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Phase Transfer Surfactant-Aided Trypsin Digestion for Membrane Proteome Analysis

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We have developed a new protocol for digesting hydrophobic proteins using trypsin with the aid of phase-transfer surfactants (PTS), such as sodium deoxycholate (SDC). SDC increases the solubility of hydrophobic proteins, enhances the activity of trypsin, and improves the accessibility to trypsin of proteins denatured during the extraction process. After digestion, SDC was successfully removed from the acidified solution containing tryptic peptides by adding a water-immiscible organic solvent, into which SDC was predominantly transferred, while the digested peptides remained in the aqueous phase. Compared with a protocol using an acid-labile surfactant, this PTS protocol increased the number of identified proteins and the recovery of hydrophobic peptides in the analysis of 400 ng of a membrane-enriched fraction of *Escherichia coli*. Application of the PTS protocol to 9.0 μg of a membrane-enriched pellet from human cervical cancer HeLa cells resulted in identification of a total of 1450 proteins, of which 764 (53%) were membrane proteins, by two-dimensional strong cation exchange (SCX)-C18 LC-MSMS with 5 SCX fractions. The distribution of the number of transmembrane domains in proteins identified in this study was in agreement with that in the IPI human database, suggesting that the PTS protocol can provide unbiased digestion of the membrane proteome.

Keywords: phase transfer surfactant • membrane proteome • sodium deoxycholate • trypsin digestion • transmembrane domain • hydrophobic peptides • HeLa cell • membrane-enriched fraction

Introduction

Since cellular membranes incorporate proteins that are involved in regulation of cell signaling and transportation of intra- and intercellular endogenous molecules, it is of importance to analyze these proteins to understand the overall cellular functions. Mass spectrometry-based proteomics has been widely used to obtain a comprehensive overview of proteins expressed in subcellular locations, or organellar proteomes, in combination with the use of biochemical techniques to purify the target organelles.[1,2] For membrane proteome analysis, however, these approaches cannot be directly applied because of the difficulties of protein extraction/solubilization and also of subsequent protease digestion for "bottom-up" shotgun proteomics.[3] Numerous protocols to improve the solubilization and the digestion of membrane proteins have been reported, including the use of organic solvents,[4–9] surfactants,[5,9–17] chaotropic reagents,[5,17–20] chemical cleavage reactions,[3,18,21,22] nonselective protease,[3,23] and a biotinyl reagent to enrich the plasma membrane.[24] Ionic surfactants, such as sodium dodecyl sulfate (SDS), are powerful tools to solubilize hydrophobic proteins, but they have to be diluted to avoid inactivation of the protease. In addition, they also influence LC separation and are incompatible with MS, so they have to be removed prior to LC-MS analysis.[25] On the other hand, organic solvents and hydrophilic chaotropic reagents, such as urea, can be easily removed.[26] Consequently, 60% methanol has often been used to extract membrane proteins,[27,28] although the solubilizing ability of organic solvents for hydrophobic proteins is generally not high.[29] Recently, acid-labile surfactants (ALS) were developed to overcome this problem.[14–16,25] For example, sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxy]-1-propanesulfonate, RapiGest SF, which is decomposed at low pH, was developed to facilitate the analysis of hydrophobic proteins. Very recently, it was reported that RapiGest SF is the most effective additive for the analysis of mammalian whole cell lysate proteins among various MS-compatible surfactants and organic solvents.[5] However, ALSs are usually expensive, and their effect on the membrane proteome is still unknown. Therefore, no "gold-standard" protocol exists for membrane proteome analysis at present, and it is still a challenging task to accomplish the unbiased digestion of membrane proteins, compared with soluble proteins.

In this study, we evaluated 27 different additives in terms of the enhancement of protease activity and solubilization ability for membrane proteins. We then introduced a new protocol using the selected additive, together with a new approach to efficiently remove the additive prior to LC-MS analysis. Finally we applied the developed protocol to the analysis of a HeLa cell membrane-enriched fraction.

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Table 1. Effect of 27 Additives on Hydrolysis of BLNA by Trypsin and Lys-C

		conc. at maximum activity[a] (activity enhancement factor[b])				upper limit of activity sustainable conc.[c]	
enhancer	ref	trypsin		Lys-C		trypsin	Lys-C
2-Propanol	5, 7	20%	3.36	20%	2.35	80%	80%
Acetonitrile	5, 7, 8	20%	2.48	10%	2.24	40%	80%
Dimethylformamide	8	10%	0.99	10%	2.06	40%	40%
Ethanol	-	20%	5.81	10%	1.79	40%	80%
HFIP	-	10%	0.33	10%	3.12	10%	20%
Methanol	4–7, 9	40%	3.88	10%	1.63	40%	80%
CHAPS	12	0.1%	5.36	0.001%	1.64	10%	10%
CHAPSO	-	0.01%	7.18	0.001%	1.65	10%	10%
CYMAL-5	10	0.01%	4.00	0.01%	1.94	10%	10%
Deoxy-BIGCHAP	-	0.01%	5.13	0.001%	2.11	10%	10%
Dodecylmaltoside	-	0.001%	2.81	0.001%	2.41	10%	10%
MEGA10	-	1%	5.08	0.01%	1.61	10%	10%
Nonylthiomaltoside	-	0.01%	4.52	0.01%	1.74	10%	10%
NP-40	-	0.1%	5.58	0.001%	1.50	5%	1%
Octylglucoside	10, 12	0.1%	6.79	0.1%	2.19	10%	10%
Octylthioglucoside	-	0.1%	6.21	1%	3.24	10%	10%
Oxatridecylmannoside	-	10%	4.56	0.001%	1.97	10%	10%
SDS	9, 11	0.01%	3.48	0.1%	1.78	0.01%	0.1%
Sodium cholate	-	0.1%	4.01	0.001%	2.01	10%	10%
Sodium deoxycholate	13, 17	0.01%	5.12	0.001%	1.74	10%	10%
Sodium glycocholate	-	0.1%	4.57	0.01%	1.86	10%	10%
Sucrose monochoalte	-	0.01%	5.15	0.001%	1.67	10%	10%
Sucrose monolaurate	-	0.01%	5.33	0.001%	1.83	10%	10%
Triton X-100	12	0.001%	4.82	0.001%	1.39	5%	1%
Tween 20	-	0.001%	4.63	0.001%	1.95	10%	10%
GuHCl	19, 20	0 M	-	0.375 M	1.02	0 M	1.5 M
Urea	5, 17, 18, 30	3 M	1.26	0.375 M	2.85	3 M	6 M

[a] The concentration at which the highest activity was observed at 1440 min. [b] The factor of the protease activity at 24 h in the presence of the enhancer to that in the absence of the enhancer. [c] The highest concentration at which more than 30% of the original activity without additives remains at 24 h min.

Materials and Methods

Materials. *n*-Decanoyl-*N*-methylglucamide (MEGA10), *n*-dodecyl-β-D-maltoside, *n*-nonyl-β-D-thiomaltoside, *n*-octyl-β-D-glucoside, sucrose monocholate, sucrose monolaurate, *n*-octyl-β-D-thioglucoside, 3-oxatridecyl-α-D-mannoside, sodium cholate (SC), sodium deoxycholate (SDC), sodium glycocholate, *N,N*-bis(3-D-gluconamidopropyl)deoxycholamide (deoxy-BIGCHAP), 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO) were obtained from Dojin (Kumamoto, Japan). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), Triton X-100, Tween 20, sodium dodecyl sulfate (SDS), sodium chenodeoxycholate, and sodium glycochenodeoxycholate were from Sigma (St. Louis, MO). Sodium ursodeoxycholate was from TCI (Tokyo, Japan). 5-Cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5) was from Anatrace (Maumee, OH). Tween 20 was from MP Biomedicals (Solon, OH). Nonidet P-40 (NP-40) was purchased from Calbiochem (Darmstadt, Germany). RapiGest SF was obtained from Waters (Milford, MA). Modified trypsin was from Promega (Madison, MA). Mass spectrometry-grade lysyl endprotease (Lys-C), *N*-benzoyl-lysine *p*-nitroanilide (BLNA), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), urea, guanidine hydrochloride (GuHCl), and other chemicals were purchased from Wako (Osaka, Japan). Water was purified by a Millipore Milli-Q system (Bedfold, MA).

Protease Activity Measurement. With BLNA as a model substrate, trypsin and Lys-C digestion (1:20 w/w enzyme-to-substrate ratio) were performed at 37 °C in 50 mM ammonium bicarbonate solutions containing 0%, 0.001%, 0.01%, 0.1%, 1.0%, 5%, and 10% surfactants, 0%, 10%, 20%, 40%, and 80% organic solvents, or 0 M, 0.375 M, 0.75 M, 1.5 M, 3 M, and 6 M chaotropic reagents. The ultraviolet absorbance at 390 nm was measured for *p*-nitroaniline, which is produced from BLNA by protease digestion, using a SPECTRA max Plus 384 spectrophotometer (Molecular Devices, MDS Sciex, Toronto, Canada). Triplicate measurements were performed under each condition.

Preparation of Membrane-Enriched Fractions of *E. coli* BW25113 Cells and HeLa Cells. A membrane-enriched fraction was prepared from *Escherichia coli* (*E. coli*) strain BW25113 cells and human cervical cancer HeLa cells. *E. coli* cells were grown in Luria–Bertani (LB) cultures at 37 °C for 8 h and were centrifuged at 4500*g* for 10 min and resuspended in 10 mL of ice-cold 1 M KCl, 15 mM Tris (pH 7.4). A protease inhibitor AEBSF was added to the final concentration of 10 mM. The solution was ultrasonicated on ice, and the unbroken cells and debris were pelleted at 2500*g* for 5 min. The supernatant was centrifuged at 100000*g* for 60 min, and the resultant pellet was resuspended in ice-cold 0.1 M Na_2CO_3 solution. After centrifugation at 100000*g* for 60 min, the pellet was collected as the membrane-enriched fraction. HeLa cells, cultured to 80% confluence in one 9-cm diameter dish (approximately 1×10^7 cells), were homogenized with a Dounce homogenizer (10 strokes) after adding protease inhibitors (Sigma) in PBS (pH 8.0). The homogenate was treated as described above for the *E. coli* cells to prepare the pellet of the membrane-enriched fraction.

Solubility Measurement for an *Escherichia coli* Membrane-Enriched Fraction. The *E. coli* BW25113 membrane fraction was solubilized with various solvents listed in Table 1 at 95 °C for 5 min followed by sonication for 10 min using a Tomy UD-201 ultrasonicator (Tokyo, Japan). After centrifugation at 10 000*g*, proteins in the supernatant solution were

quantified by UV absorbance measurement at 280 nm or by the BCA method using a Pierce BCA Protein Assay Kit.

In-Solution Digestion of the Membrane-Enriched Fractions. The membrane fraction was solubilized in a 50 mM ammonium bicarbonate solution containing 8 M urea, 1% RapiGest, or SDC. Proteins were reduced with 10 mM DTT for 30 min and alkylated with 55 mM IAA for 30 min at room temperature. Proteins solubilized in 8 M urea were digested with Lys-C, and the digest was 4-fold diluted and further digested with trypsin as described.[30] The solutions containing 1% RapiGest SF were diluted to 0.1% prior to trypsin digestion, and RapiGest SF was removed according to the manufacturer's instructions. In the case of SDC, two methods, the acid precipitation (APR) method and the phase transfer surfactant (PTS) method, were employed for SDC removal after trypsin digestion. The APR method was performed according to the reported procedure.[13] Briefly, the digested solution was acidified with 0.5% TFA (final concentration) and centrifuged at 15 700*g* for 15 min. Then, the supernatant was collected and desalted with StageTips with a C_{18} Empore disk membrane.[31] For the PTS method, 100 μL of an organic solvent was added to 100 μL of the digested solution, and the mixture was acidified with 0.5% TFA (final concentration) to be about pH 2. The mixture was shaken for 1 min, then centrifuged at 15 700*g* for 2 min to obtain aqueous and organic phases. The aqueous phase was collected and desalted using C_{18}-StageTips.

NanoLC-MS System. NanoLC-MS/MS analyses were conducted by using an LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) or a QSTAR-XL (AB/MDS-Sciex, Toronto, Canada) with a nanoLC interface (Nikkyo Technos, Tokyo, Japan), Dionex Ultimate3000 pump with FLM-3000 flow manager (Germering, Germany), and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). ReproSil-Pur C18-AQ materials (3 μm, Dr. Maisch, Ammerbuch, Germany) were packed into a self-pulled needle (150 mm length × 100 μm I.D., 6 μm opening) with a nitrogen-pressurized column loader cell (Nikkyo) to prepare an analytical column needle with "stone-arch" frit.[32] The injection volume was 5 μL, and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A three-step linear gradient of 5% to 10% B in 5 min, 10% to 40% B in 60 min, 40% to 100% B in 5 min, and 100% B for 10 min was employed throughout this study. A spray voltage of 2400 V was applied. The MS scan range was m/z 300–1500 (LTQ-Orbitrap) or 350–1400 (QSTAR). For LTQ-orbitrap, the top ten precursor ions were selected in MS scan by orbitrap for subsequent MS/MS scans by ion trap in the automated gain control (AGC) mode where AGC values of 5.00e+05 and 1.00e+04 were set for full MS and MS/MS, respectively. The normalized CID was set to be 35.0. For QSTAR experiments, MS scans were performed for 1 s to select three intense peaks, and subsequently three MS/MS scans were performed for 0.55 s each. An information-dependent acquisition function was activated for 2 min to exclude the previously scanned parent ions. The CID energy was automatically adjusted by rolling CID function.

Data Analysis and Bioinformatics. The raw data files were analyzed by Mass Navigator v1.2 (Mitsui Knowledge Industry, Tokyo, Japan) to create peak lists on the basis of the recorded fragmentation spectra. Peptides and proteins were identified by Mascot v2.1 (Matrix Science, London) against IPI human database release 3.24 (1-Dec-06) with a precursor mass tolerance of 5 ppm (LTQ-Orbitrap) or 0.25 Da (QSTAR), a fragment ion mass tolerance of 0.8 Da (LTQ-Orbitrap) or 0.25 Da (QSTAR), human taxonomy, and strict trypsin specificity allowing for up to 1 missed cleavage. Carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation was allowed as a variable modification. Peptides were rejected if the Mascot score was less the 95% confidence limit based on the "identity" score of each peptide, and a minimum of two peptides meeting the criteria was required for protein identification. False-positive rates (FPR) were estimated by searching against a randomized decoy database created by the Mascot Perl program. The grand average hydropathy (GRAVY) values for identified proteins and peptides were calculated according to a previous report.[33] Proteins and peptides exhibiting positive GRAVY values were recognized as hydrophobic. Mapping of transmembrane (TM) domains for the identified proteins was conducted using the TM hidden Markov model (TMHMM) algorithm available at http://www.cbs.dtu.dk/services/TMHMM-2.0/, to which FASTA files were submitted in batch mode.[34,35] Information on the subcellular location of identified proteins was obtained from gene ontology (GO) component terms using GOSlim (http://www.geneontology.org). Sequence coverage was visualized with the TOPO2 TM protein graphics program (http://www.sacs.ucsf.edu/TOPO2/).[36]

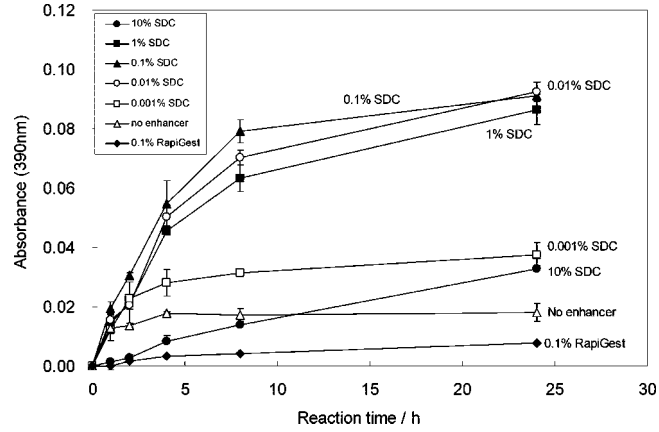

Figure 1. Trypsin activity measured in the presence of various concentrations of SDC and 0.1% RapiGest. BLNA was used as a substrate, and the hydrolyzed product, *p*-nitroaniline, was quantified by UV absorbance measurement at 390 nm. The hydrolysis reaction was performed with 4 μg of BLNA and 0.2 μg of trypsin or Lys-C. Triplicate measurements were performed. For details, see the Material and Methods section.

Results And Discussion

Various reagents have been reported as enhancers to accelerate protease digestion, as well as to improve the digestion efficiency for membrane proteins, but it is difficult to compare them because of differences in the conditions used for evaluation. Therefore, we chose 27 enhancers, including reported reagents, and examined their influence on the protease activity of trypsin and Lys-C as well as on the solubility of membrane proteins.

For the evaluation, we used the hydrolysis reaction of BLNA by trypsin and Lys-C in the presence of enhancers in the concentration range from 0.001% to 10% (wt %) for surfactants, 10 to 80% (vol %) for organic solvents, and 0.375 to 6 M for chaotropic reagents. In addition, 0.1% RapiGest as a standard ALS-type enhancer was also evaluated under the same conditions. Figure 1 shows typical reaction time-course data on the

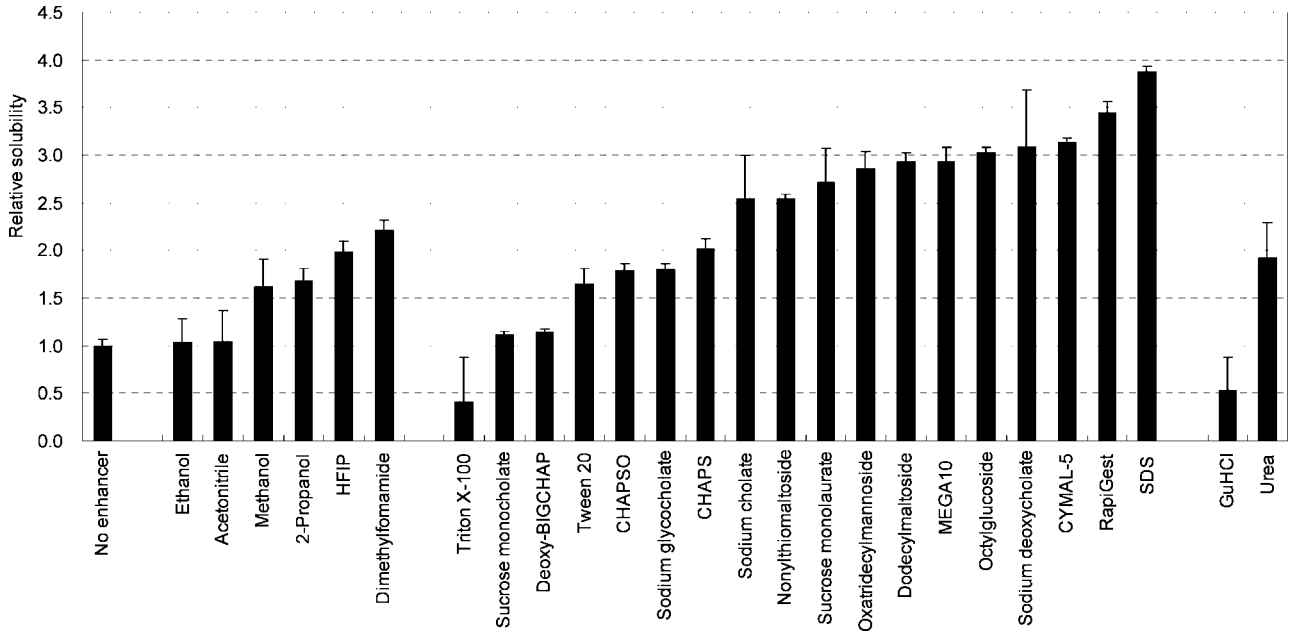

Figure 2. Solubilizing ability of enhancers for *E. coli* membrane fractions. The higher of the upper limit of ASC for trypsin or Lys-C in Table 1 was used as the concentration of the 27 enhancers. For details, see the Materials and Methods section.

protease activity of trypsin in the presence of SDC and RapiGest. The activity increased as the SDC concentration was increased and was approximately 5-fold higher at 1440 min in the presence of 0.01−1% SDC than that in the absence of SDC, though it became lower in the presence of 10% SDC. Interestingly, the addition of 0.1% RapiGest did not enhance the trypsin activity, though it was reported that 0.1% RapiGest resulted in 100% activity at 5 min using *N*-α-benzoyl-L-arginine ethyl ester as a substrate.[15] The other 26 reagents showed similar trends (Supplementary Figure S1). To assess the influence of the enhancers, we defined two parameters, activity sustainable concentration (ASC) which is the highest concentration at which more than 30% of the activity remains at 1440 min, and maximum activity concentration (MAC), which is the concentration giving the highest activity at 1440 min. Table 1 summarizes the values of these two parameters with the enhancement factors for trypsin and Lys-C. The enhancement factor was defined as the factor of the activity at 24 h in the presence of the enhancer to that in the absence of the enhancer. In general, trypsin provides larger MAC and larger activity enhancement factors than Lys-C, and surfactants gave greater enhancement of activity than organic solvents or chaotropic reagents. On the other hand, Lys-C provides higher upper limits of ASC for organic solvents and chaotropic reagents than trypsin, except in the cases of 2-propanol and dimethylformamide.

Next, we evaluated the solubilization ability of the 27 enhancers for membrane fractions of *E. coli* lysate at higher ASC for trypsin or Lys-C by measuring the UV absorbance at 280 nm. For NP-40 and octylthioglucoside, we could not obtain reproducible results owing to the high background signals caused by the absorbance of these surfactants themselves. The results are shown in Figure 2. Anionic SDS and RapiGest showed the greatest ability to solubilize *E. coli* proteins in the membrane fraction, whereas Triton X-100 and GuHCl decreased the solubility of these proteins, compared with the solubility without enhancers. In general, organic solvents did not significantly improve solubilization, whereas glyco-surfactants such as CYMAL-5 and octylglucoside, as well as bile salts such as SDC, showed more than 3-fold increases in solubility. Compared with SDS, these glyco-surfactants, as well as bile salts, are relatively mass spectrometer friendly.[37] However, it is still problematic to use them for continuous LC-MS/MS analysis, because these nonvolatile surfactants precipitate around the orifice of the mass spectrometer. In addition, some glyco-surfactants, such as octylglucoside, elute with tryptic peptides, and no peptides were detected in the elution ranges of these surfactants owing to significant ionization suppression, as well as detector saturation.[10] So, for robust LC-MSMS analysis, it is essential to eliminate the influence of residual surfactants in the injection solutions. Protocols using acid-labile surfactants (ALSs) such as RapiGest are currently the most efficient approach to analyze membrane proteins, although RapiGest does not enhance tryptic activity. Activity enhancers, such as glyco-surfactants and bile salts, would be more effective for membrane proteins if they could be easily removed from the sample solution after digestion.

It is known that bile salts, other than taurine-conjugated types, become more hydrophobic and precipitate at acidic pH owing to protonation of carboxyl groups.[13,17] Based on this property, we developed a new protocol to remove bile salts at acidic pH without precipitation. Figure 3 shows a flowchart of this protocol. An extraction solution containing a bile salt is added to the membrane-enriched pellet, and the extracted proteins are digested with trypsin in the presence of the bile salt. After digestion, a water-immiscible organic solvent such as ethyl acetate is added, and the mixture is acidified. The bile salt is transferred to the organic phase without precipitation, whereas tryptic peptides are sufficiently hydrophilic to remain in the aqueous phase if a suitable organic solvent is selected. Thus, the peptides can be separated from the bile salt prior to nanoLC-MSMS measurement. First, we examined the solubilization ability of six bile salts, sodium cholate (SC), sodium deoxycholate (SDC), sodium glycocholate, sodium chenodeoxycholate, sodium glycochenodeoxycholate, and sodium ursodeoxycholate, using BCA assay with 24 mM bile salts (corresponding to 1% SDC). Among them, SDC and sodium chenode-

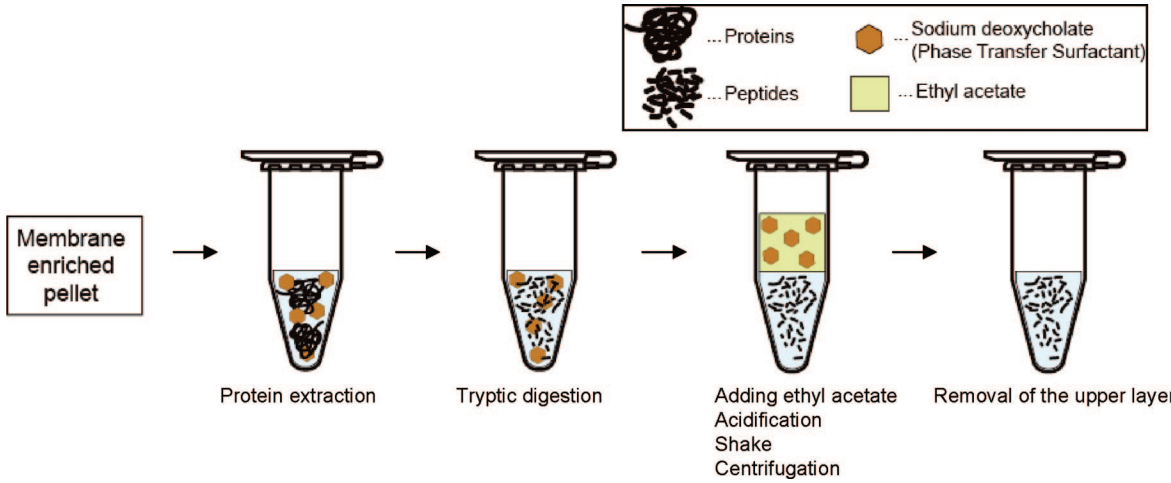

Figure 3. Flowchart for membrane proteome analysis using phase transfer surfactants. A membrane-enriched pellet was dissolved in extraction solution containing PTS (such as SDC) and digested with trypsin. SDC was separated from tryptic peptides by adding an organic solvent (such as ethyl acetate) followed by acidification. SDC is transferred to the organic phase. The aqueous phase was used for nano-LC-MS/MS analysis.

oxycholate gave the best results, and SC and sodium glycocholate were inferior to other deoxycholates (less than 50% of the result with SDC, data not shown).

Using 1% SDC as the phase-transfer surfactant (PTS) for the new protocol, we tested diethyl ether, octanol, and ethyl acetate as the organic solvents. These solvents worked well and gave almost the same result for the *E. coli* membrane fraction, although a 6-fold larger volume of diethyl ether relative to the aqueous phase was required to solubilize 1% SDC. The partition coefficients between organic and aqueous phases were 1669 (this study) and 3160[38] for ethyl acetate and octanol, respectively. Because ethyl acetate is easier to eliminate with a vacuum evaporator, we chose it for further analysis. We also confirmed that no tryptic peptide was detectable in the eliminated ethyl acetate phase in the analysis of the *E. coli* membrane fraction. Recently, it was reported that on-target liquid–liquid extraction using ethyl acetate coupled with MALDI-MS enabled us to analyze highly hydrophobic peptides including lipid-modified peptides.[39,40] In our case, however, these hydrophobic peptides could not be detected because the employed LC conditions were not suitable for these hydrophobic peptides.

Next, we compared the PTS protocol with the conventional urea protocol, in which 8 M urea is used for extraction of proteins from the membrane-enriched pellet and for digestion with Lys-C, followed by dilution to 2 M urea for trypsin digestion.[17] The acid precipitation (APR) protocol using SDC[13,17] was also investigated using 400 ng of *E. coli* membrane fraction. Note that we minimized the sample amount to emphasize the difference between protocols. The obtained peptides were evaluated in terms of the GRAVY score to categorize hydrophilic and hydrophobic peptides.[33] The identified proteins were also evaluated by use of the protein GRAVY score and the TMHMM algorithm. When either GRAVY scores were positive or TM domains were found by TMHMM, the proteins were defined as hydrophobic proteins. Triplicate analyses using each protocol were performed, and proteins were identified using the merged results, with the criteria that a minimum of two peptides with more than 95% probability scores per protein and less than 5% false positive rates for proteins were required. The results are shown in Figure 4. Compared with the urea protocol, the PTS and APR protocols using 1% SDC both gave greatly superior results in terms of both peptide and protein

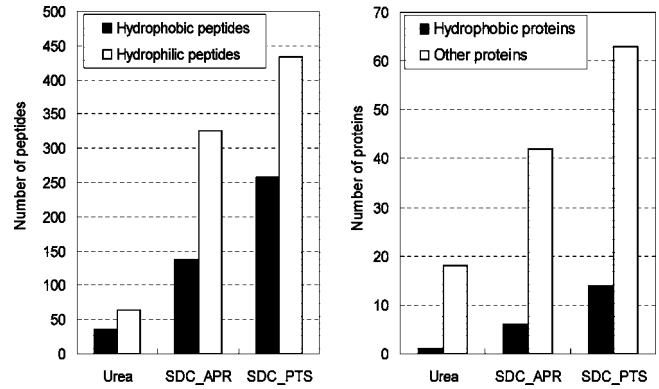

Figure 4. Comparison of different protocols for the analysis of *E. coli* membrane-enriched fractions. *E. coli* membrane proteins (400 ng) extracted with 8 M urea or 1% SDC were used for protease digestion. SDC was eliminated according to the APR or PTS protocol prior to analysis of the peptides by nano-LC-MS/MS (QSTAR). For details, see the Materials and Methods section. Hydrophobicity of identified peptides was classified by GRAVY scores. Protein with TM domains according to the TMHMM algorithm or positive GRAVY scores were classified as hydrophobic proteins. The merged results from triplicate analysis were used for comparison.

identification, and the PTS protocol showed the highest efficiency for hydrophobic peptides and proteins, as well as total numbers of identified peptides and proteins. Using the peptides identified in both the APR and PTS protocols, we also compared the ratios of peak area in the mass chromatograms to evaluate the recovery (Figure 5). The peak area ratio (PTS/APR) increased as retention time increased, indicating that the PTS protocol was more effective than the APR protocol especially for hydrophobic peptides. These results suggested that hydrophobic peptides incorporated into SDC micelles after digestion were coprecipitated with protonated SDC molecules in the APR protocol, whereas in the PTS protocol, hydrophobic peptides were released from SDC molecules when SDC was extracted with ethyl acetate.

Finally, we compared the PTS protocol and the ALS protocol with RapiGest for 5 μg of *E. coli* membrane fraction. Compared with RapiGest, SDC provides a much better enhancement factor for trypsin activity and comparable protein solubilization ability.

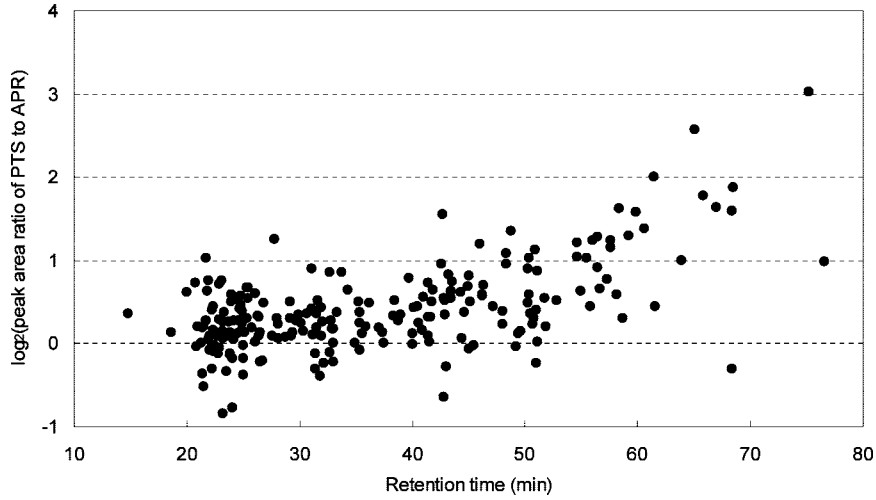

Figure 5. Comparison of the recoveries of peptides identified with the PTS and APR protocols. Peptides identified by means of both protocols in triplicate analysis were used. Peak areas were measured from the mass chromatograms, and the ratios of the peak area with the PTS protocol to those with the APR protocol were calculated. For details, see the legend to Figure 4.

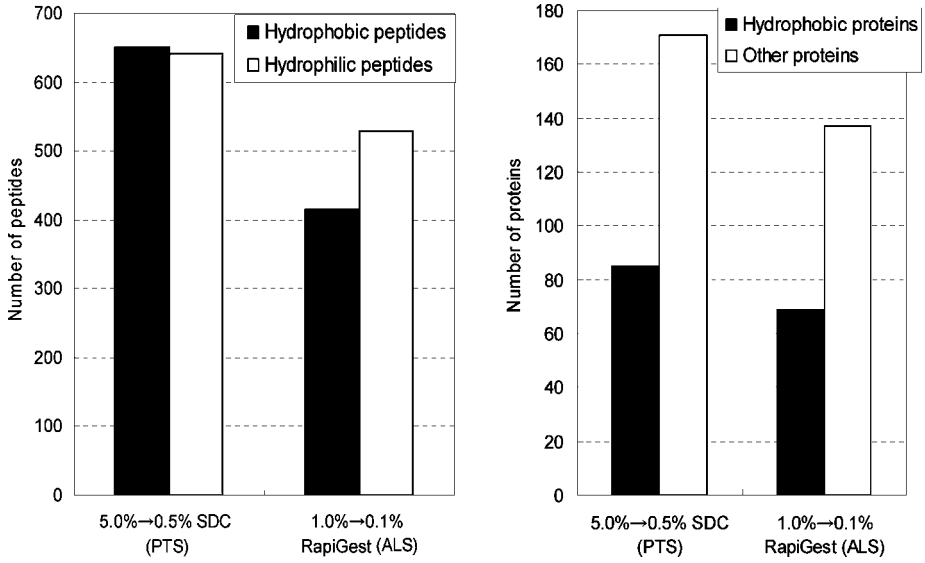

Figure 6. Comparison of the PTS protocol and the ALS protocol with RapiGest for the analysis of *E. coli* membrane-enriched fractions. *E. coli* membrane-enriched fraction (5 μg) was extracted with 5% SDC or 1% RapiGest, then diluted to 0.5% SDC or 0.1% RapiGest for trypsin digestion. Nano-LC-MS/MS was performed using QSTAR. Triplicate analyses were performed, and the merged results were used for comparison. For details, see the Materials and Methods section.

Therefore, SDC should provide better results than RapiGest if the surfactant elimination step in the PTS protocol is effective. According to the reported RapiGest protocol,[5] 1% RapiGest at 95 °C for 5 min was used for extracting hydrophobic proteins, and trypsin digestion was performed after dilution to 0.1%. For trypsin digestion in the PTS protocol, 0.5% and 1% SDC were evaluated in combination with 5% SDC at 95 °C for 5 min for protein extraction, and 0.5% SDC gave the best result (15% and 19% increase in the numbers of identified peptides and proteins, respectively, over 1.0% SDC). Thus, in the PTS protocol, 5% and 0.5% SDC were used for protein extraction and trypsin digestion, respectively. The results of triplicate analyses using the PTS protocol and ALS protocol are compared in Figure 6. At both the peptide and protein levels, the PTS protocol provided better results than the ALS protocol, especially for hydrophobic peptides. Relative standard deviation in triplicate analysis for the numbers of hydrophobic peptides and proteins in the PTS protocol was 5.8% and 0.7%, respectively, and 84% of all identified hydrophobic proteins were found in all three runs, which is in line with our experience for LC-MS replicate analysis using the same sample (the protein overlap between duplicate runs is around 80–90%), indicating the extraction/digestion steps were highly reproducible in the PTS protocol. Figure 7 shows the relationship of retention times of commonly identified peptides and the peak area ratios between these two protocols. It is clear that more hydrophobic peptides were recovered in the PTS protocol than in the ALS protocol.

Since surfactants denature proteins, including trypsin, we examined the influence of this protocol on the occurrence of semitryptic cleavage in the analysis of the *E. coli* membrane-enriched fraction. The results are listed in Table 2. We found no significant difference in the cleavage specificity between these three protocols.

A semitryptic search was performed by Mascot using the enzyme "semitrypsin". The peptide/protein identification cri-

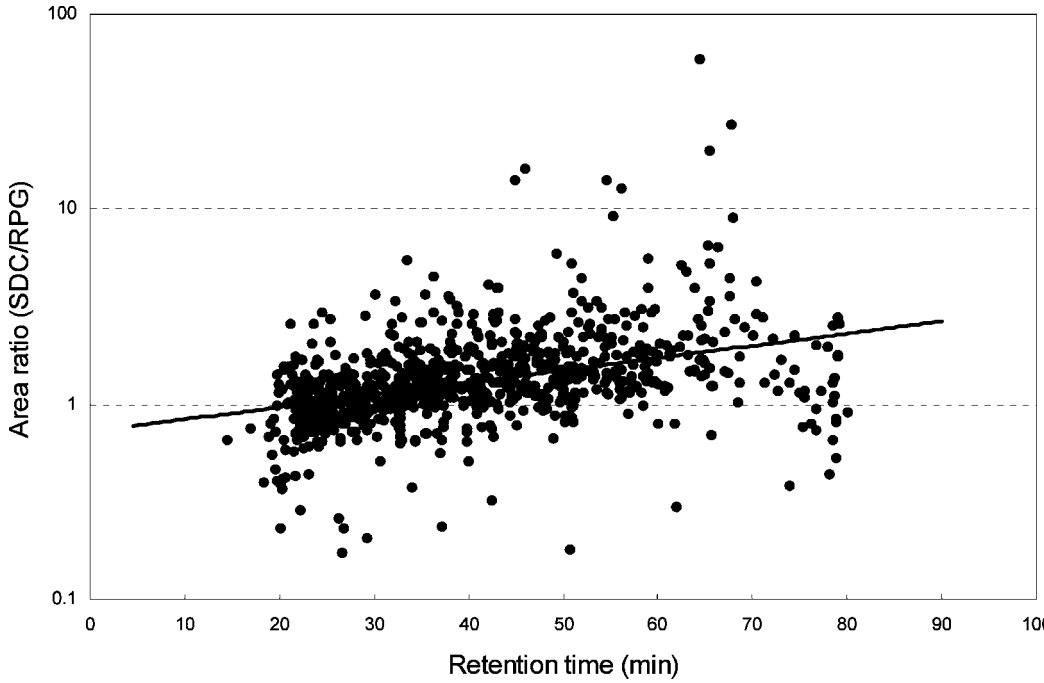

Figure 7. Comparison of the recoveries of peptides identified by use of the PTS and ALS protocols. Peptides identified according to both protocols in triplicate analysis were used. Peak areas were measured from the mass chromatograms, and the ratios of the peak area with the PTS protocol to that with the ALS protocol were calculated. For details, see the legend to Figure 6.

Table 2. Semitryptic Cleavage Contents in Trypsin Digestion with Different Enhancers

enhancer	enhancer conc. for protein extraction	enhancer conc. for protein digestion	no. of full tryptic peptides	no. of semitryptic peptides	semitryptic cleavage (%)	MS
Urea	8 M	2 M	1689	131	7.2%	Orbitrap
SDC	0.5%	0.5%	3373	176	5.1%	Orbitrap
SDC	5%	0.5%	876	51	5.5%	QSTAR
RapiGest	1%	0.1%	607	36	5.6%	QSTAR

teria were the same as for the tryptic search, as described in the Materials and Methods section.

The PTS protocol was applied to more complex samples, i.e., mammalian cells. According to the PTS protocol, 9.0 μg of the membrane-enriched fraction of HeLa whole cell lysates was treated, and the resultant tryptic peptides were applied to C18-StageTips or fractionated into five vials using SCX-StageTips.[41] The resultant six samples were analyzed by nanoLC-MS/MS in duplicate. All results from a total of 12 LC-MSMS runs were merged, and in total, 1450 proteins were identified based on 11 839 fully tryptic peptides with FPR 1.5% (Supplementary Tables S1 and S2). We employed three algorithms to predict membrane proteins, using TMHMM, GO slim, and GRAVY scoring. The results (Figure 8) indicate that more than 50% of the identified proteins (764 proteins) were membrane proteins. This data set contained at least 53 receptors, 64 transporters, 59 ion channel proteins, and 15 glycosylphosphatidylinositol anchored proteins, based on GO terms, including four G-protein coupled receptors and several growth factor-related receptor kinases (Supplementary Table S3).

One example of membrane proteins identified with high sequence coverage is shown in Figure 9. The isoform long of the sodium/potassium-transporting ATPase α-1 chain precursor, IPI00006482, with 10 predicted TM domains was identified based on 48 identified peptides (sequence coverage 44.0%). There are 7 tryptic cleavage sites inside the TM domains, and theoretically 14 peptides can be generated. Among them, 4 peptides are too short (less than 4 amino acids) and 4 peptides are too large (more than 4000 peptide mass) for the current MS scan range. Among the remaining 6 MS-detectable peptides, 3 peptides were successfully identified; the 3 unidentified peptides (3026, 2961, and

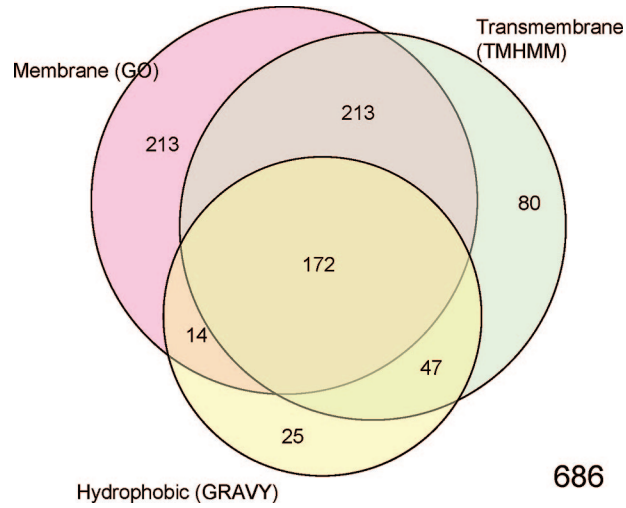

Figure 8. Venn diagram indicating total hydrophobic proteins identified from HeLa cells. The membrane-enriched fraction from HeLa cells (1.5 μg) was treated according to the PTS protocol. The resultant tryptic peptides were desalted using C18-StageTips or fractionated using SCX-StageTips (five fractions). These six samples were analyzed by nanoLC-MS/MS using the LTQ-OrbiTrap. Duplicate analyses were performed, and the merged results were analyzed.

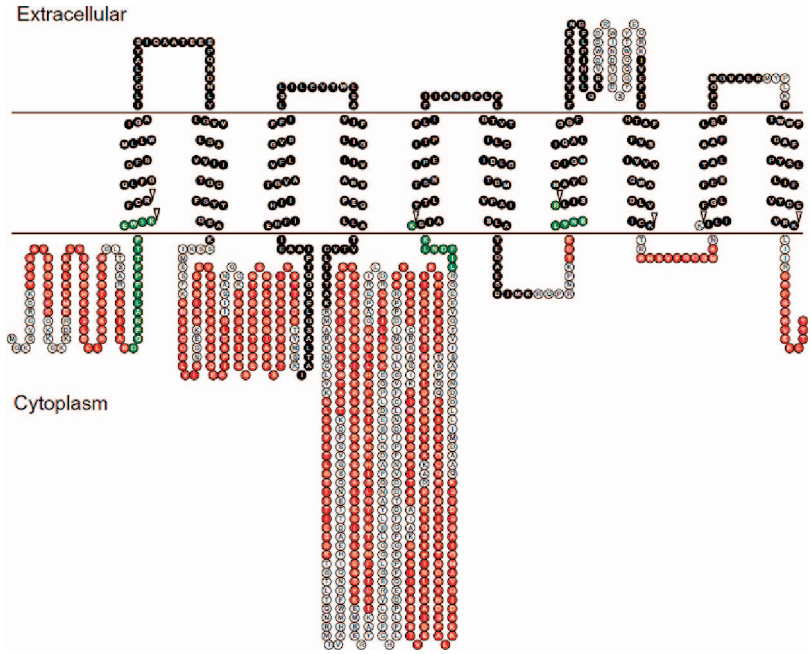

Figure 9. Protein with 10 TM domains (isoform long of sodium/potassium-transporting ATPase α-1 chain precursor, IPI00006482) identified from HeLa cells using the PTS protocol. The sequence coverage was visualized with the TOPO2 transmembrane protein graphics program. Identified tryptic peptides including TM domains are in green; identified tryptic peptides without TM domains are in red; and unidentified tryptic peptides including TM domains are in black. The tryptic cleavage sites inside the TM domains are indicated with arrows.

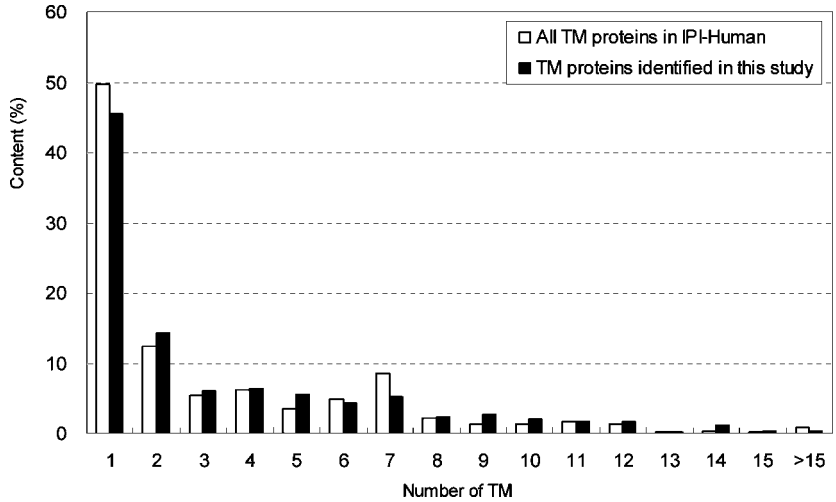

Figure 10. Comparison of the numbers of TM domains of membrane proteins identified according to the PTS protocol with database values. IPI human database version 3.24 was used to extract all human proteins with TM domains. In total, 14 950 proteins from the IPI database and 512 proteins identified from HeLa cells according to the PTS protocol were used to calculate the content (%) of proteins having various numbers of TM domains.

3344 Da) were expected to be too hydrophobic to be eluted under the current LC conditions.[42] It is known that TM domains are usually difficult to cleave with trypsin.[43] However, at least for this protein, trypsin cleavage occurred even within the TM domains, indicating the high efficiency of this protocol. To examine the effect of this protocol on the tryptic cleavage efficiency for TM domains, we compared the distribution of the number of TM domains between 512 TM proteins identified in this study and 14950 TM proteins registered in the IPI human protein database (Figure 10). The distribution pattern of PTS-identified proteins was highly consistent with that of the IPI-registered membrane proteins, suggesting that the PTS protocol shows little bias in digesting TM proteins.

In conclusion, we have developed a new protocol based on the use of phase transfer surfactant as an enhancer for protein extraction, protein solubilization, and trypsin activation. This protocol improved the efficiency of protein identification for membrane-enriched fractions of *E. coli* and HeLa cells. Application of the protocol did not introduce any bias in tryptic digestion of hydrophobic proteins with TM domains. This protocol, in combination with current "bottom-up" proteomics technologies, is expected to extend the coverage of the membrane proteome and to provide more information on membrane proteins, including post-translational modifications, which will be helpful to clarify the mechanisms of membrane-mediated cell functions.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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