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Solubilization and partial purification of a thylakoidal enzyme of spinach involved in the processing of D1 protein

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The enzyme involved in the processing of D1 precursor protein was solubilized from spinach thylakoids by Triton X-100 treatment and then partially purified in the presence of the detergent by Sephadex G-75 gel-filtration chromatography. The apparent molecular mass of the enzyme was estimated via this procedure to be about 34 kDa. The D1 precursor protein translated from the extracted spinach chloroplast RNA by a wheat germ cell-free system was used here as a substrate in measurements of the activity.

Protein, D1; Photosystem II; Protease, processing; SDS-PAGE; (Spinach thylakoid)

1. INTRODUCTION

The D1 protein is known as an integral part of the PS II reaction center [1–3] and is supposed to be responsible, together with D2 protein, for binding of components involved in the primary processes of PS II, i.e. the primary donor (P-680), pheophytin acceptor, quinone-iron acceptor complex, secondary donor (Z) and manganese cluster of the oxygen-evolving center [2–5]. On the other hand, this component is also known to be one of the most rapidly metabolizing proteins in chloroplasts in the light [6]. The light-dependent synthesis is now understood to represent the repair process of photodamaged reaction centers [7,8]. The protein is synthesized on thylakoid-bound ribosomes [9] as a precursor of size 1–2 kDa greater than that of the mature form [10,11]. The maturation process of the newly synthesized precursor protein is likely to occur through the C-terminal cleavage [12] at Ala-344 on the deduced sequence [13,14], which takes place in the thylakoidal lumen. The processing seems to be essential for the assembly of the catalytic center of water cleavage, but not for the primary photochemistry of PS II [15,16]. The enzyme involved in this processing has recently been solubilized as Triton X-100 extracts from thylakoid membranes of the wild-type strain of *Scenedesmus obliquus* [16]. Treatment with thylakoid extracts from the LF-1 mutant which is deficient in the processing of D1 protein and oxygen-evolving capacity, resulted in specific reduction of the molecular mass of LF-1 D1 to the same size as that in the wild-type membranes and enabled photoactivation of oxygen evolution to take place [16]. The processing protease in the extracts, however, has not been purified further and thus the biochemical properties of this unique enzyme have remained almost totally obscure.

In this study, we have succeeded in extracting the D1-specific processing enzyme from spinach thylakoids by Triton X-100 treatment. The partial purification, molecular mass estimation by gel-filtration chromatography and preliminary characterization of the enzyme are described herein.

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Abbreviations: Chl, chlorophyll; PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

2.1. *Preparation of intact chloroplasts, in organello radiolabeling of and chasing of the radiolabeled proteins*

Intact spinach chloroplasts were prepared according to [18]. A discontinuous gradient of Percoll (Pharmacia) of 20, 50, 80% (v/v) was used in density gradient centrifugation and the purified chloroplasts were washed twice with 50 mM Tricine-KOH buffer (pH 8.4) containing 330 mM sorbitol. Intact chloroplasts in sorbitol-Tricine buffer (1 mg Chl/ml) were incubated with [^{35}S]methionine (1 mCi/ml, 1100–1200 Ci/mmol, Trans ^{35}S-label, ICN) for 30 s at 25°C under illumination with white light (10000 lux) from a tungsten-halogen lamp. Radiolabeled chloroplasts were suspended and vigorously stirred for a brief period in 10 mM Tricine-KOH buffer (pH 7.8) containing 10 mM NaCl and 10 mM $MgCl_2$ with a vortex-mixer, then immediately washed and fractionated in the same buffer by centrifugation. Radiolabeled membrane fragments thus obtained were chased in the presence of chloramphenicol (200 mg/ml) and unlabeled methionine (5 mM) at 25°C. Samples withdrawn at the beginning of the chase period (time 0) and at definite time points of incubation were subjected to SDS-PAGE followed by fluorography (Enlightning, NEN).

2.2. *Preparation of D1 precursor protein*

High-M_r RNAs were extracted from spinach chloroplasts as in [19], with minor modifications concerning the phenol-chloroform extraction. The RNAs were translated to proteins by a wheat germ cell-free system (NEK-029 Z10, NEN) containing [^{35}S]methionine (1 mCi/ml) following the manufacturer's protocol.

2.3. *Assay of protease activity*

2 μl of the in vitro translated protein solution described above was added to 20 μl enzyme in 50 mM Tris-HCl buffer (pH 7.2) and 0.05% Triton X-100, then incubated at 25°C for 2 h. After incubation, proteins were separated by SDS-PAGE and radiolabeled proteins were visualized on fluorograms and densitometrically monitored with a dual-wavelength chromatoscanner (model CS-930, Shimadzu, Kyoto).

2.4. *SDS-PAGE*

SDS-PAGE of proteins was carried out according to Laemmli [20] using an acrylamide concentration of 15%. The analyzing gel contained 6 M urea. Samples were solubilized in a solution consisting of 62.5 mM Tris-HCl (pH 6.8), 2.3% (w/v) SDS, 10% (w/v) glycerol and 5% (v/v) 2-mercaptoethanol.

3. RESULTS AND DISCUSSION

The processing of D1 precursor protein takes place even in isolated chloroplasts, as described in [11]. The processing activity is most likely associated with thylakoids, since the membrane fragments prepared via disrupting chloroplasts by hypotonic treatment followed by washing with a low concentration of buffer solution still retained the capacity to degrade the precursor protein to the mature size (fig.1). In accordance with this observation, the supernatant from hypotonic treatment exhibited no appreciable processing activity in the in vitro assay system using D1 precursor protein translated from extracted chloroplast RNA as substrate. However, a specific reduction in molecular mass of the D1 precursor protein occurred when in vitro translated substrate was incubated with the supernatant prepared by 4% Triton X-100 treatment of spinach thylakoids [17] followed by centrifugation at 144000 × *g* for 1 h at 4°C (fig.2). The size of the breakdown product, in this case, was exactly the same as that of the mature D1 protein obtained by in organello labeling (lane 3), suggesting that this phenomenon represents specific action of a D1 processing protease in the supernatant. Specific cleavage is also demonstrated by the facts that there was no detectable radioactive protein band of size intermediate between those of the precursor and mature protein and that no appreciable further proteolytic breakdown products were observed even after prolonged incubation or when higher concentrations of supernatant were used. In addition, a preliminary partial proteolytic fingerprinting experiment using papain exhibited a similar peptide pattern for both in organello and in vitro processed proteins (not shown).

The activity in the supernatant was sensitive to pH of the reaction mixture and was highest at pH 6–7. Activity was destroyed on heating the supernatant to 70°C for 3 min. The activity was also sensitive to protease treatment (lysylendopeptidase). Activity in the supernatant was partly inhibited in the presence of Mn^{2+} (10 mM), Mg^{2+} (10 mM), and Ca^{2+} (10 mM), but not with 2 mM EDTA. Processing activity in the supernatant can be concentrated by ultrafiltration using an Amicon PM-10 membrane (exclusion limit, M_r 10000).

The peptidase in the supernatant was partially purified by fractionating on gel-filtration chromatography (fig.3). Fractionation was carried out in the presence of 0.05% Triton X-100 in order to avoid aggregation. In the experiment shown in fig.3, Triton X-100 extracts were applied directly onto a Sephadex G-75 column (1.5 × 40 cm) equilibrated with 50 mM Tris-HCl (pH 7.2) buffer containing 0.05% Triton X-100. The elution profile monitored at 280 nm exhibited a predominant peak at the void volume of the column. This frac-

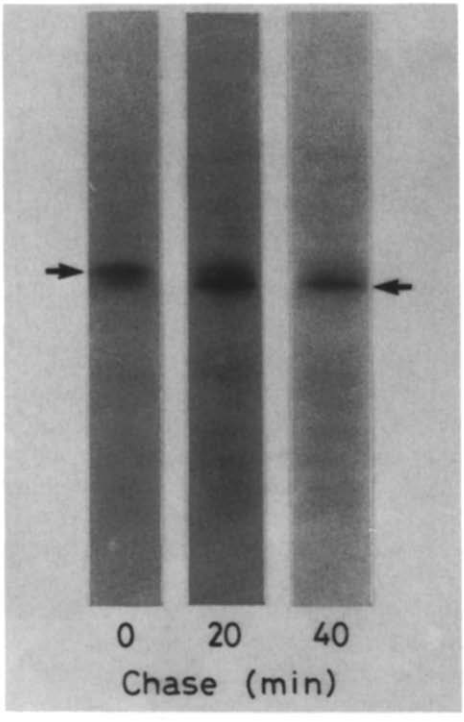

Fig.1. Processing of D1 precursor protein in membrane fragments of spinach chloroplasts. Intact chloroplasts were pulse-labeled for 30 s, then disrupted as described in section 2, and chased in the presence of chloramphenicol and unlabeled methionine for different periods. Samples were analyzed by SDS-PAGE followed by fluorography. Arrow on left indicates position of the precursor protein, that on the right denoting the mature protein.

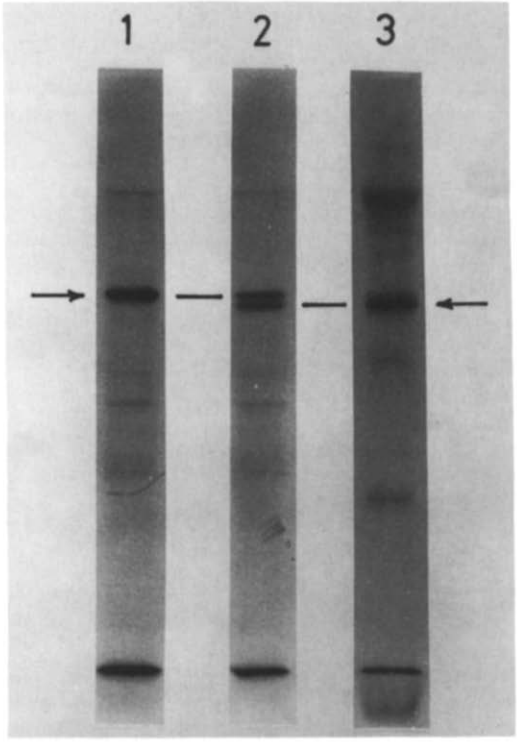

Fig.2. Processing of in vitro translated D1 precursor protein. In vitro translation products were incubated, without (lane 1) or with (lane 2) Triton X-100 extracts of spinach thylakoids, for 2 h at 25°C. Thylakoidal proteins radiolabeled in organello for 90 min in the light are also shown in lane 3.

tion contained most of chlorophyll and proteins present in extracts. Processing activity was retarded in these major components and appeared in fractions with a relatively low A_{280nm} value (fractions 21–26) as clearly indicated by fig.3B where the fluorogram around the 30 kDa region is enlarged. For convenience, the ratio $M/(M + P)$ is plotted in fig.3A in order to indicate the position of maximum activity (M, relative absorbance at the position of migration of mature protein; P, corresponding value at the position of the precursor protein) on the densitometric traces of fluorograms (unfortunately, the resolution of densitometric scanning in these measurements is insufficient to discriminate between these two bands). Using proteins of known molecular mass as standards, the size of the enzyme was estimated to be about 34 kDa in the non-denatured, active state (inset). SDS-PAGE analysis of the purified material indicated the presence of a CBB-stainable band of nearly 34 kDa, together with a few other polypeptide bands which exhibit migration patterns similar to that of the processing activity.

The enzyme described here is likely to be the processing protease of restricted reaction specificity which is engaged in maturation of D1 precursor protein. This enzyme probably corresponds to that extracted from the wild-type strain of *Scenedesmus* [16]. Both enzymes have been extracted by using the detergent Triton X-100. The *Scenedesmus* enzyme has the capacity to degrade the protein from the LF-1 mutant to the size of the mature protein of the wild-type and this accompanies acquisition of the capacity to photoactivate oxygen evolution in mutant thylakoids. On the other hand, the spinach enzyme is capable of degrading spinach D1 precursor protein to the size of the mature protein

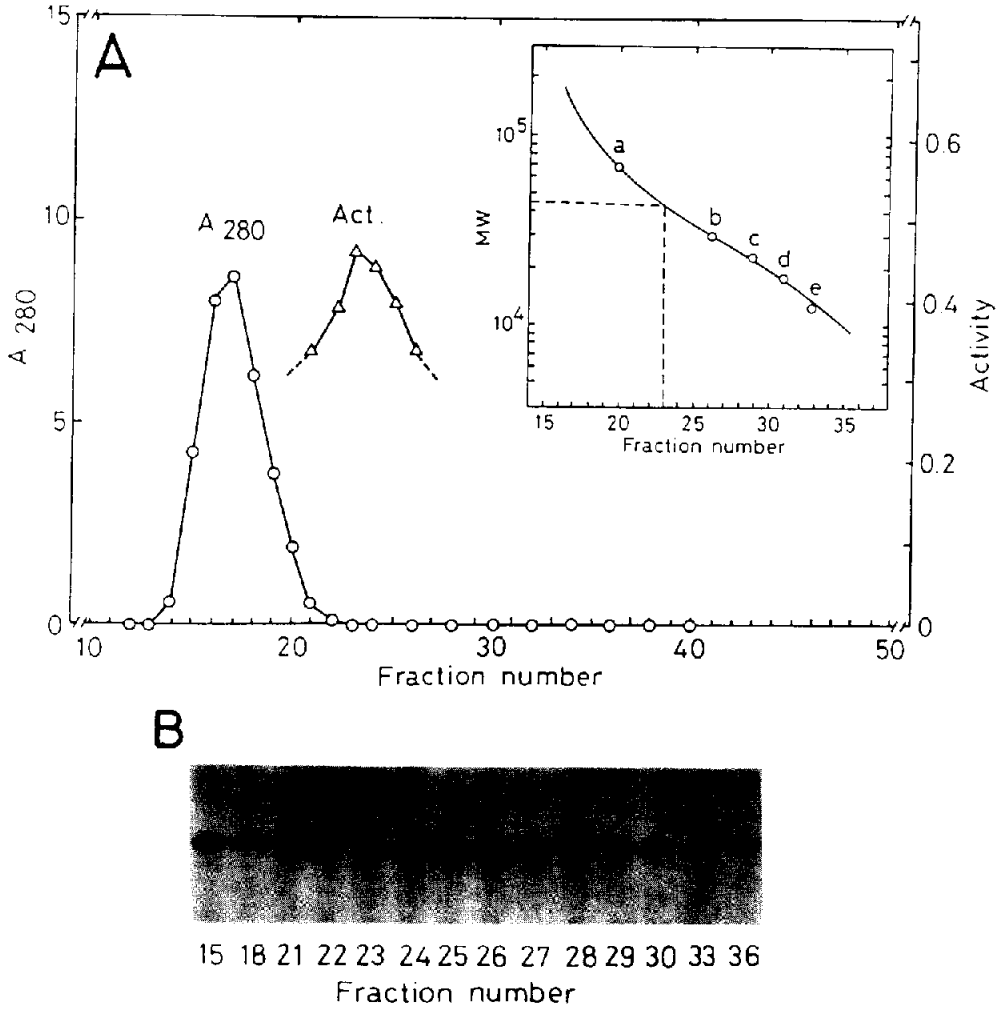

Fig.3. Sephadex G-75 gel-filtration chromatography of Triton X-100 extracts of spinach thylakoids. Elution was monitored at 280 nm (○—○). The processing activity is indicated as described in the text (△—△) in A. (Inset) Molecular mass estimation for the enzyme using as markers: bovine serum albumin (a), DNase I (b), trypsin (c), myoglobin (d) and cytochrome *c* (e). The fluorogram showing the processing activity of each fraction obtained by Sephadex gel-filtration chromatography is presented in B.

in an in vitro system. The necessity of Triton X-100 for extraction and in purification indicates that the enzyme is an integral or peripheral membrane protein, although the exact location in the thylakoids remains to be determined.

Thylakoidal protease activities involved in protein degradation have been reported [21–23] and, in some cases, the enzymes have been extracted from membranes and partially purified [23]. An example of partly purified thylakoidal proteins is provided by a plastocyanin processing enzyme [23]. This enzyme has been extracted with Triton X-100 under conditions similar to those for the D1 processing enzyme of *Scenedesmus*. Thus, it is of interest to determine whether the D1 processing protease reported here is also involved in the maturation of proteins, encoded in either chloroplast DNA or nuclear DNA, which are transported to the thylakoidal lumen. Further purification is clearly required for elucidation of the specificity as well as for attempts at gaining a better understanding of the role of D1 processing in assembly of the catalytic site of water cleavage in PS II reaction centers.

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