figs. 5, 6)

Protein	M_w (kDa)	p*I*	Spot no.	Reference
NADPH-cytochrome P450 reductase (CPR) (P450R)	75	5.1	1	[59]
Uricase (urate oxidase)	28	7.7	2	[8]
Proteasome subunit beta type 1 (proteasome component C5) (macropain subunit C5) (multicatalytic endopeptidase complex subunit C5) (proteasome gamma chain)	28	6.7	3	[60]
Proteasome subunit beta type 8 precursor (proteasome component C13) (macropain subunit C13) (multicatalytic endopeptidase complex subunit C13)	29	6.3	4	[60]
5'-AMP-activated protein kinase, gamma-1 subunit (AMPK gamma-1 chain) (AMPKg)	38	6.5	5	[55]
Sulfotransferase K2 (rSULT1C2A)	38	7.3	6	[61]
Annexin A1 (annexin I) (lipocortin I) (calpactin II) (chromobindin 9) (P35) (phospholipase A2 inhibitory protein)	35	6.6	11	[62, 63]
Annexin A2 (annexin II) (lipocortin II) (calpactin I heavy chain) (chromobindin 8) (P36) (protein I) (placental anticoagulant protein IV) (PAP-IV)	35	6.6	8	[53, 62, 63]
Tumor necrosis factor receptor superfamily member 6 precursor (FASL receptor) (apoptosis-mediating surface antigen FAS) (Apo-1 antigen) (CD95)	38	7.3	7	[54]
27 kDa Golgi SNARE protein (Golgi SNAP receptor complex member 2) (Membrin)	24	6.0	10	[64]
Phospholipase A2, membrane-associated precursor (phosphatidylcholine 2-acylhydrolase) (group IIA phospholipase A2) (GIIC sPLA2)	13	9.6	9	[55]

Table 2. Proteins identified in cation-exchange (CAX)-fraction after Triton X-100 extraction and conjoint LC (see Figs. 7b and 9)

Protein	M_w (kDa)	p*I*	Spot no.	Reference
gi|9506529|ref|NP_062057.1| cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase); cytochrome P450, 2c29 [*Rattus norvegicus*]	58.5	a)	a)	[56, 57]
gi|6978743|ref|NP_036674.1| cytochrome P450, subfamily IIA (phenobarbital-inducble)/ (cytochrome P450 IIA3) [*Rattus norvegicus*]	54.9	a)	a)	[56, 57]
Microsomal GST 1 (microsomal GST-1) (microsomal GST-I)	17	9.7	1	[65]
ssDNA-binding protein, mitochondrial precursor (MT-SSB) (MTSSB) (P16)	17	9.8	2	[66]
ssDNA-binding protein, mitochondrial precursor (MT-SSB) (MTSSB) (P16)	17	9.8	3	[66]
ssDNA-binding protein, mitochondrial precursor (MT-SSB) (MTSSB) (P16)	17	9.9	4	[66]
NADH-ubiquinone oxidoreductase 13 kDa-A subunit, mitochondrial precursor (complex I-13KD-A) (CI-13KD-A)	13	9.5	5	[67]

a) Separated by SDS-PAGE only

excised proteins by MS can be performed much easier. By use of such prefractionation methods, the enrichment of less-abundant membrane proteins can also be achieved, as shown for the alternative forms of CEACAM (*cf.* Fig. 8). After such enrichment, these proteins were accessible to identification and further characterization.

4 Discussion

Membrane proteins have structural characteristics that make their purification and analysis by 2-DE and chromatography more challenging than soluble proteins [10, 29, 32]. Integral membrane proteins have at least one membrane spanning, hydrophobic sequence, and can be

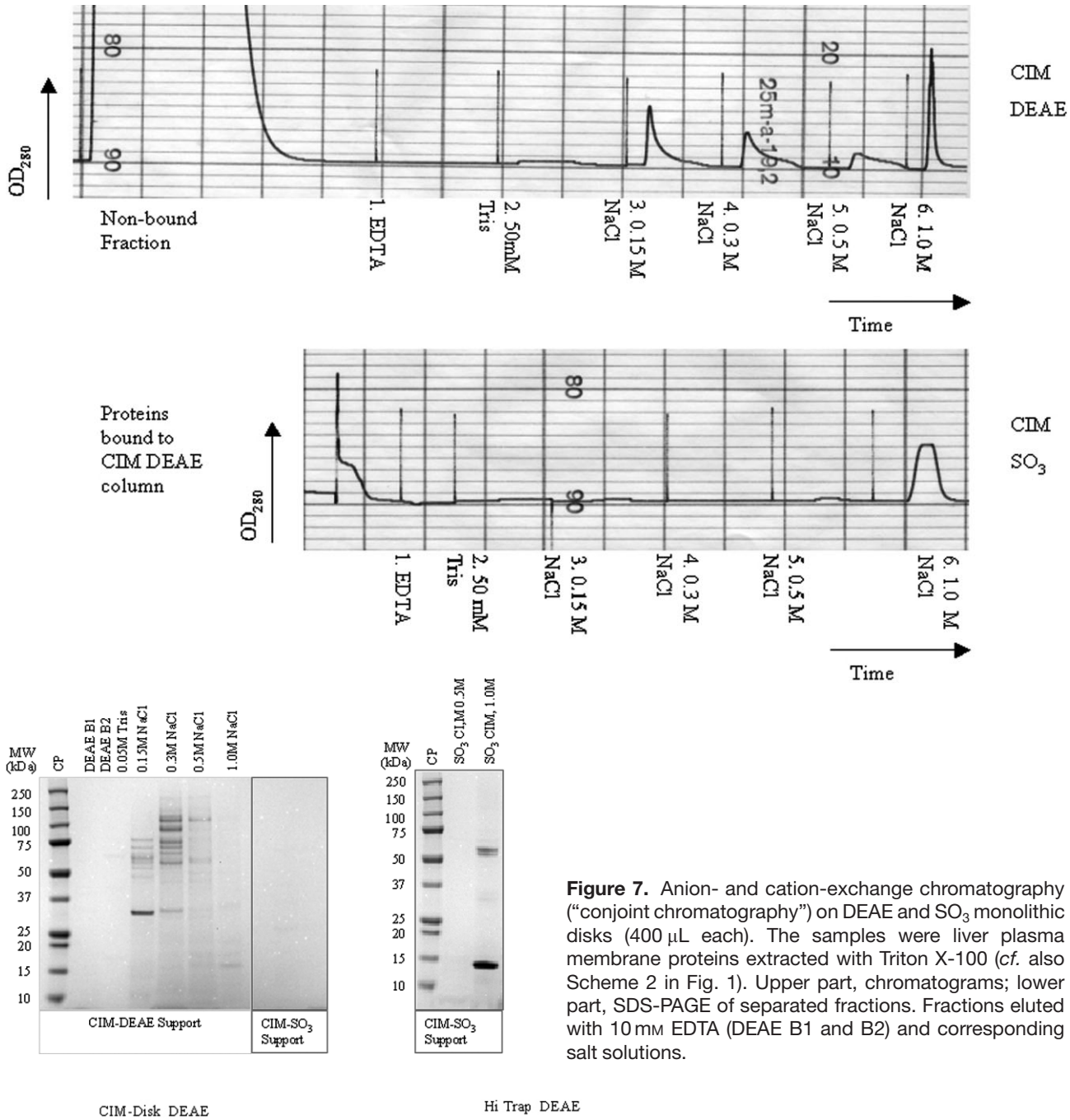

Figure 7. Anion- and cation-exchange chromatography ("conjoint chromatography") on DEAE and SO_3 monolithic disks (400 μL each). The samples were liver plasma membrane proteins extracted with Triton X-100 (*cf.* also Scheme 2 in Fig. 1). Upper part, chromatograms; lower part, SDS-PAGE of separated fractions. Fractions eluted with 10 mM EDTA (DEAE B1 and B2) and corresponding salt solutions.

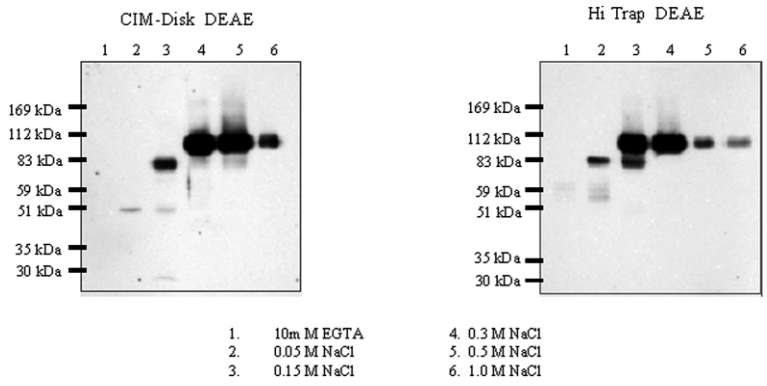

Figure 8. Immunoblots of fractions from anion-exchange chromatography (*cf.* Fig. 7). Isolated fractions were blotted with mAb against CEACAM. Left–fractions separated on CIM DEAE monolithic disk (400 μL). The main part of CEACAM was eluted with 0.5 M NaCl (lane 5). To avoid overloading, only one-fifth of protein amount was loaded in this track. Right–fractions separated on HiTrap QFF anion-exchange column.

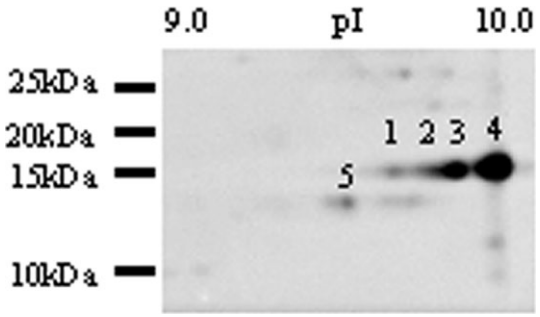

Figure 9. 2-DE of low-molecular, basic proteins from the Triton X-100 liver plasma membrane extract, eluted with 1.0 M NaCl from CIM SO_3 monolithic column. Identified proteins (*cf.* spot nos.) are listed in Table 2.

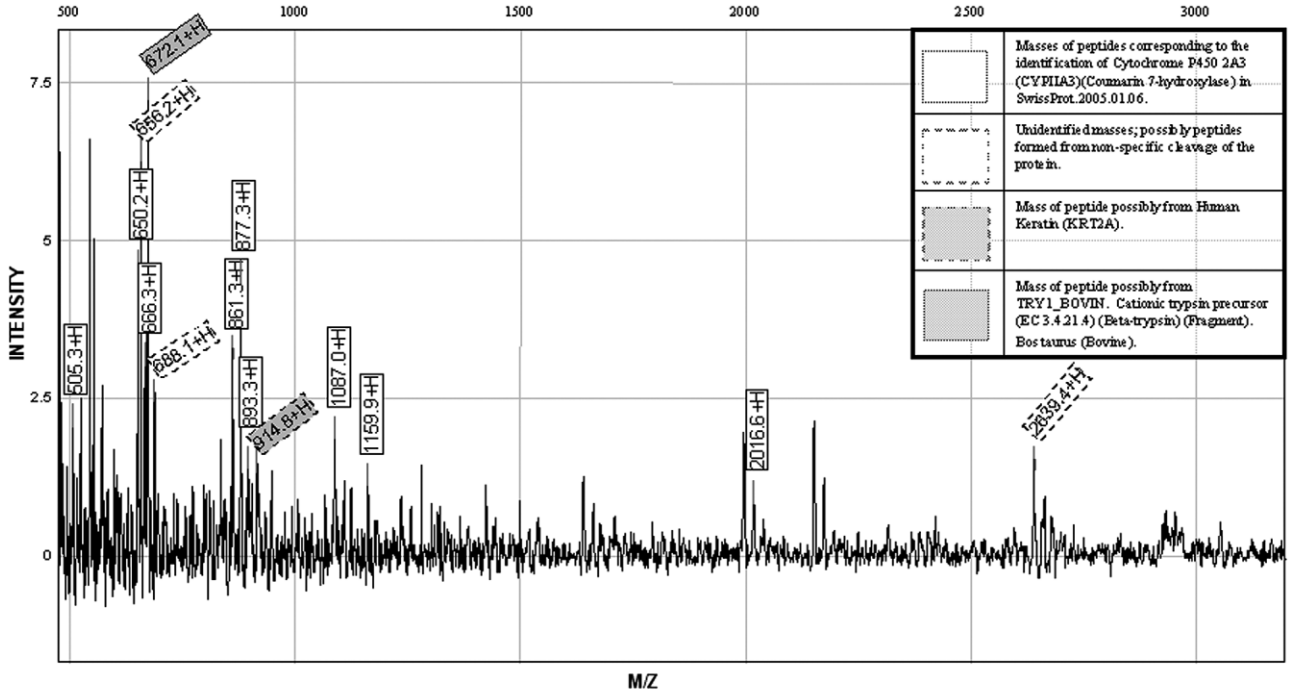

Figure 10. Spectrum of the tryptic digest of the 55 kDa band, eluted from CIM monolithic column with 1.0 M NaCl (*cf.* Fig. 7b). Focusing center mass: 2750 Da. High mass to acquire: 10 000 Da; digitizer rate: 500 MHz; ion mode: positive; chamber vacuum: 1.282×10^{-7} torr; source voltage: 20 000 V; detector voltage: 2800 V; shots fired: 330; shots kept: 314; laser intensity: 190; sensitivity: 8.

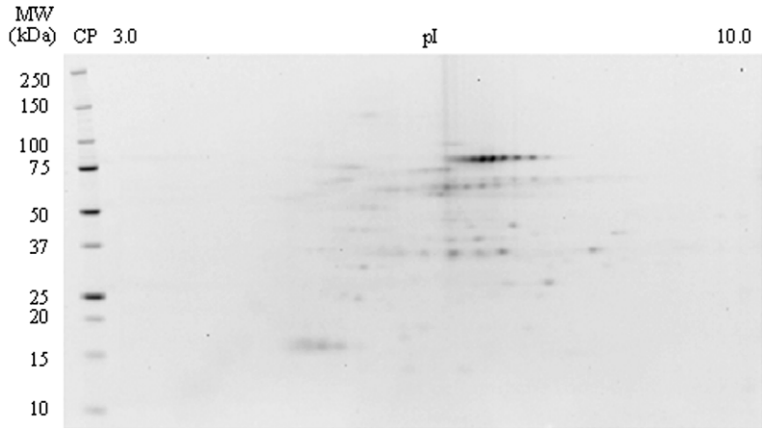

Figure 11. 2-DE of proteins extracted from purified plasma membranes with Triton X-100 after pH 11 treatment (*cf.* Scheme 2 in Fig. 1). The protein fraction was separated on a tandem containing monolithic DEAE and SO_3 disk and subsequently eluted from the DEAE disk with 0.15 M NaCl (*cf.* also Fig. 7).

highly glycosylated. Their solubilization out of the membrane bilayer can be difficult, even if strong detergents are used [29]. On the other hand, differences in their solubility enable their fractionation according to hydrophobicity [12, 29, 34, 37] and solubility in different reagents such as salts, high pH, chaotropic reagents, and different ionic, zwitterionic, and nonionic detergents [29, 34, 36].

Subsequent chromatographic separation gives us the opportunity for further protein fractionation before 2-DE and identification by MS [4, 12, 21, 47]. Monolithic chromatographic columns have advantages when they are used for this purpose. (i) They enable an extremely fast separation or detergent exchange [48, 49]; (ii) scaling up or down to miniaturized columns is possible [49]; (iii) combinations of different chromatographic columns can be placed in the same cartridge [46]; (iv) because of the low-pressure drop, the use of low-pressure pumps or even syringes is possible. Monolithic columns are suitable for the separation of hydrophobic membrane proteins because the recovery of these proteins is high [50].

In the present paper, we compared two methods for selective solubilization of liver membrane proteins using a combination of: (i) different detergents, such as sodium cholate and polydocanol [35]; (ii) different solubilization agents, such as freezing at −80°C and thawing, high pH (pH 11), and the nonionic detergent Triton X-100 [29, 34]. For solubilization of detergent-resistant proteins, the residual pellet can be subsequently extracted with calcium-chelating agents such as EDTA or EGTA [29, 36], strong detergents such as SDS, and concentrated urea solutions.

Prior to 2-DE and protein identification by MS, solubilized fractions containing membrane proteins can be further fractionated by anion-exchange chromatography or by a combination of anion- and cation-exchange chromatography utilizing monolithic supports of different sizes, from analytical 50 μL to semipreparative size 8 mL column volumes.

Prefractionation by use of selective extraction enables separation of proteins according to their solubility in different reagents. As shown here, use of different solubilization agents leads to different patterns of extracted proteins (*cf.* Fig. 2). However, a complete separation between integral and membrane-associated proteins is difficult to achieve. For example, if membranes are extracted with sodium cholate, CEACAM 1 appears in a fraction together with less-hydrophobic proteins. It was unexpected, because Lin and Fain [35] claim that the majority of this protein is solubilized by the stronger detergent polydocanol, after removal of less-hydrophobic proteins by sodium cholate. In our experiments, a significant part of CEACAM 1 was already solubilized with cholate. Using the second solubilization scheme (Scheme 2 in Fig. 1), the same protein is preferably extracted with Triton X-100 together with hydrophobic proteins, after removal of membrane-associated proteins at pH 11. However, solubilization of this protein in only one fraction could not be achieved. As shown in Fig. 3, the main part of CEACAM was extracted with Triton X-100. Traces of this protein are also present in other fractions, extracted after freezing and thawing and in small amount in the pH 11 fraction. Interestingly, this protein could also be detected in the detergent-resistant pellet, which contains the so-called membrane rafts, after intensive treatment with detergents [51]. In this fraction, an additional band with an apparent molecular mass of about 150 kDa could be seen. Compared to the Triton X 100 fraction, this band is enriched. The physiological role of CEACAM 1 is not fully clear and its involvement in cell signaling and at least temporary localization in membrane rafts cannot be excluded [41].

If the pellet remaining after extraction of a crude membrane preparation with two detergents (*cf.* Fig. 1, Scheme 1) is subsequently extracted with dilute NaOH at pH 11, one protein, with apparent molecular mass of 37 kDa in SDS-PAGE , can be highly enriched. With very high probability, this protein was identified as uricase. Uricase is an enzyme typically localized in the liver peroxisome [8, 52]. The presence was consistent with the use of a crude membrane preparation, which contained multiple types of membranes. However, it is still very intriguing that this protein was not removed in previous steps by consecutive extraction with two different detergents. In this case, as always, if a protein is unexpectedly identified in an organelle fraction in which it is not normally located, the question is raised whether it is contamination or is it an “unusual” localization in another cell compartment. It is the reason why the purity of cellular fractions is one of the crucial problems in the analysis of so-called “organelle” proteomics [10, 13, 15, 17].

After further extraction of the pellet by simple calcium chelation, some calcium-binding, membrane-associated proteins can be highly enriched [29]. In our previous work, one of these proteins was identified as annexin A6 [45]. Identification of other proteins from this fraction is ongoing. Some detergent-resistant proteins can be solubilized in the last step by use of highly concentrated urea solution in the presence of 4% CHAPS (*cf.* Figs. 6, 7). These proteins should be localized in the detergent-resistant membrane fraction, so-called rafts [29, 51]. The presence of some proteins such as annexins in membrane rafts could be as already demonstrated [53], and the presence of other membrane proteins (FASL receptor) or possibly plasma membrane-associated protein can be expected (*cf.* Table 1). However, the presence of other proteins, such as proteasome subunits C5 and C13, uricase,

membrin, and NADPH-cytochrome p450 reductase is unexpected (*cf.* Table 1). On the other hand, these proteins could be identified with high confidence, and it can be argued that they originate as contamination of the plasma membrane fraction with other organelles. The intriguing question still remains, how these proteins could stay in the pellet after previous, very harsh solubilization steps. The method for selective separation used has, at least, made the enrichment and subsequent identification of these low abundant proteins possible.

After further separation of solubilized fractions with anion-exchange chromatography on DEAE CIM disks, enrichment of less-abundant proteins prior to 2-DE was achieved. Recovery of highly hydrophobic membrane proteins after chromatographic separation is always a critical issue. It has been shown that such methacrylate disks can be used for separation of membrane proteins without significant loss [50]. As shown in Fig. 8, by use of such units, an effective separation even of different forms of a particular protein (CEACAM 1) is possible. The SDS-PAGE patterns of the fractions after chromatographic separation look much simpler. However, after separation with 2-DE, many more spots than previously expected could be detected (*cf.* Figs. 4b and c).

For further optimization of the prefractionation process prior to 2-DE, a highly enriched plasma membrane fraction was used. Because of the better results with the model integral membrane protein CEACAM 1 in previous experiments, extraction Scheme 2 was chosen. After solubilization, fractionated proteins were separated on a column-tandem, containing an anion- and a cation-exchange column. The use of monolithic supports enables rapid and simple fractionation with step gradients. The marker protein, CEACAM 1, could be highly enriched in the fraction eluted from the DEAE CIM column with 0.3 m NaCl (*cf.* Fig. 8). Separation from the other, probably truncated or differently glycosylated, form of this protein [41] was also possible. As shown in Fig. 10, indi-

Table 3. MS-Fit search results of the tryptic digest of the 55 kDa band eluted from the CAX-CIM monolithic column with 1.0 M NaCl (see Fig. 7b)

	Results summary											
	MOWSE score	#/12 masses matched	% masses matched	% coverage	% TIC	Mean error (Da)	Data total (Da)	Protein M_w (Da)	Protein p*I*	Accession no.	Species	Protein name
1	1.389 e+04	9	75%	11.0	75.0	0.601	1.70	56 511	9.32	P20812	Rat	Cytochrome P450 2A3 (CYPIIA3) (coumarin 7-hydroxylase)
2	595	7	58%	8.0	58.3	0.0230	2.73	53 429	6.4	P97834	Rat	COP9 signalosome complex subunit 1 (signalosome subunit 1) (SGN1) (JAB1-containing signalosome subunit 1) (G protein pathway suppressor 1) (MFH protein)

MS-Fit was used to search the SwissProt.2005.01.06 database for matching tryptic peptides. Note: one additional match amongst the top ten matches (at #10) was cytochrome P450 4B1 (CYPIVB1) (P450-isozyme 5) (P450 L-2). It had an MOWSE score of 106, with 4/12 masses matched and 7% coverage. Similar mass coverages and MOWSE scores were obtained for cytochrome P450 2A3 using the NCBInr.2005.01.06 database. MS-Fit and related programs were developed in the UCSF MS Facility, which is directed by Dr. Alma Burlingame, Professor of Chemistry and Pharmaceutical Chemistry at UCSF.

vidual fractions were further separated with 2-DE will be used for further identifications of excised spots. Interestingly, only a few proteins were bound to the CAX column, which was installed in the system after the anion-exchanger. These proteins bind very tightly and could be eluted only with a highly concentrated NaCl solution (*cf.* Fig. 7). Two of these strongly bound proteins, with apparent molecular masses between 50 and 60 kDa in SDS-PAGE, were identified with high confidentiality as two forms of cytochrome P450, a protein mainly localized in liver (*cf.* Tables 1, 3 and endoplasmic reticulum (ER) Fig. 10). The presence of cytochrome P450 could reflect contamination with other organelles or could represent cell surface forms of these enzymes [56, 57]. The material applied to the chromatographic columns was the Triton X-100 extract after removal of membrane-associated proteins (*cf.* Scheme 2 in Fig. 1 and [12, 29]). The question regarding the purity of organellar fraction used for characterization of their proteins can again be raised [17]. On the other hand, relocalization of proteins from other organelles into plasma membranes has been frequently observed [16, 41, 47, 57, 58]. After 2-DE, three additional low-molecular, highly basic proteins could be identified (*cf.* Table 2). None of these proteins is a typical plasma membrane protein. However, by use of such pre-fractionation method enrichment of these components, which are present at very low concentrations in the starting material, can be demonstrated.

Key questions facing proteomic analyses of target cells, tissues, or organelles are the identification of less-abundant proteins and the avoidance or, at least, tracking of possible contaminations from other organelles or cell compartments [10, 12, 14, 15]. As shown here, thorough sample preparation using a combination of well-known cell biological and biochemical methods before protein separation by 1- and 2-DE and analysis by MS is an important step in their identification.

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