Journal of Chromatography B, 901 (2012) 18–24

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Shotgun analysis of membrane proteomes using a novel combinative strategy of solution-based sample preparation coupled with liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 13 February 2012
Accepted 29 May 2012
Available online 4 June 2012

Keywords:
Membrane proteome
Sodium dodecyl sulfate
Acetone precipitation
Sodium deoxycholate
In-solution digestion
LC–MS/MS

ABSTRACT

Although much effort has been made in the field of membrane proteomics, the analysis of membrane proteins particularly integral membrane proteins with poor water solubility still presents a great challenge. In this paper, 2% SDS was used to extract membrane proteins and experimental conditions for the application of acetone precipitation method to the cleanup of SDS-solubilized membrane protein sample were optimized. For improving the re-dissolution and trypsinolysis of acetone-precipitated proteins, several commonly used additives, urea, methanol and sodium deoxycholate (SDC), were employed and compared. The results showed that, when the pre-cooled acetone-to-sample ratio was 6:1 (v/v) with one additional washing step, residual SDS in the protein sample could be lowered to below 0.01% and more than 90% of the proteins were precipitated and therefore recovered. 1% SDC-containing buffer could improve the re-dissolution and digestion of the acetone precipitated proteins more efficiently than the others. Using the combinative sample preparation strategy developed, 398 proteins were identified from the rat liver membrane-enriched fraction, including 188 membrane proteins. Compared with other three representative solution-based sample preparation methods commonly used in membrane proteomics, the newly developed combinative strategy increased the number of identified total proteins and membrane proteins on average by 29.2% and 28.5%, respectively. This combinative strategy was demonstrated to be easily operated at low cost and suitable for the analysis of membrane proteins varying in type and sample volume, etc.

1. Introduction

Membranes are critical components of cellular structure and function involving the partitioning of organelles, protecting the integrity of genome and proteome, and providing defense against foreign molecules and external conditions that may damage or destroy the cell [1]. Since the membranes incorporate a variety of proteins that perform cellular functions, it is of importance to analyze the membrane proteomes. However, despite the biological importance of membrane proteins have been widely realized and protein chemistry and proteomic technologies have been greatly developed in recent years, the analysis of membrane proteins has lagged behind that of soluble proteins and still presents a great challenge mainly because of the highly hydrophobic nature of many membrane proteins, which leads to difficulties in their extraction/solubilization and also in subsequent protease digestion in pure aqueous buffers [2,3]. To overcome these problems, a variety of methods have been developed to improve the solubilization and digestion of membrane proteins, including the use of chaotrope mixtures, detergents, organic acids, aqueous-organic solvents, etc. [4–7].

Recently, solution-based shotgun proteomic analysis has emerged as a high-throughput and powerful technique for proteomics-based biological discovery [8]. When the strategy is applied to the analysis of membrane proteomes, the additives to be used for extracting and solubilizing membrane proteins must be carefully selected because most of them may be incompatible with the subsequent protease digestion and/or mass spectrometric

Abbreviations: CapLC–MS/MS, capillary liquid chromatography–tandem mass spectrometry; HCT, high capacity ion trap; IPI, international protein index; TMD, transmembrane domain; CAP, pre-cooled acetone precipitation; SDC, sodium deoxycholate; NDD, SDD, UDD and MDD, are methods for the dissolution and digestion of acetone-precipitated proteins in pure NH_4HCO_3 buffer and NH_4HCO_3 buffers with added SDC, urea and methanol, respectively.

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1570-0232/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2012.05.035

analysis, which will lead to poor results of protein identification [1,9]. Masuda et al. evaluated the solubilization ability of 27 additives including commonly used surfactants, chaotropes or organic solvents for membrane-enriched fraction of *Escherichia coli* lysate, and the results showed that anionic detergent SDS had the strongest ability to solubilize the proteins in a membrane fraction [10]. Reynolds and Tanford also demonstrated that SDS was an extremely efficient solvent at solubilizing and denaturing proteins and could dissolve a wider range of proteins, including misfolded and precipitated proteins [11]. Unfortunately, it can be quite problematic to apply SDS in solution-based shotgun proteomics because a slightly high concentration of SDS can severely reduce the activity of proteolytic enzymes [12], interfere with the chromatographic separation of the digests [13] and suppress the ionization of the peptides by MALDI or ESI [14–16]. To overcome the problem, some researchers have attempted to replace SDS with enzyme activity- and/or mass spectrometry (MS)-compatible additives such as sodium deoxycholate (SDC) [17], urea [18] and methanol [1]. However, these alternative additives were found to have one major drawback that their ability to disrupt the membranes and extract highly hydrophobic proteins such as the proteins with multiple transmembrane domains is weaker than that of SDS [10].

In view of the facts, much effort has been made to remove the SDS from the SDS-solubilized protein samples (i.e., sample cleanup). Many conventional and newly developed methods have been employed, including dialysis, precipitation with organic solvent or organic acid, as well as column-, gel- and spin filter-based approaches (such as ion exchange, hydrophilic interaction chromatography, gel filtration, tube gel digestion and spin filter microcentrifugation) [19–23]. Although dialysis and column-based approaches have obtained some applications in the cleanup of protein samples, these methods might not be suitable for high-throughput proteomic analysis because the large volume and complex operations introduced by these methods could result in significant sample and time loss during SDS removal and/or proteins concentration [19]. The tube gel-based SDS removal method could also lead to a certain sample and time loss [22]. Furthermore, the protein samples cleaned up by the gel-based method generally had to be digested in the gel, which had some inherent limitations such as low accessibility of proteases to the deeply gel-entrapped proteins that would lead to low cleavage yields of some proteins and low recovery of proteolytic peptides of large size and/or high hydrophobicity. Although the spin filter-based SDS removal methods such as filter-aided sample preparation (FASP) strategy [23] have seen some applications to the sample preparation for shotgun proteomics, these methods have their inherent limitations such as: (1) the peptide/protein recovery is low due to the strong binding of proteins and peptides to the spin filters. Therefore, it is not suitable for the analysis of small amounts of protein samples (<50 μg); (2) there are difficulties in the removing of detergents from the highly hydrophobic proteins due to their tight binding [24]. The approach is useful in some applications, but is not necessarily "universal" as Wiśniewski et al. suggested [23,24]. In practice, all the reported methods have problems in seeking the balance point between eliminating the interfering substances and reducing the sample loss. In view of these reasons, protein sample cleanup by precipitation with organic solvents, especially with cold acetone, has attracted special attention and obtained some applications [25,26]. The method operated simply and could reduce the concentration of small-molecular-weight interfering substances in protein samples, thus reducing the effects of these substances on subsequent digestion and mass spectrometric analysis. However, for different protein samples, the required experimental conditions are different. In particular, when the method is applied to the cleanup of SDS-solubilized membrane proteome samples, a series of problems exist such as how to precipitate/recover proteins and remove SDS efficiently, how to re-dissolve and digest the precipitated proteins with high efficiency. In the present study, we sought to optimize the experimental conditions for the application of acetone precipitation method to the cleanup of SDS-solubilized membrane protein samples, use enzyme- and MS-compatible detergents to overcome the problems in the re-dissolution and digestion of acetone precipitated proteins, and thus develop a solution-based combinative strategy that comprehensively utilizes the advantages of selected detergents and optimized sample cleanup method to efficiently improve the shotgun analysis of membrane proteomes.

2. Materials and methods

2.1. Materials and chemicals

Proteomics sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Acetone, Stains-all and sodium deoxycholate (SDC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acrylamide, bisacrylamide, glycine, Tris and sodium dodecyl sulfate (SDS) were from Amresco (Solon, OH, USA). Ammonium persulfate (AP) and N, N, N′, N′-tetramethylethylenediamine (TEMED) were obtained from Amedham Pharmacia Biotech (Uppala, Sweden). Bio-Rad RC DC protein Assay kit was from Bio-Rad (Hercules, CA, USA).

2.2. Preparation and cleanup of rat liver membrane-enriched sample

Rat liver cellular membranes-enriched sample was prepared according to the procedure described previously [27,28]. Briefly, rats were killed after being starved for 18–24 h and the livers were excised. After removal of gall bladder and blood vessels, the liver pieces were homogenized on ice with four times their weight of a cooled solution (50 mM HEPES, 1.0 mM $CaCl_2$ and 0.1 mM PMSF, pH 7.4) with a Tissue Tearor (Biospec products, CE 2000, Mexico) at 20,000 rpm until completely liquefied. The mixture was added to 50-mL conical tubes and centrifuged at 600 × *g* for 20 min at 4 °C. The pellet was repeatedly treated as above and the supernatants of centrifugations were pooled and then centrifuged at 24,000 × *g* (Ti70 rotor, Beckman, Fullerton, CA, USA) for 30 min at 4 °C. The supernatants were discarded and the pellets were mixed with 69% sucrose, on the top of which 44, 41 and 37% sucrose solutions were carefully layered sequentially. After centrifugation at 100,000 × *g* (SW28 rotor, Hitachi, Tokyo, Japan) for 2.5 h, the membrane fraction at the interface between 37 and 41% sucrose solutions was immediately collected and washed with 1.0 mM sodium bicarbonate solution for three times. After centrifugation at 100,000 × *g*, the pellets were collected and stored at −80 °C until use. All procedures conformed to the Guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Beijing, China). The protein content of the sample was determined using Bio-Rad RC DC protein Assay kit with BSA as a standard protein.

For optimizing the conditions for sample cleanup and protein recovery by pre-cooled acetone precipitation, six aliquots of the membrane-enriched sample (about 90 μg of proteins each) were separately solubilized with 30 μL of 50 mM NH_4HCO_3 solution containing 2% SDS, sonicated twice in a water bath (each for 10 min) and centrifuged for 15 min at 13,000 rpm. The proteins in supernatants were precipitated for 4 h at −20 °C by addition of pre-cooled acetone at different volume ratios over the sample (1:1, 2:1, 4:1, 6:1, 9:1 and 12:1) to screen for the optimal ratio for efficient SDS removal and protein recovery. Then all the sample mixtures were centrifuged for 15 min at 13,000 rpm and the top

layers were carefully removed, leaving behind ~20 μL in the vial lest the precipitates should be aspirated. Additional washing of the pellet was executed using 400 μL of pre-cooled acetone, followed by centrifugation at 13,000 rpm and removal of the acetone layer in the same way. The residual SDS in the precipitated proteins was quantitatively assessed by a spectrophotometric assay according to the method described previously by Rusconi et al. [29]. The quantitation is based on a dye, Stains-all, the color of which changes from intense fuchsia to yellow upon addition of SDS. Briefly, 1 mg Stains-all dye was dissolved in 1 mL 50% dimethyl carbinol as stock solution. And then prepare coloration solution with stock solution, formamide and ddH_2O (1:1:18). Then 6 tubes were prepared for the establishment of SDS standard curve, which contained 1 μL, 4 μL, 8 μL, 12 μL, 16 μL, and 18 μL of 0.01% SDS stock solution, respectively. ddH_2O was added to each tube to a final volume of 20 μL. 200 μL of coloration solutions were added into each tube and the tubes followed by vortexing. The precipitated proteins and the control (without acetone precipitation treatment) were sampled and prepared as above. Light absorption values of all measurements at 438 nm were recorded with a spectrophotometer. At the same time, the protein contents in the pellet formed at the different acetone/sample volume ratios were quantitatively analyzed with a Bio-Rad RC DC protein Assay kit. All the above the experiments were repeated three times.

2.3. Re-dissolution and in-solution digestion of acetone-precipitated proteins

For investigating the re-dissolution and in-solution digestion of the proteins precipitated by optimized acetone precipitation, four different buffers were used and compared: 50 mM NH_4HCO_3, 1% SDC/50 mM NH_4HCO_3, 8 M urea/50 mM NH_4HCO_3 (diluted to 2 M prior to digestion) and 60% methanol/50 mM NH_4HCO_3. The four dissolution and digestion methods were shortly called NDD, SDD, UDD and MDD, of which N, S and U stand for NH_4HCO_3, SDC and urea, respectively, and DD for dissolution and digestion (Supplementary Fig. 1). After adding the buffer to an aliquot of precipitated protein sample, the solution was sonicated twice in a water bath (each for 10 min). Proteins were reduced with 5 mM DTT for 60 min, and then alkylated in the dark with 25 mM IAA for 45 min at room temperature. Trypsin was added at an enzyme-to-protein ratio of 1:50 and incubated at 37 °C for 16 h. Following the digestions, all reaction mixtures were acidified with 0.1% TFA to inhibit any remaining enzyme activity, and centrifuged at 15,000 × *g* for 10 min to remove insoluble materials. Then the supernatants in all methods were separately collected and concentrated in a Speed-Vac (Labconco, Kansas, MO, USA) and analyzed by CapLC–MS/MS for protein identification. It is worth noting that the surfactant SDC in the digests from the SDD method was also removed by above acidification and centrifugation because it precipitates under low-pH conditions [17].

2.4. On-line CapLC–ESI–MS/MS analysis

Tryptic digests of proteins in rat liver membrane-enriched fraction prepared with above different methods were dissolved in 30 μL of 0.1% formic acid (FA) and analyzed by an online Agilent 1200 capillary liquid chromatography system (Agilent Technologies, Waldbronn, Germany) coupled to a high-capacity ion trap mass spectrometer (HCTultra™, Bruker Daltonics, Bremen, Germany). Digests were pre-concentrated and desalted with 0.1% FA at a flow rate of 20 μL/min on a short C18 precolumn Zorbax SB (500 μm i.d., 3.5 cm length, Agilent) connected in front of an analytical capillary column (C18 PepMap, 180 μm i.d., 15 cm length, LC Packings–Dionex, Sunnyvale, CA, USA). Then peptides were separated on the analytical column with a linear gradient (5–40% solvent B in solvent A over 140 min) at a flow rate of 3.0 μL/min. Solvent A was 0.1% FA, and solvent B was 0.1% FA in ACN. The mass spectrometer was operated in positive ion mode at a 4000 V capillary voltage. Nebulizer pressure was 10 psi. Drying gas flow rate was 5 L/min. Dry gas temperature was 250 °C. The mass spectrometer was set so that one full MS scan with a scanning speed of 8100 (*m*/*z*)/s (stand enhanced mode, *m*/*z* 350–1600) was followed by four MS/MS scans at a scan rate of 26,000 (*m*/*z*)/s (ultra scan mode, *m*/*z* 100–2000) on the four most intense ions with the following dynamic exclusion settings: repeat spectrum 2, exclusion duration time 120 s. To generate fragment ions, low-energy collision-induced dissociation (CID) was performed on isolated charged peptide ions with a fragmentation amplitude of 1.05 V. System control and data collection were done by Esquire Control software (version 6.0, Bruker Daltonics).

2.5. Data processing and bioinformatics analysis

Raw spectral data were processed and Mascot compatible mgf files were created using DataAnalysis™ 3.4 software (Bruker Daltonics, Bremen, Germany) with the following parameters: compounds threshold 10,000, retention time windows 1.0 min, maximum number of compounds 100,000. Searches were performed using Mascot™ 2.2 software (Matrixscience, London, UK). The international protein index (IPI) rat database (IPI_rat_v3.70) downloaded as FASTA-formatted sequences were used for protein identification. Search parameters were set as follows: enzyme, trypsin; allowance of up to one missed cleavage peptide; MS mass tolerance, 1.2 Da and MS/MS mass tolerance, 0.6 Da; fixed modification, carbamidomethylation (C); variable modification, oxidation (M). Proteins were generally identified on the basis of two or more peptides whose ions scores exceeded the threshold, $P < 0.05$, which indicated identification at the 95% confidence level. If proteins were identified by a single peptide, the spectrum was manually inspected. For a protein to be confirmed, the assignment had to be based on four or more y- or b-series ions (e.g., y4, y5, y6, y7). The subcellular location and function annotations of these identified proteins with Gene Ontology (GO) terms were taken from UniProt Knowledgebase (UniProtKB) [30]. The average hydrophobicity expressed as grand average of hydropathy (GRAVY) value [31] for identified proteins and peptides were calculated using the ProtParam software, available at http://cn.expasy.org. Proteins and peptides with positive GRAVY values are considered to be hydrophobic and negative values, hydrophilic. Predictions for putative transmembrane domains (TMDs) in all identified proteins were carried out using the transmembrane hidden markov model (TMHMM) algorithm [32], available at http://www.cbs.dtu.dk/services/TMHMM. False positive rates were evaluated by using the reversed sequence databases search strategy [33].

3. Results

3.1. SDS removal and protein recovery by pre-cooled acetone precipitation method

In membrane proteomic researches, SDS as a significant additive has been widely used for the extraction and solubilization of membrane proteins. However, with little debate, SDS is recognized to cause significant ion suppression in mass spectrometric analyses if its concentration has not been decreased to a certain degree before Cap-LC/MS/MS analysis. Botelho et al. had showed that, as observed in the total ion chromatogram, SDS at concentrations up to 0.01% only caused a slight drop in signal intensity in a

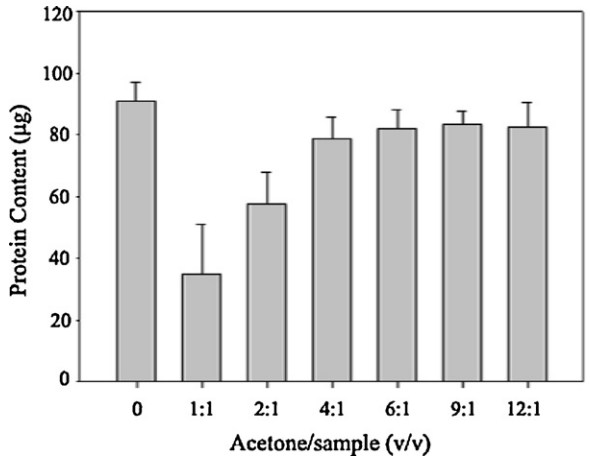

Fig. 1. Quantitative analysis of the protein levels in pellets after acetone precipitation with different acetone-to-sample ratios. The ratio of 0 represents that the sample was not treated by acetone precipitation after being solubilized by 2% SDS.

proteome experiment of the coupling of low flow HPLC with MS through electrospray interfaces. However, when the concentration was increased to 0.02%, signal suppression was evident [25]. Therefore, we believe that a suitable method for SDS reduction should reliably reduce the SDS content to below 0.01%, while maintaining a high protein recovery. In order to screen for the optimal conditions of SDS removal and protein recovery by acetone precipitation, we dissolved the membranes by 2% SDS to prepare the membrane protein samples (the final concentration of SDS was about 1.7%), and then added pre-cooled acetone in different volume ratios to the samples, each with one additional washing step. The quantitative results (Supplementary Fig. 2) showed that, as acetone-to-sample ratios (v/v) were increased, the residual SDS in the sample was gradually reduced. When the ratio was increased to 6:1, the remaining SDS was below 0.01%, which was the tolerable level by electrospray mass spectrometric analysis.

For finding out the optimal acetone-to-sample ratios for protein recovery, the contents of proteins precipitated at different acetone-to-sample ratios were comparatively determined using a Bio-Rad RC DC protein Assay kit (Fig. 1). The quantitative determination showed that the protein recovery could be raised by increasing ratios from 1:1 to 9:1. When the ratio was increased to 6:1, the content of protein in the pellet sample was close to the maximum value, with more than 90% of the proteins being precipitated and therefore recovered. For reliably reducing the SDS content to below 0.01%, maintaining high protein yield and saving the cost of the experiments, the pre-cooled acetone-to-sample volume ratio of 6:1 (with one additional washing step) was selected as the optimized acetone precipitation conditions and was employed for the further experiments.

3.2. Re-dissolution and digestion of acetone-precipitated proteins in four different methods

Re-dissolution of the precipitated proteins was a key determinant for efficient digestion and identification of the proteins. For enhancing the solubility of precipitated proteins, besides NDD method, we also used SDD, UDD and MDD methods. When aliquots of pre-cooled acetone precipitated-proteins were separately suspended and sonicated in the different buffers, it was found that most of the protein pellet could not be dissolved by the pure 50 mM NH_4HCO_3 or 60% methanol/50 mM NH_4HCO_3 and there were many small protein particles visible in the cloudy suspension, whereas 8 M urea/50 mM NH_4HCO_3 could dissolve most of the precipitated proteins, and 1% SDC/50 mM NH_4HCO_3 could completely dissolve the precipitated proteins, producing a very limpid solution. It is worth mentioning that, as the digestion went on, the suspended particles in the solutions gradually reduced or disappeared, demonstrating that the action of trypsin was helpful to the depolymerization and solubilization of suspended protein particles.

The number of identified unique peptides and the proportion of peptides containing one missed cleavage site in the total identified unique peptides (up to one missed cleavage site was allowed when searching against database) could be used as a criterion for evaluating protein digestion [34]. Under the same conditions, more unique peptides identified with lower proportion of missed cleavage peptides indicated the better digestion. CapLC–MS/MS analysis of tryptic peptides prepared in the four different methods (NDD, SDD, UDD and MDD), whose representative total ion chromatograms are shown in Supplementary Fig. 3, in combination with database search led to the identification of 1153, 1441, 1112 and 833 unique tryptic peptides (Table 1), of which 1080 (93.7%), 1346 (93.4%), 1032 (92.8%) and 762 (91.5%) were completely digested (0 missed cleavage site), respectively. A higher proportion of peptides with one missed cleavage site were generated in the UDD and MDD methods (7.2% and 8.5%, respectively) (Fig. 2). These comparative data showed that NDD and SDD methods had certain superiorities over the other two methods in facilitating the digestion of acetone-precipitated proteins.

3.3. Comparative analysis of the proteins identified based on different digestion methods

In order to evaluate the effects of different digestion methods on protein identification, the proteins and their matching peptides identified from rat liver membrane-enriched fraction based on the four methods were analyzed and compared (Table 1 and Supplementary Table S1). The false-positive rate was evaluated as below 3% by using the reversed sequence databases search strategy [33]. After removal of the false-positive results, it was shown that totals of 340, 398, 318 and 266 proteins were identified based on

Table 1
Statistical analysis of proteins and their matching peptides identified from rat liver membrane-enriched fraction based on four different digestion methods.[a]

Categorization	Strategies			
	NDD	SDD	UDD	MDD
All identified proteins	340	398	318	266
All identified peptides	1153	1441	1112	833
Peptides/proteins	3.39	3.62	3.50	3.13
Membrane proteins	158	188	154	127
Plasma membrane proteins	53	68	49	37
Hydrophobic proteins	95	110	93	75
Proteins with 1 or more predicted TMDs	116	142	105	82
Proteins with more than 1 predicted TMDs	51	64	46	35

[a] A 20-μg aliquot of tryptic digests from rat liver membrane-enriched sample was used in each experiment in all methods for CapLC–MS/MS analysis and protein identification. The merged results from triplicate analysis in each method were used for comparison.

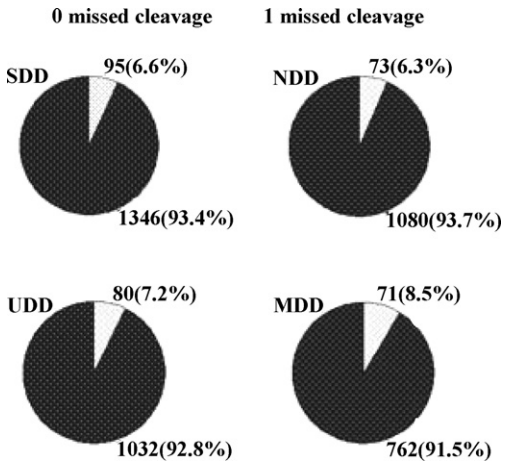

Fig. 2. Distributions of tryptic peptides with 0 and 1 missed cleavage site produced in four different methods from rat liver membrane-enriched fraction. The merged results from triplicate analysis in each method were used for comparison.

1153, 1441, 1112 and 833 peptides by NDD, SDD, UDD and MDD methods, respectively. Of the total proteins identified in the four methods, 158, 188, 154 and 127 were membrane proteins, of which 53 (33.5%), 68 (36.2%), 49 (31.8%) and 37 (29.1%) were annotated as plasma membrane proteins, respectively. Compared with the other three methods, SDD method increased the number of total identified proteins on average by 29.2%, peptides by 39.5%, membrane proteins by 28.5%, and plasma membrane proteins by 50.0%. Furthermore, when these proteins in rat liver membrane-enriched sample were identified, the average number of unique peptides per identified protein in SDD method (3.62) was higher than those in the other three methods (3.39, 3.50 and 3.13, respectively), suggesting that the proteins identified in SDD method had higher coverage and thus the reliability. These results showed that SDD method was more efficient for the re-dissolution, in-solution digestion and identification of acetone-precipitated proteins, including multi-transmembrane membrane proteins Interestingly, NDD method also had better protein identification results than UDD and MDD methods, which was presumably due to the fact that the clean NH_4HCO_3 buffer, compared with urea and methanol, had better compatibility with trypsin activity and mass spectrometric analysis.

For further probing into the possible biases of these methods in protein identification, the proteins identified in the four methods were analyzed and compared based on their main physicochemical properties (Fig. 3 and Supplementary Table S1). First, we analyzed and compared the distribution of the calculated molecular weight (MW) and isoelectric point (p*I*) of the identified proteins. As shown in Fig. 3A and B, there were no significant differences in the MW and p*I* distribution profiles among the four methods. Most of these identified proteins were distributed in the ranges of 20–80 kDa and p*I* 5–7 and 8–10. However, when compared with other methods (NDD, UDD and MDD), it was found that more proteins were identified by SDD method in nearly every part of MW and p*I* distribution range. Some proteins with extreme properties, such as very acidic or basic proteins and high MW proteins, were identified only in SDD method. For example, as shown in Supplementary Table S1, the protein 60S ribosomal protein L18 (IPI00230917) with a p*I* of about 12 was identified based on three unique peptides only in SDD method.

Furthermore, we also analyzed and compared the identified proteins on the basis of their calculated GRAVY values and predicted TMDs to assess the efficiencies of the four different digestion methods for the identification of proteins particularly integral membrane proteins with different hydrophobic properties. The identified proteins and peptides were first evaluated based on their GRAVY scores. GRAVY is a commonly used parameter to gauge the hydropathy of proteins or peptides. It is generally accepted that proteins or peptides with positive GRAVY scores are classified as hydrophobic proteins or peptides, whereas those with negative values were classified as hydrophilic [31]. In our experiments, a total of 95, 110, 93 and 75 proteins identified by the four methods (NDD, SDD, UDD and MDD, respectively) had positive GRAVY values (Table 1 and Supplementary Table S1). Then we categorized all of the identified proteins into six groups according to their GRAVY values: the proteins with GRAVY value <−0.5, −0.5 ~ −0.2, −0.2 ~ 0, 0 ~ 0.2, 0.2 ~ 0.5 and >0.5 (Fig. 3C). Compared with other three methods, SDD method led to more proteins to be identified in most groups, indicating that SDD method could improve the

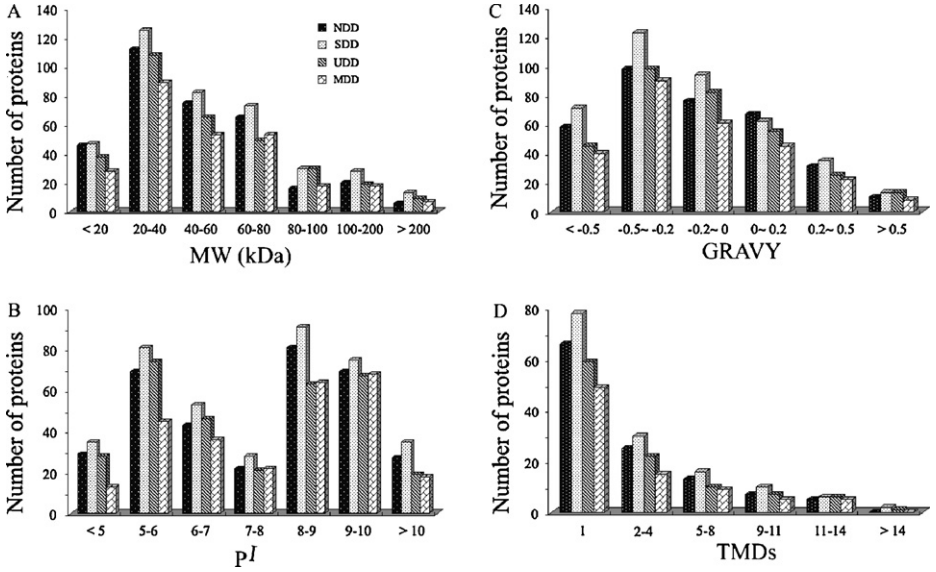

Fig. 3. Comparison of the distributions of proteins identified based on four different digestion methods. (A) Calculated molecular weight (MW). (B) Calculated isoelectric point (p*I*). (C) Calculated grand average of hydropathy (GRAVY) value. (D) Predicted transmembrane domains (TMDs). The merged results from triplicate analysis in each method were used for comparison.

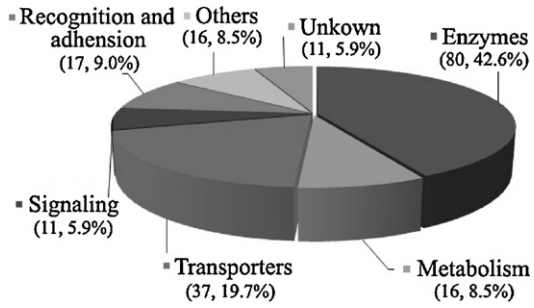

Fig. 4. Functional classification of the membrane proteins identified from rat liver membrane-enriched fraction with SDD-based combinative strategy. The merged results from triplicate analysis were used for comparison.

identification of both hydrophilic and hydrophobic proteins even better. In addition, NDD and UDD methods also gave rise to better efficiency than MDD method in the identification of both hydropholic and hydrophobic proteins.

For examining the efficiency of these methods in the identification of proteins with TMDs that usually endow the proteins with high hydrophobicity, the identified TMD-containing proteins were analyzed and compared. Prediction by the TMHMM 2.0 algorithm showed that a total of 116, 142, 105 and 82 proteins identified based on the four methods (NDD, SDD, UDD and MDD) respectively had transmembrane domains (TMDs), which contained 51 (44.0%), 64 (45.1%), 46 (43.8%) and 35 (42.7%) proteins with more than 1 predicted TMDs (Table 1 and Supplementary Table S1). The distributions of transmembrane proteins with different numbers of TMDs identified by the four methods are compared in Fig. 3D. The general distribution profiles of them were similar, and most of the identified TMD-containing proteins had 1–4 TMDs, followed by transmembrane proteins with 5–8 TMDs. More TMD-containing proteins were identified at almost every TMD number by SDD method. In particular, proteins with TMDs > 14 were identified only in SDD and UDD methods. For example, the protein NADH-ubiquinone oxidoreductase chain 5 (IPI00195892) with 15 TMDs was identified in SDD and UDD methods, and the 168 kDa protein (IPI00568685) with 16 TMDs was identified only in SDD method by four unique peptides. These data further indicated that, compared with other strategies, the SDD-based combinative strategy was even more favorable for the digestion and identification of proteins containing transmembrane domains particularly highly hydrophobic multiple transmembrane domains.

By using the optimized SDD-based combinative strategy, a total of 188 membrane proteins were identified from the rat liver membrane-enriched fraction. We categorized the GO function annotations of the identified proteins retrieved from UniProt Knowledgebase (UniProtKB) (Fig. 4), though the classification was not strict due to the fact that a protein usually has multiple functions. Of the identified proteins, 42.6% and 8.5% are enzymes and the proteins involved in metabolism respectively; 19.7% and 5.9% are involved in substance transport and signal transduction respectively; 17.5% are proteins involved in the cell recognition, adhesion and other cellular functions. In addition, 16 (8.5%) proteins have no function annotation and were classified into the group "unknown". From the data analysis we know that more than half of the identified membrane proteins are the enzymes and proteins involved in metabolism, and the proteins with transporter activity make up the second largest group, suggesting that the membrane proteome plays important roles in the substance exchange and metabolism in the cell. About 20.0% of the identified membrane proteins involve cell recognition, adhesion and signal transduction, reflecting the importance of membrane proteins in cell communication and function integration.

4. Discussion

Because current proteomic strategies limit the direct analysis of intact proteins, generation of detectable peptides by digestion is crucial to the identification of the proteins. Currently, proteins are digested primarily in solution (in-solution digestion) or in gel (in-gel digestion). Compared with the in-gel digestion, in-solution digestion can provide a liquid environment more suitable for subsequent digestion, which avoids some disadvantages of in-gel digestion method including low accessibility of proteases to the gel-entrapped proteins, low cleavage yields of some proteins and difficulties in extraction of large and/or hydrophobic peptides from the gel matrix [35]. For in-solution digestion of hydrophobic membrane proteins and other proteins difficult to dissolve, it is crucial to clean up the samples with a solution-based method after these proteins were extracted with strong detergents such as SDS.

Although in-solution digestion after acetone precipitation is not a novel concept, the systematic investigation on its application to the membrane proteome sample cleanup has not been reported. When the acetone precipitation-based strategy for membrane protein sample cleanup was used, the first concern was if SDS removal and protein precipitation were complete [25,36,37]. The former determined if the interference of SDS with the subsequent analyses could be avoided, and the latter was the main determinant of protein recovery. Acetone/sample ratio (v/v) is the most important factor affecting the efficiencies of SDS removal and protein recovery. Although different acetone/sample volume ratios were employed to precipitate proteins [25,38], our quantitative analyses demonstrated that acetone-to-sample ratio of 6:1 (with one additional washing step) was the optimal ratio. Under the optimal conditions, more than 90% protein were precipitated and the SDS concentration in membrane protein samples could be lowered to below 0.01% (Fig. 3), which would not obviously interfere with the subsequent digestion and RPLC–MS/MS analysis. Moreover, when removing the acetone layer after centrifugation, we permitted a slightly larger volume (~20 μL) of acetone supernatant to be remained in the vial, thus minimizing the risk of accidental aspiration of the protein pellet. Additionally, using properly higher centrifugal force and longer centrifugal time would be helpful to precipitate the proteins completely.

For sample cleanup strategy based on acetone precipitation, another mostly concerned issue is the efficiency of protein re-dissolution and digestion after the precipitation treatment. It was considered that some of the precipitated proteins were difficult to re-dissolve, which would affect the subsequent digestion and thus the identification of proteins [39,40]. Our experimental results demonstrated that the addition of trypsin- and mass spectrometry-compatible SDC at high concentration could more efficiently promote the re-dissolution and digestion of the precipitated proteins. Furthermore, digestion itself could improve the depolymerization and solubilization of the precipitated proteins.

The comparative analyses of the final results of protein identification showed that, compared with other methods, the SDD method had the best identification efficiency and was more suitable for the analysis of the proteins including highly hydrophobic or multiple transmembrane proteins. The explanations for this might be that the method had several advantages simultaneously. First, SDC could completely re-dissolve the acetone-precipitated proteins; second, SDC was excellently compatible with the activity of trypsin and could efficiently facilitate the depolymerization and digestion of acetone-precipitated protein; third, SDC could be efficiently removed by centrifugation following acidification, thereby avoiding its possible effect on the subsequent LC–MS/MS analysis.

In conclusion, by combining the advantages of SDS, optimized acetone precipitation method and SDC in extraction, cleanup and the re-dissolution and enzymolysis of the membrane proteins,

an entirely solution-based sample preparation strategy for the analysis of membrane proteomes was developed. This strategy overcomes some inherent limitations of the conventional sample preparation methods and is easily operated at low cost and suitable for the analysis of membrane proteomes varying in type and sample volume, etc.

Acknowledgements

This work was supported by grants from Hunan Provincial Natural Science Foundation of China (11JJ2019), National Natural Science Foundation of China (31070700, 31100502), Specialized Research Foundation for the doctorial Program of Higher Education of China (20094306110003, 20114320120004), and National Basic Research Program or "973 Program" of China (2010CB529800).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.035.

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