A Biphasic Micro-reactor for Efficient and Fast Membrane Protein Pretreatment with Combination of Formic Acid Assisted Solubilization, on-column pH adjustment, Reduction, Alkylation and Tryptic Digestion

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Preparation of membrane protein samples

Five male Sprague-Dawley rats (180–200 g) were killed by decapitation, and the cerebellums were dissected quickly and freshly frozen in liquid nitrogen followed by storage at −80 ºC. For IMP extraction, the procedure was followed the previously described method with minor modifications. Briefly, five rat cerebellums (about 1.4 g) were washed with cold PBS for 3 times, and further homogenized in 10 mL of high salt buffer (2 M NaCl, 1×PBS, pH 7.4 and 1% (v/v) protease inhibitor cocktail) using Tissue Tearor from Biospec Products (Bartlesville, OK) at approximately 20 000 rpm for 3 min, followed by ultrasonication (Cole-Parmer, Vernon Hills, IL) for 3 min on ice, at power 100% pulse duration to break cells and extract proteins. The resultant solution was centrifuged at 1 500 g for 10 min at 4 ºC to remove unbroken cells and debris. The supernatant was collected, and centrifuged at 38 000 g for 1 h at 4 ºC to collect membrane protein pellet. The pellet was re-extracted in 20 mL of high pH buffer (0.1 M Na_2CO_3, 1% (v/v) protease inhibitor, pH 11.3), and incubated on ice for 1 h to remove loosely associated peripheral membrane proteins, followed by centrifugation under the same conditions. Subsequently, the pellet was washed with 20 mL of urea buffer (4 M urea, 1×PBS, pH 7.4) to further enrich membrane proteins. After 10 min incubation on ice, the supernatant was discarded under the same centrifugation conditions. The pellet was suspended in 1.5 mL cold water and homogenized by Tissue Tearor, followed by protein quantification with Bradford assay kit (Bio-Rad, Hercules, CA) using BSA as a standard, which was further divided equally into several aliquots for analysis. Then, each aliquot of cell lysates was precipitated with methanol/chloroform to remove lipids. Finally, the pellet was lyophilized and stored at −80 ºC before use.

Preparation of on-column monolithic frit

Briefly, a fused silica capillary was washed by 1 M NaOH for 3 h, water for 30 min, 0.1 M HCl for 1 h, water for 30 min, and methanol for 30 min, respectively, followed by drying with N_2. Subsequently, the capillary was filled with 50% γ-MAPS (v/v in methanol), and kept at room temperature for 24 h. Then the capillary was washed with methanol and dried by nitrogen. The polymerization solution, composed of 30 wt%

PEGDA, 70 wt% propyl alcohol and 0.3 wt% AIBN, was purged with N_2 for 30 s, and filled into one end of the capillary about 5 cm long. After both ends of capillaries were sealed, the polymerization was performed at 50 ºC for 24 h. Finally, the prepared monolithic frit was washed with methanol.

In-solution digestion

For the control experiment, the in-solution digestion was performed for the mixture of BSA, Myo and Cyt C. Briefly, BSA, Myo and Cyt C were individually dissolved in 50 mM NH_4HCO_3 buffer, followed by mixing with equal mass to a final protein concentration of 0.2 mg/mL. Then the sample was denatured at 90 ºC for 20 min, reduced with 11 mM DTT at 56 ºC for 1 h, and alkylated with 27 mM IAA at room temperature for 30 min in the dark. After that, the sample was digested with trypsin with enzyme / protein ratio as 1:30 (m/m) at 37 ºC for 12 h. Finally, a final concentration of 1% (v/v) FA was added into the solution to terminate the reaction. The sample was stored at -20 ºC before analysis.

Fifty nanograms of Membrane proteins (1 μg/mL, 50 μL) extracted from rat cerebellums dissolved in 1%（v/v）FA was denatured at 90 ºC for 20 min, followed by dilution with 50 mM NH_4HCO_3 to adjust the pH of sample buffer to about 8, reduction with 8 mM DTT at 56 ºC for 2 h and alkylation with 20 mM IAA at room temperature for 40 min in the dark. The sample was then digested with trypsin with enzyme / protein ratio as 1:30 (m/m) at 37 ºC for 12 h. Finally, a final concentration of 1% (v/v) formic acid was added into the solution to terminate the reaction. The sample was stored at -20 ºC before analysis.

Nano-LC-ESI-MS/MS analysis

Three kinds of buffer solvents were H_2O with 2% ACN and 0.1% formic acid (A) , ACN with 2% H_2O and 0.1% formic acid (B) and 1 000 mM CH_3COONH_4, pH 3 (C).

Nano-RPLC-ESI-MS/MS analysis was carried out to analyze the mixture of BSA, Myo, and Cyt C, and trace membrane proteins from rat cerebellum, respectively. For standard protein mixture, experiments were performed with an LTQ linear ion trap mass spectrometer equipped with a quaternary Surveyor pump and an ESI probe Ion Max Source with microspray kit, controlled by Xcalibur software version 2.0.7.

(Thermo Fisher, Waltham, MA), with the gradient of mobile phase for separation as followed: from 2% to 10% B in 5 min, to 35% B in 40 min, to 80% B in 5 min and kept at 80% B for 10 min. The analysis of 50 ng membrane protein sample extracted from rat cerebellum was performed on an LTQ Orbitrap Velos MS. Before peptide separation, 1 000 mM CH_3COONH_4 was used to directly elute peptides from the membrane protein reactor onto a home-packed C18 capillary column (15 cm, 75 μm i.d. / 375 μm o.d.) packed with C18 silica particles (5μm, 100 Å), and the gradient of mobile phase for separation was set as follows: from 2% B to 10% in 5 min, to 35% B in 60 min, to 80% B in 5 min, and kept at 80% B for 10 min.

2D nano-SCX-RPLC-ESI-MS/MS analysis of membrane protein samples extracted from rat cerebellum was performed on an LTQ XL with 6-step salt elution, respectively with 30, 60, 100, 200, 300 and 1 000 mM CH_3COONH_4 to elute peptides from the biphasic micro-reactor onto a C18 capillary column (15 cm, 75 μm i.d. / 375 μm o.d.). Each salt step lasted 60 min, including 30 min for salt elution and 30 min for C18-trap column online desalting, with the flow rate of 230 nL/min. For nano-RPLC, the gradient was as followed: from 2% to 10% B in 10 min, to 40% B in 120 min, to 80% B in 10 min and maintained at 80% B for 10 min, followed by re-equilibration of the column with 2% B for 30 min.

LTQ XL was operated at positive ion mode. The MS/MS spectra were acquired in the data dependent CID mode and the normalized collision energy was set at 35%. The mass spectrometer was set that one full MS scan was followed by nine MS/MS scans on the nine most intense ions. For LTQ-orbitrap Velos analysis, MS scans were acquired in the orbitrap analyzer with resolution of 60 000 (m/z 400). Up to the 15 most intense ions with charge state ≥ 2 in each full MS scan were selected for sequencing and fragmented in the ion trap with normalized collision energy of 35%, activation Q of 0.25, activation time of 10 ms, and one microscan.

Table S-1 Transmembrane peptides identified by biphasic micro-reactor coupled with 2D LC-MS

IPI	TMDs	peptide sequence	number of amino acids belonging to the TMDs
IPI00371146	1	FLNGFLAGAVVGAAGAGLTALQFLQR	23
IPI00190020	7	LDEFGEQLSK	2
IPI00231746	1	ILLLGTAVESAWGDEQSAFR	13
IPI00231746	1	TYIISILFK	7
IPI00361872	6	LAIIFLPVFTFTAGNALK	17
IPI00231102	4	CLVGAPFASLVATGLCFFGVALFCGCGHEALTGTEK	23
IPI00188732	4	DNPLNEGTDAAR	1
IPI00370637	1	IALGLGIPASATVAYILYR	18
IPI00371518	3	SLCLGPALIHAAK	12
IPI00371518	3	FVLVFPLMYHSLNGVR	8
IPI00231774	1	LLGLTTILSATALGFLAHK	17
IPI00190772	2	FIGAGAATVGVAGSGAGIGTVFGSLIIGYAR	23
IPI00764950	1	LYSLLFR	3
IPI00948403	2	ETPFVPIGMAGFAAIVAYGLYK	18
IPI00948403	2	MSIHLIHMR	3
IPI00786838	1	ALVIAPLFGIAQGVYFIGIGER	20
IPI00230858	12	EDKTLAVGMQFMLLR	4
IPI00207065	10	VGLVSALPHLVMTIIVPIGGQIADFLR	23
IPI00207065	10	FFTSMPVYAIIVANFCR	13
IPI00209908	2	ILYMMDEINNPVLTVK	3

IPI00563013	1	VFSIVIPFLYVGTLISK	15
IPI00364850	1	TSLFTVSHVIIPLWFVHYYVK	20
IPI00951626	3	AVGVTIGCILGMFPLIFFGGGEEDEK	20
IPI00200069	2	QGIFQVVVSR	4
IPI00213618	7	LDEFGEQLSK	2
IPI00365293	1	LSIPAALALGSAALGAAFATGLFLGK	23
IPI00382312	1	VAPPFVLFYLIYTWGNQEFAQSK	15
IPI00209115	2	IIMIGTLTALQWFIYDSVK	15
IPI00230832	1	FGVAEPR	3
IPI00230832	1	VHIVGAFVVALGVAAAYK	17
IPI00562259	2	IIMIGTLTALQWFIYDSVK	15
IPI00201849	12	LSDDIGDFGEVR	4
IPI00199586	8	VAPYALQLSLAILQMALPLASLAGR	23
IPI00324377	10	MLQMLVLPLIISSLVTGMAALDSK	21
IPI00324377	10	YFSFPGELLMR	1
IPI00362563	8	TLLLPVVLAIVAAIGR	15
IPI00363807	3	LLWAYVAHLGVR	11
IPI00363807	3	LVLEGAALGVQYHLYGFK	11
IPI00231927	4	AAYFGVYDTAK	6
IPI00231927	4	GAWSNVLR	7
IPI00231927	4	GMGGAFVLVLYDEIKKYV	11
IPI00231927	4	GAWSNVLRGMGGAFVLVLYDEIKK	19
IPI00231927	4	YFAGNLASGGAAGATSLCFVYPLDFARTR	23

IPI00231927	4	GMGGAFVLVLYDEIK	11
IPI00231927	4	GMGGAFVLVLYDEIKK	11
IPI00231927	4	GLYQGFSVSVQGIIIYR	16
IPI00231927	4	YFAGNLASGGAAGATSLCFVYPL DFAR	23
IPI00231927	4	AFFKGAWSNVLR	11
IPI00213663	9	IIAIKDLEVVAR	3
IPI00213663	9	LAAPIHPDVVMLIAFPGDILMR	2
IPI00213663	9	MLILPLIISSLITGLSGLDAK	18
IPI00769139	1	WGFIPLVIYLGFTR	13
IPI00734667	1	LINGGIAGLIGVTCVFPIDLAK	20
IPI00190974	5	HALLETALTR	4
IPI00190974	5	FVPFPAVASANICNVVLMR	18
IPI00190974	5	VVLPMPILVLPPIVMSMLEK	18
IPI00205177	4	LSLATFLLGASVLALPLLTR	19
IPI00734686	2	ILYMMDEINNPVLTVK	3
IPI00231860	3	YLFNFIAAMPLISLVNNFLK	19
IPI00231860	3	LVEHLHNFIFFR	4
IPI00200472	12	AYFTSATMIIAIPTGVK	16
IPI00200472	12	VFSWLATLHGGNIK	6
IPI00208115	12	AAPILFASAALALGSSLALK	19
IPI00208115	12	AAPILFASAALALGSSLALKLPET R	19
IPI00208117	9	VALTIALFHVAGK	12
IPI00200466	2	YFAGNLASGGAAGATSLCFVYPL DFARTR	23
IPI00200466	2	YFAGNLASGGAAGATSLCFVYPL DFAR	23
IPI00331878	13	LLPMFIIVVPGMISR	14

IPI00207766	4	GAVSSLALFALMGTTVCSAFQHLGWIKPGLGSGSR	23
IPI00207766	4	VLYAYGYYTGDPSK	7
IPI00207766	4	IASGLGVAWIIGR	12
IPI00207766	4	AHQNTLEVYPPFLFFLTVGGVYHPR	18
IPI00200487	13	ELTLMALHIIPLMLLTINPK	16
IPI00200487	13	MPLYGVHLWLPK	10
IPI00200487	13	AHVEAPIAGSMILAAILLK	16
IPI00476388	9	DVNYGWLIR	4
IPI00189176	2	AYITTALGGLCGLISSAYSITHNPADSPLEAVAR	22
IPI00555227	4	ALMIAASVLGLPAILLLLTVLPCIR	23
IPI00208365	5	QVWLLFEYPESSGPAR	3
IPI00211024	12	AEDASGEAAAMLNNMR	2
IPI00193082	1	NSLLGAVAGLGPLIFWYYVIK	20
IPI00361315	7	AVGLAYILPAAYFWR	14
IPI00202111	1	KYFNSYTLTGR	4
IPI00202111	1	YFNSYTLTGR	4
IPI00202111	1	AGPESDGQFQFTGIKKYFNSYTLTGR	4
IPI00209269	4	VALGITTVLTMTTINTHLR	18
IPI00201353	1	VFIPSLFLSHVLALGLGIYIGK	20
IPI00201353	1	KGGIFSAEFLKVFIPSLFLSHVLALGLGIYIGK	23
IPI00189554	3	SIISVIHLVTAAR	11
IPI00372527	1	ATDCGPSVGLAAGIPSLLATALLVALLFTLIQR	23

IPI00205490	6	STIQNPYVAALYK	6
IPI00210125	12	VVPILLAAASLVGGGLVALR	19
IPI00210125	12	LISGIGIGGSLPIVFAYFSEFLSR	18
IPI00870007	2	VLGHVNNILISAVLPTAFR	18
IPI00364777	1	LVPFLVFVVVPFMEFLLPVVVK	20
IPI00734731	15	ADANTAALQAILYNR	1
IPI00734731	15	IIYFVTMTKPR	4
IPI00205413	3	GMAAPIIGVTPMFAVCFFGFGLG K	23
IPI00215410	2	GLVGVGLFALAHAAFSAAQHR	20
IPI00359110	2	LGTGFGLGMVFSLTFFK	16
IPI00214057	7	YFSFPGELLMR	10
IPI00957468	1	LGVIGFAGFVGLLFAR	15
IPI00957468	1	VEHFVQWGVDNYNYLQNAPPG FYPR	4

Figure S-1. Distribution of (A) TMDs of IMPs and (B) GRAVY of peptides identified from rat cerebellum (14 μg) by biphasic micro-reactor coupled with 2D nano-LC-MS/MS.

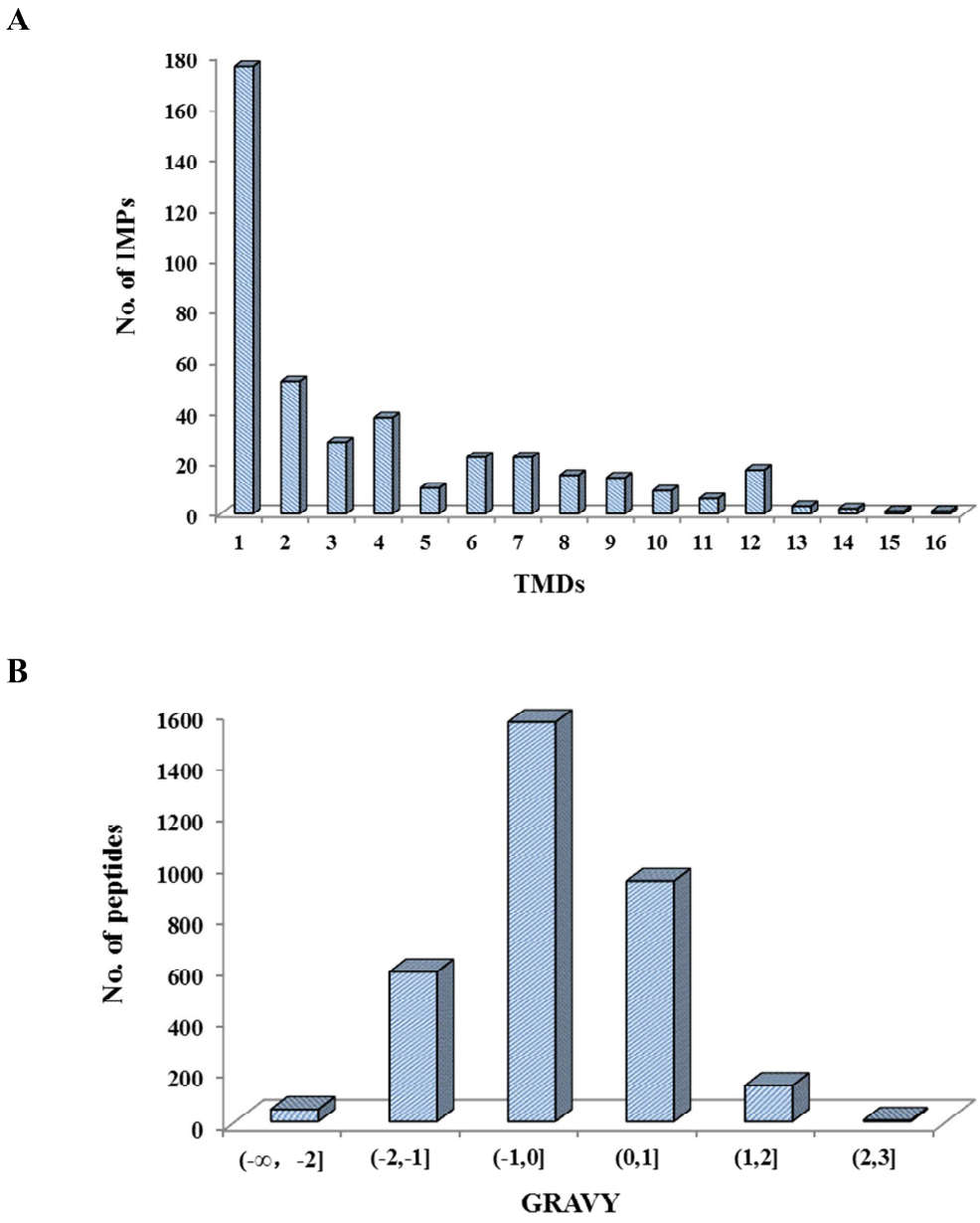
A
No. of IMPs
180
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TMDs
B
No. of peptides
1600
1400
1200
1000
800
600
400
200
0
(-∞, -2] (-2,-1] (-1,0] (0,1] (1,2] (2,3]
GRAVY

Figure S-1