

Ionic liquid 1-butyl-3-methyl imidazolium tetrafluoroborate for shotgun membrane proteomics

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Abstract The solubility and digestion efficiency are two crucial factors that might affect the identification of integral membrane proteins (IMPs). In this work, 1% (v/v) ionic liquid (IL), 1-butyl-3-methyl imidazolium tetrafluoroborate (BMIM BF₄), added in NH₄HCO₃ buffer (pH 8.3), was applied as a sample preparation buffer for IMPs analysis. Compared to the commonly used sodium dodecyl sulfate and methanol methods, the number of identified IMPs from rat brain by microcolumn reversed phase liquid chromatography (μ RPLC)-electrospray ionization tandem mass spectrometry (ESI-MS/MS) was improved by over three times, which might be due to the fact that BMIM BF₄ offered high solubilizing ability for IMPs and good compatibility for tryptic digestion. Furthermore, compared to Rapigest and urea methods, with BMIM BF₄ method, the number of identified IMPs from rat brain could be improved 25% and 80%, respectively, which might be contributed to the good solubilizing ability and high thermal stability of such IL. With the sample treated by

BMIM BF₄ method, by 2D-nanoSCX-RPLC-ESI-MS/MS, 1,450 non-redundant proteins and 7,978 unique peptides were identified from rat brain, and 418 proteins contained at least one predicted transmembrane domain, with false discovery rates of less than 1% for peptide identification, and at least two identified unique peptides per protein. All these results demonstrate that the BMIM BF₄ method is of high potential for the large-scale identification of IMPs.

Keywords Ionic liquid · 1-Butyl-3-methyl imidazolium tetrafluoroborate · Integral membrane proteins · Shotgun

Introduction

Integral membrane proteins (IMPs) lie at the critical junctions between intracellular compartments, cells, and their environments, which play unique roles in mediating various cellular processes, including intercellular communication, vesicle trafficking, ion transport, protein translocation/integration, and the propagation of signaling cascades [1–3]. However, the identification of IMPs meets great challenges due to their hydrophobicity [4–7].

Recently, shotgun approach has emerged as a powerful technique for membrane proteome identification and quantification [8–14], by which proteins should be degraded into peptides by either enzymatic or chemical digestion, followed by the separation and identification by high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS). Therefore, for IMPs analysis, improvement on the solubility of such hydrophobic proteins in suitable solvent systems that are of good compatibility with enzymatic digestion is of great significance.

In the past few years, several kinds of solvent systems have been applied in IMPs analyses [15], including chaotropes

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[16–18], detergents [7, 10, 18–21], organic solvents [7, 18, 22–26], and organic acids [8, 27]. Among them, urea is one of the most commonly used chaotropes with strong denaturation ability. Although it can be easily removed before MS analysis, its capacity to extract most IMPs, particularly polytopic proteins, is limited. Furthermore, urea can also cause problematic carbamylation of N-termini and lysine residues, when heated above 37 °C. Although sodium dodecyl sulfate (SDS), the strongest denaturation reagent, is also often used in membrane protein analysis, it is rather difficult to remove before MS analysis. In addition, MS compatible surfactants, such as Rapigest, are among the most promising additives to enhance IMPs solubilization without interfering with LC-MS. Yates et al. [10] made the global analysis of mammalian brain proteome with MS compatible surfactants, and 5,000 proteins were identified from 1.8 mg of rat brain homogenate, among which 1,897 proteins were annotated by Gene Ontology as membrane proteins, and 1,225 proteins were predicted to contain at least one transmembrane domain (TMD). However, Rapigest is expensive, and the cleavage step at low pH could result in hydrophobic peptides loss due to co-precipitation with the hydrophobic part of RapiGest [28]. Organic solvents, especially 60% (v/v) methanol, are also effective to destroy membrane structure but could attenuate enzyme activity. Organic acids with high concentration are of strongly solubilizing ability for membrane proteins, and CNBr is usually used for membrane proteins digestion. However, due to the high toxicity of CNBr, great caution should be made during sample preparation, and the high concentration of acid might lead to unexpected modifications [29].

Ionic liquids (ILs) are liquids composed completely of ions, and thus have unique physicochemical properties, including broad liquid range, low to negligible vapor pressures, high thermal stability, and high solubility for different classes of analytes [30–42]. Therefore, ILs environment is quite different from that of all polar or non-polar organic solvents [32], which is capable of forming virtually all possible types of interactions with solutes [33, 34].

Due to the above-mentioned unique properties, ILs are regarded as a new class of green solvents [30, 35] and have been successfully applied in sample extraction [31, 36, 37], chromatography separation [38, 39], and MS identification [40–42]. Anderson et al. [31] applied ILs containing tris(pentafluoroethyl)trifluorophosphate anion for the extraction of polycyclic aromatic hydrocarbons. Compared to other commonly used ILs, such ILs are substantially more hydrophobic and hydrolytically stable, making them ideal candidates for the direct immersion extraction from aqueous matrices. Chang et al. [39] obtained excellent chiral recognition ability in capillary electrophoresis, HPLC, and capillary gas chromatography

by applying (R)-*N,N,N*-trimethyl-2-aminobutanol-bis(trifluoromethanesulfonyl)imidate. Armstrong et al. [40] tried different ILs as matrices for the ultraviolet matrix-assisted laser desorption ionization MS analysis of peptides, proteins, and poly(ethylene glycol) (PEG-2000), which could produce high signal intensity, resulting in lower limits of detection than comparable solid matrices.

However, to the best of our knowledge, the application of ILs in IMPs preparation for shotgun membrane proteomics analysis has not been reported. The ideal ILs for membrane protein analysis should have the properties such as high thermal stability, good solubility and solubilizing ability, good compatibility for enzymatic digestion, and easy removal before MS analysis. Since 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM BF₄) can meet such requirements, in our recent work, it was applied for the analysis of IMPs extracted from rat brain. Compared to commonly used SDS, methanol, Rapigest, and urea methods, more IMPs were identified, which demonstrates that BMIM BF₄ method is of high potential for the analysis of IMPs.

Materials and methods

Materials

BMIM BF₄ was obtained from Shanghai Cheng Jie Chemical Co. Ltd. (Shanghai, China). Methanol and protease Inhibitor Cocktail Set I were ordered from Merck (Darmstadt, Germany). SDS was purchased from Biomol GmbH (Hamburg, Germany). Rapigest was bought from Waters Corp. (Milford, MA, USA). Urea was obtained from Shenyang Chemical Reagents (Shenyang, China). Bacteriorhodopsin from *Halobacterium salinarium* and trypsin TPKC-treated (bovine pancreas) were ordered from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was bought from Sino-American Biotech (Luoyang, China). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Acros (Morris plains, NJ, USA). Deionized water purified by a Milli-Q system (Millipore, Milford, MA, USA) was used in all experiments. SCX beads (UNI MC5SP-500, 5 μm, 500 Å pore) were purchased from Shenzhen Nano-micro Technology Inc. (Shenzhen, China). C18 silica particles (5 μm, 200 Å pore) were bought from Bona (Tianjin, China).

Sample preparation

Rat brain (~0.3 g) was homogenized by Tissue Tearor from Biospec Products (Bartlesville, OK, USA) in 2 mL of lysis buffer, composed of 2 M NaCl, PBS, and 1% (v/v) Protease Inhibitor Cocktail Set I, at approximately 10,000 rpm for

1 min. The suspension was centrifuged at 12,000×*g* for 20 min at 4 °C. With supernatant discarded, the pellet was re-extracted by vortexing in 3 mL of high pH buffer (0.1 M Na₂CO₃, 1% (v/v) protease inhibitor, pH 11.3). After 30 min incubation on ice, the soluble part was removed under the same centrifugation conditions. The pellet was treated again by high pH buffer, following by ultrasonication (Cole-Parmer, Vernon Hills, IL, USA) in 2 mL of 1% (v/v) protease inhibitor for 2 min on ice at power 100% pulse duration. After protein concentration measurement by Bradford method, the cell lysates were precipitated with methanol/chloroform to remove lipids. After centrifugation, the pellet was lyophilized in a SpeedVac (Thermo Fisher, San Jose, CA, USA), and stored at –80 °C before use.

Solubility measurement for bacteriorhodopsin

Equal aliquots of lyophilized bacteriorhodopsin (50 µg each) were solubilized in 80 µL of five solvent systems, including (a) 50 mM NH₄HCO₃ buffer, (b) 50 mM NH₄HCO₃ buffer containing 60% (v/v) methanol, (c) 50 mM NH₄HCO₃ buffer containing 2 M urea, (d) 50 mM NH₄HCO₃ buffer containing 0.1% (w/v) Rapigest, and (e) 50 mM NH₄HCO₃ buffer containing 1% (v/v) BMIM BF₄. The five samples were firstly sonicated for 0.5 h in a water bath at room temperature, and then put in the water bath at 37 °C for 10 min. After vortex for 1 min, the samples were centrifuged to remove insoluble materials, and the supernatant was respectively collected, followed by quantification by Bradford method at 595 nm with BSA as the standard protein.

In-solution digestion of BSA

BSA (6 mg/mL, dissolved in 1 mL 1 M urea, 100 mM NH₄HCO₃, pH 8.3) was denatured at 90 °C for 10 min and reduced and alkylated with standard protocol, followed by respective dilution with different solutions, including 100 mM NH₄HCO₃, 60% (v/v) methanol, 0.1% (w/v) SDS, 1% (v/v) BMIM BF₄, 2 M urea, and 0.1% (w/v) Rapigest, to the final protein concentration of 1 mg/mL. The above-mentioned six samples were digested by trypsin with a trypsin/protein ratio (w/w) of 1:40 at 37 °C for 0.5 h, respectively. The obtained digested products (10 µg for each sample) were run on a 12% acrylamide gel, and stained with Coomassie staining.

Protein solubilization and digestion

Lyophilized protein pellets (200 µg) were resuspended in five different buffers for trypsin digestion, respectively. BMIM BF₄-assisted solubilization and proteolysis of IMPs, briefly named as BMIM BF₄ method, were performed as

follows: 300 µL of 1% (v/v) BMIM BF₄ dissolved in 100 mM NH₄HCO₃ (pH 8.3) was used to re-suspend 200 µg of membrane protein pellets via vortex. The suspension was sonicated for 1 h in a water bath at room temperature, and then kept at 90 °C for 20 min for thermal denaturation. Subsequently, the sample was cooled, followed by reduction in 13 mM DTT at 56 °C for 2 h. After cysteines were alkylated in darkness in 32 mM IAA for 1 h at room temperature, tryptic digestion was performed with a trypsin/protein ratio (w/w) of 1:25 at 37 °C for 24 h. After digestion, the solution was acidified with 90% (v/v) formic acid (1% final) to stop proteolysis. Followed by centrifugation, the supernatants were desalted with a C18 solid-phase cartridge, during which ILs could be removed simultaneously. Finally, the digests were lyophilized, and re-dissolved in 80 µL of deionized water containing 0.1% (v/v) formic acid, prior to microcolumn reversed phase liquid chromatography (µRPLC)-electrospray ionization (ESI)-MS/MS analysis.

SDS and organic solvent-assisted solubilization and proteolysis of IMPs, briefly named as SDS method and methanol method, were performed according to the published protocol [7]. Rapigest-assisted solubilization and proteolysis of IMPs, briefly named as Rapigest method, were performed according to the published protocol [10] with slight modifications. Urea-assisted solubilization and proteolysis of IMPs, briefly named as urea method, were performed according to the published protocol [18]. The final digests were re-dissolved in 80 µL of deionized water containing 0.1% (v/v) formic acid, prior to µRPLC-ESI-MS/MS analysis. The details of SDS, methanol, Rapigest, and urea methods are shown in Electronic Supplementary Material “Supporting material I.”

µRPLC-ESI-MS/MS analysis

Digests obtained by the above-mentioned five methods were respectively analyzed by duplicate µRPLC-ESI-MS/MS experiments according to our previous protocols [43]. µRPLC was performed on a paradigm GM4 µHPLC system (Michrom Bioresources, Auburn, CA, USA), and ESI-MS/MS was performed on LCQ^{DUO} (Thermo-Fisher, San Jose, CA, USA). A homemade C18 column (300 µm i.d. ×35 cm) was used for peptides separation, with the flow rate of 5 µL/min. Two percent (v/v) acetonitrile with 0.1% (v/v) formic acid (buffer A) and 98% (v/v) acetonitrile with 0.1% (v/v) formic acid (buffer B) were used to generate a 420-min gradient, set as follows: 0% B for 10 min, to 5% B in 10 min, to 40% B in 380 min, to 80% B in 10 min, and kept at 80% B for 10 min. The sample injection volume was 20 µL for each µRPLC-ESI-MS/MS analysis.

LCQ mass spectrometer was operated at positive ion mode. The spray voltage was 2.5 kV, and the heated

capillary temperature was 150 °C. Total ion current chromatograms and mass spectra covering the mass range from m/z 400 to 2,000 were recorded with Xcalibur software (version 1.4). MS/MS spectra were acquired by data-dependent acquisition mode with three precursor ions selected from one MS full scan. Precursor selection was based on parent ions intensity, and the normalized collision energy for MS/MS scanning was 35%. μ RPLC solvent gradient and mass spectrometer scan were controlled by the Xcalibur data system (version 1.4).

2D-nanoSCX-RPLC-ESI-MS/MS analysis

The membrane protein pellets extracted from rat brain were solubilized and digested according to 1% (v/v) BMIM BF₄ method, as mentioned in “[Protein solubilization and digestion](#)” section, and about 160 μ g digested products were obtained. After lyophilized, the digests were re-dissolved in 100 μ L of 2% (v/v) acetonitrile with 0.1% (v/v) formic acid, prior to 2D-nanoSCX-RPLC-ESI-MS/MS analysis.

LTQ coupled with a quaternary Surveyor pump (Thermo-Fisher, San Jose, CA, USA) were used for 2D-nanoSCX-RPLC-ESI-MS/MS, similar with previous report [44]. Water solution with 0.1% (v/v) formic acid (buffer A'), acetonitrile with 0.1% (v/v) formic acid (buffer B'), and 2,000 mM NH₄Ac (pH 3) were applied. The optimized flow rate was ~200 nL/min.

The tryptic digest was loaded automatically onto an SCX trap column (2 cm, 150 μ m i.d./375 μ m o.d.) packed with polystyrene SCX particles (5 μ m, 500 Å). Then, a series of stepwise elution with 20, 60, 80, 100, 140, 180, 300, 500, and 1,000 mM NH₄Ac was used to elute peptides onto a home-packed reversed phase capillary column (14 cm, 75 μ m i.d./375 μ m o.d.) packed with C18 silica particles (5 μ m, 300 Å), and each salt step lasted 15 min. After the nanoRPLC system was re-equilibrated for 5 min with buffer A', a binary gradient was applied, from 0% to 10% B' for 10 min, to 40% B' for 75 min, and to 80% B' for 5 min. After each run, the column was flushed by 80% B' for 15 min, and further equilibrated with A' for 15 min. The 2D-nanoSCX-RPLC-ESI-MS/MS analysis was run in triplicates, and the injection volume was 20 μ L (32 μ g) for each run.

LTQ was operated at positive ion mode. The temperature of the ion transfer capillary was set at 180 °C. The spray voltage was set at 2.2 kV, and the normalized collision energy was set at 35.0%. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. The mass spectrometer was set that one full MS scan was followed by nine MS/MS scans on the nine most intense ions. The dynamic exclusion function was set as follows: repeat count 1, repeat duration 30 s, and exclusion duration 180 s. System control

and data collection were done by Xcalibur software (version 2.0).

Database search and interpretation of MS/MS data sets

For μ RPLC-ESI-MS/MS analysis, protein identification was performed using BioWorks Software 3.1 for LCQ with SEQUEST search program. The rat database was ipi.RAT.v3.26.fasta (41494 protein entries), and reversed sequences were appended to the database for the evaluation of false discovery rate (FDR). Cysteine residues were searched as static modification of 57.0215 Da. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavages sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Peptides were considered as positive identification if Xcorr was higher than 1.9 for singly charged peptides, 2.2 for doubly charged peptides, and 3.75 for triply charged peptides. Δ Cn cutoff value was set to control the FDR of peptide identification less than 3%, determined by the calculation based on the reversed database. FDRs were calculated by using the following equation: $FDR = n(\text{rev})/n(\text{forw})$, where $n(\text{forw})$ and $n(\text{rev})$ are the number of peptides identified in proteins with forward (normal) and reversed sequence, respectively.

For 2D-nanoSCX-RPLC-ESI-MS/MS analysis, protein identification was performed using BioWorks Software 3.3.1 for LTQ with SEQUEST search program. Cysteine residues were searched as static modification of +57.0215 Da, and methionine residues were searched as variable modifications of +15.9949 Da. Other parameters for database searching were same as those for μ RPLC-ESI-MS/MS analysis. After database searching, to control the FDR and reduce the apparent redundancy in protein identification, our results were aligned by a software tool (BuildSummary) using the following criterion: Δ Cn value was set at 0.1, and Xcorr values were adjusted to ensure the FDR of peptide identification less than 1%. When the same peptides were assigned to multiple proteins, the assigned proteins were clustered into a “protein group” [45]. For bioinformatics analysis, only the protein with the highest sequence coverage in each “protein group” was analyzed.

Bioinformatics analysis

For results obtained by μ RPLC-ESI-MS/MS analysis, the TMDs of the identified proteins were predicted according to the algorithm at <http://psort.ims.u-tokyo.ac.jp/form.html>. For results obtained by 2D-nanoSCX-RPLC-ESI-MS/MS analysis, the TMHMM (www.cbs.dtu.dk/services/TMHMM/) algorithm was used to predict TMDs from identified proteins. Proteins with at least one predicted TMD are regarded as IMPs. The cellular components and molecular

functions based on the Gene Ontology (GO) consortium were assigned with GoMiner. Grand average of hydrophobicity (GRAVY) values and isoelectric points (pI s) of the identified peptides were calculated with the ExPASy Server tool: ProtParam (<http://expasy.org/tools/protparam.html>).

Results and discussion

ILs are of great capacity to interact with different solutes via all possible types of interaction [33, 34] and might be helpful to solubilize IMPs. Besides good solubility, the choice of ILs for membrane protein analysis should follow additional two criterions: compatibility with tryptic digestion and facility to remove before MS/MS analysis.

Effect of additives on solubilizing ability

In order to compare the solubilizing ability of different additives for IMPs, bacteriorhodopsin was chosen as the sample, and 50 mM NH_4HCO_3 buffers, without and respectively with 60% (v/v) methanol, 2 M urea, 0.1% (w/v) Rapigest, and 1% (v/v) BMIM BF_4 added, were chosen as solvent systems. Firstly, to eliminate the effect of the four additives (60% (v/v) methanol, 2 M urea, 0.1% (w/v) Rapigest, and 1% (v/v) BMIM BF_4) on the protein concentration measurement by Bradford method at 595 nm,

the absorbance of 50 mM NH_4HCO_3 buffer containing the four kinds of additives was measured, which was further compared with that of 50 mM NH_4HCO_3 buffer. The results showed that the additives were of similar absorbance to 50 mM NH_4HCO_3 , which indicated that these additives did not affect the protein concentration measurement by Bradford method. Then, by Bradford method, the measured soluble protein concentrations were respectively as 0.03, 0.087, 0.093, 0.086, and 0.159 mg/mL. With the solubilizing ability of 50 mM NH_4HCO_3 buffer set as 1, that of the rest solvent systems was 2.9, 3.1, 2.9, and 5.3, respectively, which indicated that the solubilizing ability of 1% (v/v) BMIM BF_4 is the best among such five solvent systems.

The good solubilizing ability of BMIM BF_4 for IMPs is due to its special features. ILs are capable of most types of interactions (e.g., dispersive, π - π , n - π , hydrogen bonding, dipolar, and ionic/charge-charge) [34]. BMIM BF_4 is composed of 1-butyl-3-methyl imidazolium (cations) and BF_4 (anions). In water solution, because of hydrogen-bonding structure between imidazolium cations and BF_4 anions and π - π interaction of imidazole rings, hydrogen-bonded nanostructures might present, which contain hydrophobic domains (alkyl chains) and hydrophilic domains (ions) [46]. Therefore, IMPs might be solubilized by the hydrogen-bonded nanostructures through hydrophobic, ionic, and π - π interactions. Such interaction might exist between

Fig. 1 Schematic illustration of experimental setup for IMPs analysis

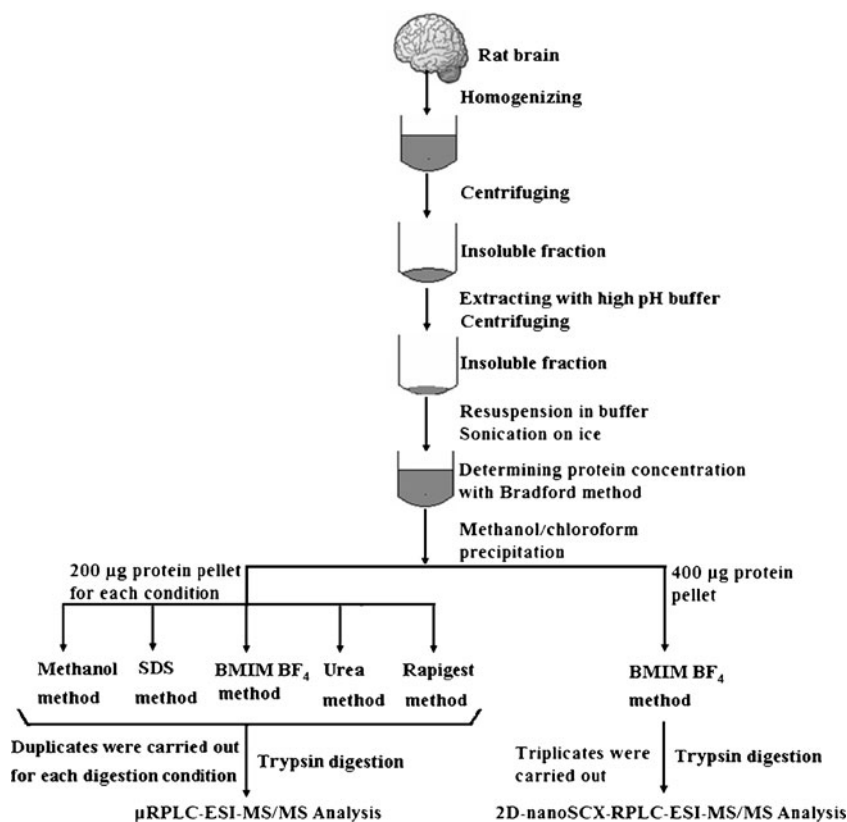


Table 1 Number of identified total peptides, total proteins, and IMPs with different sample preparation methods

	BMIM BF ₄	Rapigest	Urea	SDS	Methanol
Total peptides	452	345	282	96	152
Total proteins	290	201	194	64	79
IMPs	90	72	50	20	27
IMPs/total proteins	31%	36%	26%	31%	34%

The number of total peptides and total proteins identified in five digestion conditions is summarized in the first and second rows, respectively, with FDR less than 3% for peptides identification. IMPs predicted based on the algorithm at <http://psort.ims.u-tokyo.ac.jp/form.html> are summarized in the third row. The last row represents the ratio of IMPs vs. total proteins

alkyl chains, ions, imidazole rings, and IMPs, respectively. In addition, the hydrogen bonding in the nanostructures might be helpful to stabilize IMPs.

Effect of additives on trypsin activity

To study the effects of additives, including methanol, urea, BMIM BF₄, Rapigest, and SDS, on trypsin activity, BSA was chosen as the sample. For comparison, 100 mM NH₄HCO₃ (pH 8.3), commonly used buffer for trypsin digestion, was also used as an additive for contrast. Through the digests analysis by SDS-polyacrylamide gel electrophoresis (shown in Fig. S1 in Electronic Supplementary Material “Supporting material I”), it was found that with 1% (v/v) BMIM BF₄ and 2 M urea as additives, there was no evident BSA band, which indicated that BSA was completely digested into peptides, similar to that obtained with 100 mM NH₄HCO₃ as additive. With 0.1% (w/v) Rapigest as additive, there was no evident BSA band, but there was only one band between 20,100 and 14,400 Da. However, with 60% (v/v) methanol and 0.1% (w/v) SDS as additives, the digestion of BSA was not sufficient, and many bands between 66,200 and 14,400 Da were observed. These results demonstrate that the compatibility of 1% (v/v) BMIM BF₄, 2 M urea, and 0.1% (w/v) Rapigest with trypsin is better than that of 60% (v/v) methanol and 0.1% (w/v) SDS. Furthermore, because of very weak retention on C18 particles, BMIM BF₄ could be removed easily with a C18 trap column by 2% (v/v) acetonitrile washing, making it a good candidate for IMPs analysis.

Comparison of identified IMPs with different sample preparation methods

With the experimental setup shown in Fig. 1, insoluble proteins (200 µg) extracted from rat brain were respectively solubilized and digested with methanol, SDS, BMIM BF₄,

urea, and Rapigest methods, followed by the further analysis by µRPLC-ESI-MS/MS in duplicate runs. The base peak spectrums are shown as Fig. S2 in Electronic Supplementary Material “Supporting material I,” and the identified proteins and peptides are shown in Electronic Supplementary Material “Supporting material II.”

As shown in Table 1, although by different methods, the ratios of identified IMPs versus total proteins were similar, ranging from 26% to 36%, which might be determined by the IMPs extraction method. In addition, compared with SDS and methanol methods, over three times improvement on the identified IMPs number was obtained with BMIM BF₄ method, which might be due to its high solubilizing ability, good compatibility with trypsin, and easy removal before MS/MS identification.

Compared to Rapigest and urea methods, with BMIM BF₄ method, not only more peptides and proteins were identified but also the number of identified IMPs was improved by 25% and 80%, respectively. The results might be due to three reasons. Firstly, from the results of solubilizing ability for bacteriorhodopsin, it could be

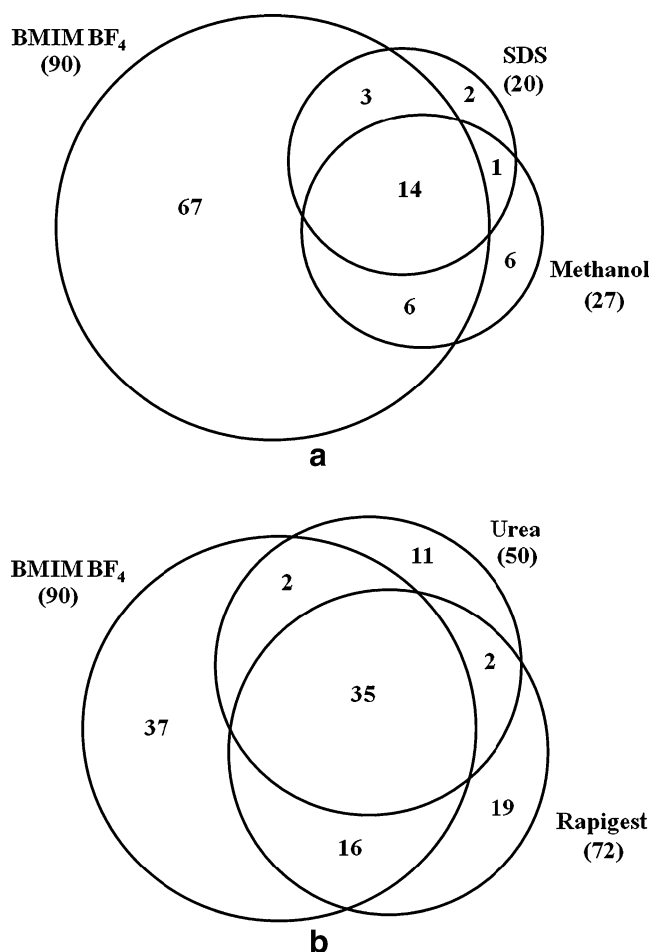
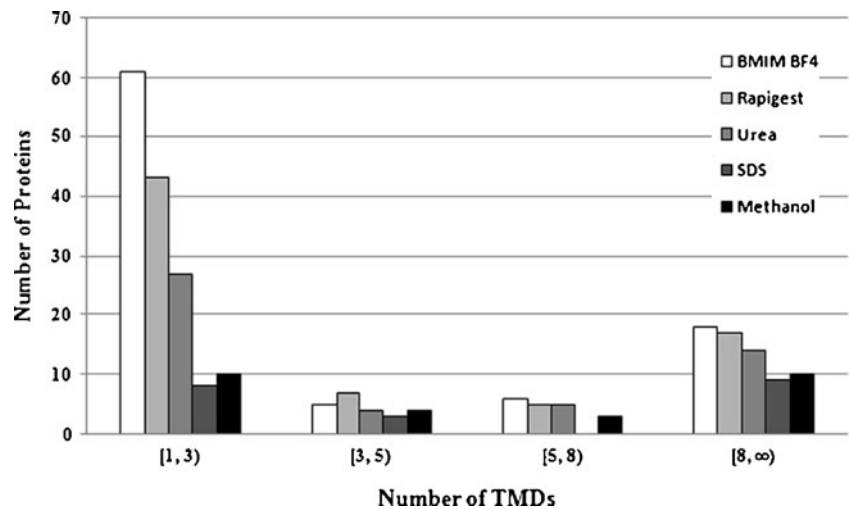
**Fig. 2** Overlapping of IMPs identified by µRPLC-ESI-MS/MS with BMIM BF₄, SDS, methanol, Rapigest, and urea methods

Fig. 3 Comparison on TMDs distribution of IMPs identified by μ RPLC-ESI-MS/MS with BMIM BF₄, SDS, methanol, Rapigest, and urea methods



concluded that 1% (v/v) BMIM BF₄ is of better solubilizing ability than those of 2 M urea and 0.1% (w/v) Rapigest. Secondly, when heated above 37 °C, urea could cause problematic carbamylation of N-termini and lysine residues. However, BMIM BF₄ is of high thermal stability, avoiding problematic modifications. Thirdly, for the removal of Rapigest before MS analysis, the cleavage step at low pH could result in hydrophobic peptides loss due to co-precipitation with the hydrophobic part of RapiGest [28]. However, BMIM BF₄ could be removed easily with a C18 trap column before MS analysis, avoiding loss of hydrophobic peptides.

The overlapping of identified IMPs by BMIM BF₄, SDS, and methanol methods is shown in Fig. 2a. A total of 99 IMPs are identified by such three methods, and IMPs identified by methanol and SDS methods are, respectively, 74% and 85% covered by those identified by BMIM BF₄ method. In addition, the overlapping of identified IMPs by BMIM BF₄, Rapigest, and urea methods is shown in

Fig. 2b. A total of 122 IMPs are identified by such three methods, and IMPs identified by Rapigest and urea methods are, respectively, 71% and 74% covered by those identified by BMIM BF₄ method. All these results show that BMIM BF₄ method is better than other four commonly used methods for the analysis of IMPs extracted from rat brain.

In addition, the identified IMPs in the insoluble fraction of rat brain by μ RPLC-ESI-MS/MS with different methods were compared with respect to the number of TMDs, predicted based on the algorithm at <http://psort.ims.u-tokyo.ac.jp/form.html>. As illustrated in Fig. 3, the identified TMDs could be divided into four sections: [1, 3), [3, 5), [5, 8), and [8, ∞), and IMPs identified by BMIM BF₄ method are distributed in all sections, with number higher than those identified by other methods.

Besides comparisons of identified IMPs, the peptides identified with the five methods were also compared with respect to the GRAVY values, as shown in Fig. 4.

Fig. 4 Comparison on GRAVY distribution of peptides identified by μ RPLC-ESI-MS/MS with BMIM BF₄, SDS, methanol, Rapigest, and urea methods

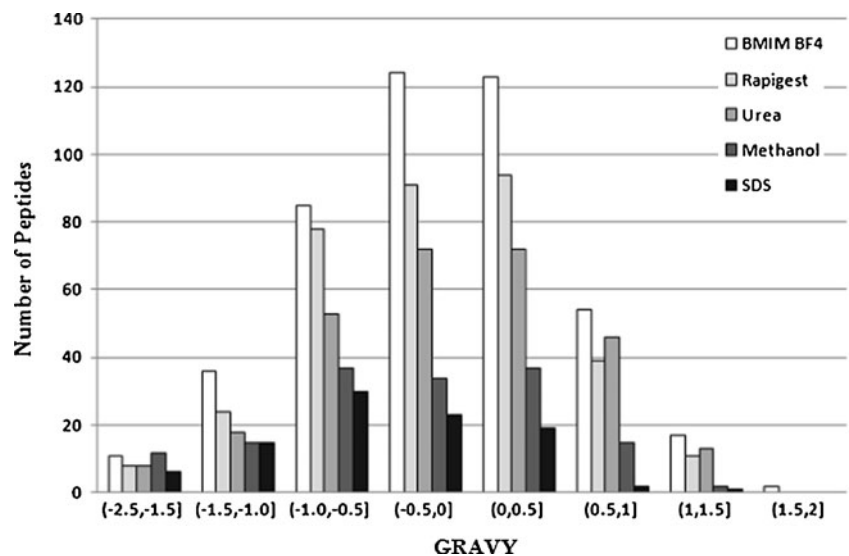
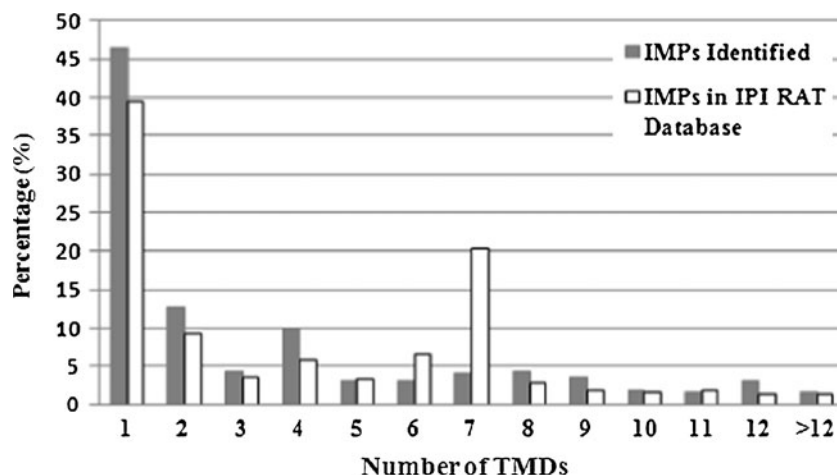


Fig. 5 Comparison on TMDs distribution of IMPs in IPI RAT database and that identified by 2D-nanoSCX-RPLC-ESI-MS/MS with BMIM BF₄ method. *White bars* refer to the IMPs in the IPI RAT database version 3.26, and *gray bars* refer to the IMPs identified with at least two unique peptides



Interestingly, by these five methods, similar peptide distribution profilings were obtained. However, with BMIM BF₄ method, more hydrophilic peptides (peptides with negative GRAVY values) and hydrophobic peptides (peptides with positive GRAVY values) were identified, when compared with those obtained by other methods. Especially, two peptides with GRAVY values higher than 1.5 were identified with BMIM BF₄ method. These results demonstrated that BMIM BF₄ method might be beneficial to the identification of peptides with high hydrophobicity.

Although by μ RPLC-ESI-MS/MS with an LCQ MS the identified peptides and IMPs number are not very high, the above-mentioned results demonstrate that compared to other presently applied methods, sample preparation with

BMIM BF₄ method is of superiority to study IMPs from rat brain.

Global analysis of IMPs in rat brain analyzed by 2D-nano-SCX-RPLC-ESI-MS/MS

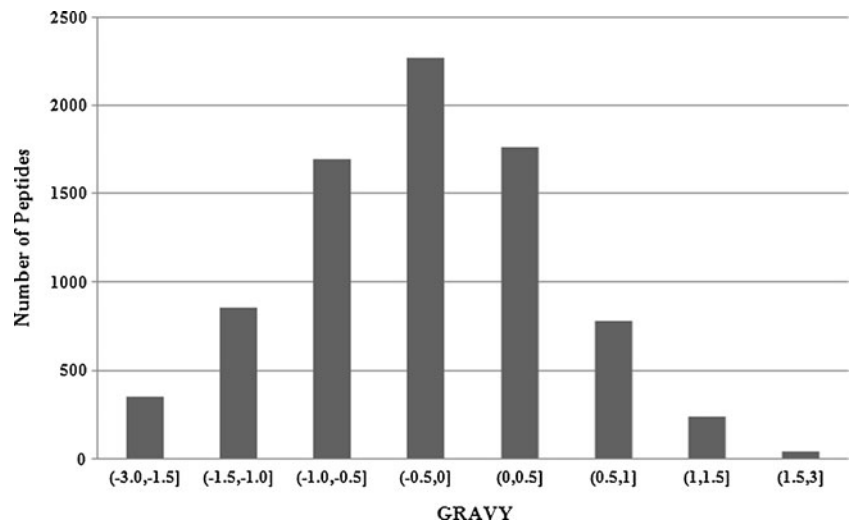
To achieve the global IMPs analysis in rat brain, 32 μ g of tryptic digests of membrane protein pellet from rat brain was automatically injected onto a 2-cm-long SCX trap column, eluted by nine salt steps, and analyzed by nano-RPLC-ESI-MS/MS, with the total analysis time of 21 h. The base peak spectrums are shown as Fig. S3 in Electronic Supplementary Material “Supporting material I.” After database searching, in triplicate runs, in total, 1,450 non-

Table 2 Ion channels identified in rat brain by 2D-nanoSCX-RPLC-ESI-MS/MS with BMIM BF₄ method

IPI	TMDs ^a	Protein name	Unique peptide number
Voltage-gated Na ⁺ channels			
IPI00189882.1	1	Sodium channel subunit beta-2	2
IPI00204294.1	1	Sodium channel subunit beta-1	2
Voltage-gated K ⁺ channels			
IPI00208362.1	3	Potassium voltage-gated channel subfamily A member 4	2
IPI00190644.1	4	Potassium voltage-gated channel subfamily A member 1	7
IPI00197991.6	4	Voltage-gated potassium channel Kv3.3EI long variant	6
IPI00208365.1	5	Potassium voltage-gated channel subfamily A member 2	6
IPI00190053.1	5	Potassium voltage-gated channel subfamily A member 6	3
IPI00394218.1	6	Potassium voltage-gated channel subfamily D member 2	4
IPI00231972.1	6	Potassium voltage-gated channel subfamily C member 1	2
Voltage-gated Ca ²⁺ channels			
IPI00207426.1	4	Voltage-dependent calcium channel gamma-8 subunit	4
IPI00201313.4	4	Voltage-dependent calcium channel gamma-2 subunit	2
IPI00211870.1	17	Voltage-dependent P/Q-type calcium channel subunit alpha-1A	7
IPI00201157.4	19	Voltage-dependent R-type calcium channel subunit alpha-1E	4

^a TMDs: number of predicted transmembrane domains obtained by TMHMM (www.cbs.dtu.dk/services/TMHMM/) algorithm

Fig. 6 GRAVY values distribution of unique peptides identified by 2D-nanoSCX-RPLC-ESI-MS/MS with BMIM BF₄ method



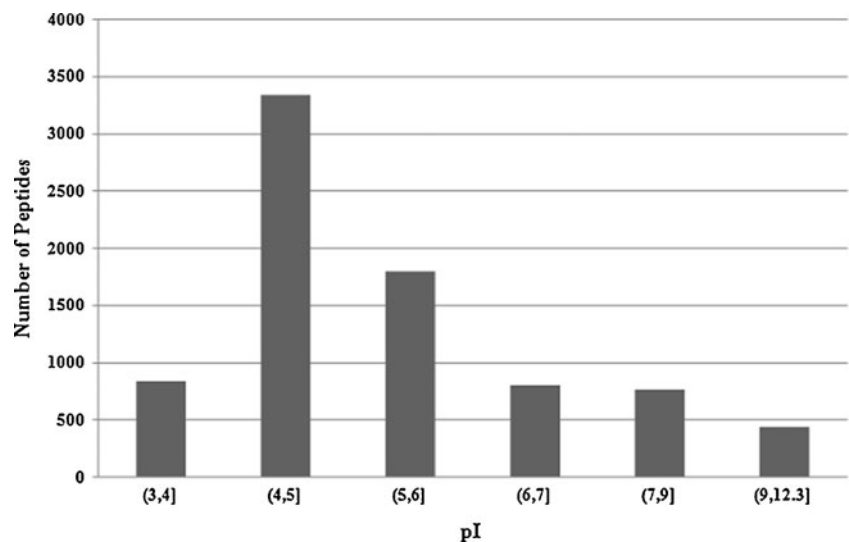
redundant proteins and 7,978 unique peptides were identified from rat brain, and 418 proteins contained at least one predicted TMD, with FDR less than 1% for peptide identification and at least two identified unique peptides per protein. The details of proteins and peptides identification are shown in Electronic Supplementary Material “Supporting material III.”

For the identified non-redundant IMPs, the distribution of identified IMPs with respect to the number of TMDs is shown in Fig. 5 and compared with those from IPI RAT database version 3.26. With at least two identified unique peptides per protein (gray bars), 418 IMPs were identified, and about 46% (194) of total IMPs are of one predicted TMD, and about 54% (224) are of two to 19 TMDs. For the IPI RAT database version 3.26 (white bars), 10,332 proteins contain at least one TMD. With respect to the number of TMDs, the percentages of identified IMPs with one to four

TMDs were higher compared with IPI RAT database version 3.26, and the percentage of identified IMPs with six or seven TMDs was lower.

The distributions of cellular components and molecular functions of identified proteins were further analyzed, according to Gene Ontology (GO) information obtained with GoMiner, and the comparison with the IPI RAT database version 3.26 was also made. As shown in Fig. S4 in Electronic Supplementary Material “Supporting material I,” for the identified proteins with at least two unique peptides per protein (gray bars), 67.14% of identified annotated proteins were mapped on membrane, among which the ratios of macromolecular complex, Golgi apparatus, cytoplasmic membrane-bounded vesicle, endoplasmic reticulum, and mitochondrion were higher than those obtained with IPI RAT database version 3.26 (white bars). In addition, as illustrated in Fig. S5 in Electronic Supplementary Material

Fig. 7 pI distribution of unique peptides identified by 2D-nanoSCX-RPLC-ESI-MS/MS with BMIM BF₄ method



“Supporting material I,” for the proteins identified with at least two unique peptides per protein (gray bars), 87.95% of the identified annotated proteins are of binding function, followed by catalytic activity (55.94%), transporter activity (20.81%), molecular transducer activity (11.29%), and structural molecule activity (9.94%), among which the percentages of proteins with binding function, catalytic activity, and transporter activity were higher compared with IPI RAT database version 3.26 (white bars).

As shown in Table 2, in total, four voltage-gated calcium ion channels, seven voltage-gated potassium ion channels, and two voltage-gated sodium ion channels were identified from rat brain. All these results show that ILs method, with BMIM BF₄ added in sample preparation buffer, is powerful to discover ionic channels on membranes.

The GRAVY values and *pI*s of the identified unique peptides were also analyzed. As shown in Fig. 6, the peptides are mainly distributed in three sections: (−1.0, −0.5], (−0.5, 0], and (0, 0.5]. The GRAVY values of the identified unique peptides range from −2.958 to 2.65, which indicates that with BMIM BF₄ method, peptides with high hydrophobicity and hydrophilicity could be identified efficiently. As shown in Fig. 7, most of identified unique peptides are acidic peptides (*pI* < 7) and are mainly distributed in (4, 5] and (5, 6] sections. The *pI*s of identified unique peptides range from 3.33 to 12.3, which shows that with BMIM BF₄ method, acidic and basic peptides could be identified efficiently.

Furthermore, with BMIM BF₄ method, 47 peptides containing partial or whole TMD were identified and recognized as transmembrane peptides, as shown in Table S1 in Electronic Supplementary Material “Supporting material I.” The TMDs number of IMPs identified with transmembrane peptides ranges from one to 12, and eight and three IMPs are of one and 12 TMDs, respectively. These results show that with BMIM BF₄ method, transmembrane peptides could be identified from IMPs with low and high TMD, which is important and challenging for IMPs analysis.

Conclusions

IL, BMIM BF₄, added in NH₄HCO₃ buffer (pH 8.3) was successfully applied for analysis of the IMPs extracted from rat brain. The experimental results indicate that compared to other commonly used membrane proteins treatment methods, with BMIM BF₄ method, more IMPs could be identified, not only due to high thermal stability, good solubilizing ability, and compatibility for trypsin digestion but also benefited from easy removal during desalting so that the interference on MS/MS detection is ignorable. By such a method, the large-scale and comprehensive analysis of IMPs could be easily achieved. Furthermore, the

systematic study on the applicability of other kinds of ILs for IMPs analysis is undergoing, which might be helpful to discover the in-depth mechanisms about the interaction between ILs and IMPs and find other suitable ILs for membrane proteomics study.

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