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Chromatographic separation of a small subunit (PsbW/PsaY) and its assignment to Photosystem I reaction center

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Abstract

By using a hydroxyapatite column, the five major Photosystem I (PSI) subunits (PsaA,-B,-C,-D,-E) solubilized by sodium dodecyl sulfate (SDS) were fractionated from a spinach PSI reaction center preparation. Another small (5–6 kDa) polypeptide was also separated, and purified to homogeneity. Mass spectroscopy yielded its molecular weight to be 5942 ± 10. This polypeptide had an N-terminal sequence homologous to those of previously reported 5-kDa subunits from spinach and wheat and a 6.1-kDa subunit of *Chlamydomonas*, which had all been assigned to Photosystem II (PSII) and designated as PsbW. However, we found similar 5-kDa polypeptides with highly conserved N-terminal sequences ubiquitously in PSI particles from other plants including Daikon (*Raphanus sativus,* Japanese radish), Chingensai (*Brassica parachinensis*, Chinese cabbage), parsley and Shungiku (*Chrysanthemum coronarium*, Garland chrysanthemum) as well. Preparations of spinach PSI particles prepared by using a mild detergent (digitonin) had this 5-kDa subunit, while PSII particles did not. Moreover, a bare-bone PSI reaction center preparation consisting of PsaA/B alone had a more than stoichiometric amount of this 5-kDa polypeptide. A mechanically (without detergent) fractionated stroma thylakoid preparation from *Phytolacca americana*, which lacked other PSII subunits, also contained this 5-kDa subunit. Thus, we propose that this 5-kDa polypeptide, previously designated as a PSII subunit (PsbW), is an integral subunit of PSI as well.

Keywords: Photosynthesis; Photosystem I; Subunit; Reaction center; Hydroxyapatite; Chromatography

1. Introduction

Since Bengis and Nelson first reported reaction center particles [1], numerous Photosystem I (PSI) preparations have been described. The advent of recombinant DNA technique and N-terminal amino acid sequencing has introduced as many as 17 polypeptides that were assigned to PSI [2]. The separation of these subunits was done almost exclusively by utilizing the supreme resolution of sodium dodecyl

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PSI, Photosystem I; PSII, Photosystem II; PVDF, polyvinylidenedifluoride; MALDI-TOF/MS, matrix assisted laser desorption/ionization time-of-flight mass spectroscopy; CBB, Coomassie brilliant blue

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sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For some years, the present authors have been aware of the ubiquitous presence of a weak band on SDS-PAGE of various PSI reaction center preparations. This small (5–6 kDa) subunit seemed to be tightly bound as it was present even in a bare-bone reaction center preparation with the two large subunits (PsaA and PsaB) alone (S. Kobayashi and T. Hiyama, unpublished observations). As the N-terminal amino acid sequence did not match any of PSI subunits reported by then, this small polypeptide was tentatively designated as PsaY [2].

In the mean time, it became clear that Photosystem II (PSII) preparations from higher plant chloroplasts are often accompanied by a small (5–6 kDa) polypeptide [3–7]. The genes for these polypeptides (*psbW*) were cloned and sequenced from spinach [8] and from *Arabidopsis thaliana* [9]. Later, we realized that the N-terminal amino acid sequences of these PsbW were almost identical with that of our PsaY.

In the present paper, we first show that a hydroxyapatite column chromatography system can separate PSI reaction center subunits including PsbW (PsaY). Then, we show that PsbW (PsaY) is present in PSI particles prepared from several different plants, in a spinach PSI particle prepared by using a mild detergent (digitonin) and also in a stroma thylakoid preparation from *Phytolacca americana* by using a non-detergent method. Furthermore, PSI reaction center preparations with a minimal number of subunits still retain substantial amounts of PsbW (PsaY). Thus, we propose that PsbW is more likely to be associated with PSI rather than PSII.

2. Materials and methods

2.1. Plant materials

Parsley, Chingensai (Pak-choi, *Brassica parachinensis*), and Shungiku (Garland chrysanthemum, *Chrysanthemum coronarium*) were purchased at a local market. Daikon (Japanese radish, *Raphanus sativus*) and spinach leaves were collected from farm-cultivated plants in Ibaraki. Wheat (Norin 61 strain) was cultivated from seeds on a vermiculite bed in the laboratory under fluorescent lamps at room temperatures. When leaves were about 10 cm, they were picked for the subsequent preparation. Leaves of *P. americana* were collected from the plants grown wild in the Urawa area from June to July.

2.2. Reaction center preparations

Spinach reaction center preparations were prepared basically according to [10]. Broken chloroplasts prepared from spinach were suspended in 50 mM sodium phosphate buffer, pH 7.0 and 10 mM NaCl. One volume of the suspension was mixed with two volumes of preheated (45°C) medium containing 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 8.8 (at 20°C) and 3% Triton X-100 (final chlorophyll concentration, 2 mg/ml) and incubated for 30 min at 45°C. The suspension was then chilled in an ice bath and centrifuged at $12\,000\times g$ for 30 min to remove debris. The supernatant (ca. 25 ml) was loaded on a DEAE-Toyopearl 650S column (3.2×10 cm) equilibrated with 10 mM Tris–HCl, pH 8.8 and 0.2% Triton X-100. The column was washed by 1000 ml of the equilibration medium supplemented with 10 mM NaCl, then by 300 ml of the equilibration medium supplemented with 50 mM NaCl, and finally eluted by 400 ml of the equilibration medium supplemented with 50–200 mM (linear gradient) NaCl. Fractions enriched with P700 activity, which was measured by using flash spectroscopy [11–13], were collected and diluted with equal volume of 10 mM Tris–HCl, pH 7.5 and 0.6 mM $CaCl_2$, and then loaded on a column (1.6×2.0 cm) of hydroxyapatite (Macro-Prep Hydroxyapatite Type II 40 μm, Bio-Rad, manufactured by Asahi Optical Co. (Pentax)Ltd., Tokyo, Japan) equilibrated with 10 mM Tris–HCl, pH 8.8, 0.3 mM $CaCl_2$ and 0.05% Triton X-100. The column was washed with 20 ml of the above medium, then eluted with a small volume (1–2 ml) of 50 mM sodium phosphate, pH 8.0 and 0.05% Triton X-100 to obtain the final PSI preparation. For storage at −80°C, the preparation was supplemented with 0.4 M sucrose before freezing. This preparation corresponds to 'psI_b' in [10]. For other plants (parsley, Chingensai, Shungiku, Daikon and wheat), the method for preparing 'psI_a' was employed, in which the heat treatment described above (45°C, 30 min) was omitted [10].

2.3. Removal of the small subunits and preparation of PsaC

The psI_b preparation (protein concentration, 4 mg/ml) was incubated in the urea medium (7.5 M urea, 10 mM Tris–HCl, pH 7.6, 0.1% Triton X-100 and 0.1% 2-mercaptoethanol) for 1 h at 4°C, then filtered through by using an ultra filtration membrane filter (50 000 cut, UK-50, Advantec, Tokyo). A small volume (less than 100 μl) of the concentrate was diluted with the medium, and the procedure was repeated once more. In another experiment, urea was replaced with 2 M NaI. Sucrose (0.4 M) was added before the storage of the concentrate at −80°C. These preparations correspond to 'cpI_a' and 'cpI_b' described in [10], respectively.

As for the preparation of PsaC, the filtrate obtained from the ultra filtration of urea-treated PSI particles stated above was passed through the hydroxyapatite column (1.6×2.0 cm) equilibrated with the urea medium, and washed with 10 ml of this medium. The passed-through fractions were combined with the washed-out fractions and concentrated by using an ultra filtration membrane filter (Molcut UFP1, Millipore). The resulted concentrate was a purified preparation of PsaC (the insert on the right in Fig. 1). The present method is much simpler and less time-consuming than our previous method for PsaC preparation [14].

2.4. Chromatographic separation of the small subunits

The hydroxyapatite column (1.6×2.0 cm) was equilibrated with 10 mM Na-phosphate, pH 6.4, 0.1% 2-mercaptoethanol and 0.1% SDS (SDS-medium). The PSI preparation was suspended in 2.5% SDS and 5% 2-mercaptoethanol and kept for 1 h at 37°C. The column was then loaded with this suspension, washed with 10 ml of the SDS-medium and then eluted with a linear gradient (0.01–0.6 M) of NaCl containing Na-phosphate, pH 6.4, 0.1% SDS and 0.1% 2-mercaptoethanol. Fractions (3 ml each) were collected, monitored for protein concentration by using absorbance at 280 nm, and subjected to

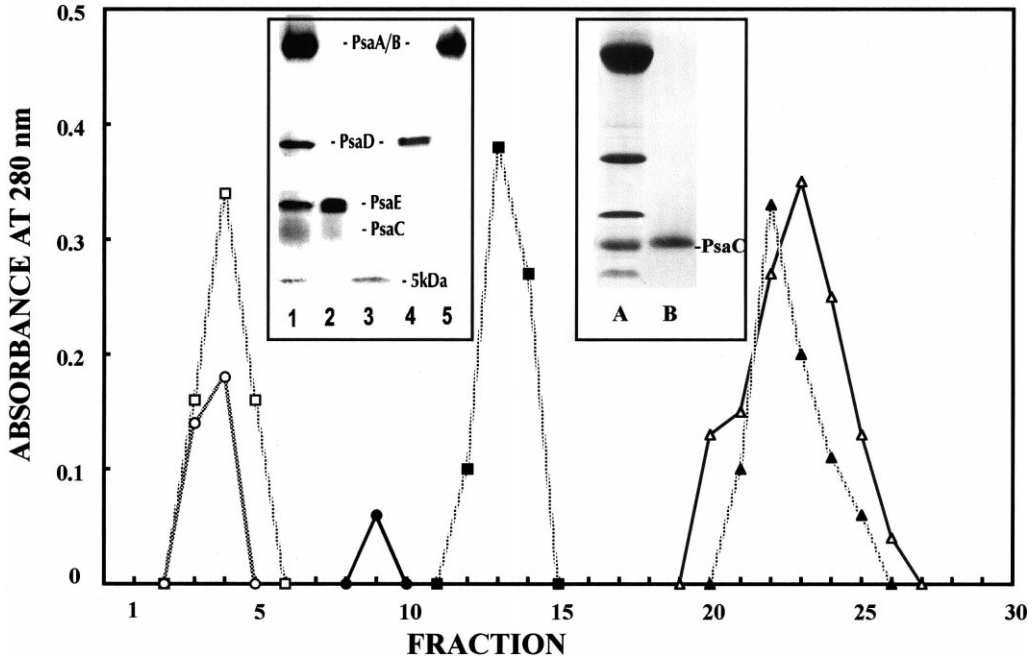

Fig. 1. An elution pattern of hydroxyapatite column chromatography and SDS-PAGE of its representative fractions. The insert on the left: lane 1, PSI particles before chromatography; lane 2, fraction 4; lane 3, fraction 9; lane 4, fraction 13; lane 5, fraction 22. The insert on the right shows SDS-PAGE of eluted fractions from a urea-containing hydroxyapatite column described in the text; lane A, PSI particles; Lane B, the fraction that run through and was not retained on the column (see the text). White squares represent the densities, as measured by a densitometer, of PsaE bands; white circles, PsaC; black circles, the 5-kDa protein; black squares, PsaD; white triangles, PsaA; black triangles, PsaB.

SDS-PAGE (Fig. 1). Most bands were identified by N-terminal amino acid sequencing. Since both PsaA and PsaB could not be sequenced due to their N-terminal blockage, these subunits were identified by migratory difference in an SDS-PAGE system where PsaB moved slightly faster [18]. More positive identifications were performed immunochemically by using specific antisera. Specific antisera for this purpose were prepared from rabbits immunized by peptides that had been synthesized according to specific inner sequences selected from those of PsaA (5–19: SPEPEVKILVDRDPV) and PsaB (234–248: AQNPDSSSHLFGTSQ). Before the injection, these synthetic peptides were crosslinked by using glutaraldehyde to rabbit serum proteins that had been extracted from the blood of those particular animals.

2.5. Digitonin-treated Photosystem I particles

Digitonin treated PSI particles were prepared basically according to [15] and [16]. Broken chloroplasts were suspended in 0.4 M sucrose, 50 mM Na,K-phosphate buffer, pH 7.2 and 10 mM NaCl to make up the chlorophyll (a+b) concentration approximately 1 mg/ml. Stock solution of digitonin (10%) dissolved in warm ethanol was diluted to 2% with the above medium and then one volume of the diluted digitonin was poured drop by drop into the chloroplast suspension under constant agitation. The mixture was then chilled and kept stirred under dim light for 1 h in a cold room, and centrifuged at $1000\times g$ for 15 min. The supernatant was centrifuged again at $10\,000\times g$ for 30 min. The resultant precipitate was resuspended in 0.4 M sucrose and 50 mM Na,K-phosphate buffer, pH 7.2 (D-10 particles). The supernatant was centrifuged at $150\,000\times g$ for 1 h, and the resultant pellet was resuspended in the sucrose-containing buffer stated above (D-144 particles).

2.6. Broken thylakoid fractions from P. americana

Stroma and grana thylakoid preparations were prepared from *P. americana* according to [17]. Four or five young leaves, after the ribs were removed, were tightly rolled to form a cigar-shaped bundle, wetted with a minimal volume of ice-cold 0.02 M sodium phosphate, pH 7.0, and pushed onto the rapidly rotating grinding disc of a fruit-juicer and ground down one at a time in a manner of grinding a slice of fruit. The resulted thick green juice was centrifuged for 15 min at $1000\times g$ to remove debris. The supernatant was centrifuged at $20\,000\times g$ for 1 h. The resultant supernatant was centrifuged at $100\,000\times g$ for 45 min, and the pellet was resuspended in the phosphate buffer containing 0.4 M sucrose and stored at −80°C. This was the stroma thylakoid fraction [17]. The grana thylakoid fraction was prepared from the precipitate from the $20\,000\times g$ centrifugation described above. This precipitate was resuspended in the phosphate buffer and then centrifuged again under the same conditions. This time, the resulted precipitate consisted of a tightly packed pellet and a fluffy loose layer on top of it. This top soft layer was carefully collected and suspended in the phosphate buffer containing 0.4 M sucrose for freezer storage. This was a grana thylakoid fraction [17].

2.7. Electrophoresis

SDS-PAGE was performed basically according to [3]. The running gel (T = 16–22% gradient; C = 1.6%) contained 7.5 M urea, 0–0.2 M sucrose gradient, and 0.62 M Tris–HCl, pH 8.4; the concentrating gel (T = 5%; C = 2.6%) contained 7.5 M urea, and 0.125 M Tris–HCl, pH 6.8. The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS. For the separation of PsaA and PsaB, the concentrating gel (T = 5%; C = 2.6%) contained 0.125 M Tris–HCl, pH 6.8, 0.1% SDS and 7.5 M urea; the running gel (T = 10%; C = 2.6%) contained 0.4 M Tris-HCl, pH 8.8, 0.1% SDS and 7.5 M urea, according to [18]. Gels were stained with Coomassie brilliant Blue (CBB) R-250.

2.8. Sequencing

N-terminal amino acid sequencing was performed by using a gas-liquid phase sequencer (PSQ-2, Shimadzu, Kyoto, Japan). Samples were spotted and dried on pieces of polyvinylidenedifluoride (PVDF) membrane (Immobilon P^{sq}, Millipore, USA). For the identification of SDS-PAGE bands, the bands on the

electroblotted membrane (Immobilon P^{sq}), after visualized by amido Black staining, were cut out and subjected to sequencing.

2.9. Mass spectroscopy

Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) was performed by using a mass spectrometer (MALDI IV tDE, Kratos/Shimadzu, Manchester, UK and Kyoto, Japan) with 3,5-dimethoxy-4-hydroxycinnamic acid as matrix.

3. Results and discussion

3.1. Chromatographic separation of PSI subunits

As shown in Fig. 1, the hydroxyapatite column chromatography employed here yielded those four fractions which contained PsaE and PsaC, PsaD, PsaA and PsaB, and a 5-kDa polypeptide. The 5-kDa polypeptide was well separated from other subunits; thus, a purified preparation could readily be prepared in quantity. As described in Section 2, PsaC free from other subunits could be purified from urea-treated PSI particles simply by passing through another hydroxyapatite column (Fig. 1, the insert on the right). PsaA and PsaB were only partially resolved. A well-resolved separation of PsaA from PsaB was achieved by a second column chromatography (data not shown).

3.2. N-terminal sequence of the 5-kDa polypeptide

We were able to determine the N-terminal sequence of the 5-kDa polypeptide up to 44 residues for the spinach preparation. This sequence (LVDERMSTEGTGLPFGLSNNLLGWILFGVFGLIWALYFVYASXL) was found to be highly homologous with those reported previously for polypeptides from spinach and wheat [5,6,19] and *Chlamydomonas* [4], as well as those translated from DNA sequences of *Arabidopsis* [9] and spinach [8]. It should be noted that all of these polypeptides have been assigned to PSII and recently designated as PsbW [8]. These were assigned so because of their frequent presence in PSII particles. No report, however, has mentioned the absence of these peptides in PSI particles. Thus, we first looked into PSI particles from other plants than spinach. We found that these PSI particles did contain more than significant amounts of highly homologous polypeptides of similar sizes. These results are summarized in Fig. 2 together with those sequences previously reported and cited above.

3.3. Molecular mass of the 5-kDa polypeptide

The molecular weight of the spinach 5-kDa poly-

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                        1         10        20        30        40        50        60        70
Arabidopsis*            MASFTASAST VSAARPALLL KPTVAISA-P VLGLPPMGKK -KGGVRCSME TKQGNVSVMG AGVS--AAAT
Spinach**               MATITASSSA SLVARASLVH NSRVGVSSSP ILGLPSMTKR SKVTCSIENK PSTTETTTTT NKSMGASLLA

                        71        80        90        100       110       120       130
Arabidopsis*            AALTAVMSNP AMALVDERMS TEGTGLPFGL SNNLLGWILF GVFGLIWTFF FVYTSSLEED EESGLSL//
Spinach**               AAAAATISNP AMALVDERMS TEGTGLPFGL SNNLLGWILF GVFGLIWALY FVYASGLEED EESGLSL//
Spinach                           LVDERMS TEGTGLPFGL SNNLLGWILF GVFGLIWALY FVYASXL...
Wheat                             LVDERMS TEGTGLSLGL SNN...
Chlamydomonas***                  LVDERMN GDGTGRPFGV NDPVLGWVLL GVFGTMWAIW FIGQKDLGDF EDADDGLKL//
Daikon radish                     LVDDRMS TEGTGLPFGL SNNLLGXI...
Chingensai                        LVDDRMS TEGTGLPFGL SNNLL...
Parsley                           LVDERLS TEGTGLPFGL SNN...
Chrysanthemum                     LVDERMS TEG...
Phytolacca americana              LVDERM...
                                  1         10        20        30        40        50
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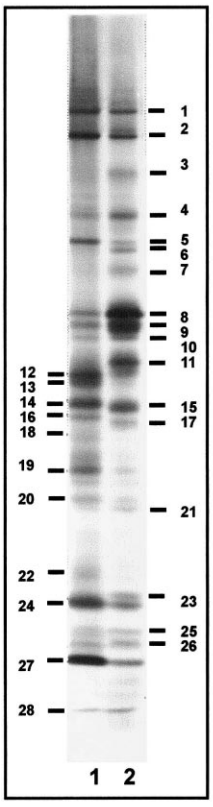
Fig. 2. Summary of amino acid sequences of PsbW (PsaY). *Translated from the gene in [9]; **translated from the gene in [8]; ***from the NIH BLAST (accession number, AF170026). The other sequences are all N-terminal sequences of the proteins determined in the present study. See text for details.



Band	Molecular Mass *kDa*	N-Terminal Sequence	Identification
1	57-56.5	VTIDADEISN...	AtpA+PsaA*+PsaB*
2	52	VSTLEKANLGRIAQGPVLD...	AtpB
3	44.5	SLPWYRVLT...	PsbC
4	38.5	YPIFAQQGY...	PetA
5	35	XLRELRERIGSVXNTQXITX...	AtpC
6	34.5	---**	
7	33	---**	
8	27.5	---**	
9	26	---**	
10	25	---**	
11	23.5	SNQLMYGPDRXKYL...	Lhb3
12	22	SADWMPGQPR...	Lha1
13	21.5	SAEWMPGQPRPPYLDGSADG...	Lha1
14	19.5	KKGEWLPGPLA...	Lha4
15	19	---**	
16	18.5	KKGEWLPGPLA...	Lha4
17	18	---**	
18	16.5	GFTPPELDPTTPSPIFAGXTT...	PsaD
19	14.3	DISGLAPLFD...	PsaF
20	13.5	AEVLLGSDSGLLAFXPGEFSVAAGE...	PetE
21	13	---**	
22	10	IXGDISVYFD...	PsaH
23	9.5	---**	
24	9	IPPPKGPIRG...	PsaE
		SHSVKIYDTX...	PsaC
		ELNPIIVISG...	PsaG
25	8	SGSTGERSFA...	PsbE
26	7.5	DFIGSSTNLIMVT...	PsaK
27	6.8	MNALIQV...	Unidentified***
28	5	LVDER...	PsbW/PsaY

Fig. 3. SDS-PAGE of mechanically prepared stroma (lane 1) and grana (lane 2) thylakoid fractions from *P. americana*. Identifications of the bands are summarized on the right. AtpA, CF_1ATPase α-subunit; PsaA, PSI subunit I_a; PsaB, PSI subunit I_b; AtpB, CF_1ATPase β-subunit; PsbC, CP43 of PSII; PetA, cytochrome f; AtpC, CF_1ATPase γ-subunit; Lhb3, LHCII Type III; Lha1, LHCI Type I; Lha4, LHCI Type IV; PsaD, PSI subunit II; PsaF, PSI subunit III; PetE, plastocyanin; PsaH, PSI subunit VI; PsaE, PSI subunit IV; PsaC, PSI subunit VII; PsaG; PSI subunit V; PsbE, Cytochrome b_{559}; PsaK, PSI subunit VIII; PsbW, 5-kDa subunit (or PsaY according to [2] and the present work). *The N-terminal was blocked and identified immunochemically; **N-terminal blocked; ***No homologous sequence has been found in the databases (DDBJ and BLAST) so far.

peptide (PsbW) was estimated from a putative sequence derived from the reported nucleotide sequence of the *psbW* gene [8] and the presently determined N-terminal, to be 5928. Then, the 5-kDa polypeptide purified as above was analyzed by using MALDI-TOF/MS, which yielded a single peak with the molecular weight of 5942 ± 20. These results confirmed that the *psbW* gene encodes the present 5-kDa polypeptide, and that the C-terminal sequence is most likely '...LSL;' as predicted from the sequence of the gene. The present result is also consistent with a previous mass spectroscopy measurement on PsbW from spinach [7].

3.4. Digitonin particles

Anderson and Boardman reported PSI and PSII particles prepared by using a milder detergent, digitonin [15]. They showed that 'PSI particles' (D-144) had PSI activity but no PSII activity, whereas 'PSII particles' (D-10) had both PSI and PSII activities. We found that the 5-kDa band was present in D-144 as well as in D-10 (data not shown).

3.5. Non-detergent particles

It was reported previously by one of the present authors (K.S.) that a simple mechanical grinding of leaves and subsequent differential centrifugation could achieve a good separation of stroma and grana thylakoids of *P. americana* chloroplasts [17]. There, it was shown that PSII activity was confined to the grana fraction while that of PSI was present in the stroma and grana thylakoid fractions as well. Our

present SDS-PAGE analysis of the two fractions (Fig. 3) supported this original conclusion: no PSII subunit was found in the stroma fraction. The 5-kDa was found in the stroma as well as in the grana fractions. The identities of other bands were summarized in Fig. 3 together with the N-terminal sequences used for identification.

3.6. Subunit compositions of bare-bone type reaction center preparations

We showed previously that the treatment of psI_b particles with chaotropic agents such as urea could remove the small subunits [10]. psI_b was treated with 7.5 M urea and then subjected to ultrafiltration in order to remove dissociated subunits. As shown in Fig. 4, the resultant preparation lacked PsaD, -E, -C, but had the 5-kDa polypeptide (cpI_a, lane 2), identified by the N-terminal sequence. The treatment with 2 M NaI had the same effect (cpI_b, lane 3). It should be noted that the intensities of the CBB-stained 5-kDa band were almost equal among these three preparations. It should also be noted that this rather faint band appeared so just because this low molecular weight polypeptide seemed to be stained poorly with CBB. Judged from the recovery yields of the N-terminal amino acids recorded for the first cycle of the Edman degradation in the sequencer, the amount of the 5-kDa protein seemed far more abundant than PsaC, PsaD or PsaE (data not shown). This indicates that this subunit is tightly and perhaps stoichiometrically bound to the PSI reaction center complex. The role of this subunit in the primary photochemistry of PSI has yet to be elucidated. Some of the PSI subunits such as PsaG, -H, and -N are found only among eukaryotes, and not in cyanobacteria [2]. So far, we have not been able to find a homologous gene in a cyanobacterial genomic database (CyanoBase for *Synechocystis* sp. PCC6803, Kazusa DNA Research Institute, Chiba, Japan: [20]).

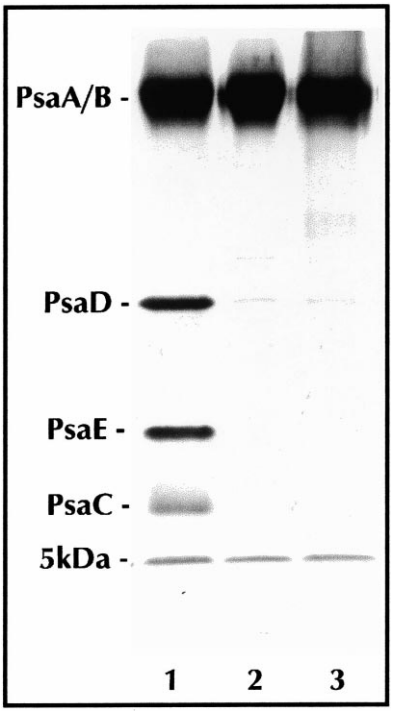


Fig. 4. SDS-PAGE of reaction center preparations. Lane 1, Triton-prepared particles (psI_b); lane 2, urea-treated Triton particles (cpI_a); lane 3, NaI treated Triton particles (cpI_b). See text for details.

Generally speaking, in PSI, those subunits whose corresponding genes are on the chloroplast DNA (PsaA, PsaB and PsaC) are highly homologous throughout plant species as well as among cyanobacteria, whereas those of nuclear encoded genes (PsaD, PsaE, PsaF etc) are not [2]. It should be noted that the present *psbW* gene is nuclear encoded [8], yet the amino acid sequence homology is remarkably high among different plant species (Fig. 2).

The reason why this subunit was frequently, but not always, seen in PSII particles is another mystery at the moment. This subunit did not exist in bare-bone PSII reaction center preparations [21,22]. On the other hand, a complex that consisted of numerous components yet could form two-dimensional crystals had one [7,23]. One possibility is that the subunit is also a part of PSII as well as of PSI; it may function structurally rather than catalytically and be common to the two photosystems. This possibility is supported by the results obtained in the PSII particles prepared from a PSI deficient *Chlamydomonas* mutant [4]. Our present results with the digitonin particles and the *Phytolacca* preparations, where the grana fractions contain substantial amounts of PsbW/PsaY, do not rule out this possibility. If that is proved to be the case, the prefix of this protein (and that of the gene) shall be either Psa- (*psa*-) or Psb- (*psb*-), or rather, a different prefix such as Psx- may be more appropriate for this small polypeptide.

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