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Analysis of mouse liver membrane proteins using multidimensional separations and tandem mass spectrometry

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ABSTRACT

In the field of proteomic investigation, the analysis of membrane proteins still faces many technical challenges. A fundamental question in this puzzle is how to maintain a proper solvent environment to allow the hydrophobic proteins to remain solubilized. We propose that the denaturation of membrane proteins in a highly concentrated urea solution enables them to be ionized such that ionic exchange chromatography can be employed to separate them. The membrane proteins prepared from the mouse liver were dissolved in 6 M guanidine hydrochloride, 20 mM Tris–HCl, pH 9.0, and loaded onto a tandem chromatography apparatus coupled with Q-Sepharose FF and Sephacryl S-200HR. These columns were able to adsorb 97.87% of the membrane protein preparations. Using a linear NaCl (0–1.0 M) gradient, the bound proteins were eluted out at 0.1–1.0 M NaCl, and examined by SDS-PAGE. Furthermore the protein bands underwent excision and digestion with trypsin, followed by reverse-phase chromatography for the separation of the digested peptides and ionic-trap mass spectrometry for the identification of the proteins. From the SDS-PAGE gels, the overlap between proteins from neighboring bands was only 21.34%, indicating that the anionic-size exclusion coupling chromatography efficiently separated these membrane proteins. Of a total of 392 proteins identified, 306 were membrane proteins or membrane-associated proteins. Based on the calculation of hydrophobicity, the GRAVY scores of 83 proteins are greater than, or equal to, 0.00. Taking all of this evidence together, our results revealed that this approach is satisfactory for studies on the membrane proteome from the mouse liver.

1. Introduction

A cell is enclosed within a protective lipid bilayer, the plasma membrane, which affords a physical boundary between the cell and its living environment [1]. The hundreds of different proteins which are involved in the functions of the plasma membrane interact with the membrane in different ways; some are embedded in the lipid bilayer as membrane-integral proteins, and some are anchored to the membrane through non-covalent interactions as membrane-associated proteins [2]. These membrane proteins play important roles in many fundamental biological processes such as cell–cell interactions, signal transduction, and material transport [3]. The plasma membrane proteins participating in drug recognition have been extensively studied, accounting for ~70% of all known drug targets located on the plasma membrane [4]. Nearly all of the drugs are modified or degraded in the liver; therefore, the identification of the liver plasma membrane proteins could provide protein targets for the design of either therapeutic monoclonal antibodies or small-molecule drugs [5,6].

The study of membrane proteins is a tough challenge, particularly with respect to the separation techniques [7]. Most membrane proteins possess strong hydrophobic domains which are rarely dissolved in aqueous solutions [8]. Even though detergents enable some membrane proteins to be solubilized, the micelle formed may lead to protein dysfunction or prevent the further separation and purification of these proteins [9–12]. Another question that often bothers investigators is how to maintain the soluble status of the membrane proteins during a long process of experimentation. In many cases, a membrane protein, well solubilized in one solution, can be precipitated in another solution [8]. This is the main cause for the loss of membrane proteins during the experimental procedures. To circumvent the problems associated with the insolubility of the membrane proteins, a "shotgun" digestion approach has been proposed and employed in many analyses [13–16]. With

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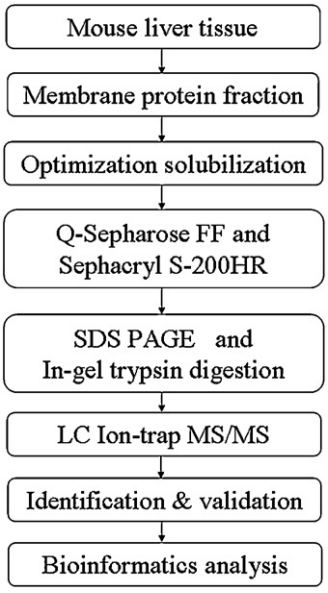
this approach, a separation system no longer deals with the separation of the intact proteins, but focuses on the separation of the digested peptides. Hence, it is more effective at preventing low abundant membrane protein extraction loss and high abundant membrane protein precipitation. However, a number of technical barriers are not overcome by this method, in particular during proteomic analysis. When faced with the huge numbers of peptides generated from shotgun digestion in a protein mixture, liquid chromatography provides a powerful capacity for peptide separation. In spite of reverse phase materials which perform well in peptide separation, the current chromatography system is still limited in its effectiveness in separating the enormous amount of peptides generated by the shotgun system. Multiple-chromatography is a way to improve the separation efficiency in liquid chromatography [17–19]; nevertheless, this technique has the drawback that peptide loss may be significantly increased due to the multiple changes of columns as well as buffers. Additionally, the membrane proteins are of relatively low abundance in the cell [20]. If these proteins and their peptides cannot be partially enriched, the weak signals are likely to be suppressed during the process of protein separation or identification. For these reasons, the shotgun technique has to be considered and specifically designed for a given sample in proteomic analysis. Generally, three principles ought to be fully estimated in the analysis of membrane proteins: (1) a strong solvent to maximally solubilize the membrane proteins, (2) a consistent system to maintain the solubilized state of the membrane proteins, and (3) a careful procedure to enrich the low abundance of the membrane proteins.

The proteomic strategy has paved the way for protein expression profiling. There are several reports describing new approaches to conducting membrane proteomics [11,21–23]. Blonder et al. used, Triton X-100 or Brij-96, to extract the membrane proteins in rat basophilic leukemia cells. They quantitatively compared the extracted membrane proteins with a combination of $^{(16)}O/^{(18)}O$ and isotope coded affinity tag (ICAT) labeling. The comparative analysis revealed that the proteins were more readily extracted using Triton X-100 than Brij-96 [22]. Fandino et al. developed a new method by which the membrane proteins were first separated by blue-native gel electrophoresis to prevent protein aggregation of the hydrophobic regions during electrophoresis. The separated protein bands were then subjected to in-gel tryptic digestion, and further analyzed by liquid chromatography–tandem mass spectrometry [23,24]. In this communication, we propose and examine a new strategy for the analysis of the membrane proteomes. In contrast to the earlier reports, the mouse liver membranes prepared by ultracentrifugation were treated in a denaturant solution for the extraction of the membrane proteins, and the extracted proteins were separated through an ionic exchange column. After collecting these eluted proteins, each fraction was further analyzed by SDS-PAGE, followed by an in-gel tryptic digestion of the excised bands and protein identification using LC–MS/MS. The experimental procedure is depicted in Scheme 1. Of the total 392 proteins identified, 306 were membrane proteins or membrane associated proteins based on the literature. Following the estimation of hydrophobicity, 83 proteins have GRAVY scores over 0.00, which is a criterion for determining the hydrophobicity of the proteins. The success of this strategy in separating and identifying membrane proteins makes it a potential technique for analyzing insoluble protein fractions.

2. Materials and methods

2.1. Chemicals

Guanidine-HCl, urea, thiourea, protease inhibitor cocktail, TCA, TEMED, glycine, PMSF, acetonitrile, and Brilliant Blue R-250 were obtained from Sigma–Aldrich (Steinheim, Germany). Sodium dodecyl sulphate, ammonium persulphate, and all chromatography resins used in this experiment were obtained from Amersham Biosciences (Uppsala, Sweden). Modified trypsin (Sequence grade) and dithiothreitol were obtained from Promega (Madison, WI, USA). All other reagents were of analytical reagent grade or the highest purity available.



Scheme 1. Schematic overview of multiple separation strategy for analyzing the mouse liver membrane protein.

2.2. Preparation of mouse liver membrane

Male mice (7–9 weeks of age) of the strain C57BL/6J were purchased from the Beijing Laboratory of Animal Center and held under specific pathogen-free conditions. The protocols for animal handling were approved by the Committee of Animal Experimentation, CAS. The mice were anesthetized with pentobarbital sodium and sacrificed to remove the livers. The liver tissue was first cut into cubes (each of about 3 mm^3 in size) and washed three times with cold PBS buffer to eliminate excessive blood elements (1 g of liver tissue required at least 20 ml of wash buffer for one wash). The liver cubes were then combined into one tube which was immediately snap frozen in liquid nitrogen. The samples remained frozen until they were used.

The liver tissue pieces (1.0 g) were suspended in 5 ml of sample buffer (50 mM mannitol, 200 mM sucrose, 2 mM EDTA, 1 mM PMSF, 0.2 mM Na_2VO_3, 1 mM NaF, and protease inhibitor cocktail, 10 mM Tris–HCl pH 7.4). The suspension was gently homogenized three times with a hand-held Dounce homogenizer, and the membrane fraction was extracted. First, the suspension was centrifuged at 400 × *g* for 10 min, and the pellet was discarded. The suspension was then centrifuged at 800 × *g* for 10 min, and the pellet was again discarded. The suspension was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the pellet was discarded. Finally, the suspension was centrifuged at 150,000 × *g* for 60 min, and the pellet was retained for the crude membrane fraction. This crude membrane fraction was then washed twice with the sample buffer.

2.3. Extraction of the membrane proteins

Six extraction solutions were used to examine the extraction efficiency of the membrane proteins. These solutions contain 20 mM Tris–HCl with various denaturants, detergents and pH val-

ues: (1) 7 M urea and 2 M thiourea, pH 9.0, (2) 6 M guanidine-HCl, pH 9.0, (3) 7 M urea, 2 M thiourea, and 4.5 M guanidine-HCl, pH 9.0, (4) 3.5 M urea, 1 M thiourea, and 5% SDS, pH 9.0, (5) 7 M urea, 2 M thiourea, 4.5 M guanidine-HCl, and 2 M NaOH, pH 13.5, and (6) 8 M urea, pH 11.0. The samples of the mouse liver membranes were divided into groups and subjected to ultrasonication for 3 min in the different extraction solutions, and then incubated for 1.0 h at room temperature. After incubation, these samples were centrifuged at 40,000 × *g* for 20 min, and the resultant supernatants were transferred to clean tubes for protein concentration measurements using a 2-D QUANT KIT (Amersham Biosciences). All of the extraction experiments were repeated at least three times, and the protein concentration measurements for each extraction were performed in triplicate. The protein extraction in the solution containing 7 M urea, 2 M thiourea, 4.5 M guanidine-HCl, and 2 M NaOH, pH 13.5, was used as a baseline control for all the extractions.

2.4. The protein binding capacities of different ionic exchange resins

Four ionic exchange resins were examined for their protein binding capacity, DEAE-Sepharose FF, Q-Sepharose FF, CM-Sepharose FF, and SP-Sepharose FF. Binary resin mixtures were used for these studies as well, including DEAE-Sepharose FF with CM-Sepharose FF, DEAE-Sepharose FF with SP-Sepharose FF, Q-Sepharose FF with CM-Sepharose FF, Q-Sepharose FF with SP-Sepharose FF, the mixing proportion for each mixture was 1:1. For the resins of type DEAE-Sepharose FF or Q-Sepharose FF, the binding buffer used contained 20 mM Tris–HCl, pH 9.0 and 4 M urea. For the resins of type CM-Sepharose FF and SP-Sepharose FF, the binding buffer used contained 20 mM NaAc-HAc, pH 6.0 and 4 M urea. For the binary resin mixtures, the binding buffer contained 20 mM Tris–HCl, pH 9.0 and 4 M urea. An equal amount of the extracted membrane proteins (40 μl) were mixed with an equal volume of the swollen resins (100 μl) and placed at room temperature for 30 min. The resins and binding solutions were separated by centrifugation at 2000 × *g* for 10 min. The concentration of the protein in the supernatants was determined, and the binding efficiencies were estimated based on the ratio of the protein concentration before and after resin treatment.

If a resin was found to possess a high capacity for protein binding, the protein extracts of different concentrations were incubated with the resin to ascertain the proper loading.

2.5. The separation of membrane proteins through fast performance liquid chromatography

A chromatography column (1 cm × 40 cm) was adopted for FPLC analysis by packing with two resins, Sephacryl S-200HR and Q-Sepharose FF. The resins were swollen and equilibrated thoroughly in buffer A containing 20 mM Tris–HCl and 4 M urea, pH 9.0. The swollen Sephacryl S-200HR was first packed into the column with about 16 ml of column volume. After the Sephacryl S-200HR had settled down completely, the equilibrated Q-Sepharose FF was poured into the column using 10 ml of column volume, to constitute the upper layer. The packed column was fully equilibrated with buffer A using a flow rate of 12 ml/h. Ten milligrams of the membrane proteins were loaded onto the column and washed with buffer B, containing 20 mM Tris–HCl, 1 M NaCl and 4 M urea, pH 9.0. The bound proteins were eluted with a step-wise gradient of NaCl increasing from 0.2 M to 1 M in a buffer of 20 mM Tris–HCl and 4 M urea, pH 9.0. The eluted fractions were collected in volumes of 2.0 ml/tube or 3.0 ml/tube. And the eluted fractions are precipitated by adding 4 times volume acetone, the suspension was centrifuged at 40,000 × *g* for 15 min at 4 °C, and the pellets were retained.

2.6. The separation of the membrane proteins by SDS-PAGE

The aliquots from the collected FPLC fractions were reduced and denatured by boiling in SDS loading buffer for 3 min. Then these samples were resolved via SDS/PAGE using a 12% polyacrylamide gels and a BioRad electrophoresis device. The separated proteins were visualized by staining with Coomassie Brilliant Blue R-250. Each lane on the gel was evenly excised into 7 gel slices which were subjected to a complete tryptic digestion.

2.7. In-gel digestion with trypsin

The excised gel bands were destained and dehydrated with 50% acetonitrile followed by a reduction with 10 mM DTT at 56 °C for 1 h and alkylation with 55 mM iodoacetamide in the dark at room temperature for 45 min *in situ*. The gel slices were thoroughly washed with 25 mM ammonium bicarbonate in a water/acetonitrile (1:1, v/v) solution and were completely dried in a SpeedVac. The proteins were digested in 25 μl of a modified trypsin solution (10 ng/μl in 25 mM ammonium bicarbonate) and incubated overnight at 37 °C. The peptides were released with vigorous shaking and were extracted in 50 μl of 50% acetonitrile containing 0.1% formic acid.

2.8. Separation of the digested peptides by HPLC

The digested peptides were further separated on reverse-phase (C_{18}) capillary columns [5 μm, 300 Å (1 Å = 0.1 nm) particles, 0.15 mm × 100 mm or 0.15 mm × 150 mm; MicroTech] using a Surveyor LC system with a consistent flow rate of 100 μl/min. The bound peptides were eluted using an acetonitrile gradient. The buffer A was composed of 0.1% formic acid in water, and the buffer B was composed of 0.1% formic acid in acetonitrile. A linear gradient of 2–80% buffer B was employed within a gradient period of 80 min.

2.9. Protein identification using mass spectrometry

The peptides separated by reverse phase HPLC were delivered to an ion-trap tandem mass spectrometry (LC–MS/MS) using a LCQ Deca^{XP} ion-trap mass spectrometer. The mass spectrometer retained 3.2 kV of spray voltage and 150 °C at the heated desolvation capillary. The *m*/*z* range from 400 to 2000 was scanned in 1.2 s, and the ions were detected with a high energy conversion dynode detector. The LC–MS/MS data were analyzed using the SEQUEST algorithm to identify the proteins in the mouse IPI database (http://www.ebi.ac.uk/IPI/IPIhelp.html) [25]. All the accepted results had a $^{\Delta}$Cn of 0.1 or greater. To be treated as an acceptable result, a singly charged peptide must be tryptic, and the cross-correlation score (Xcorr) had to be at least 1.9. The tryptic or partially tryptic peptides with a charge state of +2 must have a Xcorr of at least 2.2. Triply charged tryptic or partially tryptic peptides with a +3 charge state were accepted if the Xcorr was ≥3.7.

2.10. Statistical analyses

The average values of the parallel experiments are given as the mean ± SD. The comparison of differences between the groups was performed using the Student's *t* test. The significance was defined as $p < 0.05$.

Table 1
Membrane protein extraction yields.

	1	2	3	4	5	6
Membrane proteins ± SD (mg)	2.304 ± 0.166	3.263 ± 0.305	2.408 ± 0.242	2.953 ± 0.516	2.506 ± 0.274	1.790 ± 0.178
Recovery of membrane proteins (%)	92	130	96	118	100	72
Yield of membrane proteins (%)	4.12 ± 0.29	5.84 ± 0.55	4.31 ± 0.44	5.29 ± 0.93	4.49 ± 0.49	3.20 ± 0.32

Notes: (1) 7 M urea and 2 M thiourea, 20 mM Tris–HCl, pH 9.0, (2) 6 M guanidine-HCl, 20 mM Tris–HCl, pH 9.0, (3) 7 M urea, 2 M thiourea, and 4.5 M guanidine-HCl, 20 mM Tris–HCl, pH 9.0, (4) 3.5 M urea, 1 M thiourea, and 5% SDS, 20 mM Tris–HCl, pH 9.0, (5) 7 M urea, 2 M thiourea, 4.5 M guanidine-HCl, and 2 M NaOH, 20 mM Tris–HCl, pH 13.5, and (6) 8 M urea, 20 mM Tris–HCl, pH 11.0, respectively.

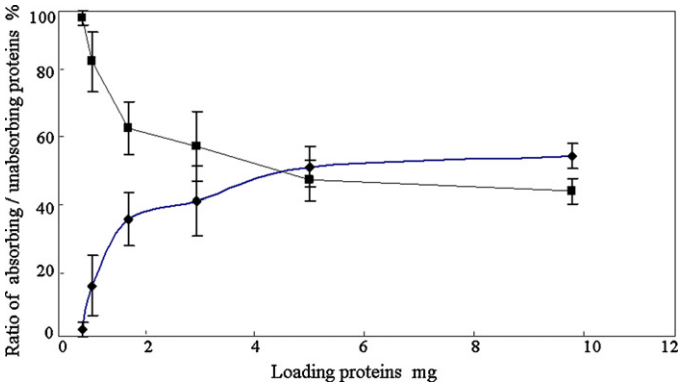


Fig. 1. The capacity of Q-Sepharose FF to adsorb membrane proteins. The different amounts of the extracted membrane proteins (10–0.47 mg) were mixed with an equal volume of the Q-Sepharose FF swollen resins (100 μl) and placed at room temperature for 30 min. The resins and binding solutions were separated by centrifugation at 2000 × *g* for 10 min. The supernatants were used to determine the concentrations of the proteins, and the binding efficiencies were estimated based on the ratio of the protein concentrations before and after resin treatment. The symbols "■", and "♦", represent the ratio of adsorbed and unadsorbed proteins, respectively.

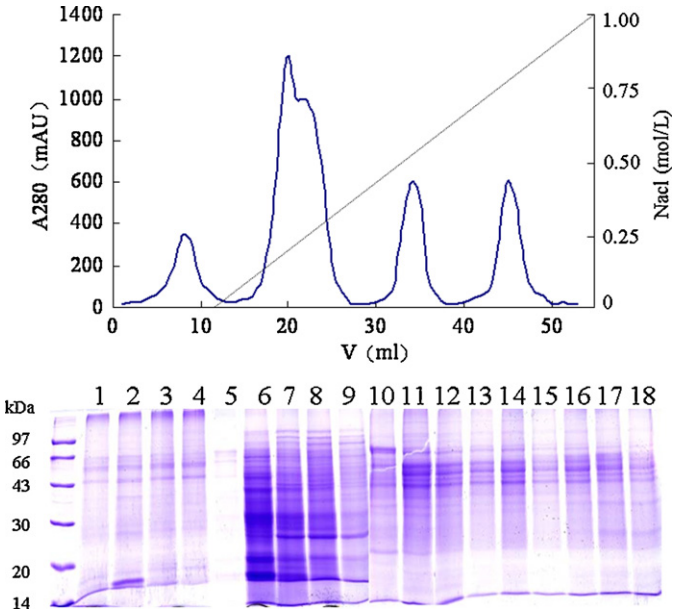


Fig. 2. Separation of the solubilized membrane proteins using the tandem column of Sephacryl S-200HR and Q-Sepharose FF as the first dimension, and SDS-PAGE as the second dimension. The upper panels represent the elution profiles of membrane proteins monitored at 280 nm. The lower panels represent the eluted fractions analyzed by SDS-PAGE (12%T, 1%C) with Coomassie Brilliant Blue staining. Each fraction contained 3.0 ml eluant. Mw values are given in kDa. mAU, milli-absorbance units.

3. Results and discussion

3.1. Comparison of different techniques for the extraction of membrane proteins

The mouse liver membrane was dissolved fully in all six of the extraction solutions, and all the suspensions generated a few pellets after centrifugation at 40,000 × *g*. Moreover, the extraction efficiencies of the membrane proteins in these solutions, listed in Table 1, were not significantly different except in the 8 M urea alone, which had the lowest yield of membrane proteins. Of all the extraction solutions, 6 M guanidine-HCl produced the highest yield of membrane proteins, and this solution was chosen for use in later experiments. The pH values in the extraction solutions were a critical factor that seriously affected the efficiency of the extraction. When the pH dropped to seven, precipitation occurred in the extraction suspension. Almost 40% of the membrane proteins were lost in the extraction solution at pH 6.0; and 55% were lost in the pH 3.0 solution. The solutions containing 7 M urea, regardless of other components in the solutions, were able to achieve quite similar extraction effects with almost identical extraction efficiencies (Table 1). Furthermore, there was no precipitation in this solution even though the urea concentration in this protein suspension was diluted to 4 M. Considering the destructive impact of the high concentrations of denaturant on the chromatography materials, the 6 M guanidine-HCl suspension was chosen for later experiments.

3.2. A comparison of the binding capacities of different ionic exchange resins

There were two factors which were considered prior to our series of binding experiments. First of all, as mentioned above, the low pH in the binding buffer can bring about protein precipitation. Since the binding capacity of a cation exchange resin depends on the samples being ionized in a low pH environment, this kind of resin is not suitable for the binding of the membrane proteins. Secondly, the level of protein binding to an anion exchange resin is affected by the presence of guanidine even with concentrations as low as 100 mM. This chemical therefore had to be eliminated from

Table 2
The capacity of some resins to adsorb membrane proteins.

	1	2	3	4	5	6	7	8
Loading proteins (mg)	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Unadsorbed proteins ± SD (mg)	6.35 ± 0.37	5.55 ± 0.39	6.84 ± 0.33	6.74 ± 0.36	7.54 ± 0.29	7.44 ± 0.30	6.12 ± 0.37	6.58 ± 0.29
Adsorbed proteins ± SD (mg)	3.65 ± 0.37	4.45 ± 0.39	3.16 ± 0.33	3.26 ± 0.36	2.46 ± 0.29	2.56 ± 0.30	3.88 ± 0.37	3.42 ± 0.29
Ratio of adsorbed proteins (%)	36.5 ± 3.7	44.5 ± 3.9	31.6 ± 3.3	32.6 ± 3.6	24.6 ± 2.9	25.6 ± 3.0	38.8 ± 3.7	34.2 ± 2.9

Notes: (1) DEAE-Sepharose FF, (2) Q-Sepharose FF, (3) CM-Sepharose FF, (4) SP-Sepharose FF, (5) DEAE-Sepharose FF and CM-Sepharose FF, (6) DEAE-Sepharose FF and SP-Sepharose FF, (7) Q-Sepharose FF and CM-Sepharose FF, and (8) Q-Sepharose FF and SP-Sepharose FF.

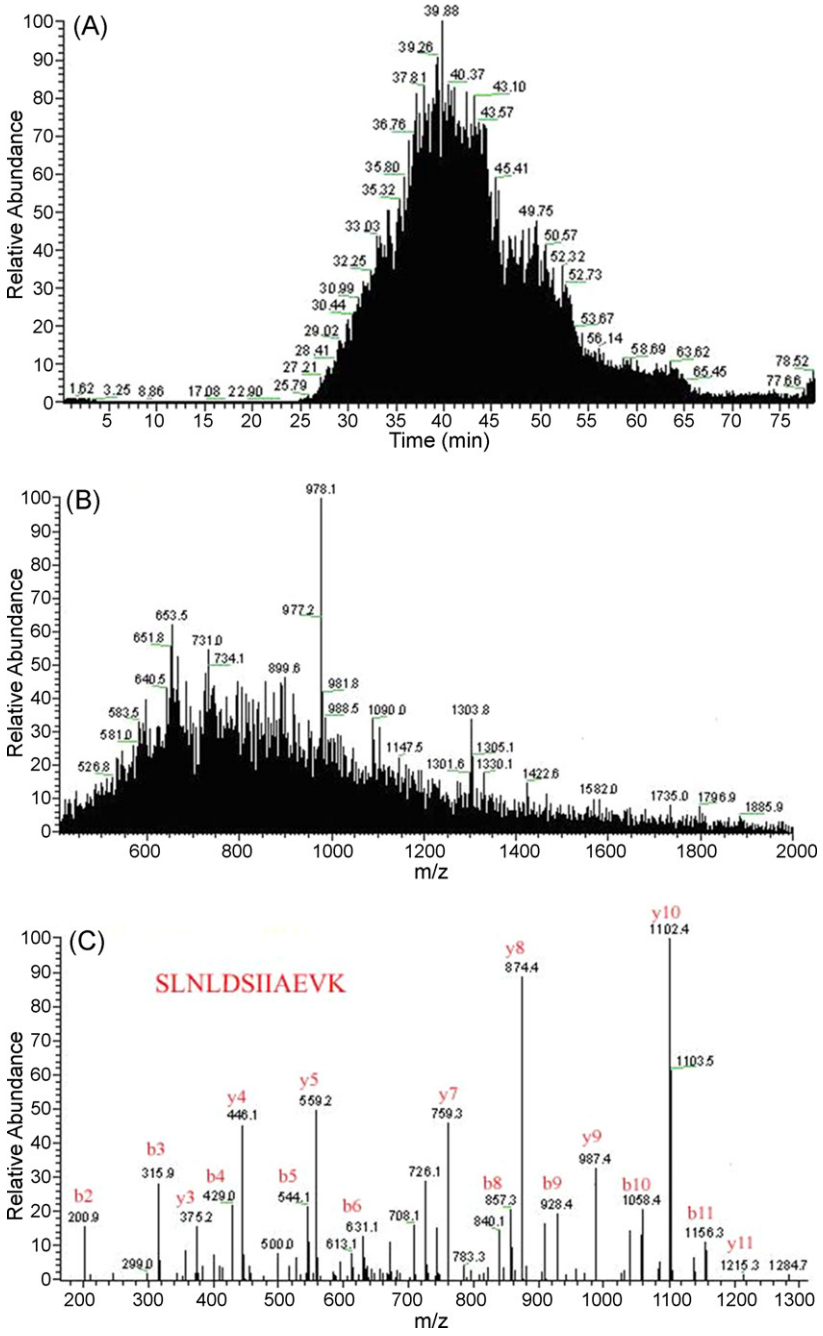


Fig. 3. Identification of membrane protein fractions by LC–MS/MS. (A) A full-scan mass spectrum from LC–MS/MS analysis of the peptides identified as the membrane proteins. (B) An MS survey scan at an elution time of 45.15 min during LC–MS analysis. The parent ion 651.78^{2+} (RT = 45.15 min) was selected for further MS/MS. (C) The MS/MS spectra for the parent ion 651.78^{2+}. The amino acid sequence, SLNLDSIIAEVK, was confirmed by analyzing b- and y-ions derived from the peptide ion.

the binding buffer. The guanidine-HCl solution was dialyzed three times overnight against the column buffer.

Table 2 shows the results of protein binding to the eight different ionic resins. Compared with other resins, Q-Sepharose FF exhibited the strongest binding capacity to this protein preparation. Based on the binding experiments, three points merit attention. (1) On loading the same volume of resin for protein binding, the binary mixtures composed of two resins did not demonstrate any significant improvement in the binding capacity (Table 2). (2) Although the commercial company stated a high capacity of protein binding for Q-Sepharose (120 HSA mg/ml), the binding capacity of the membrane proteins to this resin was only 45 mg/ml. (3) The binding capacity was influenced by the binding periods or the protein concentrations. An extension of the resin–protein incubation time considerably enhanced the resin's protein binding capacity, and the diluted protein solution also responded similarly. Therefore, a series of experiments were carried out to demonstrate the relationship between the resin and the protein concentrations. As shown in Fig. 1, when a fixed amount of resin was mixed with various concentrations of the membrane proteins, the proteins adsorbed by this resin could be quantitatively estimated. A solution containing 10 mg of the membrane proteins was only 45% adsorbed in a resin volume of 0.1 ml, whereas 500 μg of the membrane protein was almost completely adsorbed (97.87%) by the same amount of resin. Based upon these values, the optimum adsorbance ratio of Q-Sepharose resin to membrane proteins was calculated to be 0.5 mg protein *per* 0.1 ml packed resin.

3.3. Fast performance liquid chromatography for the separation of membrane proteins

A chromatography column packed with Q-Sepharose FF in 10 ml of column volume was used to separate the membrane proteins. The chromatographic optical density at 280 nm and the SDS-PAGE images of the collected fractions are depicted in Supplementary Fig. 1A. The outcome of the separation was not satisfactory because the bound proteins were eluted within a short range of NaCl concentrations, with most of the proteins eluted around 0.5 M NaCl.

The chromatography column packed with Sephacryl S-200HR in 30 ml column volume was utilized in the protein separation as well. This column performed well on standard proteins from 10 to 100 kDa, separating them with good resolution; however, the membrane proteins were separated poorly, as shown in Supplementary Fig. 1B and C. The results were understandable because size-exclusion chromatography was previously shown to be unable to enrich proteins and to resolve proteins in a complicated protein mixture.

The hydrophobic medium, Pheny-Sepharose FF, was also employed for protein separation using an acetonitrile gradient. Unfortunately, the profile resolution for these membrane proteins was not acceptable (data not shown).

Based on the differences in the chromatographic media, we wanted to determine how a chromatographic procedure could enhance the advantages and avoid the weaknesses for each medium with regard to protein separation. The approach of establishing a tandem column has been proposed and widely practiced for proteomic analysis. In this project, a FPLC tandem column was constructed, as described in Section 2, in which the proteins were allowed to first go through an ionic exchange layer, then to pass through a gel-filtration layer. The chromatographic results indicated that the protein separation was significantly improved using this technique compared with using one resin alone. Based on the optical density profile at 280 nm, there were only three broad peaks visible after an increase in the NaCl concentration. This was confirmed by the SDS-PAGE (Fig. 2).

3.4. The use of SDS-PAGE to separate the membrane proteins

The elution buffer with 4 M urea enables the eluted proteins to remain solubilized. Further protein separation has to be performed with deliberate care in order to avoid protein precipitation. SDS-PAGE is the proper method to apply following tandem chromatography. This technique monitors the chromatographic profile of protein resolution and functions as an additional separation means for the membrane proteins (Fig. 2D). Generally, all of the eluted fractions including the unadsorbed portion were collected and run on SDS-PAGE. The fractions for the subsequent analyses were alternately selected according to the extent of the overlap between the neighboring fractions.

3.5. The reverse-phase HPLC separation of the digested peptides and their subsequent identification by ion-trap mass spectrometry

During the two initial steps of tandem chromatography and SDS-PAGE, the intact proteins from the mouse liver membranes were resolved, and at the next step, a reverse-phase HPLC was applied to separate the peptides generated from these resolved proteins. Since there was a limited amount of digested peptides from each excised band, the peptide profiles resulting from HPLC analysis were monitored by mass spectrometry. A full-scan mass spectrum from the LC–MS/MS analysis of the membrane protein peptide fragments is shown in Fig. 3A, a single MS survey scan at the elution time of 45.15 min is represented in Fig. 3B, and the MS/MS scan of the precursor ion with a m/z value of 651.78 generated by the survey scan is depicted in Fig. 3C. Depending on the population of the constituent peptides, an average of 2497 MS/MS spectra was automatically collected for each slice. Following the acquisition of the MS/MS spectra, the proteins or peptides were identified using the software algorithm from SEQUEST. From all of the gel slices that underwent tryptic digestion and were delivered to ion-trap mass spectrometry, 653 peptides (472 unique peptides) were identified corresponding to 392 unique proteins. From the SDS-PAGE results, the overlap of the proteins from each neighboring lane was only 21.34% (data not shown). The information regarding the identified proteins, such as the matched peptide sequences, accession numbers, theoretical pI and Mw, their charge states, and m/z values, are given in Supplementary Table I.

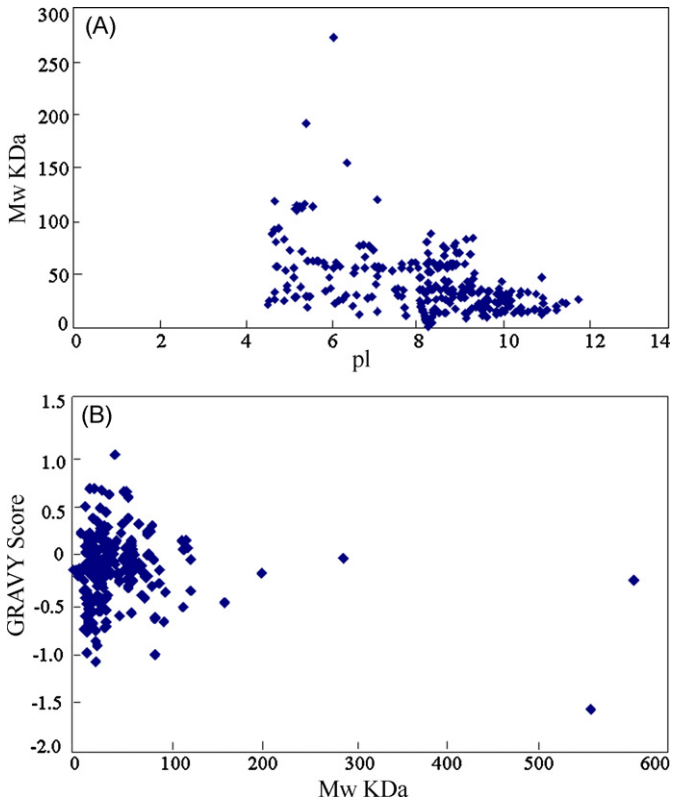


Fig. 4. (A) Virtual 2D map of the membrane proteins identified in this study. (B) The distribution of GRAVY scores for the membrane proteins identified in this study.

3.6. The analysis of the identified proteins based on a literature search and hydropathy plot

We predicted whether an identified protein belonged to the group of membrane proteins, using the GO classification (http://www.geneontology.org/) and the calculated grand average of hydropathicity (GRAVY score, http://ca.expasy.org/tools/protparam.html). We classified proteins as membrane or membrane-associated proteins when the results of the GO component determination or reference showed it to be a membrane or membrane-associated protein, or when the calculated GRAVY score of the protein was more than 0. According to this method of analysis, there are 306 membrane or membrane-associated proteins amongst the total of 392 unique identified proteins (Supplementary Table I). Of these, 19.3% (59/306) were identified with functional categories, 55.9% (171/306) matched the theoretical predictions for the membrane proteins according to the references used in GO, and 24.8% (76/306) had GRAVY scores which were higher than 0. The detailed information is described in Supplementary Table II.

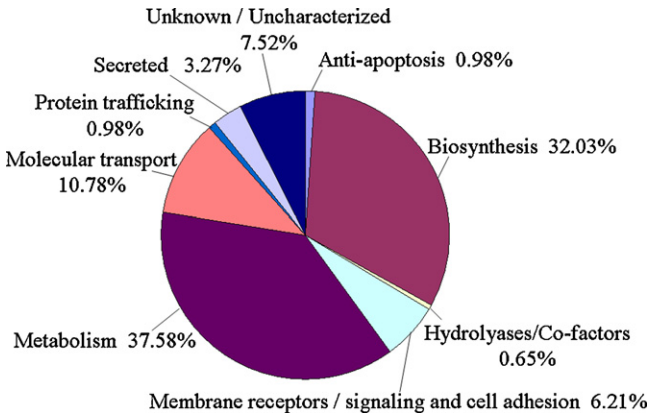


Fig. 5. Functional classification of the identified membrane proteins.

On further analysis of the GRAVY scores of all the identified membrane proteins, the number of proteins with a GRAVY score more than or equal to 0, −0.1, −0.2, and −0.5, were 83, 130, 199, and 265, respectively. Furthermore, we have drawn the analogue 2-DE gel of all the identified membrane proteins, by using p*I* as the horizontal axes and Mw as the vertical axes. These proteins had a pH range from 4 to 12 (Fig. 4A), and the molecular weight of the proteins identified was generally lower than 120 kDa (Fig. 4B).

Using the mouse liver genomic data and GO analysis, we classified the 306 identified membrane or membrane-associated proteins into nine categories as shown in Fig. 5. As shown in the figure, it is obvious that the proteins involved in metabolism comprise the biggest category (37.58%, 115/306), while the second highest category includes proteins involved in biosynthesis (32.03%, 98/306). The remaining proteins were classified into molecular transport (10.78%, 33/306), unknown/uncharacterized (10.52%, 23/306), membrane receptors/signaling and cell adhesion (6.21%, 19/306), secreted (3.27%, 10/306), anti-apoptosis (0.98%, 3/306), protein trafficking (0.98%, 3/306), and hydrolases/Co-factors (0.65%, 2/306) groups.

4. Concluding remarks

In this study, we were able to separate and identify 306 unique mouse liver membrane proteins. The experimental results showed that our multidimensional separation system has high resolution and peak capacities, may serve as a general method for membrane protein research and be suitable for the preparation of samples from other organisms. To our knowledge, the 306 proteins reported here represent the largest number of proteins identified from insoluble fractions of the mouse liver. As shown in Supplementary Table II, amongst the identified membrane proteins, there were some proteins involved in energy metabolism and respiration, such as ATP synthase [26], ATP-citrate synthase, Na+/K+-ATPase, Cytochrome c, NADH dehydrogenase (ubiquinone), Fe–S protein [27] and Cytochrome P450 [28], which are typical membrane or membrane-associated proteins. We also observed many proteins of the endoplasmic reticulum, for example, laminin receptor, RER1 protein and reticulum calcium ATPase2 [29]. In addition, there were some lipid metabolism and ribosomal proteins, such as the fatty acid transport protein, and long chain fatty acid CoA ligase [30–32]. As integral membrane proteins of the lysosome and the proteasome [30,31], the lysosome membrane protein II, the proteasome subunit, and the 26S protease regulatory subunit were also identified in our results. Another interesting finding was that some well known cancer markers were identified in the insoluble fraction, such as cadherin and microsomal glutathione S-transferase [33–37].

The membrane proteome can provide information on cell functions; however, the techniques for the extraction as well as the separation of the membrane proteins are restricted in their methodology by the special characteristics of these proteins. It was known that traditional 2D technology was very limited in the separation and resolution of the membrane proteins [11,38], and this made it difficult to perform automated high throughput analyses. So in recent years, multidimensional analysis of proteins identification technology (MUDPIT) was used to separate the mixed protein peptides [39–42], in which the peptides were separated by multidimensional chromatography and then analyzed by mass spectrometry. But this method was rarely employed in membrane proteomics, because it was difficult to get a satisfactory resolution in the separation of the digested peptides by liquid chromatography, and the separation and MS identification were affected by the detergents in the solution. An innovation was urgently required in this field. Using the mouse liver membrane as a target, a new strategy was proposed and tested in this communication. Briefly, there are two important issues raised from the experimental results. First, the procedure of membrane proteomic analysis has to fully take into account the importance of the solvent system adopted, especially for the extraction of the intact proteins. Using the proper denaturants or detergents, the mouse liver membrane proteins were effectively extracted in the basic extraction solution and well resolved by tandem chromatography and electrophoresis. Second, the analysis of the membrane proteins should be based upon multiple separations, which enable the enrichment of low abundant proteins and increase the resolution of the protein mixture. Three steps, tandem-FPLC, SDS-PAGE and reverse-HPLC, have been employed for the separation of the membrane proteins here. The data acquired from LC–MS/MS showed that the mouse liver proteins were well resolved following these multiple separations, and most of them were membrane-integral or membrane-associated components with high GRAVY scores. This strategy is expected to be potentially useful in the analysis of the insoluble proteins. And it is also useful for membrane biomarker discovery and measurement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.005.

References

[1] D. Josic, J.G. Clifton, Proteomics 7 (2007) 3010.
[2] W. Dormeyer, D. van Hoof, S.R. Braam, A.J. Heck, C.L. Mummery, J. Krijgsveld, J. Proteome Res. (2008) 2936.
[3] S. Tan, H.T. Tan, M.C. Chung, Proteomics 8 (2008) 3924.
[4] A.L. Hopkins, C.R. Groom, Ernst Schering Res Found Workshop, 2003, p. 11.
[5] A. Overbye, M. Fengsrud, P.O. Seglen, Autophagy 3 (2007) 300.
[6] J. Xie, B.F. Yu, J. Xu, Y.H. Zhang, N.L. Cheng, B. Niu, X.N. Hu, Q. Xiang, Z.G. Zhang, Hepatobiliary Pancreat. Dis. Int. 4 (2005) 90.
[7] S.P. Gygi, G.L. Corthals, Y. Zhang, Y. Rochon, R. Aebersold, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 9390.
[8] M. Chevallet, V. Santoni, A. Poinas, D. Rouquie, A. Fuchs, S. Kieffer, M. Rossignol, J. Lunardi, J. Garin, T. Rabilloud, Electrophoresis 19 (1998) 1901.
[9] T. Rabilloud, Methods Mol. Biol. 112 (1999) 9.
[10] R.P. Zahedi, C. Meisinger, A. Sickmann, Proteomics 5 (2005) 3581.
[11] I. Lehner, M. Niehof, J. Borlak, Electrophoresis 24 (2003) 1795.
[12] R. Henningsen, B.L. Gale, K.M. Straub, D.C. DeNagel, Proteomics 2 (2002) 1479.
[13] A. Intoh, A. Kurisaki, Y. Yamanaka, H. Hirano, H. Fukuda, H. Sugino, M. Asashima, Proteomics 9 (2009) 126.

[14] B. Rietschel, T.N. Arrey, B. Meyer, S. Bornemann, M. Schuerken, M. Karas, A. Poetsch, Mol. Cell. Proteomics (2008).
[15] N. Zhang, R. Chen, N. Young, D. Wishart, P. Winter, J.H. Weiner, L. Li, Proteomics 7 (2007) 484.
[16] J. Zhao, T. Izumi, K. Nunomura, S. Satoh, S. Watanabe, Biochem. J. 408 (2007) 51.
[17] M.P. Washburn, D. Wolters, J.R. Yates III, Nat. Biotechnol. 19 (2001) 242.
[18] D.A. Wolters, M.P. Washburn, J.R. Yates III, Anal. Chem. 73 (2001) 5683.
[19] M.J. MacCoss, W.H. McDonald, A. Saraf, R. Sadygov, J.M. Clark, J.J. Tasto, K.L. Gould, D. Wolters, M. Washburn, A. Weiss, J.I. Clark, J.R. Yates III, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 7900.
[20] T. Prinz, J. Muller, K. Kuhn, J. Schafer, A. Thompson, J. Schwarz, C. Hamon, J. Proteome Res. 3 (2004) 1073.
[21] H. Everberg, U. Sivars, C. Emanuelsson, C. Persson, A.K. Englund, L. Haneskog, P. Lipniunas, M. Jornten-Karlsson, F. Tjerneld, J. Chromatogr. A 1029 (2004) 113.
[22] J. Blonder, L.R. Yu, G. Radeva, K.C. Chan, D.A. Lucas, T.J. Waybright, H.J. Issaq, F.J. Sharom, T.D. Veenstra, J. Proteome Res. 5 (2006) 349.
[23] A.S. Fandino, I. Rais, M. Vollmer, H. Elgass, H. Schagger, M. Karas, J. Mass Spectrom. 40 (2005) 1223.
[24] T.T. Tran, V.C. Phan, Nanosci. Nanotechnol. 1 (2010) 1.
[25] P.J. Kersey, J. Duarte, A. Williams, Y. Karavidopoulou, E. Birney, R. Apweiler, Proteomics 4 (2004) 1985.
[26] I. Panfoli, L. Musante, A. Bachi, S. Ravera, D. Calzia, A. Cattaneo, M. Bruschi, P. Bianchini, A. Diaspro, A. Morelli, I.M. Pepe, C. Tacchetti, G. Candiano, J. Proteome Res. 7 (2008) 2654.
[27] K. Bych, S. Kerscher, D.J. Netz, A.J. Pierik, K. Zwicker, M.A. Huynen, R. Lill, U. Brandt, J. Balk, EMBO J. 27 (2008) 1736.
[28] H.J. Lee, M.S. Kwon, E.Y. Lee, S.Y. Cho, Y.K. Paik, Proteomics 8 (2008) 2168.
[29] Y.Y. Hwang, M.D. Li, Proteomics 6 (2006) 3138.
[30] R.D. Bagshaw, D.J. Mahuran, J.W. Callahan, Mol. Cell. Proteomics 4 (2005) 133.
[31] H. Zhang, X. Fan, R. Bagshaw, D.J. Mahuran, J.W. Callahan, Methods Mol. Biol. 432 (2008) 229.
[32] H. Riezman, Cell 130 (2007) 587.
[33] P.J. Kowalski, M.A. Rubin, C.G. Kleer, Breast Cancer Res. 5 (2003) R217.
[34] K.A. Voutilainen, M.A. Anttila, S.M. Sillanpaa, K.M. Ropponen, S.V. Saarikoski, M.T. Juhola, V.M. Kosma, J. Clin. Pathol. 59 (2006) 460.
[35] E. Ferruzzi, R. Franceschini, G. Cazzolato, C. Geroni, C. Fowst, U. Pastorino, N. Tradati, J. Tursi, R. Dittadi, M. Gion, Eur. J. Cancer 39 (2003) 1019.
[36] L. Schumaker, N. Nikitakis, O. Goloubeva, M. Tan, R. Taylor, K.J. Cullen, Clin. Cancer Res. 14 (2008) 5877.
[37] J.G. Clifton, X. Li, W. Reutter, D.C. Hixson, D. Josic, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 849 (2007) 293.
[38] C. Pasquali, I. Fialka, L.A. Huber, Electrophoresis 18 (1997) 2573.
[39] J. Peng, J.E. Elias, C.C. Thoreen, L.J. Licklider, S.P. Gygi, J. Proteome Res. 2 (2003) 43.
[40] R.J. Chalkley, P.R. Baker, L. Huang, K.C. Hansen, N.P. Allen, M. Rexach, A.L. Burlingame, Mol. Cell. Proteomics 4 (2005) 1194.
[41] C.G. Huber, W. Walcher, A.M. Timperio, S. Troiani, A. Porceddu, L. Zolla, Proteomics 4 (2004) 3909.
[42] A.K. Ottens, F.H. Kobeissy, R.A. Wolper, W.E. Haskins, R.L. Hayes, N.D. Denslow, K.K. Wang, Anal. Chem. 77 (2005) 4836.