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Improvement of hydrophobic integral membrane protein identification by mild performic acid oxidation-assisted digestion

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ABSTRACT

Integral membrane proteins (IMPs) are critical for the maintenance of biological systems and represent important targets for the treatment of disease. The hydrophobicity and low abundance of IMPs make them difficult to analyze. In proteomic analyses, hydrophobic peptides including transmembrane domains are often underrepresented, and this reduces the sequence coverage and reliability of the identified IMPs. Here we report a new strategy, mild performic acid oxidation treatment (mPAOT), for improvement of IMP identification. In the mPAOT strategy, the hydrophobicity of IMPs is significantly decreased by oxidizing their methionine and cysteine residues with performic acid, thereby improving the solubility and enzymolysis of these proteins. The application of the mPAOT strategy to the analysis of IMPs from human nasopharyngeal carcinoma CNE1 cell line demonstrated that many IMPs, including those with high hydrophobicity, could be reliably identified.

The plasma membrane (PM)[1] functions as a barrier and communication interface. It is characterized by the presence of specific integral membrane proteins (IMPs), which play important biological and pharmacological roles in cell–cell interactions and signal transduction. These proteins represent important targets for the treatment of disease [1]. It is well known that many IMPs have intrinsic hydrophobic character and are of poor water solubility. As a result, the digestion of IMPs usually presents in low sequence coverage that could be explained by the nature of poor accessibility for proteolytic attack and low detection ability for hydrophobic peptides [2]. To address this problem, various digestion strategies for membrane proteins have been reported [3,4], including solubilization of the membrane proteins in aqueous solution containing detergents such as sodium dodecyl sulfate (SDS) [5] and sodium deoxycholate (SDC) [6] before trypsin digestion. However, high concentrations of detergents could suppress enzymatic activity and interfere with subsequent mass spectrometry (MS) measurements. We have developed a gel embedment method that allows the use of a high concentration of detergents to achieve efficient solubilization of very hydrophobic membrane proteins while avoiding interference with the subsequent liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis [7]. Although this method is very suitable for the comprehensive analysis of membrane proteins, the coverage of IMPs is still unsatisfactory. An alternative to detergent addition is the use of organic solvent for sample treatment [8–10]. For example, Blonder et al. [8] demonstrated that 60% methanol could efficiently solubilize the hydrophobic IMPs from complex mixtures. Fischer et al. [9] improved this method and demonstrated that the number of identified IMPs could be increased significantly by chymotrypsin/trypsin digestion in 60% methanol. In their method, the membranes were first incubated with trypsin in aqueous buffer to remove hydrophilic domains and membrane-associated proteins, followed by chymotrypsin/trypsin digestion in 60% methanol to release peptides from transmembrane helices. Blackler et al. showed that cyanogen bromide/formic acid (CNBr/FA) digestion could release peptides from transmembrane domains (TMDs) [10]. Recently, the elastase digestion method was used to increase transmembrane coverage in membrane proteome analysis [11]. Despite the suitability of chymotrypsin, proteinase K, and elastase for the shotgun analysis of membrane proteomes, trypsin is the prevalent enzyme choice in most current proteomic strategies because of its strict cleavage behavior.

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[1] *Abbreviations used:* PM, plasma membrane; IMP, integral membrane protein; MS, mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; CNBr, cyanogen bromide; FA, formic acid; TMD, transmembrane domain; FBS, fetal bovine serum; CM, crude membrane; PPM, purified plasma membrane; BSA, bovine serum albumin; IAA, iodoacetamide; mPAOT, mild performic acid oxidation treatment; SIMPLE, specific integral membrane peptide level enrichment; GRAVY, grand average hydropathy; 1D, one-dimensional.

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Performic acid oxidation, a method developed during the 1960s, could completely oxidize methionine to sulfone and cysteine to cysteic acid [12,13]. In addition, it shifts the hydrophobicity of transmembrane peptides, increases the hydrophilic nature of the oxidized peptides, and thereby increases accessibility for proteolytic attack [14]. Two recent proteomic research studies used performic acid oxidation to cleave the disulfide bond before hydrolysis with a protease in proteomic research [15,16]. Unfortunately, the traditional performic acid oxidation can affect other amino acids as well [17,18], and this restricts its application in proteomics. Pesavento et al. described and applied a mild performic acid oxidation method in a top-down proteomic study and found no side reactions [19]. Eichacker et al. showed that nearly 50% of all TMDs in the eukaryotic proteome contain a Met and proposed that methionine modification (e.g., by hyperoxidation or CNBr digestion) could be a tool to detect an increased number of TMDs by MS [14]. In the current article, we present a new strategy that greatly improves the identification of a larger number of hydrophobic multi-spanning IMPs by oxidation of methionine. The method focuses on reducing the hydrophobicity of the peptides instead of the length of the transmembrane peptides. The mild performic oxidation provides an obvious increase in the number of identified hydrophobic multi-spanning membrane proteins and is a good method for the analysis of close to complete sequences of IMPs.

Materials and methods

Cell culture and PPM purification

The human nasopharyngeal carcinoma CNE1 cell line was provided by the Cancer Research Institute of Xiangya Medicine College at Central South University (Changsha, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin, and 100 U/ml streptomycin. Harvested cells were homogenized by a Dounce homogenizer (~30 strokes) and centrifuged at 1000*g* for 5 min to pellet unbroken cells and nuclei. The crude membranes (CMs) obtained by sucrose density centrifugation were pelleted by ultracentrifugation at 100,000*g* and 4 °C for 30 min. The PMs were further purified as described before [20]. Briefly, the CM pellets were transferred to the 16-g two-phase systems (6.4% [w/w] PEG 3350, 6.4% [w/w] dextran T500, and 0.2 M potassium phosphate buffer, pH 7.2). After mixing and phase separation, the upper phases containing purified plasma membrane (PPM) were combined and diluted fivefold with 1 mM sodium bicarbonate. The PPM was finally pelleted by centrifugation at 100,000*g* for 1.5 h in a SW40 rotor. The pellet was resuspended in 1 ml of wash buffer (0.2 M NaBr, 0.2 M KCl, and 50 mM Tris–HCl, pH 8.0). After 30 min of incubation on ice, the soluble material was removed by centrifugation as above. The pellet was resuspended in 1 ml of 4 M urea and 100 mM Tris–HCl (pH 8.0). After 1 h of incubation on ice, the soluble material was removed by centrifugation as above. The pellet was resuspended in 1 ml of 100 mM Na_2CO_3 (pH 11.3). After 30 min of incubation on ice, the soluble material was removed by centrifugation as above.

Oxidation of BSA

To optimize mild performic acid oxidation for mass spectral analysis in membrane proteomics, 50 μg of bovine serum albumin (BSA) was dissolved in a freshly prepared mixture of 0.1% H_2O_2 and 3% FA, 3% H_2O_2 and 3% FA, 6% H_2O_2 and 6% FA, or 5% H_2O_2 and 95% FA. After 4 h of incubation on ice or at room temperature, all reactions were quenched by the addition of five volumes of cold Milli-Q water. The resulting solution was lyophilized. The oxidized BSA was dissolved in 50 mM NH_4HCO_3, and proteolysis was carried out overnight with a protein-to-trypsin ratio of 50:1 at 37 °C. BSA was alternatively modified with iodoacetamide (IAA) to compare the modification efficiency.

Classical 60% methanol/trypsin digestion

The classical 60% methanol/trypsin digestion protocol was carried out essentially as reported by Blonder et al. [8]. High pH washed membranes were resuspended in 60% methanol/50 mM NH_4HCO_3, and 5 μg of trypsin was added. Next, 6 h later, membranes were sedimented by centrifugation at 20,000*g* for 20 min (in the previous study [8], membrane debris was removed by a C18 reversed-phase solid-phase extraction [SPE] column), and the supernatant was collected as methanol_classical.

Trypsin predigestion of PPM fraction

The pellet was resuspended in 200 μl of 25 mM NH_4HCO_3 buffer and sonicated for 5 s. Then 5 μg of sequence-grade-modified trypsin (Promega, Madison, WI, USA) was added, and the membranes were incubated overnight at 37 °C for a first-time digestion. The supernatant was removed to a clean tube as the predigestion sample, and the pellets were divided into three parts. Subsequently, the predigested membrane pellets were collected as "shaved" membranes. The first part of shaved membranes was used for trypsin/chymotrypsin digestion. The second part was used for mild performic oxidation/trypsin digestion. The third part was further resuspended in 60% methanol/50 mM NH_4HCO_3.

Mild performic acid oxidation and trypsin digestion

After predigestion, the shaved membrane pellet was washed twice with ice-cold deionized water. The washed membrane pellets were dissolved in a final volume of 500 μl with a final concentration of 3% (v/v) H_2O_2 and 3% (v/v) FA (both from Thermo Fisher Scientific Inc., IL, USA) (200 μl of 30% H_2O_2, 60 μl of FA, and 740 μl of water, heated to 50 °C for 3 min). The solution was incubated on ice for 4 h, five volumes of cold Milli-Q water was added, and the resulting solution was lyophilized. Oxidized sample was dissolved in 50 mM NH_4HCO_3, and then tosyl phenylalanyl chloromethyl ketone (TPCK)-modified porcine trypsin (Promega) was added at a protease-to-protein ratio of 1:25 (w/w), followed by incubation at 37 °C overnight. Subsequently, the supernatant was collected as mild performic acid oxidation/trypsin digestion (mPAOT: mild performic acid oxidation treatment).

Chymotrypsin/trypsin digestion

The chymotrypsin/trypsin digestion was carried out as described previously [9]. Briefly, the membranes were predigested with trypsin overnight and then sedimented by ultracentrifugation at 20,000*g* for 20 min. After washing with ice-cold 0.1 M ammonium carbonate buffer (pH 11.0) and deionized water, the shaved membrane pellet was resuspended in 60% methanol/25 mM ammonium bicarbonate buffer. Chymotrypsin and trypsin (4 μg each) were added to the samples, and proteolysis was carried out overnight at 37 °C. The membranes were then removed by centrifugation, and the supernatant was named as SIMPLE (specific integral membrane peptide level enrichment) as described previously.

Modified 60% methanol/trypsin digestion

An aliquot of shaved membrane pellets was resuspended in 50 mM NH_4HCO_3 via intermittent vortex and sonication. The sample was then thermally denatured and methanol was added