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Systematic cyanobacterial membrane proteome analysis by combining acid hydrolysis and digestive enzymes with *nano*-liquid chromatography–Fourier transform mass spectrometry

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ABSTRACT

The identification of membrane proteins is currently under-represented since the trans-membrane domains of membrane proteins have a hydrophobic property. Membrane proteins have mainly been analyzed by cleaving and identifying exposed hydrophilic domains. We developed the membrane proteomics method for targeting integral membrane proteins by the following sequential process: in-solution acid hydrolysis, reverse phase chromatographic separation, trypsin or chymotrypsin digestion and *nano*-liquid chromatography–Fourier transform mass spectrometry. When we employed total membrane proteins of *Synechocystis* sp. PCC 6803, 155 integral membrane proteins out of a predictable 706 were identified in a single application, corresponding to 22% of a genome. The combined methods of acid hydrolysis-trypsin (AT) and acid hydrolysis-chymotrypsin (AC) identified both hydrophilic and hydrophobic domains of integral membrane proteins, respectively. The systematic approach revealed a more concrete data in mapping the repertoire of cyanobacterial membrane and membrane-linked proteome.

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1. Introduction

Far from the predictable number of 20–30% genomic complements of integral membrane proteins (IMPs), the identifiable number of membrane proteins is experimentally under-represented in typical proteomic approaches [1]. The main reason for underestimation is the authentic property of the hydrophobic trans-membrane domains (TMDs). For membrane proteomic analysis, *Synechocystis* sp. PCC 6803 (hereafter, referred to as *Synechocystis*) was adopted as a model organism since *Synechocystis* possesses multilayered lamellate structures: outer membrane, plasma membrane, and thylakoid membrane. Thylakoid membranes occupy larger volumes for the residence of respiratory and photosynthetic protein machinery than plasma or outer membranes [2]. Contrary to predictable 706 IMPs, so far only 66 IMPs were experimentally identified from the purified membrane fractions using gel-based proteomics [3,4]. For gel-based membrane proteomics, technical limitation for obtaining large number of proteins has been encountered. In-gel tryptic digestion covers only exposed hydrophilic domains from IMPs, leaving the hydrophobic TMDs embedded intact [5]. For cleaving the TMDs, chymotrypsin–trypsin double-digestion [6] and extraction in 60% methanol followed by chymotrypsin digestion [7] were applied to increase the sequence coverage of IMPs. Several methods have been proposed for better identification of membrane proteins, i.e., the delipidation by an organic solvent [8] or detergent [9], the disintegration by an organic solvent and basic chemical [10], and a modified cleavage strategy for integral membrane proteins [11].

In order to increase the number of peptides derived from membrane proteins by tandem mass analysis, we developed a new digestion and separation strategy which included acid hydrolysis reaction of a highly solubilizing chemical cleavage with hydrophobic membrane fraction followed by trypsin or chymotrypsin digestion. In addition, this new membrane proteomics method compromises detergent-free improved sample

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preparation for LC–MS/MS analysis. This systematic membrane proteomics increased the sequence coverage of hydrophilic as well as hydrophobic peptides of IMPs.

2. Experimental

2.1. Materials and chemicals

All chemicals used in the present experiment were of analytical grade or the highest purity available. Proteomics-grade trypsin and sequencing-grade modified bovine chymotrypsin were from Sigma–Aldrich (St. Louis, MO, USA) and Princeton Separations (Adelphia, NJ, USA), respectively. Picotip^{TM} was purchased from New Objective (Woburn, MA, USA). Sleeve and fused silica tubing were from Upchurch Scientific (Oak Harbor, WA, USA). Ferrule and union were purchased from Valco Instrument (Houston, TX, USA). HPLC-grade acetonitrile and water were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA).

2.2. Preparation of total membrane proteins

Total membranes of *Synechocystis* were isolated with minor modification as reported previously [12]. *Synechocystis* cells were harvested from 1 L culture medium of BG11 supplemented with glucose up to early-log phase (OD_{750} = 1.0–1.2) by centrifugation. Cells were washed three times with 20 mM Tris–HCl (pH 8.0) buffer. The harvested cells were resuspended in Tris–HCl buffer and disrupted by high pressure cell disrupter (30 000 psi, Stansted Fluid Power Ltd., UK). Cell-free crude extracts were treated with 150 U of DNase I and protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA) at 37 °C for 30 min. The protein solution was obtained by centrifuging two times at 15 000 × *g* for 20 min. Subsequently membranous proteins were pellet-down by ultracentrifugation at 105 000 × *g* at 4 °C for 30 min and the resultant pellets were freeze-dried and stored at −80 °C prior to MS analysis.

2.3. Chemical cleavage of membrane proteins by acid hydrolysis reaction

The procedure of the new membrane proteomics is illustrated in Fig. 1. The key steps of systematic membrane proteomics

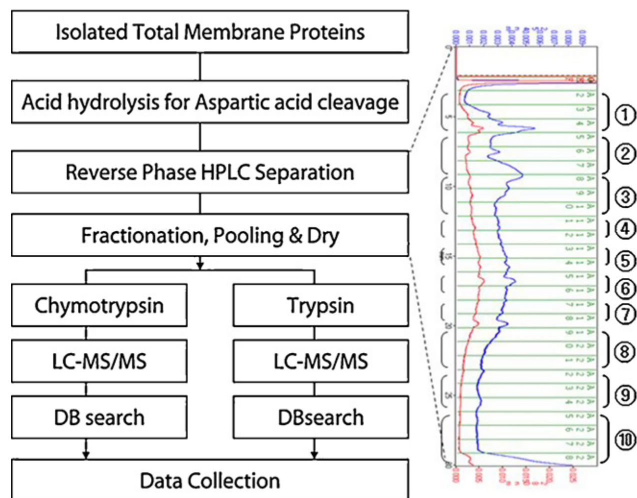

Fig. 1. Schematic procedure of acid hydrolysis reaction combined with trypsin or chymotrypsin treatment. (i) Acid hydrolysis reaction contained disulfide cleavage, thermal denaturation, and cleavage at aspartic acids. (ii) Separation of peptide mixture in RP-HPLC, dryness of the isolated peptides, and alkylation prior to enzymatic digestion. (iii) The independent digestion by trypsin and chymotrypsin. (iv) The generated peptide fragments were analyzed by *nano*-FT-MS analysis.

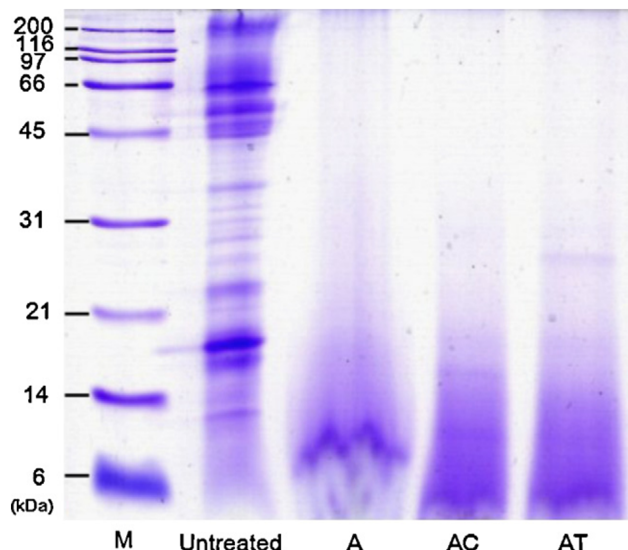

Fig. 2. SDS-PAGE of digested membrane proteins by acidic cleavage (A) and further treatment of enzyme, chymotrypsin (AC) or trypsin (AT). Cleavage and digestion conditions were described in the text. Untreated membrane proteins were loaded 30 μg and other digested samples were loaded 10 μg each. The SDS-PAGE gel was stained with Coomassie brilliant blue R250.

consist of acid hydrolysis at aspartyl residues followed by digestion with either chymotrypsin or trypsin. The acid hydrolysis solution was composed of a 1:1 mixture of 10 mM tris(2-carboxyethyl)phosphine (TCEP) in 30% acetonitrile and 25% formic acid in water. These chemicals are water-miscible organic solvents for solubilizing hydrophobic membrane proteins and reducing reagents working well at low pH, cleaving at amino- or carboxyl-terminals of aspartyl residues. Reaction was initiated with 150 μl of reactants poured into 1 mg of the dried *Synechocystis* membrane fraction. The reaction solution was heated at 95 °C for 4 h. The solvent in a reaction tube was removed by evaporation using Speed vacuum (SC 110A, GMI Inc., MI, USA). During the thermal acid hydrolysis, peptides less than 10 kDa were produced (Fig. 2).

2.4. Trypsin or chymotrypsin digestion of chemically cleaved peptides

The post-acid hydrolysis reactants were cooled at room temperature and centrifuged at 10 000 × *g* for 10 min to discard non-solubilized pellets. The resulting peptide mixture was separated with reverse phase-LC (Poros perfusion chromatography EYELA, DC-1000, Tokyo, Japan) equipped with column (Poros R2/M 4.6/100, EYELA, Tokyo, Japan) followed by collecting 28 fractions. According to the peptide content of each fraction, samples were pooled into 10 fractions. All the proteins were dried in the freeze dryer. After alkylation of each fraction with 10 μl of 100 mM iodoacetamide in 50 mM ammonium bicarbonate at 40 °C for 2 h, each fraction was subjected to digest with either trypsin or chymotrypsin (each 500 ng enzyme) at 37 °C for 12 h.

2.5. nano-Liquid chromatography–Fourier transform mass spectrometry analysis

For the separation and identification of double-digested peptides, 7-Tesla LTQ-Fourier Transform mass spectrometer (Thermo, Waltham, MA, USA) equipped with a *nano*-electrospray ionization source was used. Peptide mixture produced by thermally acidic cleavage combined with trypsin or chymotrypsin was loaded on a home-made trap column to remove salts and concentrate the peptides. The trapped peptides were directly applied on an analytical

column (75 μm × 150 mm, C_{18} silica of 5 μm in particle size) at a flow rate of 200 μl/min. The LC gradient program and operation mode were processed as reported previously [13].

2.6. Database search and protein identification

Protein identification was carried out using an internal Mascot server (version 2.2, Matrix Science Inc., London, UK). A database search against the *Synechocystis* database retrieved from Cyanobase (http://bacteria.kazusa.or.jp/cyanobase/) including 3661 entries was performed with merged dta file using the home-made Perl script including data generated separately from FT-LTQ (Thermo, Waltham, MA, USA). Peptide tolerance of parent ion was set at 1.2 Da and MS/MS tolerance set at 0.8 Da. During Mascot search, the oxidation of methionine (+16 Da) and N-terminal modification of Pyro-Gln (−17 Da) and Pyro-Glu (−18 Da) were chosen as a variable modification. In addition, the carbamidomethylation of cysteine (+57 Da) was chosen as a fixed modification. Four missed cleavages were allowed for identifying the peptides cleaved at either Asp-X or X-Asp or both side directions by Mascot search engine. Redundant peptides were removed and related peptides were filtered by matching the candidate protein with validation tool, PROVALT [14]. For the protein identification, the minimum MOWSE score for more informative peptides was set at 10.

2.7. Bioinformatic analysis of Synechocystis membrane proteins

Possibly all of the open reading frames from *Synechocystis* genome were accounted for 3661 proteins so that the physiochemical properties were computationally analyzed. A total of 751 proteins were predicted more than single TMD by TMHMM 2.0 [15] and 45 out of 751 proteins were detected single TMD and signal sequence by Signal P 3.0 [16]. However, these 45 proteins are considered as false integral membrane proteins because these proteins with signal sequences were actually misrecognized as single transmembrane protein. Thus, the finally filtered 706 proteins either with two more TMDs or with single TMD in the absence of signal sequence were considered the authentic IMPs. All peptide physical properties were calculated using Biopython (http://biopython.org).

3. Results and discussion

3.1. Overview of the combined methods of AC and AT

In general, the analysis of membrane protein complex requires the following steps such as the solubilization with detergents, purification, and chromatographic or electrophoretic separation as reviewed previously [17,18]. Instead of the complicated procedure prior to LC–MS analysis, we attempted to develop the systematic membrane proteomic method for the potential usefulness. The method we developed was illustrated in Fig. 1. In brief, isolated whole membrane proteins of *Synechocystis* were cleaved at both amino- and carboxyl-terminals of aspartic acid residue with chemical digestion reagents at high temperature. The generated peptide mixture was separated with HPLC column for the lesser complexity and the elimination of hydrophilic contaminants. The resulting peptides from membrane proteins were fully scanned for 30 min of retention time and detected at the absorbance 254 nm and 280 nm (Fig. 1). A majority of peptides were evenly digested. Each fraction of RP-LC was dried *in vacuo* followed by the treatment of trypsin or chymotrypsin as described. Chemical cleavage method at aspartyl residues was first attempted in proteomics by Li et al. [19], in which method revealed more efficient and specific than using single trypsin digestion. However, little has been reported for the application of the chemical cleavage to the large-scale membrane proteomics.

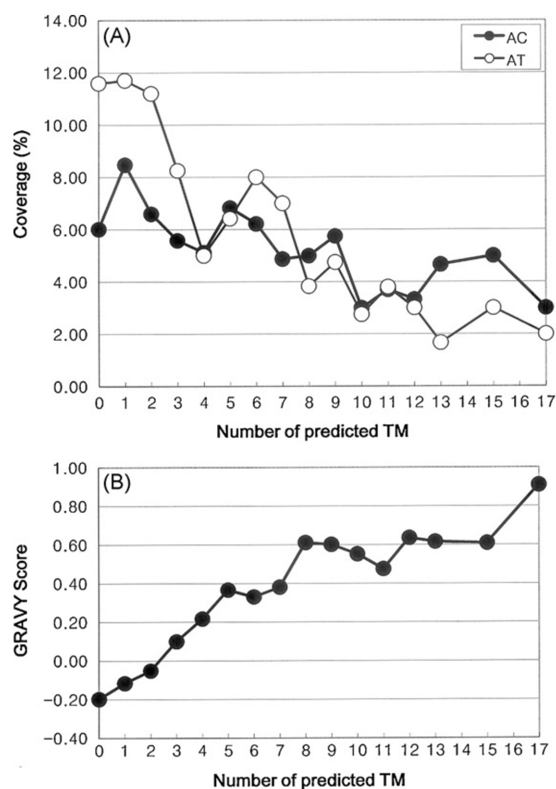

Fig. 3. Relationship between number of trans-membrane domain and sequence coverage by AC and AT methods (A). Relationship between number of trans-membrane domain and GRAVY score (B).

Herein, we improved the chemical cleavage method for the better identification of membrane proteins. Basically chemical reaction is based on the thermal acid hydrolysis for cleaving at aspartyl site so that it can produce the peptides less than 10 kDa in 4 h. In addition to acid hydrolysis, the following enzyme digestion produced the smaller peptides less than 5 kDa in size (Fig. 2). The acid hydrolysis is resistant to a high concentration of detergent, SDS and NaCl up to final concentration of 10% (data not shown). Since the thermal acid hydrolysis is not required for the prior solubilization step of membrane proteome, it saves time for the preparation of peptides prior to LC–MS analysis. In particular, acid-tolerant reducing reagent, TCEP is a derivative compound of trialkylphosphine which is an odorless reducing agent and essentially non-reactive toward other functional groups of proteins, and selectively reduces disulfide bonds of proteins [20]. TCEP was used to cleave the disulfide bonds of proteins and transformed to the oxide form, TCEPO, during this reaction [21]. TCEPO is so hydrophilic that it can be easily removed from RP-HPLC without further purification step. The separation of peptides by RP-HPLC was performed in our protocol for considering two reasons: (i) reducing the complexity of the peptide mixtures cleaved at aspartyl sites by acid hydrolysis reaction and (ii) facilitating the clearly purified peptides fractions to further digest with trypsin or chymotrypsin.

The newly developed systematic membrane proteomics showed the characteristic feature of hydrophobic coverage by AT and AC methods. The extent of TMD coverage can be examined by overlaying the identified peptides whether the peptides were found in TMD. Relationship between the number of TMD and sequence coverage revealed that AC method was better for identifying IMPs with more than 12 TMDs (Fig. 3A). In addition, the hydropathy analysis using GRAVY (grand average of hydropathicity) score [22] was performed with the totally identified IMPs according to the number

of trans-membrane domains. GRAVY score greater than +0.3 was considered as an indicative hydrophobic membrane protein [23]. In the present study, overall hydrophobic proteins in average based on GRAVY score were observed from five more TMD-containing IMPs (Fig. 3B). From the prepared *Synechocystis* membrane fractions, the systematic membrane proteomics method could identify the membrane-embedded or membrane-associated peripheral proteins up to 191 (Table 1).

3.2. Comparison of in silico prediction and experiment of membrane proteins

High sequence coverage including TMD is prerequisite for the membrane proteomics, which is applicable for the analysis of post-translational modifications of protein such as phosphorylation, acetylation, ubiquitination and cysteine oxidation [24]. For the maximal identification of membrane proteins with different combinations of digestion methods, there is no straightforward method for large-scale proteomics, but it is rather empirical. To evaluate the feasibility of the systematic membrane proteomics we developed, the data generated by bioinformatic prediction and experimentation were systematically compared.

The membrane-associated proteins identified by systematic AT and AC methods are listed in Table 1. A total of 785 unique proteins were identified out of 4695 peptides MS/MS data (Supplementary Table S1) that were collected by the AT method (65%, an exclusive identification rate from 2958 peptides) and AC method (25%, from 1615 peptides). Based on the rule of TMHMM 2.0 and Signal P, 155 proteins were finally assigned, ≥1 (# of TM), as IMPs (Table 1). Here, our method generated 352 peptides (49%) with terminal aspartyl residues out of 722 peptides from the finally identified 155 IMPs. In addition, 36 proteins had no TMDs, however, these suggest to be peripheral proteins since they are linked to the IMP complex by KEGG metabolic pathway analysis [25]. The 155 IMPs were classified into hypothetical proteins (31%, 48 IMPs), transport and binding proteins (21%, 32 IMPs), and photosynthesis and respiration (14%, 22 IMPs) by functional category.

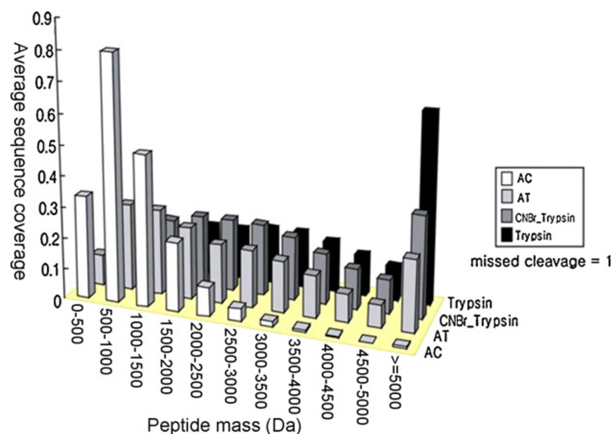

Fig. 4. Characteristics of theoretical peptide products by trypsin, CNBr-trypsin, AT, and AC digestion. The predictable 706 *Synechocystis* integral membrane proteins were queried for the analysis of digestion patterns and the resulting peptide fragments were achieved by the peptide mass at the interval of 500 Da-unit.

In order to evaluate the our membrane proteomics, we compared *in silico* digestion patterns of 706 theoretical membrane proteins in *Synechocystis* protein database among four different digestion strategies, i.e., AT (cleavage at D,K,R), AC (cleavage at D,F,W,Y,L), and trypsin (cleavage at K,R), CNBr/trypsin (cleavage at M,K,R) for the comparison of digestion protocols frequently cited [26]. As shown in Fig. 4, the fragmented peptides by single trypsin treatment were widely formed in the range of 500–5000 Da. However, the tryptic peptides were mainly observed over 5000 Da. Single application of trypsin to membrane proteome is not a good

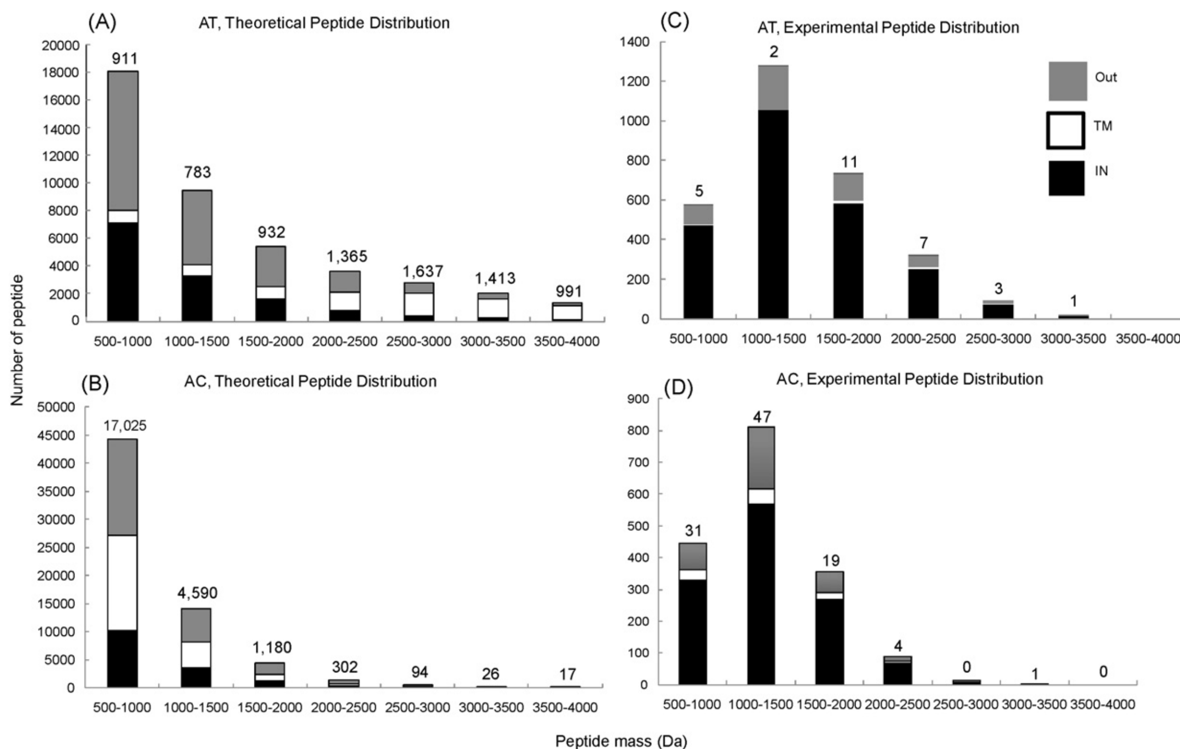

Fig. 5. Size distribution and predicted region of theoretically identifiable and experimentally identified peptides by AT and AC methods. Peptide masses were displayed at the interval of 500 Da-unit. The distribution of peptides produced by the theoretical prediction (A and B) was compared to that of peptides produced by experiment (C and D). Each peptide was annotated as extracellular (gray), trans-membrane (white), and cytoplasmic domain (black). The number of peptide including trans-membrane domain are annotated on the top of bar graph.

Table 1

List of membrane-associated proteins identified by AC and AT methods from *Synechocystis* membrane fraction. PI, Previously identified protein from gel-based proteomics method. Details are described in the main text.

No.	Protein name	Gene locus number	Functional category	# of spectra (AC/AT)	GRAVY score	# of TM[a]	Membrane complex
1	NADH dehydrogenase subunit L	Sll1732	Photosystem/respiration	3/2	0.91	17	–
2	NADH dehydrogenase subunit L	Slr0844	Photosystem/respiration	5/3	0.61	15	–
3	Cytochrome b subunit of nitric oxide reductase	Sll0450	Amino acid biosynthesis	6/1	0.37	13	–
4	Melibiose carrier protein	Sll1374	Transport/binding proteins	4/2	0.63	13	–
5	Virulence factor MviN homolog	Slr0488	Cellular processes	4/2	0.85	13	–
6	NAD(P)H-quinone oxidoreductase subunit 4	Slr0331	Photosystem/respiration	3/2	0.81	12	–
7	Cation or drug efflux system protein	Slr0454	Transport/binding proteins	2/2	0.45	12	–
8	Putative membrane protein for bicarbonate uptake	Slr1515	Hypothetical	5/5	0.65	12	–
9	Cation or drug efflux system protein	Sll0142	Transport/binding proteins	2/1	0.38	11	–
10	Na + /H + exchanger	Sll0273	Transport/binding proteins	4/2	0.79	11	–
11	Hypothetical protein	Sll0862	Hypothetical	2/2	0.57	11	–
12	Quinolene resistance protein, NorA	Sll1154	Other categories	2/4	0.71	11	–
13	Chloride channel protein	Sll1864	Transport/binding proteins	2/1	0.26	11	–
14	44.5 kDa bacteriochlorophyll synthase subunit	Sll1906	Hypothetical	3/9	0.81	11	–
15	Cation or drug efflux system protein	Slr0369	Transport/binding proteins	1/1	0.29	11	–
16	Cation or drug efflux system protein	Slr0794	Transport/binding proteins	3/2	0.34	11	–
17	P700 apoprotein subunit 1b	Slr1835	Photosynthesis/respiration	16/13	0.21	11	Photosystem I
18	Probable cation efflux system protein, CzcA homolog	Slr6043	Hypothetical	2/3	0.40	11	–
19	Cation-transporting ATPase (E1–E2 ATPase)	Sll0672	Transport/binding proteins	1/1	0.24	10	–
20	Hypothetical protein	Slr0643	Hypothetical	3/3	0.40	10	–
21	Probable Na^{+}/Ca^{2+} exchanger protein	Slr0681	Hypothetical	3/4	0.96	10	–
22	Nickel resistance	Slr0796	Transport/binding proteins	5/3	0.53	10	–
23	Na^{+}/H^{+} antiporter	Sll0689	Transport/binding proteins	5/4	1.02	9	–
24	Hypothetical protein	Slr0913	Unknown	1/2	0.53	9	–
25	Sulfate permease	Slr1229	Transport/binding proteins	3/1	0.63	9	–
26	P700 apoprotein subunit Ia	Slr1834	Photosynthesis/respiration	14/12	0.23	9	Photosystem I
27	Hypothetical protein	Sll0360	Hypothetical	12/3	0.82	8	–
28	NADH dehydrogenase subunit H	Sll0519	Photosynthesis/respiration	2/13	0.83	8	NADH dehydrogenase
29	Zinc exporter	Sll1076	Transport/binding proteins	2/1	0.23	8	–
30	Hybrid sensory kinase	Sll1672	Regulatory functions	6/1	0.19	8	–
31	High-affinity branched-chain amino acid transporter	Slr1200	Transport/binding proteins	5/2	0.97	8	–
32	Pyridine nucleotide transhydrogenase beta subunit	Slr1434	Biosynthesis of cofactors, prosthetic groups, carriers	3/3	0.63	8	–
33	Hypothetical protein	Sll0060	Hypothetical	3/7	0.75	7	–
34	Photosystem II CP43 protein	Sll0851	Photosynthesis/respiration	13/24	0.27	7	Photosystem II
35	Hypothetical protein	Sll1151	Unknown	4/2	0.21	7	–
36	Hypothetical protein	Sll1166	Hypothetical	2/7	0.86	7	–
37	Nitrate transport protein, NrtB	Sll1451	Transport/binding proteins	9/6	0.81	7	–
38	Hypothetical protein	Sll1608	Hypothetical	3/2	−0.04	7	–
39	Hypothetical protein	Slr0104	Hypothetical	1/3	−0.09	7	–
40	Potassium-transporting ATPase subunit B	Slr1729	Transport/binding proteins	4/5	0.28	7	–
41	OppB in a binding protein-dependent transport system	Sll0312	Transport/binding proteins	4/5	0.54	6	Ribosomal proteins
42	ABC transporter	Sll0778	Transport/Binding proteins	4/4	0.16	6	–
43	Photosystem II D2 protein	Sll0849	Photosynthesis/respiration	24/19	0.36	6	Photosystem II
44	Sensory transduction histidine kinase	Sll1353	Regulatory function	1/1	0.08	6	–
45	Probable permease protein of ABC transporter	Slr0347	Transport/Binding proteins	3/5	1.13	6	–
46	Cation-transporting ATPase (E1–E2 ATPase)	Slr0797	Transport/binding proteins	2/1	0.34	6	–
47	Photosystem II CP47 protein	Slr0906	Photosynthesis/respiration	16/31	0.12	6	Photosystem II
48	Haemolysin secretion ATP-binding protein	Slr1651	Transport/binding proteins	1/2	0.21	6	–
49	Hybrid sensory kinase	Slr2098	Regulatory function	1/4	0.04	6	Pyrimidine nucleotide biosynthesis
50	ABC transporter	Sll0739	Transport/binding proteins	6/1	0.07	5	Ribosomal proteins

Table 1 (*Continued*)

No.	Protein name	Gene locus number	Functional category	# of spectra (AC/AT)	GRAVY score	# of TM[a]	Membrane complex
51	OppC in a binding protein-dependent transport system	Sll0833	Transport/binding proteins	3/3	0.52	5	Ribosomal proteins
52	Hypothetical protein	Sll1040	Unknown	3/2	−0.13	5	–
53	ATP synthase subunit A	Sll1322	Photosynthesis/respiration	5/7	0.67	5	ATP synthase
54	Hypothetical protein	Sll1477	Hypothetical	2/4	−0.06	5	–
55	Hypothetical protein	Slr0232	Hypothetical	11/4	0.51	5	–
56	Cytochrome b6	Slr0342	Photosynthesis/respiration	25/24	0.56	5	Cytochrome b6/f complex
57	Protein export protein SecD	Slr0774	Cellular processes	2/2	0.31	5	Protein/peptide secretion
58	ABC transporter	Slr1149	Transport/binding proteins	2/3	0.2	5	–
59	Hypothetical protein	Slr1215	Hypothetical	9/10	0.65	5	–
60	Photosystem II D1 protein	Slr1311	Photosynthesis/respiration	9/13	0.3	5	–
61	Aquaporin Z	Slr2057	Transport/binding proteins	5/4	0.8	5	–
62	Adenylate cyclase	Sll0646	Regulatory functions	3/1	0.05	4	–
63	TRAP-type permease to mediate Na^+-dependent glutamate transport, GtrA	Sll1102	Transport/binding proteins	11/3	0.52	4	–
64	HlyB family	Sll1180	Transport/binding proteins	3/13	0.05	4	Ribosomal proteins
65	Short chain dehydrogenase	Sll1376	Hypothetical	4/2	0.15	4	–
66	Probable glycosyltransferase	Sll1377	Other categories	3/5	−0.14	4	–
67	Hypothetical protein	Slr0594	Hypothetical	7/3	0.43	4	–
68	Mechanosensitive ion channel homolog	Slr0639	Transport/binding proteins	3/8	0.41	4	–
69	Hypothetical protein	Slr1918	Hypothetical	7/5	0.27	4	–
70	Haemolysin	Sll0260	Hypothetical	5/2	0.15	3	–
71	Hypothetical protein	Sll0298	Hypothetical	4/8	0.2	3	–
72	Hypothetical protein	Sll0804	Hypothetical	2/4	−0.06	3	–
73	Cell division protein, FtsH	Sll1463	Cellular processes	12/31	−0.09	3	–
74	Hypothetical protein	Sll1757	Hypothetical	4/6	0.16	3	–
75	Cytochrome b6-f complex subunit 4	Slr0343	Photosynthesis/respiration	16/24	0.57	3	Cytochrome b6/f complex
76	Ethylene response sensor protein	Slr1212	Regulatory functions	2/1	−0.24	3	–
77	Mg^{2+} transporter	Slr1216	Transport/binding proteins	6/7	0.25	3	–
78	Hypothetical protein	Slr1413	Hypothetical	4/1	−0.19	3	–
79	Hypothetical protein	Slr1462	Hypothetical	4/2	−0.04	3	–
80	Putative inner membrane protein translocase component YidC	Slr1471	Hypothetical	4/9	0.02	3	Transposon-related function
81	Hypothetical protein Ycf81	Slr1972	Hypothetical	4/4	0.47	3	–
82	Hypothetical protein	Sll0154	Hypothetical	3/8	0	2	–
83	Hypothetical protein	Sll0412	Hypothetical	5/2	0.15	2	–
84	Probable glycosyltransferase	Sll0501	Other categories	4/8	0.06	2	–
85	Hypothetical protein	Sll0505	Hypothetical	3/4	0.15	2	–
86	Drug sensory protein A	Sll0698	Other categories	5/1	−0.08	2	–
87	Photosystem I subunit III	Sll0819	Photosynthesis/respiration	23/45	0.08	2	Photosystem I
88	Methyl-accepting chemotaxis-like protein	Sll1294	Cellular processes	1/5	−0.2	2	–
89	Apocytochrome f precursor	Sll1317	Photosynthesis/respiration	6/35	0.06	2	Cytochrome b6/f complex
90	Hypothetical protein	Sll1365	Unknown	4/5	−0.03	2	–
91	RfbJ protein	Sll1457	Cell envelope	5/2	0.12	2	–
92	Sensory transduction histidine kinase	Sll1590	Regulatory functions	5/2	0.07	2	–
93	Hypothetical protein	Sll1681	Unknown	2/6	−0.28	2	–
94	Hypothetical protein	Slr0016	Hypothetical	10/4	0.16	2	–
95	Hypothetical protein	Slr0483	Hypothetical	17/35	−0.01	2	–
96	Hypothetical protein Ycf66	Slr0503	Hypothetical	2/7	−1.1	2	–
97	PleD-like protein	Slr0829	Unknown	2/2	−0.18	2	–
98	Methyl-accepting chemotaxis protein	Slr1044	Cellular processes	1/1	−0.31	2	–
99	Hypothetical protein	Slr1257	Unknown	14/8	0.16	2	–
100	Photosystem I reaction center protein subunit XI	Slr1655	Photosynthesis/respiration	18/41	0.4	2	Photosystem I
101	Hybrid sensory kinase	Slr1759	Regulatory functions	2/3	−0.18	2	–
102	Cell division protein Ftn2 homolog	Sll0169	Cellular process	1/2	−0.38	1	–

103	Hypothetical protein	Sll0178	Hypothetical	1/2	−0.39	1	–
104	Alkaline phosphatase	Sll0222	Other categories	4/3	−0.4	1	–
105	Hypothetical protein	Sll0441	Unknown	2/3	−0.42	1	–
106	Hypothetical protein	Sll0478	Unknown	10/8	0.1	1	–
107	Asparaginyl-tRNA synthetase	Sll0495	Translation	3/2	−0.2	1	–
108	Hypothetical protein	Sll0606	Hypothetical	2/6	−0.06	1	Transposon-related function
109	Eucaryotic protein kinase	Sll0776	Regulatory functions	2/2	−0.33	1	–
110	PleD-like protein	Sll0779	Unknown	4/3	−0.33	1	–
111	Hypothetical protein Ycf22	Sll1002	Unknown	2/5	−0.17	1	–
112	Hypothetical protein	Sll1053	Hypothetical	3/5	−0.37	1	–
113	Hypothetical protein	Sll1106	Hypothetical	7/4	0.23	1	–
114	Hypothetical protein	Sll1155	Hypothetical	1/1	0.08	1	–
115	Penicillin-binding protein 4	Sll1167	Unknown	4/2	−0.11	1	–
116	Plastoquinol-plastocyanin reductase	Sll1316	Photosynthesis/respiration	18/26	−0.09	1	–
117	ATP synthase subunit B	Sll1323	Photosynthesis/respiration	10/50	−0.43	1	ATP synthase
118	Hypothetical protein	Sll1390	Hypothetical	8/4	0.1	1	–
119	Nitrate transport protein, NrtD	Sll1453	Transport/binding proteins	6/13	−0.17	1	–
120	ABC-transporter DevB-like protein	Sll1481	Transport/binding proteins	6/4	−0.29	1	–
121	Hypothetical protein	Sll1495	Hypothetical	2/5	−0.08	1	–
122	Molybdopterin biosynthesis protein, MoeB	Sll1536	Biosynthesis of cofactors, prosthetic groups, carriers	11/15	−0.05	1	–
123	Hypothetical protein	Sll1586	Unknown	4/2	−0.31	1	–
124	Hypothetical protein	Sll1665	Unknown	9/5	−0.54	1	–
125	Penicillin-binding protein	Sll1833	Other categories	1/3	−0.29	1	–
126	Exopolysaccharide export protein-like	Sll5052	Other categories	2/4	−0.23	1	–
127	Hypothetical protein	Sll7069	Hypothetical	10/11	0.4	1	–
128	Hypothetical protein	Slr0226	Unknown	2/5	−0.14	1	–
129	Hypothetical protein	Slr0404	Hypothetical	6/30	0.07	1	–
130	Soluble lytic transglycosylase	Slr0534	Cell envelope	3/1	−0.59	1	–
131	ThiG protein	Slr0633	Biosynthesis of cofactors, prosthetic groups, carriers	1/5	0.02	1	–
132	Biopolymer transport ExbD-like protein	Slr0678	Transport/binding proteins	18/6	−0.39	1	–
133	Regulation of penicillin-binding 5 production	Slr0883	Hypothetical	2/7	−0.05	1	–
134	8-Amino-7-oxononanoate synthase	Slr0917	Biosynthesis of cofactors, prosthetic groups, carriers	4/3	−0.14	1	–
135	Serine protease, HtrA	Slr1204	Translation	5/2	−0.05	1	–
136	Hypothetical protein	Slr1275	Hypothetical	2/6	−0.05	1	Transposon-related function
137	Hypothetical protein	Slr1276	Hypothetical	5/4	−0.21	1	Transposon-related function
138	Processing protease	Slr1331	Translation	5/10	−0.18	1	–
139	UDP-N-acetylmuramate-L-alanine ligase	Slr1423	Cell envelope	7/6	−0.07	1	–
140	Hypothetical protein	Slr1470	Hypothetical	6/16	0	1	–
141	Penicillin-binding protein 1B	Slr1710	Other categories	2/3	−0.32	1	–
142	Hypothetical protein	Slr1799	Hypothetical	2/2	−0.42	1	–
143	Mercuric reductase	Slr1849	Other categories	2/2	0.05	1	–
144	ICFG protein	Slr1860	Regulatory functions	5/1	−0.03	1	–
145	Hypothetical protein	Slr1968	Unknown	3/2	−0.28	1	–
146	Hypothetical protein	Slr2013	Hypothetical	2/2	−0.01	1	–
147	Hybrid sensory kinase	Slr2104	Regulatory functions	1/1	−0.26	1	–
148	Probable cation efflux system protein, CzcB homolog	Slr6042	Transport/binding proteins	2/1	−0.38	1	–
149	Photosystem I PsaM subunit	Smr0005	Photosynthesis/respiration	32/84	1.19	1	Photosystem I
150	Cytochrome b559 subunit beta	Smr0006	Photosynthesis/respiration	70/98	0.6	1	Photosystem II
151	Hypothetical protein	Ssl0788	Hypothetical	14/15	−0.27	1	–
152	Hypothetical protein	Ssl1498	Hypothetical	10/15	0.24	1	–

Table 1 (*Continued*)

No.	Protein name	Gene locus number	Functional category	# of spectra (AC/AT)	GRAVY score	# of TM[a]	Membrane complex
153	Photosystem II reaction center protein, PsbH	Ssl2598	Photosystem/respiration	14/22	0.71	1	Photosystem II
154	Hypothetical protein	Ssl5114	Unknown	17/11	−0.07	1	–
155	Cytochrome b559 subunit alpha	Ssr3451	Photosynthesis/respiration	70/65	−0.21	1	–
156	PatA subfamily	Sll0038	Regulatory functions	4/13	−0.03	0	Type IV pili biosynthesis
157	CheA like protein	Sll0043	Regulatory functions	2/5	−0.13	0	Type IV pili biosynthesis
158	Plastocyanin precursor	Sll0199	Photosynthesis/respiration	12/14	0.29	0	Membrane-associated electron carrier
159	ABC transporter	Sll0385	Transport/binding proteins	3/4	0.01	0	Ribosomal proteins
160	Photosystem II manganese-stabilizing polypeptide	Sll0427	Photosynthesis/respiration	4/18	−0.21	0	Photosystem II
161	Translocase	Sll0616	Cellular processes	6/13	−0.51	0	Protein/peptide secretion
162	Phosphate transport ATP-binding protein, PstB	Sll0683	Transport/binding proteins	8/4	−0.35	0	Ribosomal proteins
163	Allophycocyanin B	Sll0928	Photosynthesis/respiration	28/71	−0.25	0	Phycobilisome complex
164	Cytochrome b6/f complex iron–sulfur subunit	Sll1182	Photosynthesis/respiration	13/13	−0.15	0	Cytochrome b6/f complex
165	ATP synthase subunit D	Sll1325	Photosynthesis/respiration	8/42	0.1	0	ATP synthase
166	ATP synthase subunit A	Sll1326	Photosynthesis/respiration	14/45	−0.03	0	ATP synthase
167	ATP synthase subunit C	Sll1327	Photosynthesis/respiration	10/44	−0.11	0	ATP synthase
168	Twitching motility protein	Sll1533	Cellular processes	5/8	−0.42	0	Type IV pili biosynthesis
169	Phycocyanin b subunit	Sll1577	Photosynthesis/Respiration	44/88	0.09	0	Phycobilisome complex
170	Phycocyanin a subunit	Sll1578	Photosynthesis/respiration	72/94	−0.3	0	Phycobilisome complex
171	Phycocyanin associated linker protein	Sll1579	Photosynthesis/respiration	14/49	−0.5	0	Phycobilisome complex
172	ABC transporter	Sll1927	Transport/binding proteins	1/4	−0.21	0	Ribosomal proteins
173	General secretion pathway protein E	Slr0063	Cellular processes	3/1	−0.35	0	Protein/peptide secretion
174	NADH dehydrogenase delta subunit	Slr0261	Photosynthesis/respiration	2/28	−0.25	0	NADH dehydrogenase
175	Phycobilisome LCM core-membrane linker polyopeptide	Slr0335	Photosynthesis/respiration	26/57	−0.39	0	Phycobilisome complex
176	Glucosylglycerol transport system substrate-binding protein, GgtB	Slr0529	Transport/binding proteins	5/1	−0.08	0	Ribosomal proteins
177	Photosystem I subunit II	Slr0737	Photosynthesis/respiration	23/40	−0.66	0	Photosystem I
178	ATP-binding subunit of an ABC-type osmolyte transporter	Slr0747	Transport/binding proteins	5/8	−0.17	0	Ribosomal proteins
179	ABC transporter	Slr0982	Transport/binding proteins	3/2	−0.2	0	Ribosomal proteins
180	Membrane protein	Slr1274	Cellular processes	2/12	−0.1	0	Type IV pili biosynthesis
181	General secretion pathway protein D	Slr1277	Cellular processes	11/31	−0.11	0	Protein/peptide secretion
182	ATP synthase subunit B	Slr1329	Photosynthesis/respiration	19/40	−0.06	0	ATP synthase
183	Phycobilisome core component	Slr1459	Photosynthesis/respiration	7/27	−0.17	0	Phycobilisome complex
184	Ferredoxin-NADP oxidoreductase	Slr1643	Photosynthesis/respiration	7/41	−0.57	0	Membrane-associated electron carrier
185	Photosystem II 11 kDa protein	Slr1645	Photosynthesis/respiration	16/19	−0.31	0	Photosystem II
186	Allophycocyanin b chain	Slr1986	Photosynthesis/respiration	37/75	0.11	0	Phycobilisome complex
187	Allophycocyanin a chain	Slr2067	Photosynthesis/respiration	50/71	−0.05	0	Phycobilisome complex
188	Ycf32 gene product	Sml0007	Photosynthesis/respiration	54/85	0.89	0	Photosystem II
189	Photosystem I subunit VII	Ssl0563	Photosynthesis/respiration	35/22	0.09	0	Photosystem I
190	Phycocyanin associated linker protein	Ssl3093	Photosynthesis/respiration	13/70	−0.63	0	Phycobilisome complex
191	Photosystem I reaction center subunit IV	Ssr2831	Photosynthesis/respiration	28/47	−0.3	0	Photosystem I

[a] Numbers of TM were determined by the rule of TMHMM 2.0 and Signal P 3.0.

choice since arginine and lysine are less frequent in TMDs. In contrast to trypsin digestion, AC-generated peptides were mainly populated in the peptides with molecular weight of smaller than 3 kDa. The peptides contain hydrophilic and hydrophobic domains of IMPs produced by AT and AC, respectively, in which the produced peptides were distributed under 3 kDa. Our finding coincided well with the previous study of *in silico* digestion prediction. From a theoretical perspective, we suggest that acid hydrolysis cleaves first hydrophilic aspartic acids at the extracellular or intracellular loops and trypsin digests further hydrophilic lysine and arginine sites. Likewise the introduction to AC cleaves hydrophobic peptides between 1 and 3 kDa, facilitating the peptides of TMDs to be adequate for MS/MS analysis.

When the pattern of identifiable IMPs by theoretical analysis was compared with those by experimental analysis, the regional distribution of peptide masses is shown in Fig. 5. The theoretical average sequence coverage of peptides containing TMDs by AT was increased as increasing the peptide masses while that of TMD-containing peptides by AC was decreased inversely (Fig. 5A and B). The frequency of TMD peptides by AT was theoretically observed 4041 out of 8032, corresponding to 50.3%, in the peptides greater than 2500 Da. In contrast, the population of TMD peptides by AC was rarely observed over 2500 Da, in which 137 peptides were predicted out of total generated 23 234 peptides with one missed cleavage, corresponding to 0.6%. The experimental occurrence of TMDs was exclusively identified by AC as expected (Fig. 5C and D). Therefore, the theoretical TMD-containing 23 097 peptides (99.4% hit below 2500 Da) could be experimentally detected by AC, suggesting to be given more likelihood of multiple spanning TMD proteins by AC method. The membrane proteins captured by non-TMDs like extracellular or intracellular loops, can be given more likely by AT. Interestingly, the higher abundance of intracellular domains of experimentally identified *Synechocystis* membrane proteins (Fig. 5C and D) was similar to the predominant observation of C-terminal intracellular membrane proteins in yeast and *E. coli* [27,28].

4. Conclusion

AT and AC methods are ideally complementary to identify IMPs at the hydrophilic and hydrophobic domains, respectively. The systematic membrane proteomics method was comparable to proven methods such as trypsin/chymotrypsin and trypsin/cyanogen bromide in *Corynebacterium glutamicum* [29] and chemical cleavage at cysteine/trypsin digestion in *E. coli* [30]. This membrane proteomics method contains the advantageous chemistry for membrane proteome: use of acid-tolerant detergent and thermal denaturation for solubilization of membrane as described previously [31]. The membrane topologies of experimentally identified peptides by AC and AT methods are presented in Supplementary Fig. S1. The combination of acid hydrolysis with enzymatic digestion including peptides fractionation and separation by preparative HPLC and *nano*-LC was shown to be very efficient and useful for the systematic analysis of large-scale membrane proteome. The new membrane proteomics we developed can be applicable to the retractable Gram-negative bacterial membrane proteome as well as the comparatively easily accessible animal cells in the absence of cell wall.

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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.chroma.2009.11.045.

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