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# Prefractionation and separation by C8 stationary phase: Effective strategies for integral membrane proteins analysis

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ABSTRACT

Analysis of integral membrane proteins (IMPs) presents great challenges due to their hydrophobic nature. Recently, much attention has been paid to improve the solubilization of IMPs. However, besides that, the separation of hydrophobic peptides with high recovery is also a dominating factor, but with rare report. Here, the prefractionation of the digests by reverse phase trap column during desalting was presented to efficiently decrease the complexity of samples, with the identified hydrophobic peptides and IMPs increased by more than 43%. Furthermore, the effect of C18 and C8 stationary phases on the separation of membrane protein digests was studied. A total of 301 proteins (536 peptides) with C18 stationary phase and 398 proteins (703 peptides) with C8 stationary phase were identified by μRPLC–ESI-MS/MS using an LCQ instrument in duplicate runs, with false discovery rate (FDR) less than 5% at peptide level. In addition, with C8 stationary phase, the number of identified hydrophobic peptides and IMPs was obviously improved by 29% and 20%, respectively, compared with that identified by C18 stationary phase, indicating that the polarity of stationary phase has evident effect on the analysis of membrane protein digests. All these results show that the prefractionation by reverse phase trap column during desalting and the separation by C8 stationary phases could facilitate the efficient identification of IMPs.



## 1. Introduction

Membrane is the natural barrier between cells, their environment and various subcellular compartments. Therefore, integral membrane proteins (IMPs) play critical roles in regulating a host of cellular processes, including intercellular communication, vesicle trafficking, ion transport, protein translocation/integration, and propagation of signaling cascades [1–3]. However, the analysis of IMPs presents great challenges due to the hydrophobic nature [4–7]. Recently, much attention has been paid to the solubilization of IMPs, by using chaotropes [8–10], detergents [9–17], organic solvents [9,16,18–20], organic acids [21–23] and ionic liquid [24]. Besides, the separation of hydrophobic peptides with high recovery also plays a crucial role in IMP identification. Zhang et al. [16] compared the performance of SDS and methanol for IMP identification, and it was demonstrated that the differences in compatibility of reagents with protein digestion, downstream peptide separation, and mass spectrometry identification were critical for the analytical performances of IMPs, rather than the solubilization potential of solvent.

Due to high resolution and good compatibility with mass spectrometry (MS), reverse-phase liquid chromatography (RPLC) is preferred for peptide separation prior to the identification by MS/MS [25–29]. However, the digests of IMPs contain large amount of hydrophobic peptides. Therefore, the conventional C18 stationary phase may reduce the recovery of hydrophobic peptides due to its long alkyl chain, which further affected the MS identification capability. To solve this problem, Wu et al. applied RPLC at elevated temperature prior to identification by electrospray ionization tandem mass spectrometry (ESI-MS/MS) [30], to reduce the retention of hydrophobic peptides and increase their recovery [31,32]. However, for this strategy, an additional device for adjusting the column temperature and heat-stable packing materials are indispensable. Moreover, another viewpoint was presented that the choice of RP materials might affect the efficiency of hydrophobic peptide separation [19]. However, to the best of our knowledge, the in-depth study on the separation of IMP digests by RPLC with different carbon chain lengths has not been reported. In addition, it is well-known

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that peptide prefractionation prior to LC–MS analysis is a valuable method to simplify the complexity of sample digests, allowing the improved number of identified peptides and proteins [33–37].

In this work, firstly, ionic liquids (ILs) based protocol developed by our group [24] was used to solubilize the membrane protein pellet from rat hippocampus. Secondly, the prefractionation of membrane protein digests with a reverse phase trap column during desalting was performed to decrease the complexity of the sample. Finally, the effect of polarity of reverse stationary phase on separation of membrane protein digests was studied, by comparing the performance of C18 and C8 stationary phases. The experimental results demonstrate that, the developed strategy, with the combination of peptide prefractionation during desalting, and separation by C8 stationary phase is promising for the large-scale membrane proteome analysis.

## 2. Materials and methods

### 2.1. Reagents and materials

1-Butyl-3-methyl imidazolium tetrafluoroborate (BMIM $BF_4$) was obtained from Shanghai Cheng Jie Chemical (Shanghai, China). Trypsin TPCK treated (bovine pancreas) was ordered from Sigma–Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Acros (Morris Plains, NJ, USA). Protease inhibitor cocktail set I and acetonitrile (ACN, HPLC grade) were ordered from Merck (Darmstadt, Germany). Urea was obtained from Invitrogen (Carlsbad, CA, USA). Water was purified by a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals and solvents were analytical grade.

Fused-silica capillaries (150 μm i.d. × 365 μm o.d.) were brought from Sino Sumtech (Handan, China). Hypersil C18 silica particles (5 μm, 300 Å pore) and Hypersil C8 silica particles (5 μm, 300 Å pore) were obtained from Thermo Electron (San Jose, CA, USA). The rat hippocampus was obtained from Dalian Medical University (Dalian, China). A paradigm GM4 μHPLC system (Michrom Bioresources, Auburn, CA, USA) coupled with an LCQ$^{DUO}$ quadrupole ion trap mass spectrometer (LCQ-IT MS, Thermo Fisher, San Jose, CA, USA) was used for protein identification. A SpeedVac (Thermo Fisher, San Jose, CA, USA) was used to lyophilize samples.

### 2.2. Sample preparation

Three male Sprague-Dawley rats (180–200 g) obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China) were killed by decapitation, and their hippocampus were dissected quickly and flashly frozen in liquid nitrogen followed by storage at −80 °C. For IMP extraction, the procedure was performed according to the previously described method [10], with minor modifications. Briefly, three rat hippocampus tissues (~1.6 g) were washed with cold PBS for 3 times, and further homogenized in 3 mL of high salt buffer (2 M NaCl, 180 mM PBS, pH 7.4 and 1% (v/v) protease inhibitor cocktail set I) using Tissue Tearor from Biospec Products (Bartlesville, OK, USA) at approximately 20,000 rpm for 2 min, followed by ultrasonication (Cole-Parmer, Vernon Hills, IL, USA) for 2 min on ice at power 65% pulse duration to break cells and extract proteins. The resultant solution was centrifuged at 20,000 × *g* for 40 min at 4 °C to collect membrane protein pellets. The pellets were re-extracted in 2 mL of high pH buffer (0.1 M $Na_2CO_3$, 1% (v/v) protease inhibitor, pH 11.3) and incubated on ice for 30 min to remove loosely associated peripheral membrane proteins, followed by centrifugation under the same conditions. Subsequently, the pellets were washed with 2 mL of urea buffer (4 M urea, 180 mM PBS, pH 7.4) to further remove loosely associated peripheral membrane proteins. After 15 min incubation on ice, the supernatant was discarded under the same centrifugation conditions. The pellets were suspended in 2 mL cold PBS and homogenized by Tissue Tearor, followed by protein quantification with Bradford assay kit (Bio-Rad, Hercules, CA, USA) using BSA as a standard, and divided equally into several aliquots for analysis. Then, the cell lysates were precipitated with methanol/chloroform to remove lipids, followed by centrifugation at 20,000 × *g* for 40 min. Finally, the pellets were lyophilized and stored at −20 °C before use.

The solubilization and digestion of membrane proteins were performed according to our recent work [24] with slight modification. In brief, one aliquot of the membrane pellets (containing ~100 μg of proteins) was resuspended in 37.5 μL of 20% (v/v) BMIM $BF_4$ in 100 mM $NH_4HCO_3$ buffer (pH 8.3), and sonicated for 40 min, followed by heating at 90 °C for 20 min for denaturation. Subsequently, the sample was reduced in 27 mM DTT for 2 h at 56 °C. After cooled to room temperature, the cysteines were alkylated in 67 mM IAA for 40 min in the dark at room temperature. Then the resultant solution was diluted to 150 μL with 100 mM $NH_4HCO_3$ buffer (pH 8.3), and the tryptic digestion was performed with a trypsin/protein ratio (w/w) of 1:25 at 37 °C for 12 h. Subsequently, the solution was acidified with 1% (v/v) formic acid (final concentration) to stop proteolysis and centrifuged to save supernatant. Two aliquots were subjected to treatment as described above, one for prefractionation experiment and the other for conventional experiment, without prefractionation.

### 2.3. Membrane protein prefractionation

The digests from membrane proteins were loaded onto a homemade C18 trap column (4.6 mm i.d., 1 cm length) packed with C18 silica particles (5 μm, 300 Å pore). The digests with prefractionation were desalted and fractionated with a 20 min gradient from 2% to 80% acetonitrile at a flow rate of 1 mL/min using a SHIMADZU Prominence LC-20A system. Solvent A was 95% $H_2O$ with 5% ACN and 0.1% TFA, and solvent B was 95% ACN with 5% $H_2O$ and 0.1% TFA. The gradient was as follows: 2% B was kept for 6 min and the eluate was discarded; the concentration of solvent B was increased to 20% in 0.1 min, and maintained for 3 min, with the eluate named as fraction 1; the concentration of solvent B was increased to 30% in 0.1 min, and kept for 3 min, with the eluate named as fraction 2; then solvent B was changed to 80% in 0.1 min, and for 3 min, and the eluate named as fraction 3. Without prefractionation, the digests were desalted by the C18 trap column with 2% B for 6 min, followed by the concentration of solvent B increased to 80% in 0.1 min, and then kept for 14 min. The eluate was collected (without fractionation). The amount of the digests was evaluated by ultraviolet detection (214 nm) according to Ref. [38]. The collected fractions were lyophilized, and re-dissolved with deionized water containing 0.1% formic acid, prior to μRPLC–ESI-MS/MS analysis. Without specific statement, all percentage shown in this work was volume ratio.

### 2.4. μRPLC–ESI-MS/MS analysis

Experiments were performed on μRPLC–ESI-MS/MS, by the coupling of a MAGIC MS4 dual solvent delivery system with an LCQ detector equipped with either a C18 or C8 reversed-phase column, controlled by Xcalibur software version 1.4. Before separation on the reverse phase capillary column, the sample was concentrated on a C18 pre-column (0.3 mm i.d., 5 cm length). Digests of all the three fractions from prefractionation experiment and digests without prefractionation were separated by both C18 and C8 columns in duplicate runs. Two kinds of solvents were used for the gradient separation of digests, $H_2O$ with 2% ACN and 0.1% formic acid (A), and ACN with 2% $H_2O$ and 0.1% formic acid (B). For C18 column

**Table 1**
Optimized gradients of C18 and C8 based μRPLC separation for prefractionated and unprefractionated samples.

| | *t* (min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 10 | 20 | 140 | 160 | 170 | 180 |
| **C18 column (B%)** | | | | | | | |
| Fraction 1[a] | 0 | 0 | 10 | 18 | 40 | 80 | 80 |
| Fraction 2[b] | 0 | 0 | 20 | 24 | 40 | 80 | 80 |
| Fraction 3[c] | 0 | 0 | 30 | 33 | 40 | 80 | 80 |
| Without fractionation[d] | 0 | 0 | 10 | – | 40 | 80 | 80 |
| **C8 column (B%)** | | | | | | | |
| Fraction 1[a] | 0 | 0 | 5 | 21 | 30 | 80 | 80 |
| Fraction 2[b] | 0 | 0 | 15 | 28 | 30 | 80 | 80 |
| Fraction 3[c] | 0 | 0 | 25 | – | 30 | 80 | 80 |
| Without fractionation[d] | 0 | 0 | 5 | – | 30 | 80 | 80 |

[a] The loaded amount of digests was 3.7 μg.
[b] The loaded amount of digests was 2.3 μg.
[c] The loaded amount of digests was 0.6 μg.
[d] The loaded amount of digests was 6.6 μg.

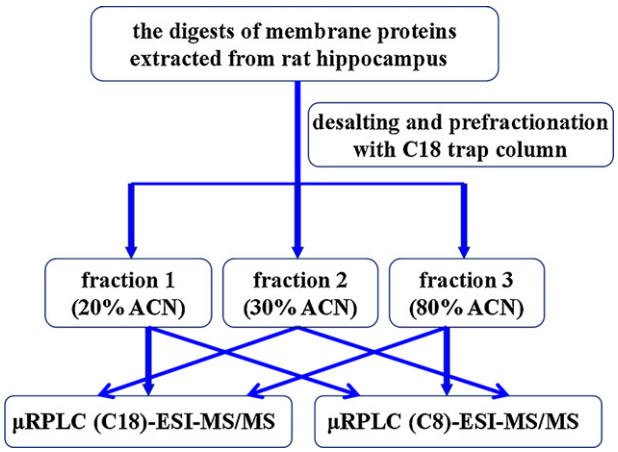


**Fig. 1.** Schematic illustration of experimental setup for prefractionation experiment of membrane protein digests from rat hippocampus analyzed by μRPLC–ESI-MS/MS.

(5 μm, 300 Å pore, 150 μm i.d. × 27 cm including 3 cm C12 frit) separation, the flow rate after splitting was 1.05 μL/min, and for C8 column (5 μm, 300 Å pore, 150 μm i.d. × 27 cm including 3 cm C12 frit) separation, the flow rate after splitting was 1.03 μL/min. The separation gradient of C18 and C8 capillary columns for each fraction is shown in Table 1.

The LCQ instrument was operated at positive ion mode with spray voltage of 3 kV, and the temperature of heated capillary was set at 150 °C. Total ion current chromatograms and mass spectra covering the mass range from *m*/*z* 400 to 2000 were recorded with Xcalibur software version 1.4. MS/MS spectra were acquired at data-dependent acquisition mode. A full MS scan was followed by three MS/MS events and precursor selection was based on parent ion intensity. The normalized collision energy for MS/MS scanning was 35%. μRPLC solvent gradient and mass spectrometer scan were controlled by Xcalibur data system (version 1.4).

### 2.5. Data analysis

The resulting data was analyzed by BioWorks Software 3.1 with SEQUEST search program. The database ipi.RAT.v3.26.fasta (41,494 protein entries) and its reverse database were separately searched to evaluate false discovery rate (FDR). Cysteine residues were searched as static modification of 57.0215 Da, and methionine residues were searched as variable modifications of 15.9949 Da. Peptides were searched using fully tryptic cleavage constraints and up to two internal cleavage sites were allowed. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. For peptide identification, Xcorr was higher than 1.9 for singly charged peptides, 2.2 for doubly charged peptides, and 3.75 for triply charged peptides, and ΔCn was adjusted to keep the FDR less than 5% in all runs.

The grand average of hydropathicity (GRAVY) values of the identified peptides was calculated using the ProtParam program (http://tw.expasy.org/tools/protparam.html). The peptides with positive and negative GRAVY values are named as hydrophobic and hydrophilic peptides, respectively. The TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) algorithm was used to predict transmembrane domains (TMDs) of identified proteins. Proteins with at least one predicted TMD were regarded as IMPs [19,30]. The cellular components and molecular functions based on Gene Ontology (GO) consortium were assigned with GoMiner.

## 3. Results and discussion

### 3.1. Evaluation of prefractionation efficiency

To simplify the complexity of digests from rat hippocampus, peptide prefractionation by a reverse phase trap column during the desalting was performed, followed by μRPLC–ESI-MS/MS analysis of each fraction with both capillary C18 and C8 columns in duplicate runs, as shown in Fig. 1.

For μRPLC (C18)–ESI-MS/MS, the number of identified peptides and proteins was increased by 66% (536 versus 322) and 58% (301 versus 191), respectively, compared with that identified without prefractionation. In addition, the number of IMPs and hydrophobic peptides identified after prefractionation was increased by 58% (79 versus 50) and 44% (184 versus 128). The remarkable increase benefited from the simple and efficient prefractionation strategy with little overlap between fractions, less than 7% (as shown in Fig. 2), which obviously decreased the complexity of samples. Similar conclusion was obtained by μRPLC (C8)–ESI-MS/MS analysis, shown in S-Table 1. All the results demonstrated that prefractionation strategy we presented in this work, taking no additional time than conventional desalting, was efficient to improve the identification of IMPs.

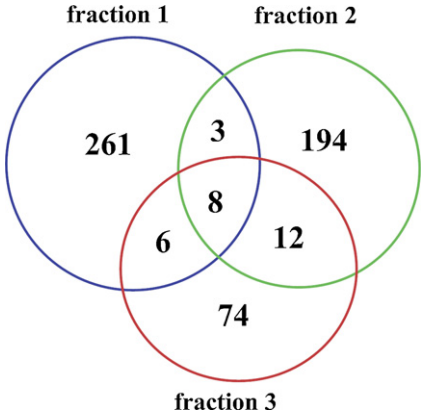


**Fig. 2.** Venn-diagram of peptides overlapping identified in three fractions with sample analyzed by C18 reverse phase column.

**Table 2**
Identified protein, peptide, IMP and hydrophobic peptide number with prefractionation.

| Item | C18 column | | | | C8 column | | | |
|---|---|---|---|---|---|---|---|---|
| | Fraction 1 | Fraction 2 | Fraction 3 | Total results | Fraction 1 | Fraction 2 | Fraction 3 | Total results |
| Proteins | 169 | 157 | 82 | 301 | 193 | 207 | 104 | 398 |
| Peptides | 278 | 217 | 100 | 536 | 330 | 280 | 138 | 703 |
| IMPs | 52 | 39 | 21 | 79 | 47 | 53 | 29 | 95 |
| Hydrophobic peptides | 68 | 85 | 54 | 184 | 79 | 109 | 69 | 238 |

### 3.2. Evaluation of analysis reliability of C18 and C8 for IMP digest separation

To ensure the reliability of the comparison, Hypersil C18 and C8 silica particles with the same particle diameter (5 μm) and pore diameter (300 Å) were chosen from the same manufacturer, and packed under the same conditions. The reproducibility of μRPLC–ESI-MS/MS was also evaluated. Duplicate runs were performed for analysis of the digests without prefractionation by both C18 and C8 columns, and more than 41% of the total proteins/peptides were identical in both runs, as shown in S-Fig. 1. The results showed that the analysis reproducibility was quite good. Therefore, the difference of analysis results was dominantly due to separation capacity of C18 and C8 stationary phases.

### 3.3. Comparison of C18 and C8 on IMP digest separation

To perform in-depth comparison of C18 and C8 stationary phases for the analysis of IMP digests, the digests was prefractionated before separation. Each fraction was analyzed by μRPLC–ESI-MS/MS in duplicate runs. For the analysis of each fraction, the gradient of C18 and C8 capillary columns was optimized to identify more peptides and proteins. Under the optimized conditions, with C8 column, 398 proteins and 703 peptides were identified from the three fractions, which were improved by 32% and 31%, respectively, compared with that identified with C18 column (301 and 536), as shown in Table 2. With C8 column, 95 IMPs and 238 hydrophobic peptides were identified, improved by 20% and 29%, respectively, compared with those identified with C18 column. Especially, 25 and 17 peptides with high hydrophobicity (GRAVY > 1) were identified with C8 and C18 stationary phases, respectively, of which 10 and 2 peptides were exclusively identified by C8 and C18 stationary phases, respectively. These results further demonstrated that C8 stationary phase was preferable for the analysis of the IMP digests since more IMPs and hydrophobic peptides were identified, compared with C18 stationary phase.

To further analyze the identified peptides and proteins in three fractions analyzed with C18 and C8 stationary phase, the distribution of GRAVY values and TMDs of identified peptides and proteins was analyzed. As shown in Fig. 3, for fraction 1, with C8 and C18 stationary phases, difference on the identified hydrophilic and hydrophobic peptide was not noticeable. However, for fractions 2 and 3, the superiority of C8 stationary phase for the analysis of the hydrophobic peptides, with GRAVY values over −1 was shown obviously. These results indicated that C8 stationary phase was of high efficiency for the analysis of hydrophobic peptides from IMP digests, which might be attributed to the different retention capability of C8 and C18 stationary phases for hydrophobic peptides. For C18, the hydrophobic peptides were strongly retained, tending to lower hydrophobic peptide recovery, which was in accordance with previous study by Wu et al. [30–32].

In addition, the distribution of TMDs of proteins identified by C8 and C18 stationary phases was also compared, as shown in Fig. 4. For fraction 1, the number of IMPs with at least one predicted TMDs identified with C8 stationary phase was not obviously more than that identified with C18 stationary phase, except IMPs with three and four predicted TMDs. However, more IMP proteins with larger TMD number were identified with C8 stationary phase than those identified by C18 in the second and third fractions. In addition, for fraction 2, the IMPs with 12, 16 and 20 TMDs were exclusively identified with C8 stationary phase.

### 3.4. Functional characterization of identified proteins

To characterize 398 proteins identified with C8 stationary phase, from membrane protein sample of rat hippocampus, the distributions of cellular components and molecular functions of identified proteins were further analyzed, according to Gene Ontology (GO)

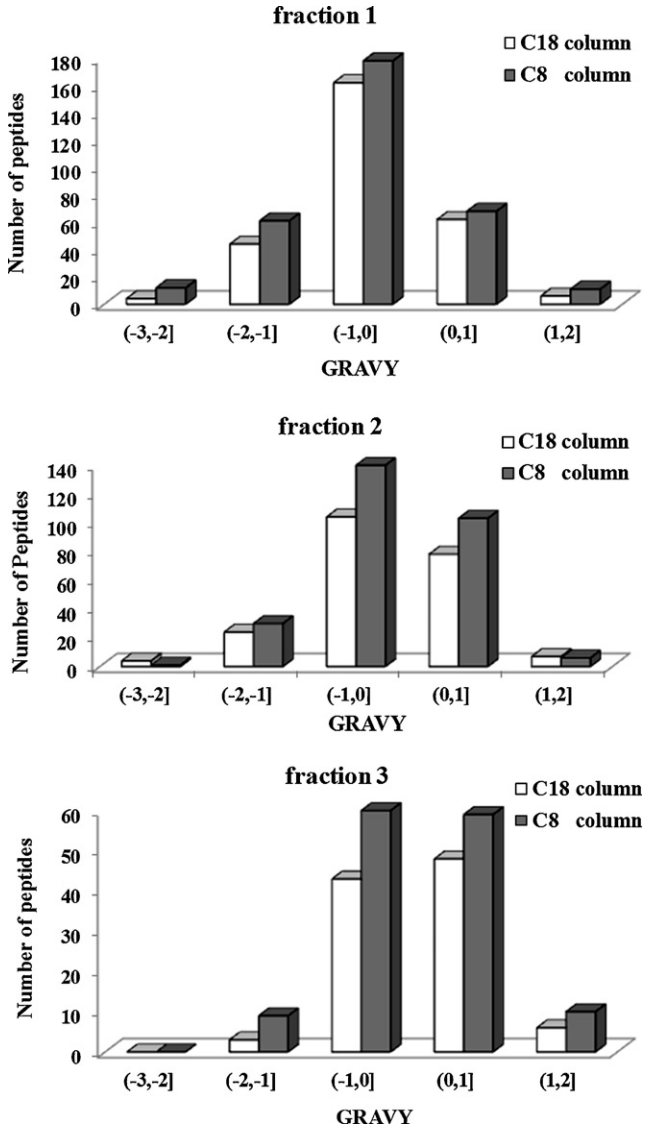


**Fig. 3.** Comparison on GRAVY distribution of peptides identified by C18 and C8 stationary phases.

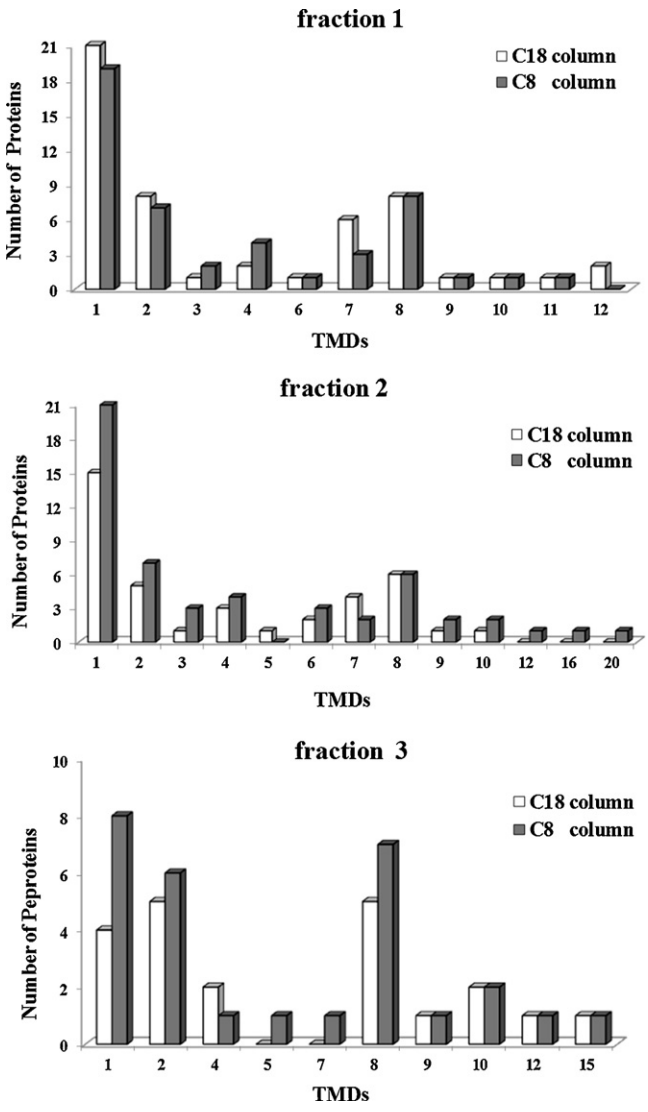


**Fig. 4.** Comparison on TMD distribution of IMPs identified by C18 and C8 stationary phases.

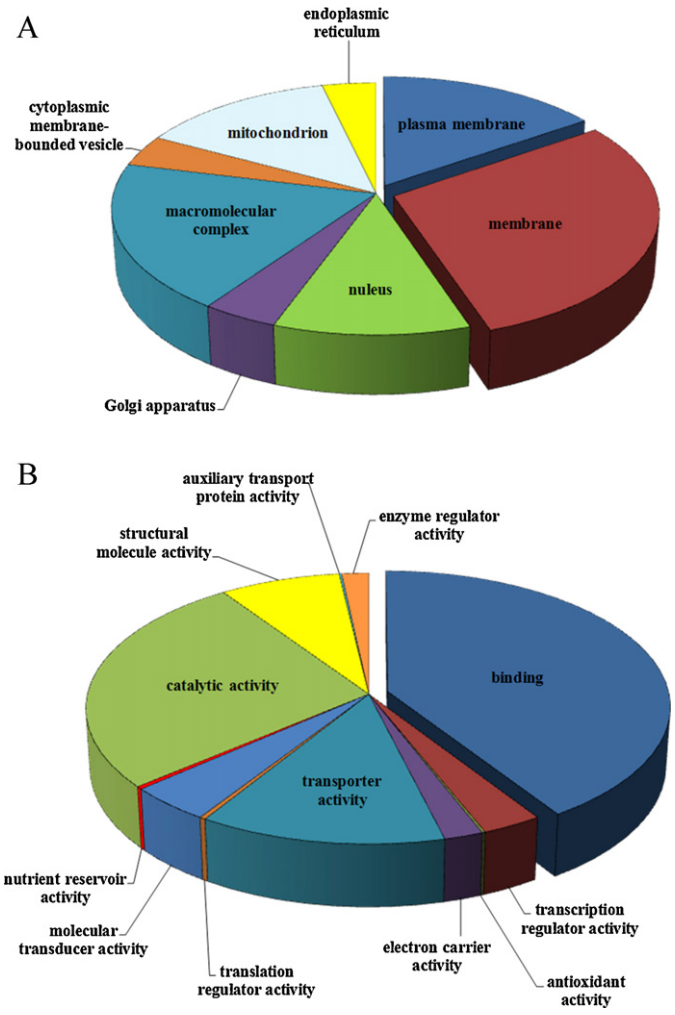


**Fig. 5.** Distribution of (A) cellular component and (B) molecular function for the proteins identified by C8 stationary phase. The cellular localization and molecular functions for identified proteins based on Gene Ontology (GO) consortium were assigned with GoMiner.

information obtained with GoMiner. 303 (76%) proteins were of annotated cellular component, and 310 (78%) were of annotated molecular function. Among them, as shown in Fig. 5A, 184 (61%) of identified annotated proteins were mapped on membrane, among which 96 (52%) was mapped on plasma membrane. Besides, 119 (39%) of identified annotated proteins were located in macromolecular complex. Other proteins were from organelles, such as Golgi apparatus, cytoplasmic membrane-bounded vesicle, endoplasmic reticulum, and mitochondrion. In addition, GO molecular function annotation analysis revealed that 262 (85%) of the identified annotated proteins were of binding function, followed by catalytic activity (171, 55%), transporter activity (82, 27%), and structural molecule activity (49, 16%), as shown in Fig. 5B. According to the different biological functions, 49 identified annotated proteins with structural molecule activity might be classified as fibrous proteins, which were the structural constituent of cytoskeleton, ribosome, myelin sheath, and so on. In addition, 261 annotated proteins might be classified as globular proteins with catalytic activity, transporter activity, transcription regulator activity, and so on.

A more detailed characterization of IMPs, with TMDs over 9 exclusively identified with C8 stationary phase, was performed based on Uniprot annotation (http://www.uniprot.org). Canalicular multispecific organic anion transporter 2, an IMP with 16 TMDs, 1522 amino acids and molecular weight 168,978 Da, might be glycosylated and phosphorylated, acting as an inducible transporter in the biliary, intestinal excretion of organic anions and an alternative route for the export of bile acids and glucuronides from cholestatic hepatocytes. Adenylate cyclase type 8 with 10 TMDs, 1248 amino acids and molecular weight 168,978 Da, a membrane-bound, calcium-stimulable adenylyl cyclase, was revealed to be glycosylated and may be involved in learning, in memory and in drug dependence [39].

## 4. Conclusions

The strategy with the combination of peptide prefractionation by reverse phase trap column during desalting and μRPLC separation with C8 stationary phase was proposed for the analysis of IMPs from rat hippocampus, and showed the superiority to decrease the complexity of membrane protein digests, and improve the recovery of hydrophobic peptides, and thus IMPs, compared to conventionally applied C18 stationary phase. In addition, ionic liquids for solubilizing membrane proteins are easy and cheap to apply. C8 stationary phase is widely used in HPLC separation. Furthermore, the prefractionation of digests presented in this work could be performed during desalting, without additional time spent. Therefore, such a strategy is economical, practical and time-saving, which

might be promising to promote the large-scale membrane proteome profiling.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.11.035.

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