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Shotgun analysis of membrane proteomes by an improved SDS-assisted sample preparation method coupled with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Analysis of the membrane proteins, particularly the integral membrane proteins, is limited by the inherent membrane hydrophobicity. Sodium dodecyl sulfate (SDS) is one of the most efficient reagents used for the extraction of membrane proteins, but its presence in samples interferes with LC–MS-based proteomic analyses because it affects RP-LC separations and electrospray ionization. In this paper, we present an improved sample preparation strategy based on SDS-assisted digestion and peptide-level SDS-removal using an optimized potassium dodecyl sulfate (KDS) precipitation method (SSDP method) for shotgun analysis of the membrane proteome. This method utilizes a high concentration of SDS (1.0%) to lyse the membranes and to solubilize the hydrophobic membrane proteins, resulting in a more complete protein digestion in the diluted SDS buffer (0.1% SDS), and a high efficiency of SDS removal and peptide recovery by the optimized KDS precipitation for protein identification. The SSDP method provides evidence that proteins can be efficiently digested, and the SDS can be decreased to <0.01% allowing >95% peptide recovery. Compared to other sample preparation methods commonly used in shotgun membrane proteomics, the newly developed method not only increased the identified number of the total proteins, membrane proteins and integral membrane proteins by an average of 33.1%, 37.2% and 40.5%, respectively, but also leading to the identification of highest number of matching peptides. All the results showed that the method yielded better recovery and reliability in the identification of the proteins especially the highly hydrophobic integral membrane proteins, and thus providing a promising tool for the shotgun analysis of membrane proteome.

1. Introduction

Membrane proteins are critical components of cellular structure and function, playing central roles including cellular homeostasis, signal transduction, cell–cell recognition and host defense [1–3]. A comprehensive analysis of membrane proteins would facilitate our understanding of their roles in cell life. However, the highly hydrophobic nature of many membrane proteins, especially integral membrane proteins (IMPs) brings great difficulties to their extraction, solubilization, enzymolysis and identification [4,5]. Recently, the solution-based shotgun approach has emerged as a powerful technique for membrane proteome identification and quantification [6–9], avoiding some of the limitations of the gel-based approach, such as low accessibility of proteases to deeply gel-entrapped proteins, leading to a low cleavage yield and low recovery of large and/or highly hydrophobic tryptic peptides [10,11]. In the method, proteins can be cleaved into small peptides using in-solution enzymatic digestion, followed by the separation and identification by high performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS). Therefore, for solution-based shotgun analysis of membrane proteome, improving the solubility of such hydrophobic proteins in a suitable solvent system with good compatibility with enzymatic

Abbreviations: KDS, potassium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RPLC–MS/MS, reversed-phase liquid chromatography–tandem mass spectrometry; GO, gene ontology; GRAVY, grand average hydropathy value; TMD, transmembrane domain; SSD, SRD, SUD or SMD, sample preparations based on SDS-, RapiGest-, urea- or methanol-assisted digestion; SSDP, sample preparation based on SDS-assisted digestion followed by KDS precipitation; FASP, filter-aided sample preparation.

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digestion and mass spectrometric analysis is of great significance.

In order to improve the solubilization and digestion of membrane proteins, a variety of additives have been investigated, including surfactants, organic solvents and chaotropic reagents [12]. Among them, sodium dodecyl sulfate (SDS) is the most efficient in the extraction/solubilization of membrane proteins [13], and is particularly useful for IMPs, which are usually difficult to extract and solubilize using other reagents. Unfortunately, it can be quite problematic to apply SDS in solution-based shotgun proteomics because the increase of SDS concentration can reduce the activity of proteolytic enzymes [14], interfere with the chromatographic separation of the digestion products [15] and severely suppress ionization of the peptides by electrospray ionization (ESI) [16,17]. To overcome this problem, enzyme activity- and/or mass spectrometry (MS) compatible additives, such as RapiGest SF (an acid-labile surfactant), urea, and methanol have been widely applied to shotgun membrane proteomics. RapiGest SF, as a representative of enzyme-friendly and MS-compatible surfactants, is among the most promising additives to improve shotgun analysis of the membrane proteome. It efficiently solubilizes proteins without inhibiting trypsin activity, and does not interfere with mass spectrometric analysis as it degrades rapidly under low-pH conditions [14]. Urea is a chaotrope that can bind to the proteins and compete with native interactions [18], thereby actively participating in the unfolding process of proteins without interfering with peptide analysis by standard LC–MS/MS because it does not bind to reversed-phase (RP) resins and is readily removed before the peptides are eluted from the column. Moreover, the use of many organic solvents for protein solubilization and proteolysis has also been reported [19,20]. For example, 60% methanol has often been used to extract membrane proteins and Blonder et al. [21] demonstrated it could be used to efficiently isolate and solubilize the hydrophobic integral membrane proteins from complex mixtures. However, compared to SDS, the major drawback of these alternative additives is that their weaker ability to solubilize membranes and extract highly hydrophobic proteins [13].

In view of these finding, the benefit of SDS for protein extraction, solubilization and denaturation can still be realized in shotgun membrane proteomics if we able to find an appropriate method to reduce the concentration of SDS prior to digestion and RPLC–MS/MS analysis. Dilution is a commonly used method, where the concentration of SDS is diluted to 0.1% from high concentrations (e.g. 1.0%) for effective tryptical digestion [20,22,23]. However, to avoid interference with the subsequent LC–MS/MS analysis, the SDS content must be diluted to below 0.01% [24]. This dilution creates a very large volume of sample, making it not suitable for high-throughput analysis of small amounts of proteins or peptides. Theoretically, many conventional methods such as dialysis and chromatography could be used to remove small molecules like SDS from the protein or peptide samples. However, in practice these methods might not be good for high-throughput analysis because of the large volume, time-consuming operations and significant sample loss [24]. Recently, the spin filter-based SDS removal methods, such as the filter-aided sample preparation (FASP) strategy [25] have been developed and used in the field of proteomics. Nevertheless, these methods might not be more suitable for the analysis of membrane proteins and/or hydrophobic proteins as they have some inherent limitations including: (1) the recovery of the peptides/proteins, especially large or hydrophobic peptides/proteins, is low, due to the strong binding of these proteins and peptides to the spin filters; (2) there are difficulties in removing the SDS from the highly hydrophobic peptides/proteins due to their tight binding [26].

Very recently, Zhou et al. [27] reported a highly effective peptide-level SDS removal strategy using potassium dodecyl sulfate (KDS) precipitation from the SDS-solubilized whole cell lysate protein samples. With this method, SDS was effectively (>99.9%) removed from the peptides following digestion. Peptide recovery was >95%, and the method provided proteome coverage that was higher than that achieved by the FASP method. However, for different samples, the required experimental conditions were different, so the application of this method in the membrane protein sample cleanup is controversial, and their effect on the membrane proteome is still unknown.

Based on these observations, in the present study, we tried to evaluate the digestion efficiency and sought to optimize the conditions of the KDS precipitation method for SDS-solubilized membrane protein samples. Through comparison and optimization, we found that the SDS-assisted digestion method (e.g. extracted in 1.0% SDS and digested in 0.1% SDS) could provide the highest digestion efficiency, and KCl (0.5 M) added into the digests was enough to remove most of the dodecyl sulfate (DS) (residual SDS <0.01%) leading to a peptide recovery of >95%. High efficiencies of digestion, SDS-removal and peptide recovery and low concentration salt added into the samples also improved the results of subsequent RPLC–MS/MS analysis. Thus an improved SDS-assisted sample preparation strategy that comprehensively utilizes the advantages of the SDS and optimized KDS precipitation method to efficiently improve the analysis of membrane proteomes is developed. Compared to other commonly used sample preparation methods in the analysis of the rat liver membrane-enriched sample, this method has obvious superiorities in the recovery and identification of membrane proteins, especially the highly hydrophobic membrane proteins with multiple transmembrane domains, which demonstrates that the SSDP method has high potential for shotgun analysis of the membrane proteome.

2. Materials and methods

2.1. Materials

Potassium chloride (KCl), trifluoroacetic acid (TFA), ammonium bicarbonate (NH_4HCO_3), phenylmethylsulfonyl fluoride (PMSF), urea, methanol, dithiothreitol (DTT), iodoacetamide (IAA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), $CaCl_2$, BSA, sucrose, formic acid (FA) and Stains-all were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acrylamide, bisacrylamide, glycine, Tris and SDS were from Amresco (Solon, OH, USA). RapiGest SF was purchased from Waters Corp. (Milford, MA). Ammonium persulfate (AP) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from GE Healthcare (Piscataway, NJ, USA). BCA protein assay kit and Coomassie brilliant blue G-250 were from Thermo Scientific Pierce (Rockford, IL). Proteomics sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Acetonitrile (ACN, HPLC-grade) was purchased from Hunan Fine Chemistry Institute (Changsha, Hunan, China). Ultrapure 18.2-MΩ water obtained from a Millipore Milli-Q system (Bedford, MA, USA). All other regents were domestic products of highest grade available. Rats were purchased from Medical Academy of Central South University (Changsha, Hunan, China).

2.2. Enrichment of rat liver plasma membrane fraction

Preparation of rat liver membrane-enriched sample was performed according to the methods described [28,29]. Briefly, rats were killed after being starved for 18–24 h. The livers were excised and homogenized in a cold buffer containing 50 mM HEPES (pH 7.4), 1.0 mM $CaCl_2$ and 0.1 mM PMSF. The homogenate was centrifuged at $600 \times g$ for 20 min at 4 °C and the supernatant was collected. The pellet was repeatedly homogenized and centrifuged

as above and the supernatants were pooled, followed by centrifugation at 24 000 × *g* (Ti70 rotor, Beckman, Fullerton, CA, USA) for 30 min at 4 °C. The supernatant was discarded and the pellet was mixed with 69% sucrose and then placed in a centrifuge tube, 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 Animal.

2.3. Digestion of proteins in rat liver plasma membrane fraction for sample preparation

2.3.1. Sample preparations based on SDS-, RapiGest-, urea- and methanol-assisted digestion (SSD, SRD, SUD and SMD)

For comparison, the membrane protein sample was aliquoted and separately solubilized under four different conditions: 1.0% SDS/50 mM NH_4HCO_3 (diluted to 0.1% prior to digestion) (SSD), 1.0% RapiGest/50 mM NH_4HCO_3 (diluted to 0.1% prior to digestion) (SRD), 8 M urea/50 mM NH_4HCO_3 (diluted to 2 M prior to digestion) (SUD) and 60% methanol/50 mM NH_4HCO_3 (SMD). The proteins were reduced with 5 mM DTT in 50 mM NH_4HCO_3 at 56 °C for 60 min, and alkylated in the dark with 25 mM IAA in 50 mM NH_4HCO_3 at room temperature for 45 min. Trypsin was added using an enzyme-to-protein ratio of 1:50 and incubated at 37 °C for 16 h. Following digestion, 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. The supernatants in all methods were separately collected and concentrated using a Speed-Vac (Labconco, Kansas, MO, USA) and analyzed by SDS-PAGE to evaluate the protein digestion efficiency or by RPLC–MS/MS for protein identification. It is worth noting that the surfactant RapiGest SF in the digests from the SRD method was also removed by the acidification and centrifugation because it degrades rapidly under low-pH conditions, which eliminates surfactant interference [14].

2.3.2. Sample preparation based on SDS-assisted digestion followed by KDS precipitation (SSDP)

The SSDP method includes two main steps: SDS-assisted digestion and SDS removal following the digestion by KDS precipitation. The protocol of SDS-assisted digestion is the same as described above. The SDS was removed by KDS precipitation [27]. The concentration of KCl was varied to obtain optimal conditions for SDS removal and peptide recovery in the membrane-enriched sample. Seven aliquots of the membrane-enriched samples were mixed with an equal volume of KCl at different concentrations (0.02, 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 M) and incubated at room temperature for 5 min to allow the KDS precipitates to form. The KDS precipitates were pelleted by centrifugation at 14 000 × *g* for 10 min, and the SDS remaining in the supernatant was quantitatively determined by a Stains-all spectrophotometric assay following a previously described method [30]. Peptide recovery was also assessed following KDS precipitation based on BCA assay measurements [27]. For RPLC–MS/MS analysis, the peptides in the supernatant were collected and desalted using C18-SPE columns, and concentrated using a Speed-Vac (Labconco, Kansas, MO, USA).

2.3.3. Filter-aided sample preparation (FASP) method

The spin filter-based FASP protocol was the same as previously described [25]. Briefly, the aliquot membrane protein sample was solubilized in 4.0% SDS/50 mM NH_4HCO_3, retained and concentrated into microliter volumes in an ultrafiltration device. The filter unit acts as a 'proteomic reactor' for detergent removal, buffer exchange, chemical modification and protein digestion.

2.4. SDS-PAGE analysis

SDS-PAGE was used to evaluate digestion efficiency of the four methods including different additives (SSD, SRD, SUD and SMD methods). Briefly, the digests were firstly concentrated in a Speed-Vac, and then respectively dissolved by loading buffer containing 4.0% SDS and heated at 90 °C for 10 min. All samples were separated by SDS-PAGE in parallel lanes on a 4.8% stacking gel and a 10% separation gel (thickness 1 mm, containing 10 wells) run, as described by Laemmli with minor changes [31]. After electrophoresis, gels were fixed in a fixing solution (50% methanol, 10% acetic acid) for 10 min and subsequently washed with water for at least 30 min. Proteins/peptides bands were visualized using Coomassie brilliant blue G-250 staining.

2.5. RPLC–ESI-MS/MS analysis

All the tryptic digests prepared with above different methods were dissolved in 30 μL of 0.1% 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). Peptides were separated and eluted with a C18 PepMap column (180 μm i.d., 15 cm length, LC-Packings, Amsterdam, Netherlands) at a flow rate of 3 μL/min. Gradients for separation were programmed from 5% to 35% B in 150 min, 35% to 80% B in 10 min, followed by 80% B for 10 min, then by 5% B in 10 min. Solvent A was 0.1% FA in water, 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 300 °C. The full MS scan mode was standard-enhanced (m/z 350–1600). Four most abundant ions detected in each MS scan were selected for collision-induced dissociation (CID) with collision energy of 1.0 V. The peptides were analyzed using the data-dependent MS/MS mode over the m/z range of 200–2000. Instruments were controlled using Chemstation B01 (Agilent) and EsquireControl™ 6.0 (Bruker Daltonics) software.

2.6. 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 15 000, retention time windows 1.0 min, maximum number of compounds 80 000. 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, 1.0 Da and MS/MS mass tolerance, 0.5 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). False positive rates were evaluated using the reversed sequence databases search strategy [32]. Each identified proteins in rat liver membrane-enriched fraction has an IPI database

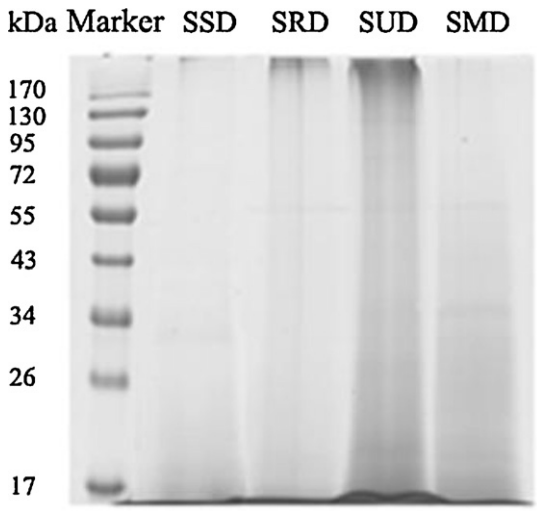

Fig. 1. SDS-PAGE analysis of digestion products of proteins from the rat liver membrane-enriched fraction comparing four different methods. Each experiment used 80 μg of protein.

accession number and many of these proteins have assigned gene ontology (GO) numbers [33], which was used to retrieve the protein information in the databases such as UniProt Knowledgebase (UniProtKB). Predictions for putative transmembrane domains (TMDs) in all identified proteins were carried out using the transmembrane hidden markov model (TMHMM) algorithm [34], available at http://www.cbs.dtu.dk/services/TMHMM. The average hydrophobicity values expressed as grand average of hydropathy (GRAVY) values [35] for identified proteins were calculated using the ProtParam software, available at http://cn.expasy.org. Proteins with positive GRAVY values are considered to be hydrophobic and negative values, hydrophilic. Sequence coverage was visualized with the TOPO2 transmembrane protein graphics program (http://www.sacs.ucsf.edu/TOPO2/) [36].

3. Results

3.1. Evaluation of digestion efficiency of the proteins from the rat liver membrane-enriched fraction based on different methods

For solution-based shotgun proteomics, the solubility and digestion efficiency are two crucial factors that might affect the identification of the proteins, especially IMPs in membrane-enriched samples. To improve the solubility and digestion of these proteins, a number of additives are used in sample preparation buffers (SDS, RapiGest, urea and methanol mostly used). The level of protein digestion by the four different methods including above additives was evaluated using SDS-PAGE analysis (Fig. 1). The experiments were performed in triplicate and were highly reproducible. A representative image is shown in Fig. 1, from which it can be observed that the digests in the SSD method were nearly undetectable in the molecular weight range indicated by the standard protein markers, suggesting the sizes of nearly all the proteins/peptides in the digests were smaller than the lower limit of the range. In contrast, the SUD method gave rise to the worst digestion effect, because the staining intensity of the corresponding lane indicated that there might be a significant amount of large proteins/peptides in the digest. The SRD method was less effective compared to the SSD method but more effective than the SMD method in improving the digestion efficiency of the proteins from the rat liver membrane-enriched sample. These results were also supported by the statistics analysis of identified peptides by LC–MS/MS in terms of the proportion of peptides containing one missed cleavage site (up to one missed cleavage site was allowed when searching against database). It is all known that the peptides with missed cleavage sites result in the generation of larger peptides, and the lower proportion of missed cleavage peptides indicated the better digestion. As shown in Supplementary Fig. 1, the SSD and SUD methods led to the lowest and highest proportions (6.5% and 8.3%), respectively, and the proportions in the SSD and SRD methods were much lower than those in the SUD and SMD methods. These comparative data further showed that the SSD and SRD methods had certain superiorities over the other two methods in facilitating the digestion of the proteins from membrane samples.

The above analyses showed the relative effects of the four different methods in improving the trypsinolysis of the proteins from the membrane-enriched sample. It is important to note that the proteins extracted/dissolved in a typical 1.0% SDS buffer and digested in 0.1% SDS buffer gave the most complete digestion, which demonstrates that the conditions in the SSD method are the most efficient and more suitable for membrane proteome analysis. Compared to the SSD method, it is possible the other methods have a weaker ability to disrupt the membranes, limiting the extraction and digestion of these proteins. However, the digestion results did not completely represent the identification results of the proteins because it is also possible that these additives have different effects on mass spectrometric analyses of the digests. To investigate this possibility the proteins identified by RPLC–MS/MS based on the four methods were further analyzed and compared.

3.2. SDS removal and peptide recovery by KDS precipitation

The SSD method (extracted in 1.0% SDS, and then diluted to 0.1% for digestion) has a higher digestion efficiency compared to the other three methods. However, SDS causes significant ion suppression in RPLC–MS/MS analysis if its concentration is too high. Botelho et al. [24] showed that in a proteome experiment of the coupling of low flow RPLC with MS through electrospray interfaces, SDS concentrations less than 0.01% caused only a slight drop in signal intensity, as observed in the total ion chromatogram. However, when the concentration was increased to 0.02%, signal suppression was evident. Therefore, we know that a suitable method for SDS reduction is one that can reliably reduce the SDS content to below 0.01%, while maintaining a high peptide recovery. Recently, Zhou et al. [27] reported a very effective method of SDS removal and peptide recovery by KDS precipitation from SDS-solubilized whole cell lysate. With this method, SDS removal and peptide recovery were nearly maximal, and it provided improved proteome coverage compared with the FASP method. However, for different samples, the required experimental conditions are different, making the method difficult to transfer.

In order to screen for optimal conditions of SDS removal and peptide recovery by KDS precipitation for membrane samples, we dissolved the rat liver membrane-enriched samples using 1.0% SDS/50 mM NH_4HCO_3, and the samples were digested in 0.1% SDS buffer for 16 h after dilution, in a volume of 100 μL. We then added 100 μL KCl at a number of concentrations (0.02–4 M) to remove the SDS from the peptide mixtures. The residual SDS in the sample was measured by Stains-all spectrophotometric assay. The quantitative results (Fig. 2A) showed that as the concentration of KCl increased, the residual SDS in the digest was gradually reduced. When the concentration was increased to 0.5 M, the remaining SDS was below 0.01% (the ratio of residual SDS weight-to-volume of sample loading in our LC–MS analysis), which was the tolerable level of ESI mass spectrometric analysis.

To identify the optimal concentration of KCl for peptide recovery, the contents of the peptides in the samples following KDS precipitation were comparatively determined based on the BCA

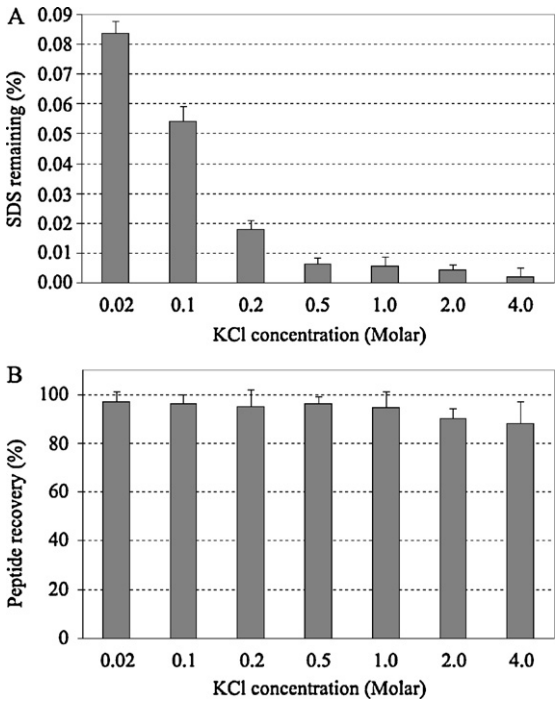

Fig. 2. Optimization of KDS precipitation for SDS removal and peptide recovery, displaying (A) residual SDS contents and (B) peptide recovery ratios after treatment with KCl at different concentrations. Each experiment used 20 μg of protein from the rat liver membrane-enriched fraction. It was solubilized initially in 1.0% SDS, digested in 0.1% SDS after dilution, and then treated with KCl of different concentrations (0.02–4 M). 100 μL of KCl of the indicated concentration was added to 100 μL of the digest for inducing KDS precipitation. The residual SDS content was calculated and evaluated according to the ratio of the residual SDS weight-to-volume of sample loading in our LC–MS analysis. Error bars, representative of the standard deviation of the mean for the triplicate experiments are presented.

assay. As shown in Fig. 2B, the quantitative determination showed that the absorbance at 562 nm of the supernatant was almost constant at different concentrations of the added KCl from 0.02 to 1.0 M, and the peptide recovery >95%. However, when the concentration was increased to 2.0 M, the peptide recovery was obviously decreased, possibly due to the peptides being salted out in very high salt conditions. For further confirming above observations, the samples treated with KCl of two selected concentrations (0.5 and 2.0 M) were comparatively analyzed by LC–MS/MS. The base peak chromatograms in the LC–MS analysis of the triplicates and protein/peptide identification results were shown in Supplementary Fig. 2. In most regions, the chromatograms of the samples with the treatment of 0.5 M KCl show more base peaks with higher intensities compared to those of 2.0 M KCl. Then, when these chromatograms were processed with data analysis and database search, more proteins and their matching peptides were also identified in the samples with 0.5 M KCl treatment than those with 2.0 M KCl treatment (Supplementary Fig. 2). These comparative results further demonstrated that, even if there was a cleanup step, too high concentration salt (e.g. 2.0 M) added to the sample might affect the ionization of peptides in mass spectrometric analysis except for peptide recovery during SDS removal. To reliably reduce the SDS content below 0.01%, while maintaining a high peptide yield and decreasing the interference in mass spectrometric analysis, 0.5 M KCl was selected as the optimized KDS precipitation condition and was used for the further experiments.

3.3. Comparative analysis of the proteins identified based on sample preparation

In order to evaluate the effects of the different sample preparation methods on protein identification, the proteins and their matching peptides identified from rat liver membrane-enriched fractions based on the six methods (SSD, SSDP, SRD, SUD, SMD and FASP methods) were analyzed and compared (Table 1). Using the reversed sequence database search strategy the false-positive rate was <3% [32]. After removal of the false-positive results, it was shown that in total 189, 385, 351, 306, 263 and 337 proteins were identified based on 611, 1462, 1336, 1079, 870 and 1259 peptides using SSD, SSDP, SRD, SUD, SMD and FASP methods, respectively. Of the total proteins identified by the six methods, 90 (47.6%), 197 (51.2%), 178 (50.7%), 151 (49.3%), 132 (50.2%) and 167 (49.6%) were membrane proteins, of which 55, 136, 120, 103, 94 and 112, respectively, were annotated as integral membrane proteins with 1–16 transmembrane domains. Compared with the other five methods, the SSDP method had the highest efficiency for protein identification and increased the number of total proteins identified on average by 33.1%, peptides by 41.8%, membrane proteins by 37.2%, and integral membrane proteins by 40.5%. These comparative results demonstrated that the SSDP method was more efficient for protein identification including membrane proteins, particularly integral membrane proteins. Meanwhile, the other statistical analysis of identified proteins using the average values and standard deviations from the triplicates also indicated the similar results and conclusions (data shown in the brackets of Table 1). It is worth noting that, as shown in Table 1 and Fig. 3, the SSD method had the worst identification results, but the SSDP method greatly improved the efficiency of protein identification by adding the SDS-removal step, because virtually nearly all the identified

Table 1
Comparison analysis of proteins and their matching peptides identified from rat liver membrane-enriched fraction based on six different sample preparation methods.[a]

Categorization	Sample preparation methods					
	SSD	SSDP	SRD	SUD	SMD	FASP
All identified proteins	189 (114 ± 16)	385 (191 ± 15)	351 (176 ± 17)	306 (153 ± 20)	263 (131 ± 17)	337 (169 ± 22)
All identified peptides	611 (347 ± 51)	1462 (720 ± 42)	1336 (657 ± 39)	1079 (511 ± 57)	870 (403 ± 36)	1259 (613 ± 46)
Peptides/proteins	3.2 (3.0 ± 0.4)	3.8 (3.8 ± 0.2)	3.8 (3.7 ± 0.2)	3.5 (3.3 ± 0.4)	3.3 (3.1 ± 0.3)	3.7 (3.6 ± 0.3)
Membrane proteins	90 (58 ± 9)	197 (113 ± 10)	178 (104 ± 9)	151 (85 ± 13)	132 (76 ± 11)	167 (96 ± 11)
Integral membrane proteins	55 (37 ± 5)	136 (81 ± 7)	120 (73 ± 8)	103 (60 ± 10)	94 (54 ± 10)	112 (68 ± 8)
Hydrophobic proteins	49 (35 ± 6)	116 (74 ± 8)	102 (65 ± 8)	87 (55 ± 9)	75 (49 ± 6)	92 (61 ± 10)

[a] 20 μg of proteins from rat liver membrane-enriched sample were used in each experiment of all methods for protein identification. Except the merged results from triplicate analysis in each method were used for comparison, the average values of all parameters and standard deviations from the triplicates were also shown in the brackets.

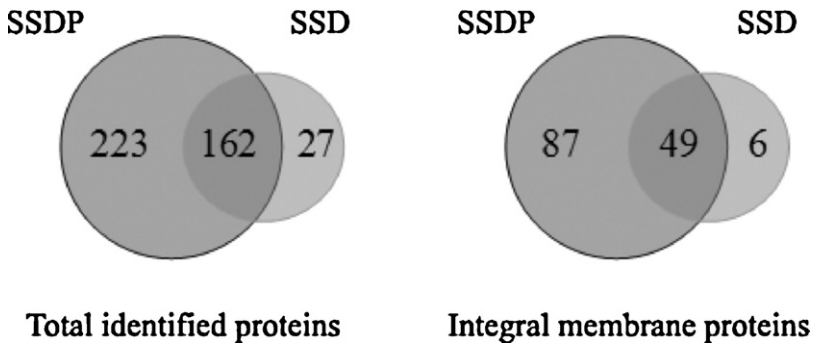

Fig. 3. Comparison of (A) all proteins and (B) integral membrane proteins identified by the SSDP and SSD methods. The merged results from triplicate analysis in each method were used for comparison.

proteins by the SSD method, especially the IMPs, were identified by the SSDP method (Fig. 3). Also, the number of total identified proteins in the SSDP method was almost double that of the SSD method (Table 1).

Furthermore, the number of matching peptides for an identified protein could be used as a criterion for evaluating the confidence of protein identification. When the number of matching peptides per identified protein in all methods were compared, it was shown that the average number in the SSDP method (3.8) was comparable to the SRD method (3.8) and higher than that in the other methods (3.2, 3.5, 3.3 and 3.7, respectively) (Table 1). This suggests that the proteins identified in the SSDP and SRD methods had higher coverage and thus reliability. At present, it is generally accepted that proteins with a positive GRAVY value are hydrophobic, and a negative value as hydrophilic [35]. In our experiments, we have also compared the GRAVY values of the total proteins identified by the different methods. The results showed that the number (as well as percentage of hydrophobic proteins) (GRAVY value >0) identified based on the SSDP method were the highest (116 and 30.1%), followed sequentially by 102 (29.0%), 75 (28.5%), 87 (28.4%), 92 (27.3%) and 49 (25.9%) on the SRD, SMD, SUD, FASP and SSD methods, respectively (Table 1). These experimental results further demonstrated that the SSDP method improved the identification of proteins, particularly the hydrophobic integral membrane proteins more efficiently than the other commonly used methods. Moreover, the statistical analysis of identified proteins using the average values and standard deviations from the triplicates was also essentially consistent with above results (Table 1).

Generally, in the field of biological research, the analysis of membrane proteins, especially IMPs, presents a special challenge due to their hydrophobic nature and low abundance, seriously complicating their solubilization, digestion and identification. In this context, for further probing the difference between the methods in membrane protein identification, we separately compiled and then categorized the identified membrane proteins based on their GO function annotations, though the classification was not strict due to the fact that a protein usually has multiple functions. As shown in Fig. 4, the proteins with ion channel/transport and signaling/receptor functions, which were generally considered to have low abundance and are hydrophobic, accounted for a large proportion of the proteins. This is consistent with the main functions of the membranes in cells. The remaining proteins were enzymes, and binding and structural proteins. Importantly, in most of the groups of proteins, particularly those with transport and signaling functions, the SSDP method identified more proteins compared with other methods, and was superior in the identification of membrane proteins, especially those with low abundant and/or highly hydrophobic characteristics.

3.4. Comparative analysis of physicochemical characteristics

In addition, to further assess the possible analytical bias of the SSDP method in protein identification, we analyzed and compared several physicochemical characteristics of identified proteins using bioinformatics. We selected the SRD method as the control because it also greatly improved the digestion efficiency and identification result (Fig. 1, Supplementary Fig. 1 and Table 1). First, we analyzed and compared the distribution of the calculated molecular weight (MW) and the isoelectric point (p*I*) of the identified proteins. As shown in Fig. 5A and B, there were no significant differences in the MW and p*I* distribution profiles between the two methods. Most of these identified proteins were distributed in the MW range of 20–80 kDa and in the p*I* range of 5–7 and 8–10. However, when compared with the SRD method in detail, it was found that more proteins were identified by the SSDP method in nearly every MW and p*I* distribution range. Some proteins with extreme properties, such as very high MW proteins (MW > 500 kDa) and basic proteins (p*I* > 10), were only identified in the SSDP method. For example, as shown in the Supplementary Table S1, the protein similar to AHNAK nucleoprotein isoform 1 (IPI00368391) with a MW of about 900 kDa was identified based on five unique peptides in the SSDP method.

Furthermore, we also analyzed and compared the identified proteins using their calculated GRAVY values and predicted transmembrane domains (TMDs) to assess the efficiency of the SSDP methods for the identification of proteins, particularly integral membrane proteins with different hydrophobic properties. In the experiments, we categorized the identified proteins into six groups according to their GRAVY values: <−0.5, −0.5 to −0.25,

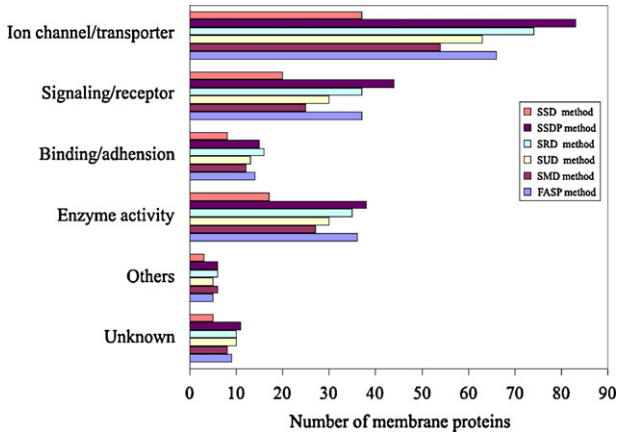

Fig. 4. Comparison of the distributions of membrane proteins based on their function annotations identified by the six methods. The merged results from triplicate analysis in each method were used for comparison.

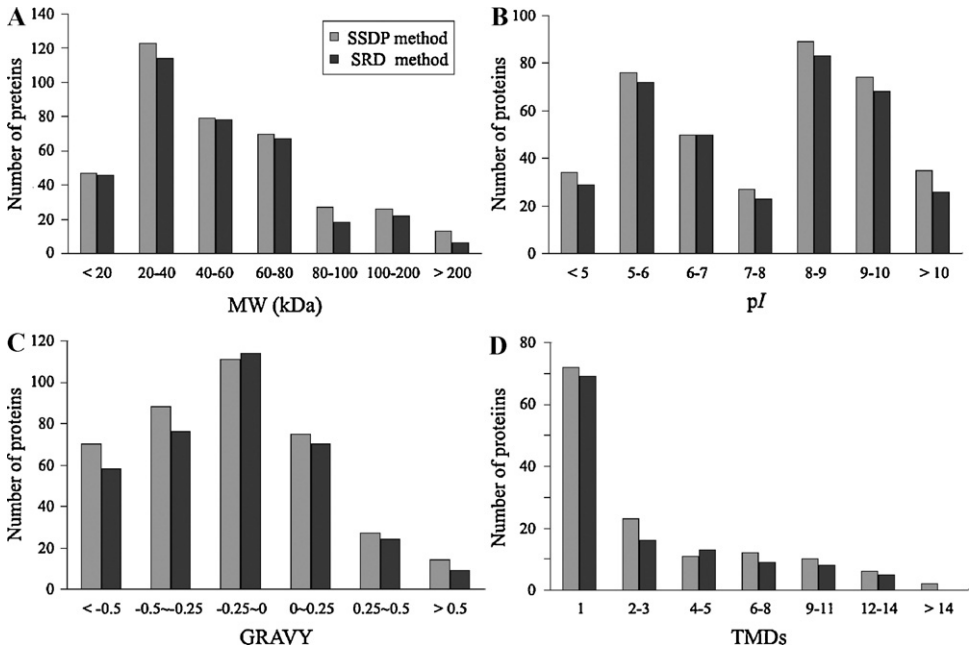

Fig. 5. Comparison of the distribution of proteins identified based on the SSDP and SRD methods as a function of (A) calculated molecular weight (MW), (B) calculated isoelectric point (p*I*), (C) calculated grand average of hydropathy (GRAVY) value, and (D) predicted transmembrane domains (TMDs). The merged results from triplicate analysis in each method were used for comparison.

−0.25 to 0, 0–0.25, 0.25–0.5 and >0.5 (Fig. 5C). Compared with the SRD method, the SSDP method led to more proteins being identified in most groups, indicating that the SSDP method could improve the identification of both hydrophilic and hydrophobic proteins. To further examine the efficiency of the SSDP method to identify proteins with TMDs, the numbers of TMDs were predicted using the TMHMM 2.0 algorithm and compared with those by the SRD method. As shown in Fig. 5D and Supplementary Table S1, the general distribution profiles were similar, and most of the identified TMD-containing proteins had 1–3 TMDs, followed by proteins with 4–8 TMDs. More TMD-containing proteins were identified at almost every TMD number by the SSDP method. In particular, proteins with >14 TMDs were identified only in the SSDP method, such as the 168 kDa protein (IPI00568685) with 16 TMDs and NADH-ubiquinone oxidoreductase chain 5 (IPI00195892) with 15 TMDs. As shown in Fig. 6 the sequence coverage of the 168 kDa protein was visualized by the TOPO2 transmembrane protein graphic program. The identified tryptic peptides are indicated

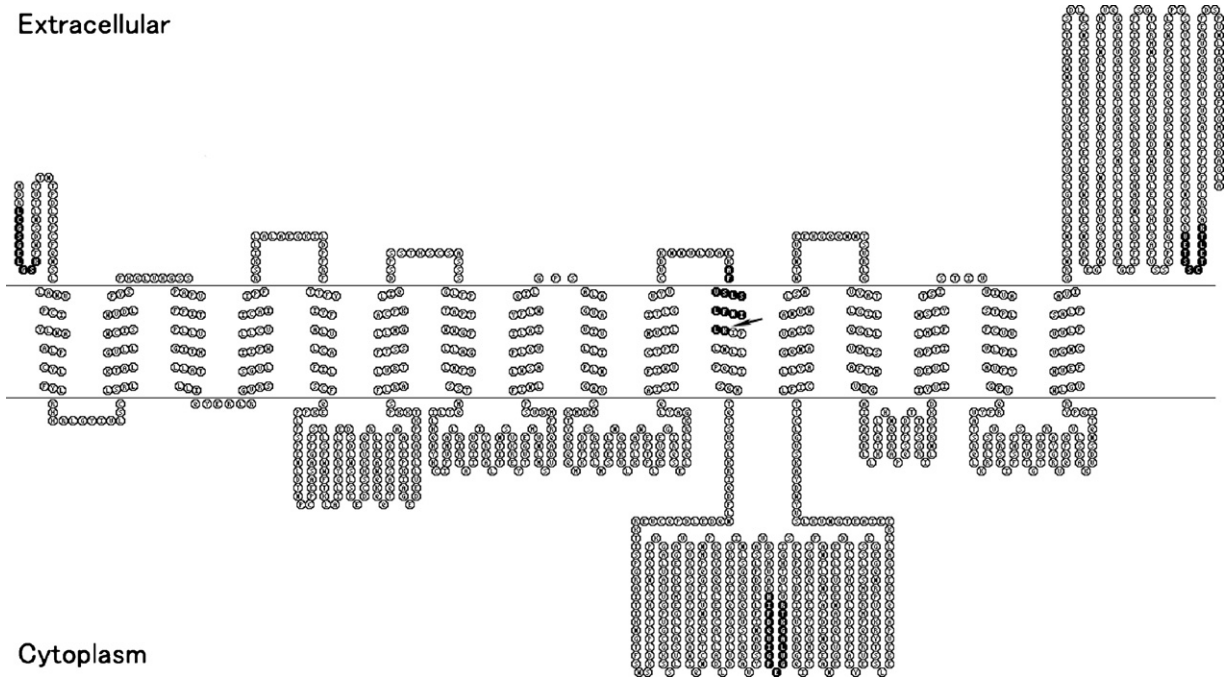

Fig. 6. Protein with 16 TM domains (168 kDa protein, IPI00568685) identified only by the SSDP protocol from the rat liver membrane-enriched fraction. The sequence coverage was visualized with the TOPO2 transmembrane protein graphics program. Identified tryptic peptides are represented by black circles and unidentified tryptic peptides by white circles. The tryptic cleavage site inside the TM domains of one identified peptide is indicated with a black arrow.

by black circles. Four unique peptides, including one peptide (AFVSLSLFNILK) with a tryptic cleavage site inside the TMD were only identified by the SSDP method. These data further indicated that compared with other strategies, the SSDP method was more favorable for the digestion and identification of proteins containing TMDs, particularly highly hydrophobic multiple TMDs.

4. Discussion

The "bottom-up" approach has become a key technology in mass spectrometry (MS)-based proteomic analysis, involving the reduction of intact proteins into a collection of peptides suitable for MS analysis. When the approach is applied to large-scale analysis of highly complex protein mixtures, it is often known as "shotgun proteomics" [37,38]. In shotgun proteomics analysis, the sample preparation process as the initial step is also the most critical step, which directly affects the final identification results of the proteins.

The SDS-assisted sample preparation method for shotgun proteomics is not a novel concept, and it had been used in a number of applications in the field of membrane proteomics [20,22], which is particularly useful for integral membrane proteins that are difficult to extract/solubilize by other reagents such as urea and methanol. In this method, the proteins were first solubilized/denatured using SDS at a high concentration, digested in a diluted SDS buffer, and then separated/identified by LC–MS/MS. In our experiments, we also showed that the proteins from the membrane-enriched fraction extracted/dissolved in a typical SDS containing buffer (1.0% SDS) and digested in 0.1% SDS provided the most complete digestion, demonstrating that the SDS-assisted digestion method is of high potential for the analysis of membrane proteome. However, the worst identification results by the SSD method also demonstrated that the SDS in digests must be removed before LC–MS/MS analysis for improving the identification results.

Although it has been shown that KDS precipitation is highly efficient in SDS-removal and peptide recovery during MS-based proteomic sample processing, the application of this method in the membrane protein sample cleanup is controversial, and their effect on the membrane proteome is still unknown. When the strategy for membrane protein sample cleanup was used, the main concern was whether the SDS removal and peptide recovery were complete. The former determined if the interference of SDS with the subsequent analyses could be avoided, and the latter was the main determinant of the sensitivity of the method. KCl concentration for KDS precipitation is the most important factor affecting the efficiencies of SDS removal and peptide recovery. To identify an optimal concentration of KCl for membrane-enriched samples, different concentrations were used to precipitate DS from the digests. Our quantitative analyses demonstrated that when the concentration of added KCl was 0.5 M, not only were >95% of the peptides recovered and the remaining SDS lowered to <0.01% (Fig. 2), but it also decreased the salt concentration in the digests, which would not obviously interfere with the subsequent digestion and/or RPLC–MS/MS analysis. Additionally, using a higher centrifugal force and longer centrifugal time would be helpful in precipitating the KDS completely.

The comparative analyses of protein identification results showed that, compared to other methods, the SSDP method had the highest identification efficiency and was more suitable for the analyses of the proteins, particularly highly hydrophobic or multiple transmembrane proteins. The explanations for this might be that the method had several advantages. First, SDS has stronger solubility compared with other additives, which would be helpful to extract, dissolve and denature membrane proteins completely; second, the low SDS concentration might be compatible with the activity of trypsin and could efficiently facilitate the digestion of these proteins; third, SDS could be efficiently removed by the optimized KDS precipitation and centrifugation, thereby avoiding its possible effect on the subsequent RPLC–MS/MS analysis.

In conclusion, we have investigated an improved SDS-assisted sample preparation method based on SDS-assisted digestion and peptide-level SDS-removal by an optimized KDS precipitation for shotgun analysis of the membrane proteome. This strategy includes the extraction/solubilization of membrane proteins by SDS, more complete protein digestion in the diluted SDS buffer, and the high efficiencies of SDS removal and peptide recovery following the digestion by an optimized KDS precipitation method. This strategy overcomes some inherent limitations of the conventional sample preparation methods and is easy to perform at low cost and more suitable for solution-based shotgun analysis of membrane proteomes.

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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.2012.10.016.

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