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Development and evaluation of an entirely solution-based combinative sample preparation method for membrane proteomics

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ABSTRACT

The hydrophobic nature of many membrane proteins, especially integral membrane proteins, brings great difficulties to their analysis. To improve the analysis of membrane proteins, an entirely solution-based combinative sample preparation (CSP) method was developed and its application to the shotgun analysis of rat liver membrane proteomes was evaluated in this study. This CSP method comprehensively uses the strong ability of sodium dodecyl sulfate (SDS) to lyse the membranes and solubilize hydrophobic membrane proteins, the high efficiency of the optimized acetone precipitation method in sample cleanup and protein recovery, and the advantages of sodium deoxycholate (SDC) in improving protein solubilization/digestion as well as being compatible with trypsin activity. Compared with two other representative sample preparation methods, the SDC-assisted membrane-lysing method and the tube gel method, the newly established CSP method exhibited superiority in the recovery and identification of hydrophobic peptides, larger peptides, and highly hydrophobic membrane proteins with multiple transmembrane domains. The CSP method has characteristics of easy operation, low cost, and suitability for treating protein samples in various volumes, particularly large volumes, thereby having potential in the analysis of membrane proteomes with mass spectrometry.

Biological membranes incorporate many proteins that involve a variety of cellular functions such as regulation of cell signaling and transportation of intra- and intercellular endogenous molecules; therefore, it is of significance to analyze these proteins [1,2]. However, the highly hydrophobic nature of many membrane proteins, especially integral membrane proteins (IMPs),[1] brings great difficulties to their extraction, solubilization, enzymolysis, and identification. Recently, the solution-based shotgun method has become one of the efficient methods for membrane proteome identification and quantification [3–6]. In this method, proteins are degraded into a peptide mixture by enzymatic digestion, followed by separation and identification by high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS). Therefore, for an efficient membrane proteomic analysis, it is important to develop a suitable solvent system that not only enhances the solubility of hydrophobic proteins but also has good compatibility with protease activity and mass spectrometry (MS).

During the past few years, to improve the solubilization of membrane proteins, a variety of additives have been used. Of these, detergent sodium dodecyl sulfate (SDS) is realized as the most efficient reagent [7]. However, SDS has certain limitations in its application to the analysis of membrane proteomes because SDS of slightly higher concentrations interferes with the subsequent digestion, chromatographic separation, and MS analysis [8–12]. To address the problem, other additives have also been applied for membrane proteomic analyses. Protease- and MS-compatible surfactants, such as sodium deoxycholate (SDC), are among the most promising additives to improve shotgun analysis of membrane proteomes. SDC can be used at high concentrations without interfering with trypsin

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[1] *Abbreviations used:* IMP, integral membrane protein; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; SDS, sodium dodecyl sulfate; SDC, sodium deoxycholate; ALP, acid-labile surfactant; PFOA, perfluorooctanoic acid; CSP, combinative sample preparation; SDCA, SDC-assisted; TG, tube gel; TMD, transmembrane domain; NH_4HCO_3, ammonium bicarbonate; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; IAA, iodoacetamide; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; FA, formic acid; AP, ammonium persulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; ACN, acetonitrile; PAGE, polyacrylamide gel electrophoresis; CapLC, capillary liquid chromatography; CID, collision-induced dissociation; IPI, international protein index; GO, gene ontology; TMHMM, transmembrane hidden Markov model; GRAVY, grand average of hydropathy; ESI, electrospray ionization.

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activity and LC–MS/MS analysis to enhance the solubilization, enzymolysis, and identification of membrane proteins, including IMPs and proteolytically resistant proteins [10,13]. Acid-labile surfactant (ALS, trade name RapiGest SF) is another protease- and MS-compatible surfactant that solubilizes proteins without inhibiting trypsin or other common endopeptidase activity and does not interfere with MS analysis because it degrades rapidly at low-pH conditions [8]. However, the major drawback of the alternative additives is that their ability to lyse membranes and extract highly hydrophobic proteins is weaker than that of SDS [14]. In addition, ALS is chemically synthesized and is expensive, thereby limiting its extensive application to membrane proteomics. Recently, Kadiyala and coworkers [15] described the novel use of a surfactant, perfluorooctanoic acid (PFOA), for shotgun membrane proteomics. They found that PFOA was able to solubilize membrane proteins as effectively as SDS and that PFOA concentrations up to 0.5% did not significantly inhibit trypsin activity. However, although the authors defined PFOA as a volatile surfactant, the volatility of surfactant in fact is very low due to its high boiling point (189–192 °C) [16], bringing about difficulties in its removal from the digests prior to MS analysis. Furthermore, PFOA is a stable and toxic polyfluorinated compound, and its continuous use presents a challenge to the environment and public health [17]. In view of the facts, finding new and appropriate methods for removing SDS from the samples after using high concentrations of SDS to lyse membranes and extract the membrane proteins has special significance. Theoretically, many conventional methods such as dialysis and chromatography can be used to remove small molecules such as SDS from the protein samples. However, in practice, these methods might not be suitable for high-throughput analysis of small amounts of proteins because the large volume and complex operations introduced by these methods could result in significant sample and time loss during SDS removal and/or protein concentration [18]. Recently, a tube gel-based SDS removal method has been developed and obtained some applications in the field of membrane proteomics [11,19]. Nevertheless, this method may also lead to a certain sample and time loss [11]; furthermore, the protein samples cleaned up by the gel-based method generally need to be digested in the gel, and this has some limitations such as low accessibility of proteases to some deeply gel-entrapped proteins that would lead to low cleavage yields of these proteins and low recovery of large and/or highly hydrophobic tryptic peptides [20,21]. In addition, such gel-based methods are not suitable for the treatment of large-volume protein samples.

In consideration of the disadvantages of the above SDS removal methods, acetone precipitation as a potential method attracted our special attention. The method not only operates simply, costs little, and applies to the treatment of large-volume samples but also can avoid some disadvantages of gel-based protein analysis. However, for different protein samples, the required experimental conditions are different, so the application of this method in the membrane protein sample cleanup is controversial. The main concerns are whether the proteins can be precipitated completely and whether the precipitated proteins can be efficiently redissolved and digested. Based on the above analyses, in the current study, we first optimized the experimental conditions of the acetone precipitation method for the cleanup of SDS-solubilized membrane protein samples and then, for efficiently redissolving and digesting the acetone-precipitated proteins, we used an SDC-containing buffer and demonstrated its validity. Thus, an entirely solution-based combinative sample preparation (CSP) method that comprehensively uses the advantages of SDS, SDC, and the optimized acetone precipitation method has been developed. Compared with the SDC-assisted (SDCA) and tube gel (TG) sample preparation methods, the CSP method exhibited obvious superiority in the recovery and identification of hydrophobic peptides, larger peptides, and highly hydrophobic membrane proteins with multiple transmembrane domains (TMDs), demonstrating that the method is of high potential for the shotgun analysis of membrane proteomes.

Materials and methods

Materials

Ammonium bicarbonate (NH_4HCO_3), phenylmethylsulfonyl fluoride (PMSF), trifluoroacetic acid (TFA), bovine serum albumin (BSA), sucrose, dithiothreitol (DTT), iodoacetamide (IAA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), $CaCl_2$, formic acid (FA), acetone, Stains-All, and SDC were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acrylamide, bisacrylamide, glycine, Tris, and SDS were obtained from Amresco (Solon, OH, USA). Ammonium persulfate (AP) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were obtained from Amedham Pharmacia Biotech (Uppsala, Sweden). Coomassie G-250 and a Bio-Rad RC DC protein assay kit were purchased from Bio-Rad (Hercules, CA, USA). Proteomic sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). Acetonitrile (ACN, HPLC grade) was purchased from Hunan Fine Chemistry Institute (Changsha, Hunan, China). Ultrapure 18.2-MΩ water was obtained from a Millipore Milli-Q system (Bedford, MA, USA). All other reagents were domestic products of the highest grade available. Rats were purchased from Medical Academy of Central South University (Changsha, Hunan, China).

Enrichment of rat liver plasma membranes

The rat liver membrane sample was prepared using a modification of an earlier procedure [22,23], Briefly, rats were killed after being starved for 18 to 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*g* for 20 min at 4 °C, and the supernatant was collected. The pellet was repeatedly homogenated 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 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.

Membrane protein extraction and optimization of sample cleanup conditions

The protein content of membrane-enriched fraction of rat liver was quantified with a Bio-Rad RC DC protein assay kit with BSA as a standard protein. Extraction/solubilization of the proteins in membrane-enriched fraction was carried out according to the procedure described previously with minor modifications [20]. To optimize the conditions for the cleanup of membrane protein samples by the acetone precipitation method, aliquots of the membranes were separately solubilized with 50 mM NH_4HCO_3 solution containing 2% SDS, followed by sonication and centrifugation. The supernatants were collected, and the proteins in supernatants were precipitated by precooled acetone (previously cooled to −20 °C in a freezer) for 4 h at 4 °C with different acetone/sample