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Specific release of the extrinsic 18-kDa protein from spinach Photosystem-II particles by the treatment with NaCl and methanol and its application for large-scale purification of the three extrinsic proteins of Photosystem II without chromatography

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The extrinsic 18-kDa protein in spinach Photosystem-II particles was specifically released from the membrane by the treatment with 0.5 M NaCl and 20% methanol at pH 6.5. The NaCl-methanol treatment was used in combination with the treatments with 2 M NaCl (pH 6.5) and 0.8 M Tris-HCl (pH 8.4) for developing a new procedure for the purification of the subunit proteins (the extrinsic 33-, 24- and 18-kDa proteins) of the oxygen-evolution enzyme complex from spinach chloroplasts. The three extrinsic proteins were liberated from the membranes almost completely and specifically by the simple washing procedure employed here. As no chromatographic step was required for the purification of the proteins, the time for the purification was considerably shortened and the yields of the proteins, especially of the 24- and 18-kDa proteins, were significantly improved.

Three peripheral proteins of PS II having molecular masses of 33, 24 and 18 kDa are comprised in the so-called oxygen evolution enzyme complex in chloroplasts (for reviews, see Refs. 1 and 2). Of these proteins, the 33-kDa protein was first isolated from spinach PS-II particles with sonic oscillation and isoelectric focusing [3]. The protein was shown to be associated with PS II, but its exact role was not known at that time. Later, the 33-kDa protein as well as the 24- and 18-kDa proteins were found to be released from the PS-II membranes by Tris treatment which inhibited oxygen evolution specifically [4], and since then considerable attention has been attracted for the purification and characterization of the proteins.

The isoelectric points of the three proteins are different from each other by more than 1.5 pH unit [5], and isoelectric focusing or ion-exchange chromatography has been used for the purification of the proteins [3,5–8]. As to the 33-kDa protein, the condition for the purification was optimized both in the efficiency of the procedure and the yield of the protein by the use of butanol/water phase partitioning and anion-exchange column chromatography [8]. On the other hand, the purification of the 24- and 18-kDa proteins was laborious because the repeated chromatography was required for the reasonable purification of the proteins. As the procedures employed for the purification of these proteins needed usually a long

Abbreviations: PS II, Photosystem II; PS-II particles, membrane fragment enriched in Photosystem II; Mes, 4-morpholineethanesulfonic acid; HPLC, high-performance liquid chromatography.

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time, the proteins were sometimes partially degraded in the purification. In order to avoid these problems, we developed a new procedure which enable us to purify large amounts of the three peripheral proteins in a short time.

Oxygen-evolving PS-II particles were prepared from spinach chloroplasts by brief sonication (30 s at 0 °C) and Triton X-100 treatment as previously described [8]. The PS-II particles were suspended in the minimum volume of the solution containing 0.33 M sorbitol, 50 mM Mes and 0.12 M NaCl (pH 6.5) and stored at liquid nitrogen before use. For the treatment of the PS-II particles with NaCl and methanol, the PS-II particles were once washed with a solution containing 0.33 M sorbitol and 50 mM Mes by the centrifugation at 35 000 × *g* for 30 min and incubated in a solution containing 50 mM Mes (pH 6.5), NaCl, and methanol at the chlorophyll concentration of 1 mg/ml for 10 min at 4 °C. The concentrations of NaCl and methanol were varied from 0 to 1 M and from 0 to 20%, respectively. The PS-II particles treated with NaCl/methanol were then centrifuged at 35 000 × *g* for 30 min. The supernatnat containing the 18-kDa protein exclusively was dialyzed against distilled water and concentrated by ultrafiltration with an Amicon ultrafiltration cell model 52 with a YM 10 filter. The precipitate of the centrifugation was incubated with a solution containing 50 mM Mes (pH 6.5) and 2 M NaCl at 1 mg chlorophyll/ml for 10 min to remove the 24-kDa protein from the PS-II particles. The suspension was recentrifuged at 35 000 × *g* for 30 min.

The supernatant was dialyzed and concentrated by ultrafiltration, and the precipitate was finally incubated with 0.8 M Tris (pH 8.4) at 1 mg chlorophyll/ml for 10 min. After the centrifugation of the suspension at 35 000 × *g* for 30 min, the supernatant was collected, dialyzed against distilled water and concentrated. For the estimation of the yields of the proteins, the supernatant fraction in each washing step extensively dialyzed against distilled water was lyophilized and the amounts of the proteins were weighed. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli's method with a 4% stacking and a 12.5% resolving gels [9].

Of the three peripheral proteins involved in oxygen evolution, the 24- and 18-kDa proteins are known to be removed from the membranes by the treatment of the PS-II membranes with high concentration of NaCl (250 mM–1 M) [7,10,11]. With such a high concentration of NaCl, however, both the proteins were released together from the membranes and further chromatographic steps were required to separate the two proteins from each other. As the 24- and 18-kDa proteins have different p*I* values and were expected to have different sensitivity to the salt, we first examined the effects of the lower concentrations of NaCl in removing the two proteins from the PS-II membranes (Fig. 1A). Apparently there was difference in the susceptibility of the two proteins to the NaCl-treatment, and the 18-kDa protein was easily released from the membranes at the lower concentration of NaCl as compared with the 24-kDa protein. The half-maximum concentrations of NaCl effective in releasing the 18- and 24-kDa proteins were 0.15 and 0.5 M, respectively. Under these conditions, however, the separation of the 18-kDa protein from the 24-kDa protein was still poor, and another strategy was required to solve the problem. We examined the effects of washing the PS-II particles with various concentrations of NaCl in the presence of methanol (Fig. 1B).

Alcohol is known to alter the inter and/or intra molecular interaction in the membrane proteins and induces changes in the stability of these components on the membranes. Methanol itself was not effective in releasing the peripheral proteins from the membranes when it was used alone. As to the 18-kDa protein, there was no change in the pattern of release of the protein between the NaCl-treatment and the NaCl/methanol treatment of the PS-II particles (Fig. 2A). However, methanol suppressed the release of the 24-kDa protein from the membranes significantly when it was included in the NaCl solution containing 50 mM Mes (pH 6.5) for the NaCl-treatment of the PS-II particles (Fig. 2B). The concentration of methanol optimum for the suppression was 10–20%. A higher concentration of methanol was not used here to avoid the solubilization of the other membrane components such as chlorophyll from the membranes.

The 33-kDa protein was not removed from the membranes at all under the present conditions. Thus the 18-kDa protein was easily obtained in

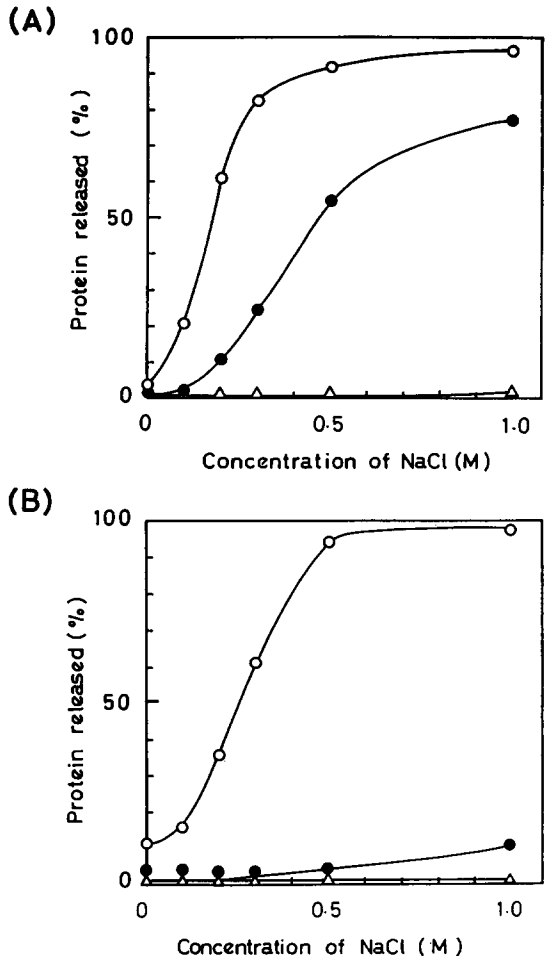

Fig. 1. Release of the peripheral 33-, 24- and 18-kDa proteins from the membranes of the spinach PS-II particles by the treatment with NaCl and methanol. The PS-II particles were washed with NaCl solution of 0–1 M in the absence (A) or presence (B) of 20% methanol at the chlorophyll concentration of 1 mg/ml. The amounts of the proteins released were estimated from the densitogram of SDS-polyacrylamide gel electrophoresis for the supernatant fractions obtained by centrifugation of the NaCl or NaCl-methanol treated PS-II particles. Complete release of the proteins from the membranes was attained by washing the PS-II particles with 0.8 M Tris (pH 8.4) twice, and the amounts of the three proteins released by this procedure were taken as 100% in the ordinate. △, the 33-kDa protein. ●, the 24-kDa protein, ○, the 18-kDa protein.

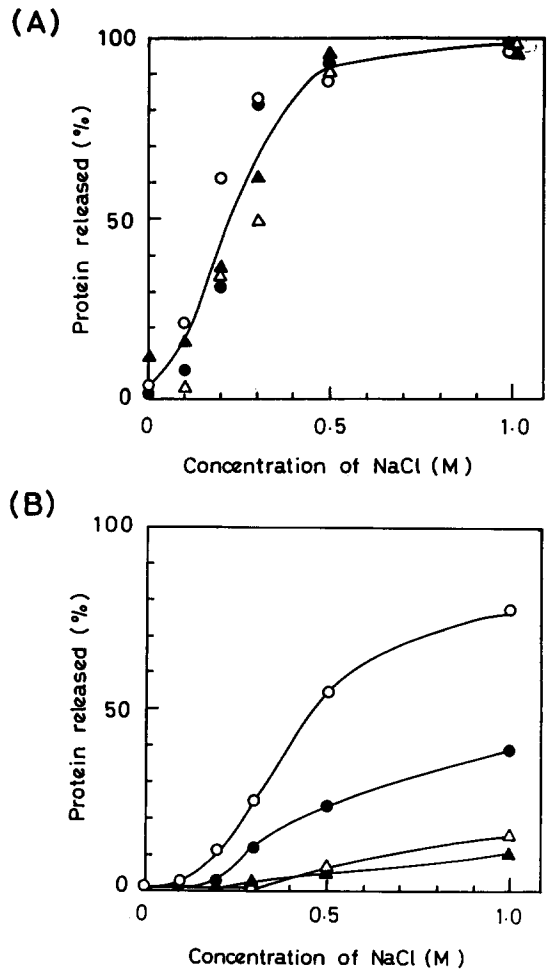

Fig. 2. Dependence of the release of the 24- and 18-kDa proteins from the PS-II membranes on the concentrations of NaCl and methanol in the NaCl-methanol treatment. (A) The 18-kDa protein. (B) The 24-kDa protein. The amounts of the 24- and 18-kDa proteins released from the membranes were estimated as described in Fig. 1. The concentrations of methanol used were 0% (○), 5% (●), 10% (△) and 20% (▲).

the supernatant of the centrifugation after the NaCl/methanol treatment of the PS-II particles. (Fig. 2A and Fig. 3, lane 2). After the 18-kDa protein was removed from the membranes by NaCl/methanol treatment, the PS-II particles were washed with 2 M NaCl to release the 24-kDa protein (Fig. 3, lane 4). In the previous work, 1 M NaCl was used to remove the protein from the membranes [7], but a higher concentration of NaCl was found to be more appropriate for the complete removal of the 24-kDa protein from the membranes. The 33-kDa protein remained in the membranes after the NaCl/methanol and the NaCl treatments was isolated by the treatment of the PS-II particles with 0.8 M Tris (pH 8.4) (Fig. 3, lane 6). The yields of the three proteins ob-

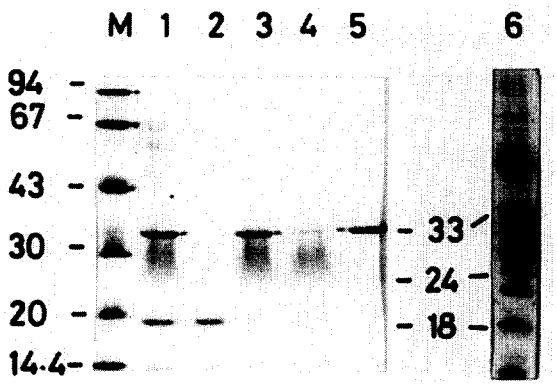

Fig. 3. An SDS-polyacrylamide electrophoresis gel showing the purification of the three proteins from the PS-II particles. The PS-II particles were washed stepwise with 0.5 M NaCl-20% methanol (pH 6.5), 2 M NaCl (pH 6.5), and 0.8 M Tris (pH 8.4), and the supernatant obtained after each treatment was applied to SDS-polyacrylamide gel electrophoresis. To determine the proteins which remained on the membranes after each treatment, the precipitates obtained after the treatments were washed with 0.8 M Tris and the supernatants were also applied to SDS-polyacrylamide gel electrophoresis. Lane 1, the supernatant of Tris-washing of the PS-II particles showing the peripheral proteins (the 33-, 24- and 18-kDa proteins) in PS II. The 24-kDa protein was usually detected as a diffused band. Lane 2, the supernatant of the NaCl-methanol-treated PS-II particles. Lane 3, the supernatant of Tris-washing of the NaCl-methanol-treated PS-II particles. Lane 4, the supernatant of 2 M NaCl-treated PS-II particles. Lane 5, the supernatant of Tris washing of the NaCl-treated PS-II particles. Lane 6, the PS-II particles. Far left lane shows marker proteins. The molecular masses are shown in kDa.

tained by the present procedure is summarized in Table I with the yields obtained by other methods [8,12]. Judging from the densitograms of SDS-polyacrylamide gel electrophoresis, the amounts of the 33-, 24- and 18-kDa proteins purified by this procedure were 80–90% of the total proteins originally present in the PS-II particles.

Incubation of the PS-II particles in NaCl-methanol, NaCl, and Tris for more than 10 min did not affect the yield of the proteins. It was also demonstrated that 10 min centrifugation at 35 000 $\times g$ was enough for obtaining the supernatant fractions after washing the PS-II particles with NaCl-methanol, NaCl, and Tris, although we used 30 min centrifugation for this purpose.

In the present procedure, we used the PS-II

TABLE I

COMPARISON OF THE YIELDS OF THE THREE PROTEINS PURIFIED BY THE NaCl-METHANOL-, NaCl-, AND TRIS-TREATMENTS OF THE PS-II PARTICLES WITH THOSE PURIFIED BY OTHER PROCEDURES

Molecular mass of proteins (kDa)	Amounts of proteins obtained (μg/mg chlorophyll)		
	this method [a]	ion-exchange chromatography [b]	HPLC [c]
33	71	83	58
24	59	–	15
18	25	–	8

[a] The supernatant fractions obtained by the treatment of the PS-II particles (equivalent to 1 mg chlorophyll) with NaCl-methanol, NaCl, and Tris were dialyzed for 5 days against distilled water. The extensively dialyzed fractions were then lyophilized and the amounts of the proteins were weighed.

[b] From Ref. 8.

[c] From Ref. 12.

enriched particles from spinach chloroplasts as the starting material and employed a simple stepwise washing procedure with NaCl/methanol, NaCl and Tris buffer for liberating the three proteins from the PS-II membranes. Suppression of the release of the 24-kDa protein from the PS-II membranes observed in NaCl-methanol treatment was the basis for separating the 18-kDa protein from the 33- and 24-kDa proteins of PS II. These effects of NaCl and alcohol were not prominent when methanol was replaced by ethanol or propanol. It is probable that the solvent used here exerted an influence on the stability of the proteins on the membranes. The new procedure for the purification of the three proteins did not require the complicated and time-consuming chromatography steps. In spite of the simplicity of the whole procedure, the yields of the purified proteins were quite high and no degradation of the proteins was detected during the purification judging from the SDS-polyacrylamide gel electrophoresis of the proteins.

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References

1 Govindjee, Kambara, T. and Coleman, W. (1985) Photochem. Photobiol. 42, 187–210

2 Ghanotakis, D.F. and Yocum, C.F. (1985) Photosynthesis Res. 7, 97–114

3 Kuwabara, T. and Murata, N. (1979) Biochim. Biophys. Acta 581, 228–236

4 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268

5 Yamamoto, Y., Shimada, S. and Nishimura, M. (1983) FEBS Lett. 151, 49–53

6 Jansson, C., Åkerlund, H.-E. and Andersson, B. (1983) Photosynthesis Res. 4, 271–279

7 Kuwabara, T. and Murata, N. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G., and Satoh, K., eds.), pp. 223–228, Academic Press Japan, Tokyo

8 Yamamoto, Y., Hermodson, M.A. and Krogmann, D.W. (1986) FEBS Lett. 195, 155–158

9 Laemmli, U.K. (1970) Nature 227, 680–685

10 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10

11 Yamamoto, Y. and Nishimura, M. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G., and Satoh, K., eds.), pp. 229–238, Academic Press Japan, Tokyo

12 Yamamoto, Y., Nakayama, S., Cohn, C.L. and Krogmann, D.W. (1987) Arch. Biochem. Biophys. 255, 156–161