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Targeted proteome analysis of microalgae under high-light conditions by optimized protein extraction of photosynthetic organisms

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Cell disruption and protein solubilization protocols for the relative quantification of individual subunits in photosystems were developed for photosynthetic organisms including cyanobacterium *Synechocystis* sp. PCC 6803, green-algae *Chlamydomonas reinhardtii*, and seed plant *Arabidopsis thaliana*. The optimal methods for the disruption of *Chlamydomonas*, *Synechocystis*, and *Arabidopsis* cells were sonication, microbeads (Φ approximately 0.1 mm), and large beads (Φ = 5.0 mm), respectively. Extraction of the total proteins exceeded 90% using each optimal cell disruption method. Solubilization efficiency of membrane proteins was improved by the phase transfer surfactant (PTS) method. Ninety seven and 114 proteins from *Chlamydomonas* and *Synechocystis*, respectively, including membrane proteins such as photosystem proteins, ATP synthase, and NADH dehydrogenase, were successfully analyzed by nano-liquid chromatography tandem mass spectrometry. These results also indicated the improved efficiency of solubilization and trypsin digestion using PTS buffer. The results of the relative quantitative evaluation of photosystem subunits in *Chlamydomonas* and *Synechocystis* grown under high-light conditions were consistent with those of previous studies. Thus, the optimal cell disruption and PTS methods allow for comprehensive relative quantitative proteome analysis of photosynthetic organisms. Additionally, NdhD1 and NdhF1, which are NDH-1 subunit homologs, were increased under high-light conditions, suggesting that the NDH-1L complex, including NdhD1 and NdhF1, is increased under high-light conditions. The relative quantitative proteome analysis of individual subunits indicates the diverse functions of NDH-1 protein.

[**Key words:** Nano-liquid chromatography-tandem mass spectrometry; Phase transfer surfactant; Photosynthetic organisms; Protein solubilization; High-light condition; NDH-1]

Plants and microalgae have many acclimation systems to adapt to various environmental changes such as light, temperature, and nutrients (1–7). Under high-light conditions, photosynthesis is reduced to protect cells from oxidative damage. One method to investigate novel acclimation systems is relative quantitative proteome analysis (8–12). Mass spectrometry (MS)-based proteomics have been widely used to obtain a comprehensive overview of expressed proteins. The usual direct approaches cannot be used for the analysis of the membrane proteomes, because of the difficulties in protein extraction and solubilization, and because of subsequent protease digestion. Various protocols to improve the solubilization and digestion of membrane proteins include the use of organic solvents (13) and ionic surfactants (14,15). Ionic surfactants such as sodium dodecyl sulfate (SDS) are powerful tools to solubilize hydrophobic proteins, but they inhibit the protease activity, influence liquid chromatography (LC) separation, and are incompatible with MS. Recently, a new protocol was developed to dissolve and digest membrane proteins with the aid of a removable phase transfer surfactant (PTS) (16,17). The PTS method for nano-LC tandem MS (nano-LC-MS/MS) analysis has been used with mammalian cells and *Escherichia coli* (18). However, there have been no reports of its use with other organisms.

Chloroplasts in green algae and land plants, and cyanobacteria contain photosynthetic thylakoid membranes with four multi-subunit protein complexes (photosystem I [PSI], photosystem II [PSII], ATP synthase complex, and cytochrome b_6f complex) and multiple cofactors, which perform the photosynthetic reactions (19–21). The plant proteome database (PPDB; http://ppdb.tc.cornell.edu) houses 154 peripheral and 162 integral *Arabidopsis* thylakoid membrane proteins (22), indicating the important roles of integral membrane proteins in photosystems. However, in previous studies (14,23), *n*-dodecyl β-D-maltoside and SDS were used to solubilize proteins in the targeted proteome analysis of photosynthetic organisms. Thus, it is necessary to confirm the effectiveness of the PTS method for a comprehensive relative quantitative proteome analysis of photosynthetic organisms. Additionally, a complete cell disruption method is required. For the analysis of the transcriptome and proteome, cells of photosynthetic organisms were disrupted with a sonicator, a bead crusher, and a French press

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(14,23–26). However, it is not clear which method is the most efficient to disrupt each cell type.

The aim of this study was to establish a membrane proteomics method for photosynthetic organisms that have many membrane protein complexes, such as photosystems, which is unbiased with respect to protein solubility, hydrophobicity, and protein abundance. We examined protocols for cell disruption and protein solubilization for three photosynthetic organisms; cyanobacterium *Synechocystis* sp. PCC 6803, green-alga *Chlamydomonas reinhardtii*, and plant *Arabidopsis thaliana*. We determined the optimal cell disruption method for each photosynthetic organism and improvements in protein solubilization efficiency by the PTS method. In *Chlamydomonas* and *Synechocystis*, proteins of the photosynthetic apparatus (mainly integral membrane proteins) were efficiently detected compared with the conventional method. The findings support the value of the PTS method for the relative quantitative analysis of individual photosystem subunit proteins. Additionally, we demonstrated the decreased contents of individual photosystem subunit proteins of *Chlamydomonas* and *Synechocystis* under high-light conditions. Conversely, some subunits of type I NADH dehydrogenase (NDH-1) were increased preferentially in *Synechocystis* cell under high-light conditions.

MATERIALS AND METHODS

Photosynthetic organisms and growth conditions Cells of *Synechocystis* sp. PCC 6803 glucose-tolerant strain (GT) (27) were grown in BG-11 medium (28) at 32 °C under continuous light-emitting diode (LED) white light (approximately 20 μmol m^{-2} s^{-1}) in flasks that were constantly shaken at 120 rpm. Cells of *C. reinhardtii* 137c (29) were grown in tris-acetate-phosphate (TAP) medium (30) at 25 °C under continuous light (approximately 50 μmol m^{-2} s^{-1}) in flasks constantly shaken at 120 rpm. For the high-light experiment, *Chlamydomonas* and *Synechocystis* cells were grown under a light intensity of approximately 20 μmol m^{-2} s^{-1} for 3 days [OD_{730} of 1.7–2.0 (*Chlamydomonas*) and 0.5–0.7 (*Synechocystis*)] and then grown under a light intensity of approximately 150 μmol m^{-2} s^{-1} for 1 day [OD_{730} of approximately 4.0 (*Chlamydomonas*) and approximately 1.2 (*Synechocystis*)]. For the preparation of ^{15}N-labeled standard from *Chlamydomonas*, TAP medium containing ^{15}N-labeled ammonium chloride (99% ^{15}N; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) was used for the pre- and main cultures. For the harvest of leaves and stems, *A. thaliana* Columbia-0 was grown on a plate supplemented with Murashige and Skoog salt, 1.5% sucrose, and 0.7% agar at 22 °C under alternating 12-h periods of light (white LED, 100 μmol m^{-2} s^{-1}) and dark for 24 days after germination.

KCl method Total proteins were extracted as described previously (31). *Chlamydomonas* cells were harvested by centrifugation such that the OD_{730} × volume (mL) = 50, and then frozen at −80 °C until analysis. Cell pellets were resuspended in 1 mL lysis buffer (50 mM HEPES at pH 7.5, 5% v/v glycerol, 15 mM dithiothreitol [DTT], 100 mM KCl, and 5 mM EDTA). Resuspended cells were divided into three aliquots and disrupted using a bead crusher (model μT-12; TAITEC Co., Saitama, Japan) with zirconia beads (Φ = 0.5 and 5.0 mm) at 3000 min^{-1} for 5 min, or using a sonicator (model UD-100; TOMY, Tokyo, Japan) for five cycles of 30 s with a 30-s interval five times on ice, and then centrifuged at 20,000 ×*g* for 5 min. The supernatants were used for protein quantitation using the Qubit Protein Assay kit (Thermo Fisher Scientific Life Science, Waltham, MA, USA) and the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Life Science). Next, 50 μg of total *Chlamydomonas* protein was supplemented with denaturing buffer (500 mM Tris–HCl at pH 8.5, 10 mM EDTA, 7 M Guanidine HCl) to total volume of 220 μL. One microliter of 50 mg mL^{-1} DTT was added and mixed by vortexing at room temperature for 1 h. Then, proteins were alkylated with 2.5 μL of 50 mg mL^{-1} iodoacetamide (IAA) with vortex mixing in the dark at room temperature for 1 h. Protein were purified as described previously (32). Six hundred microliters of ice-cold methanol, 150 μL of chloroform, and 450 μL of water were consecutively added to the lysates and mixed gently after the addition of each component. After centrifugation at 20,000 ×*g* for 5 min at 4 °C, the upper phase was discarded. Subsequently, 450-μL of methanol was added to the bottom phase as well as the interphase, and proteins were precipitated by centrifugation under the same conditions. Trypsin/LysC digestion was performed as described previously (33). Proteins were dissolved in 9 μL of 6 M urea for 10 min by vortex mixing. Then, 36 μL of 0.1 M Tris–HCl (pH 8.5) was added to the protein solution and mixed via sonication. Proteolytic digestion into peptides was performed using 1 μL of 0.5 mg mL^{-1} lysyl endopeptidase (Lys-C; Wako, Osaka, Japan) at a final concentration of 1% (w/w) Lys-C per sample protein and 2.5 μL of 1% w/v ProteaseMax Surfactant Trypsin Enhancer (Promega, Madison, WI, USA) at 25 °C for 3 h, followed by 1 μL of 0.5 mg mL^{-1} L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Promega) at a final concentration of 1% (w/w) trypsin per sample protein at 37 °C overnight. Following trypsin digestion, 7.5 μL water and 3 μL of 50% v/v formic acid were added to the protein mixture, which was then centrifuged at 20,000 ×*g* for 5 min. Finally, 12 μL of the sample and 20 μL ^{15}N labeled internal standard (extracted by the PTS method as next described) were mixed with 36 μL of 5% formic acid, and the mixtures were desalted with C_{18}-StageTips (34–36).

PTS method Total proteins were extracted as described previously (21,22). *Chlamydomonas* and *Synechocystis* cells were harvested by centrifugation. Aliquots in which the OD_{730} × volume (mL) = 50 were frozen at −80°C until analysis. Cell pellets were resuspended in 1 mL PTS buffer (12 mM sodium deoxycholate, 12 mM sodium lauroylsarcosinate, and 100 mM Tris–HCl at pH 8.5). Resuspended cells were divided into three aliquots and disrupted using a beads crusher with glass beads (Φ = 0.105–0.125 mm) or a sonicator. The resulting preparation was centrifuged at 20,000 ×*g* for 5 min. *Arabidopsis* leaves and stems (approximately 15 mg fresh weight) were disrupted with 500 μL PTS buffer using a bead crusher with zirconia beads (Φ = 5.0 mm) and a sonicator, and centrifuged at 20,000 ×*g* for 5 min. The supernatants were used for protein quantitation using the Pierce BCA Protein Assay Kit. Next, 50 μg of total protein was supplemented with PTS buffer to a total volume of 100 μL, and then reduced with 3 μL of 50 mg mL^{-1} DTT at room temperature for 30 min. Then, proteins were alkylated with 20 μL of 50 mg mL^{-1} IAA in the dark at room temperature for 30 min. The protein mixture was diluted 5-fold with 50 mM ammonium bicarbonate and then digested with 1 μL of 0.5 mg mL^{-1} Lys-C at a final concentration of 1% (w/w) Lys-C per sample protein and 5 μL of 0.1 mg mL^{-1} TPCK-trypsin at a final concentration of 1% (w/w) trypsin per sample protein. The equivalent volume of ethyl acetate and 5 μL of formic acid (0.5% final concentration) were added to the digestion solution and mixed by vortex for 1 min. After centrifugation, the ethyl acetate upper phase was discarded, and the bottom phase and interphase were dried via evaporation. The dried pellets were resuspended with 100 μL of 5% formic acid and centrifuged at 20,000 ×*g* for 5 min at 4°C. Finally, 20 μL of the sample and 20 μL ^{15}N labeled internal standard were mixed with 20 μL of 5% formic acid and the mixtures were desalted with C_{18}-StageTips (34–36).

Targeted proteome analysis using nano-LC-MS/MS and data analysis Samples prepared via the KCl and PTS methods were analyzed by nano-LC-MS/MS. The nano-LC-MS/MS system comprised a LC-20ADnano and a LCMS-8060 triple-quadrupole mass spectrometer with an electrospray ionization ion source (Shimadzu, Kyoto, Japan). Electrospray ionization was performed and sample separation was done by nano-LC (LC-20ADnano, Shimadzu). All analytical methods used were performed as described previously (37–39). For *Chlamydomonas*, the amino acid sequences of 117 target proteins including photosynthesis-related proteins and metabolic enzymes, were obtained from NCBI (https://www.ncbi.nlm.nih.gov/genome/). To evaluate solubilization efficiency, 245 peptides from 97 proteins were identified (Table S1). The multiple reaction monitoring (MRM) method used to relatively quantify these 97 proteins was created by the open software Skyline version 4.1 (40). For *Synechocystis*, 127 proteins including photosynthesis and respiration-related proteins were targeted and 396 peptides from 114 proteins were identified (Table S2). Peptides were relatively quantified by the peak area ratio of the ^{14}N sample to the ^{15}N sample. For the high-light condition experiment, 40 peptides from 10 proteins and 239 peptides from 86 proteins were measured in *Chlamydomonas* and *Synechocystis*, respectively.

Prediction of localization, hydrophobicity, and transmembrane helices We estimated the subcellular localization of the detected proteins in *Chlamydomonas* cells using the WoLF PSORT prediction tool for subcellular localization (41). The average hydrophobicity and number of transmembrane helices were predicted using SOSUI, a classification and secondary structure prediction system for membrane proteins (42).

RESULTS

Evaluation of protein extraction Effective protein extraction and solubilization methods were examined for photosynthetic model organisms including the cyanobacterium *Synechocystis* sp. PCC 6803, green algae *C. reinhardtii*, and the seed plant *A. thaliana*. Performances of two buffers (KCl and PTS buffers) and two extraction methods (beading and sonication) were compared by repeated protein extractions. Although KCl buffer was used to solubilize soluble proteins, PTS buffer was used for the solubilization of membrane proteins. Crude proteins were extracted from the model organisms, whose total protein concentrations were compared among the extraction methods employed.

In *Chlamydomonas*, total protein was extracted from approximately 3 mg of dry cell mass using micro- or zirconia beads (Φ = 0.5 and 5.0 mm, respectively) or by sonication. The total content of extracted proteins with beads in KCl buffer (Fig. 1; *Chlamydomonas*,

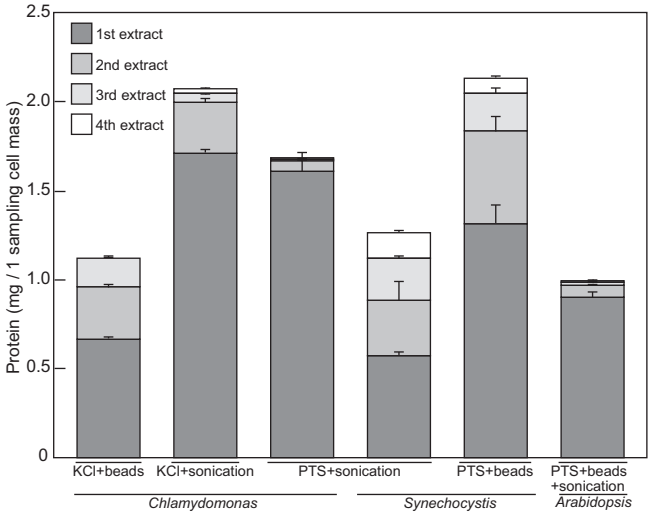

FIG. 1. Comparison of protein extraction methods for *Chlamydomonas*, *Synechocystis*, and *Arabidopsis* cells. Protein extractions from *Chlamydomonas*, *Synechocystis*, and *Arabidopsis* cells were repeated three or four times using beads and/or sonication with KCl or phase transfer surfactant (PTS) buffer. Each value represents the average ± SD of the results obtained from three independent experiments.

KCl + beads) was 1.12 ± 0.03 mg. Although the cell debris remained after three treatments, proteins were not extracted by the fourth treatment. On the other hand, the content of the extracted proteins by sonication with KCl buffer (Fig. 1; *Chlamydomonas*, KCl + sonication) was 1.71 ± 0.02 and 2.08 ± 0.05 mg after the first and all extractions, respectively. Cell residue was not observed after the fourth treatment. Thus, approximately 90% of total protein was extracted by one sonication treatment. The value of total extracted protein was approximately 60% of the cell dry weight, which was similar to those observed in previous studies (42–45). These values were not significantly different from that of proteins extracted with PTS buffer (Fig. 1; *Chlamydomonas*; PTS + sonication). These results indicated that sonication was preferred over the use of beads for protein extraction from *Chlamydomonas* cells, and that the total protein was extracted more efficiently by one sonication treatment.

In *Synechocystis*, total protein was extracted from approximately 3 mg of dry cell mass via glass beads (Φ approximately 0.1 mm) or by sonication. The total content of extracted proteins with the beads in PTS buffer (*Synechocystis*; PTS + beads) was 2.14 ± 0.08 mg (Fig. 1). Protein recovery was approximately 60% of the extracted dry cell mass, which was consistent with a previous study (46). The content of proteins at the first, second, third, and fourth extraction with the beads were 1.32 ± 0.10, 0.52 ± 0.08, 0.21 ± 0.03, and 0.09 ± 0.01 mg, respectively. Consequently, over 90% of the total protein was extracted three times using the beads. On the other hand, the content of extracted proteins by four sonication treatments (*Synechocystis*; PTS + sonication) was 1.27 ± 0.09 mg (Fig. 1). These results indicated that the beads were preferred over sonication for protein extraction from *Synechocystis* cells, and total protein was extracted efficiently after three treatments with the beads.

For *Arabidopsis*, 0.90 ± 0.04 mg of total protein was extracted from approximately 15 mg of leaves and stems with zirconia beads (Φ = 5.0 mm) and one sonication treatment (Fig. 1; *Arabidopsis*; PTS + beads + sonication). The total content of extracted proteins was 1.00 ± 0.04 mg, which was approximately 7% of the starting material. Cell debris was not observed after four treatments. Thus, approximately 90% of total protein was extracted by one treatment with beads and sonication. However, treatment with micro and large beads, or by following the method for protein extraction from *Chlamydomonas* cells, the cell debris remained after four treatments (data not shown). Additionally, *Arabidopsis* leaves and stems were barely able to disrupt by sonication (data not shown). These results demonstrated that *Arabidopsis* proteins were extracted efficiently by treating cells with large beads. The results indicated that the total protein extraction method should utilize a suitable disruption method based on the cell characteristics such as cell size and cell wall components. Furthermore, these results suggest that PTS extraction using beading and sonication may be universal protocol as a pretreatment process for protein extraction of various cell types.

Solubilization of photosystem membrane proteins The effect of cell disruption and solubilization method on the detectability of individual subunits of the photosynthetic apparatus by nano-LC-MS/MS was examined as a target of *Chlamydomonas*. Since the structures of membrane proteins in the photosynthetic apparatus are very similar, we considered proteins extracted from *Chlamydomonas* to be representative. The peptides were detected and relative quantified using nano-LC-MS/MS to evaluate the solubilization of membrane proteins including photosystem- and ATP synthase-associated proteins via the PTS method (Tables 1 and S1, and Figs. 2 and 3). Additionally, the effect of trypsin digest time was evaluated (Fig. S1). Equal amounts (50 μg) of crude proteins were extracted from *Chlamydomonas* cells with beads in KCl buffer (KCl + Beads), sonication with KCl buffer (KCl + Sonication), and sonication with PTS buffer (PTS + Sonication). The crude proteins were reduced, alkylated, and digested by trypsin. The effect of additional trypsin digestions on peptide recovery was also investigated for the PTS + Sonication method (Table 1, 1 and 2 days). The recovery of tryptic peptides was determined by a targeted proteome analysis. In this analysis, the abundance of preselected peptides were determined using the MRM mode of triple-quadrupole MS. The multiple reaction monitoring assay method employed in this study is shown in Table S3. ^{15}N labeled peptides were used as the internal standard because it is difficult to create fully labeled internal standards on ^{13}C since photosynthetic organisms fix carbon dioxide in the air. The peak of ^{14}N peptides were not detected in analysis of ^{15}N sample using nano-LC-MS/MS. Thus, we confirmed that ^{14}N had been completely replaced to ^{15}N in ^{15}N sample. Peptides were relative quantified by the peak area ratio of ^{14}N sample to ^{15}N sample.

For the KCl + beads treatment, 46 peptide signals from 26 membrane proteins (including 37 peptides from 22 chloroplast proteins, and three peptides from one mitochondrial protein) and 178 peptides from 64 soluble proteins (including 118 peptides from 41 chloroplast proteins, and 13 peptides from six mitochondrial proteins) were observed by the targeted proteome analysis using nano-LC-MS/MS (Table 1). These numbers were slightly increased by employing sonication (KCl + Sonication), yielding peptides derived from 28 membrane proteins and 65 soluble proteins. Furthermore, these numbers were increased by employing sonication with PTS buffer with trypsin digestion for 1 day (PTS + Sonication [trypsin 1 day]), since peptides derived from 32 membrane proteins and 65 soluble proteins were observed. These results were not different from the results for the 2-day treatment (PTS + Sonication [trypsin 2 days]) (Table 1). These results indicate that the efficiency of membrane protein solubilization was improved by the use of PTS buffer.

Figs. 2 and 3 show the relative intensities of signals for peptide extracted by sonication with PTS buffer compared to those extracted by sonication with KCl buffer. The relative signal intensity of peptides for the membrane and soluble proteins extracted using PTS buffer was increased by an average of 3- and 2-fold, respectively, with respect to that using KCl buffer (Fig. 2A). These results

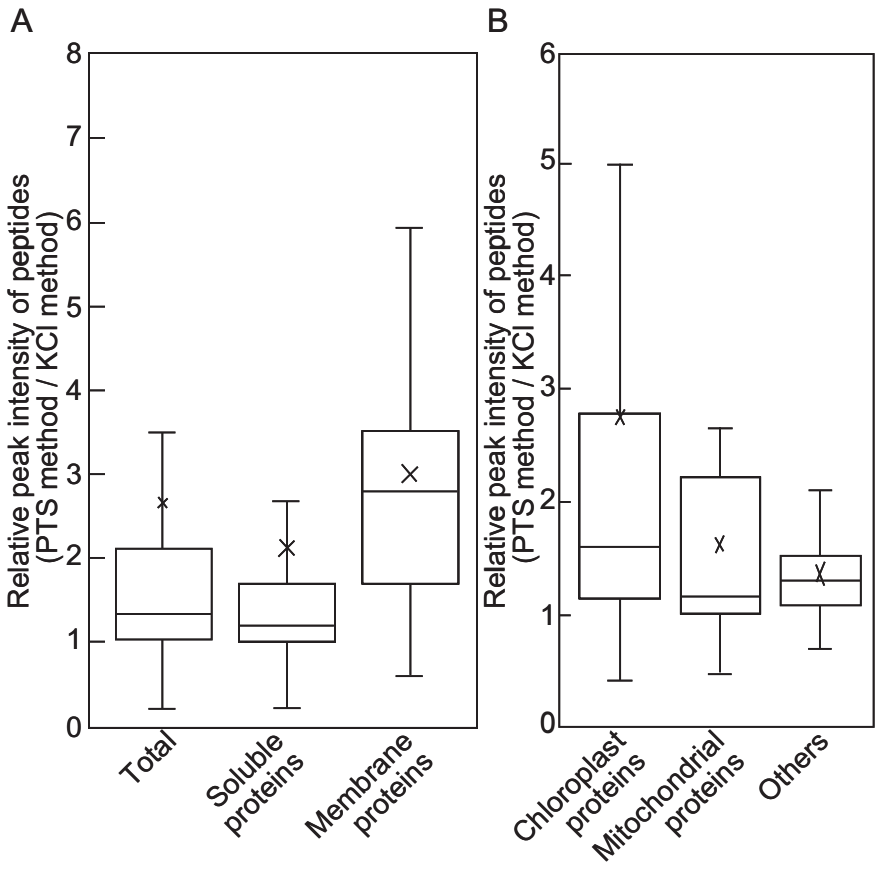

FIG. 2. Comparison of peak intensity of peptides identified using phase transfer surfactant (PTS) and KCl methods in *Chlamydomonas* cells. Peptides were extracted from *Chlamydomonas* cells as shown in Fig. 1. Peptides were identified according to each protocol, and three independent experiments were performed. Peak areas were measured using nano-LC-MS/MS, and the ratios of the peak area of peptides extracted by sonication with PTS buffer with one-day trypsin digestion to that by sonication with KCl buffer were calculated. The ratios of the peak area of peptides are shown as a box plot, in which the heavy horizontal line represents the median, the box represents interquartile range, bars represent the 95% confidence intervals, and the cross mark represents the mean. (A) Proteins including detected peptides were predicted and classified into soluble and membrane protein using the SOSUI system. (B) Subcellular localizations of proteins including the detected peptides were predicted using WoLF PSORT. Cp, chloroplast localization; Mt, mitochondrion localization; Others, chloroplast, mitochondrion and/or cytosol localization. The numbers and details of the detected peptide are shown in Tables 1 and S1, respectively.

also indicated that efficiency of solubilization and trypsin digestion were improved with PTS buffer. The relative signal intensity of peptides from chloroplast and mitochondrial proteins extracted using PTS buffer increased to an average of about 3- and 1.5-fold, respectively, with respect to that obtained using KCl buffer (Fig. 2B). For example, for the chloroplast proteins, three peptides (FGQEEETYNIVAAHGYFGR, LIFQYASFNNSR, and VLNTWADIINR) of PsbA, which has a hydrophobicity of approximately 0.285 and five transmembrane helices, were detected (Table S1). The peak area ratios ($^{14}N/^{15}N$) of these peptides were 0.15, 0.20, and 0.25, respectively, in the KCl + sonication extraction, and 1.27, 1.13, and 0.85, respectively, in the PTS + sonication extraction, indicating that the content of PsbA increased by about 6-fold using PTS buffer compared to KCl buffer (Fig. 4). Furthermore, for PsbA, the relative contents of photosystem II associated membrane proteins PsbB, PsbC, PsbD, PsbE, PsbF, PsbL, PsbN, and PsbR were increased significantly using PTS buffer (Table S1). Especially, the peptides from PsbC, PsbE and PsbF, which could not be detected from the proteins extracted using KCl buffer, were detected from proteins extracted using PTS buffer. Additionally, the peptide relative contents of the cytochrome b_6f complex, photosystem I-associated proteins, and ATP synthase subunit proteins were also increased.

Furthermore, the peptides from metabolic enzymes such as RbcLS, GAP3, FBA3, PGK1, TPIC, SEBP1, RPI1, FBP1, and PGL2 were also detected sufficiently (Table S1). For the mitochondrial proteins, the detected protein NAD5 displayed a hydrophobicity of 0.838 and 15 transmembrane helices. The peak area ratios of the three detected peptides (SPVNPIFDLGR, ITWAIGDR, and GLLSVGNLR) were 0.38, 0.37, and 0.34, respectively, with the KCl + sonication extraction, and 1.01, 0.88, and 0.84, respectively, with the PTS + sonication extraction. The NAD5 relative content increased about 3-fold using PTS buffer. For NAD5, the peptide relative contents of COB, NAD2, and COX1, which have many transmembrane regions, were increased, whereas those of the soluble proteins MDH1, MDH4, ACH1, and SCLB1 were not significantly different (Table S1). Furthermore, the peptides from COB and NAD2, which were not detected from proteins extracted using KCl buffer, were detected from proteins extracted using PTS buffer. For other proteins such as soluble protein from the cytoplasm, the peptide relative contents were not significantly different (Table S1). The peptide relative contents from the proteins digested for one day were not significantly different from those digested for 2 days (Figs. 2, 3, and S1, and Table S1). These results indicate that the PTS method improved the solubilization efficiency of membrane proteins from the chloroplast and mitochondrion.

Additionally, we detected *Synechocystis* sp. PCC 6803 proteins solubilized by PTS method (Tables S2 and S4). 114 proteins of photosynthetic apparatus and respiration were identified, and included chlorophyll metabolic enzymes, subunits of NADH dehydrogenase (NDHs), and respiration chain complexes. In the

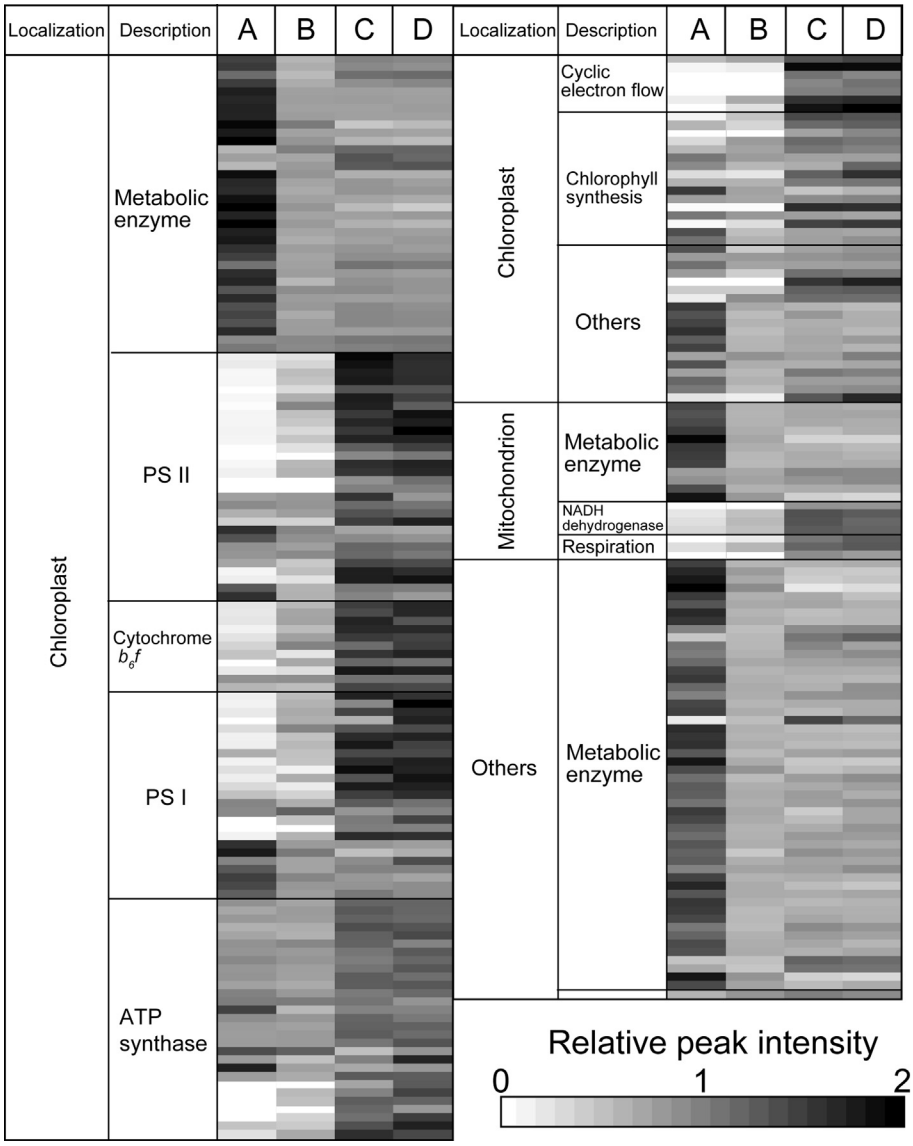

FIG. 3. Relative peak intensity of peptides identified using phase transfer surfactant (PTS) and KCl methods in *Chlamydomonas* cells. The relative peak intensities were normalized as the average of the peak intensities of individual peptides in each experiment as 1.0. (A) Extraction via beads with KCl buffer. (B) Extraction via sonication with KCl buffer. (C) Extraction via sonication with PTS buffer after a trypsin digestion for 1 day. (D) Extraction via sonication with PTS buffer after a trypsin digestion for 2 days.

previous method using KCl for solubilization, 59 of 114 proteins were not detected (47). Proteins with an average of hydrophobicity exceeding 0.4 were only marginally detected using the KCl method, but those proteins were detected using the PTS method (Table S4). Twenty PSII-associated proteins were detected and three proteins (PsbF, PsbI, and PsbZ) were not detected, excluding small proteins that were not digested by trypsin. All PSI and antenna proteins were detected, excluding PsaI and PsaK that were not digested by trypsin. Furthermore, subunits of huge membrane protein complexes, including NDH and respiration complexes, were detected. In

TABLE 1. Number of detected peptides and proteins extracted from *Chlamydomonas* cells by the various method.

Localization	Property	Number of detected peptides/Number of detected proteins			
		KCl+beads	KCl+sonication	PTS+sonication (trypsin 1 day)	PTS+sonication (trypsin 2 days)
Cp	Soluble	118 peptides/41 proteins	122/42	129/42	129/42
	Membrane	37/22	41/23	44/25	44/25
Mt	Soluble	13/6	13/6	13/6	13/6
	Membrane	3/1	4/2	6/4	6/4
Others	Soluble	47/17	47/17	47/17	47/17
	Membrane	6/3	6/3	6/3	6/3
Total	Soluble	178/64	182/65	189/65	189/65
	Membrane	46/26	51/28	56/32	56/32

The soluble and membrane proteins were predicted using SOSUI system. Subcellular localization of proteins was predicted using WoLF PSORT. Cp, chloroplast localization; Mt, mitochondrion localization; Others, chloroplast, mitochondrion and/or cytosol localization.

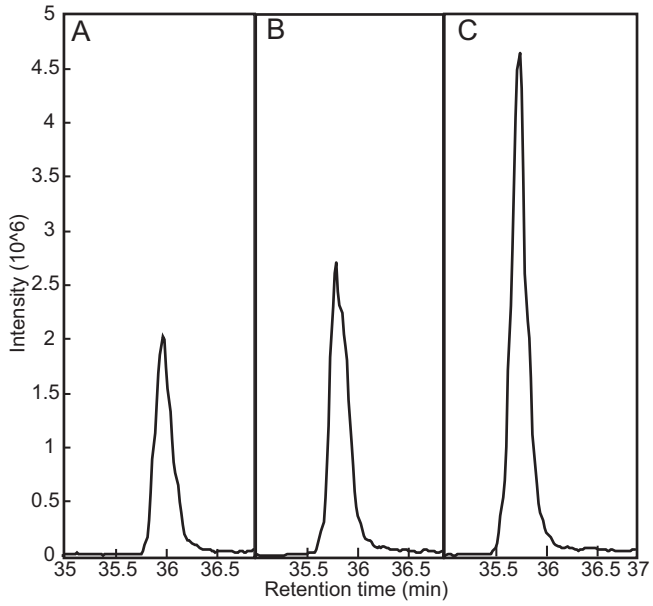

FIG. 4. Selected reaction monitoring spectra of peptide VLNTWADIINR (PsbA) identified using phase transfer surfactant (PTS) and KCl methods in *Chlamydomonas* cells. Peptides were extracted from *Chlamydomonas* cells as shown in Fig. 1. Peak areas were measured using the nano-LC-MS/MS. (A) Extraction via beads with KCl buffer. (B) Extraction via sonication with KCl buffer. (C) Extraction via sonication with PTS buffer.

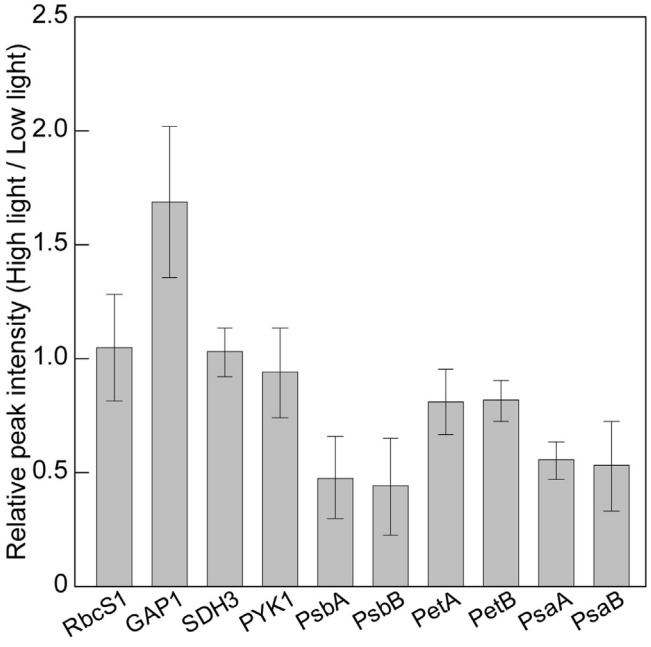

FIG. 5. Relative change in protein content in *Chlamydomonas* cells grown under high-light conditions. Proteins were extracted according to the phase transfer surfactant (PTS) method via sonication after a one-day trypsin digestion. Peak areas were measured using the nano-LC-MS/MS, and the ratios of the peak area with the high-light condition to that with the low-light condition were calculated (High light/Low light). The value of each protein is shown as the average of the detected peptides. Each value represents the average ± SD of the results obtained from three independent experiments.

particular, subunits of NDH-1 (NdhA/B/D/E/F/G) with high hydrophobicity (>0.6) and many transmembrane helices (3–18 helices) were detected. Homologs of subunits of NDH-1, including NdhD1/2/3/4/5/6 and NdhF1/3/4, were also identified. These results indicated that the PTS method also improved the solubilization efficiency of membrane proteins in *Synechocystis* sp. PCC 6803.

Relative quantitative changes of photosystem proteins from *Chlamydomonas* and *Synechocystis* sp. PCC 6803 cells grown under high-light condition Changes in the relative content of proteins from *Chlamydomonas* cells grown under the high-light condition were measured by nano-LC-MS/MS analysis after the sonication treatment and PTS method to assess the usability of relative quantitative proteomics analysis for photosynthetic organisms. The contents of chloroplast proteins including PsbA, PsbB, PetA, PetB, PsaA, and PsaB, and metabolite enzymes including RbcS1, GAP1, SDH3, and PYK1 were quantified relatively by averaging the values of multiple peptides constituting those proteins (Fig. 5 and Table S5). These chloroplast proteins extracted by sonication with PTS buffer were detected better than when KCl buffer was used (Table S1). The contents of these chloroplast proteins were thought to change in response to the light condition. Under the high-light condition, the relative contents of the photosystem-associated proteins (PsbA, PsbB, PetA, PetB, PsaA, and PsaB) decreased, whereas those of metabolic enzymes (RbcS1, GAP1, SDH3, and PYK1) were not significantly different (Fig. 5 and Table S5). This was consistent with results from a previous study (48), in which the content of D1 protein (PsbA) in *Chlamydomonas* cells grown under a high-light condition was decreased. These results suggest that the relative quantitative proteomics protocol using the PTS method and nano-LC-MS/MS analysis is reliable.

Additionally, changes in the relative content of proteins even in *Synechocystis* sp. PCC 6803 cells grown under the high-light condition were measured (Fig. 6). Like in *Chlamydomonas*, the relative contents of subunit of photosynthetic apparatus decreased in *Synechocystis* sp. PCC 6803 under the high-light condition, whereas those of metabolic enzymes were not significantly different. Furthermore, the relative contents of subunits of ATP synthase (Atp1, AtpA, AtpC, AtpD, AtpE, AtpF and AtpG) and NDH-1 (NdhA, NdhD, NdhE, NdhG, NdhH, NdhI, NdhJ, NdhK, NdhM, NdhN and NdhO) increased significantly. NdhD has multiple homologs in the genome of *Synechocystis*. One of those (NdhD1) was greatly increased under the high-light condition. Similarly, NdhF1, one of multiple homologs of NdhF, increased more than the other NdhF homologs under the high-light condition.

DISCUSSION

Suitable cell disruption method for each photosynthetic organism In this study, we examined the various protocols of cell disruption for efficient total protein extraction (Fig. 1). For the disruption of *Chlamydomonas*, *Synechocystis*, and *Arabidopsis* cells, the optimal method was the use of sonication, microbeads, and large beads, respectively. *Chlamydomonas* cells were disrupted by sonication more efficiently compared with the use of beads, which was consistent with the results in *Chlorella* (25). On the other hand, *Synechocystis* cells were disrupted using microbeads more efficiently compared with sonication. *Chlamydomonas* cells may be weak against pressure and strong against physical forces because of their cell wall. In contrast, *Synechocystis* cells may be weak against physical forces and strong against pressure because of their cyanobacterial extracellular polysaccharide sheath. More than 90% of the proteins in the cells were extracted using each cell disruption method (Fig. 1). These results suggest that the total protein extraction method should utilize a suitable disruption method based on the cell characteristics such as cell size and cell wall components.

Solubilization of membrane proteins for unbiased quantitative proteome The *Chlamydomonas* cell disruption methods affected the comprehensiveness of proteins detected by

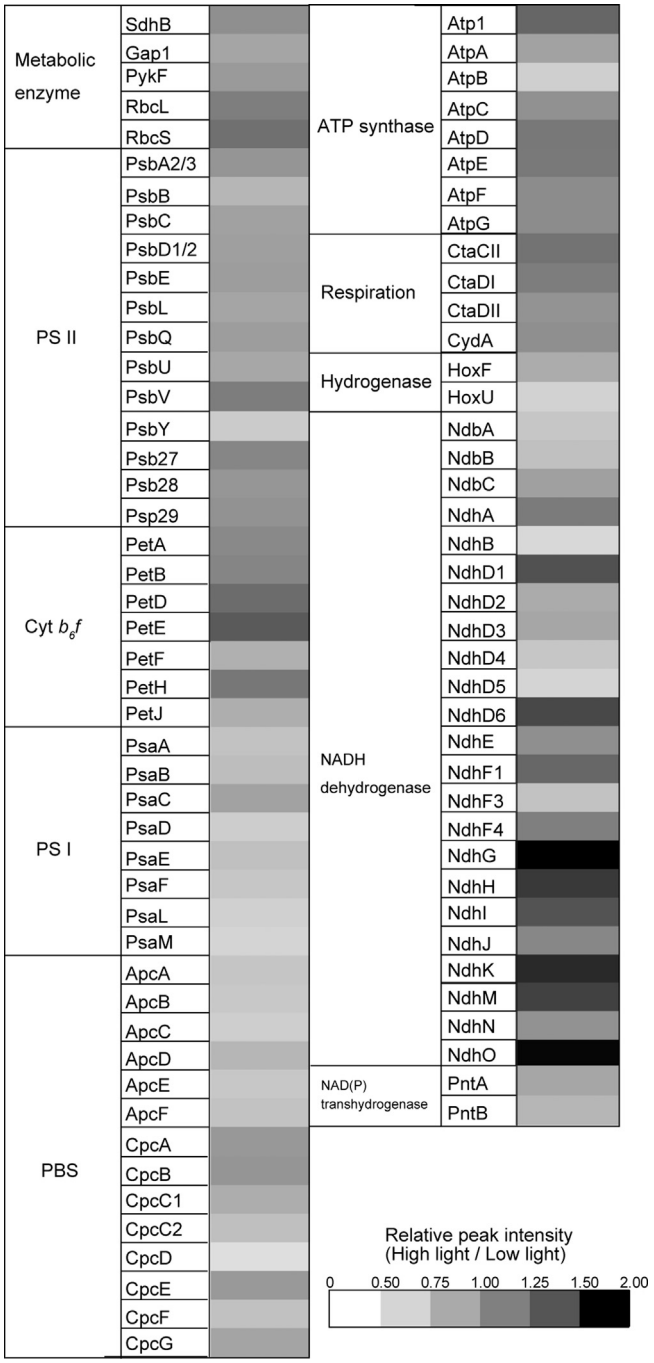

FIG. 6. Relative change in protein content in *Synechocystis* sp. PCC 6803 cells grown under high-light conditions. Proteins were extracted according to the phase transfer surfactant (PTS) method via sonication after a one-day trypsin digestion. Peak areas were measured using the nano-LC-MS/MS, and the ratios of the peak area with the high-light condition to that with the low-light condition were calculated (high light/low light). The value of each protein is shown as the average of the detected peptides. Each value obtained from three independent experiments.

nano-LC-MS/MS (Tables 1 and S1). Compared with the extraction using the beads, the number of detected peptides from chloroplast proteins extracted by sonication was increased. This result might indicate that the chloroplast envelope membrane was disrupted completely by sonication. In fact, *Chlamydomonas* cells were not observed in the suspension of cells disrupted by sonication, whereas the cells were observed in the suspension after the bead treatments (data not shown). If major proteins aggregated by sonication and their yields were decreased, the huge peaks corresponding to major proteins in nano-LC-MS/MS might be weakened, resulting in the appearance of many new peaks. The possibility of protein aggregation was negated by the correlation between the abundance of mRNAs and identified proteins in *E. coli* in a previous study (21). Although many proteins often remain aggregated in spite of an attempt to re-solubilize with surfactants, their careful lysis procedures and surfactant treatments can significantly increase the recovery yield. This possibility was likely low in our study because sonication was performed on ice and denatured protein was released by surfactant. Additionally, proteins tend to aggregate as the molecular mass of proteins increases (49) although we did not observe a negative correlation between the molecular mass and the peak intensity of proteins. On the other hand, solubilization of membrane proteins, such as photosystem-associated proteins, was also important for unbiased relative quantitation of the photosynthetic organism proteome. Our results showed that the detectability of individual subunits of the photosynthetic apparatus and other large protein complexes was increased by the PTS method (Table 1 and S1, Figs. 2 and 3). These results indicated that the PTS method facilitated the peptidization of the photosynthetic apparatus proteins by trypsin treatment. Thus, the PTS method is useful for unbiased relative quantitative proteomics of photosynthetic organisms using nano-LC-MS/MS.

In our results, various characteristic proteins with different hydrophobicities and different numbers of transmembrane helices were detected (Table S1). The maximum hydrophobicity and number of transmembrane helices for NAD2 was 1.07 and 15, respectively. This is equal to the detectability reported in a previous study, in which the membrane proteins were solubilized with SDS and separated by SDS-PAGE, and then analyzed by reversed phase chromatography and LC-MS/MS (23). Additionally, compared with the KCl method, the detectability of membrane and soluble proteins was increased with the PTS method (Fig. 2A). These results indicate that PTS buffer improved not only the solubilization efficiency of membrane proteins but also the trypsin digestion efficiency of soluble proteins, consistent with the results of a previous study (21). Also, the detectability of membrane proteins in chloroplast and mitochondria of *Chlamydomonas* increased by cell disruption using sonication with the PTS method (Fig. 2). These results indicated that the cell wall and membranes of organelles of *Chlamydomonas* cells were efficiently disrupted by sonication, and that membrane proteins were solubilized by the PTS method. Additionally, solubilization by PTS method enabled the pronounced detection of membrane proteins, even in *Synechocystis* sp. PCC 6803, compared with a previous study (47) (Table S4). Especially, subunits of huge complexes, such as NDH-1 and cytochrome oxygenase, were detected by the PTS method (Table S4). Thus, the optimal cell disruption method and PTS method allow for comprehensive relative quantitative proteome analysis of photosynthetic organisms.

Although degradation of D1 protein (PsbA) under high-light is established (48,50–54), quantitative analysis of changes of subunit of other photosynthetic apparatus complex were not clarified. Our results of proteome analysis of *Chlamydomonas* and *Synechocystis* showed that other photosynthetic apparatus decreased along with D1 protein, whereas proteins related to intracellular metabolism were not significantly different (Figs. 5 and 6). These results indicate that energy transfer is limited by decrease of the D1 protein but also the other components of the photosynthetic apparatus under the high-light condition.

To investigate the relationship between different layers of omics data, the relative protein abundance levels of *Synechocystis* sp. PCC 6803 were compared with the gene-expression data obtained by the microarray analysis reported in a previous study (55).

Correlation coefficient value of all plots was 0.31 ($p = 0.003$; Fig. S2) and weak similarities between abundance changes of proteins and mRNAs were observed. The abundance of proteins obtained in this study was quantitatively measured. Protein expressions of subunits of NDH-1 (NdhF1, NdhD1, NdhD6, NdhO, and NdhG) increased, whereas mRNAs were not significantly different. It could suggest that NDH-1 proteins synthesis is regulated at the post-transcriptional level. In *Arabidopsis*, multiple RNA editing sites were found in NDH-1 subunits, and post-transcriptional regulation of NDH-1 subunits has been described (56).

NdhD and NdhF have some homologs in the genome of *Synechocystis* sp. PCC 6803 (57). The cyanobacterial NDH-1 complex has different functions depending on the combinations of the NdhD and NdhF subunits constituting the complex (57,58). For example, the NDH-1L complex containing NdhD1 and NdhF1 was demonstrated to function as a component of cyclic electron transport by extensive reverse genetics and functional proteomic studies (57–64). Presently, protein expression of NdhF1 and NdhD1 under high-light conditions was significantly increased, whereas the difference of mRNA expression of homologs of NdhD and NdhF was not significant (Figs. 6 and S2). This observation of protein layer supports the previous finding of reverse genetics that the NDH-1L complex selectively acts under the high-light condition.

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