Cleavage of a synthetic COOH-terminal oligopeptide of D1 precursor protein by a purified processing enzyme

S. Fujita, N. Inagaki, T. Ono+, Y. Inoue+ and K. Satoh

Department of Biology, Faculty of Science, Okayama University, Okayama 700 and +*Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*

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A synthetic COOH-terminal oligopeptide of D1 protein deduced from the spinach *psb*A gene (Asn-325–Gly-353) was subjected to proteolytic digestion by purified processing enzyme of D1 protein [(1989) FEBS Lett. 246, 218–222] and the following two fragments were obtained as cleavage products: a COOH-terminal 9-amino-acid fragment (Ala-345–Gly-353) and an NH_2-terminal 10-amino-acid fragment (Asn-325–Arg-334). It was concluded that: (i) the oligopeptide consisting of the COOH-terminal 29-amino-acid sequence deduced from the spinach *psb*A gene provides the recognition domain for the processing enzyme; (ii) the cleavage takes place at the predicted processing site of native D1 precursor protein (COOH side of Ala-344); and (iii) another cleavage takes place at an additional site (COOH side of Arg-334) for the synthetic substrate, but not for the native D1 precursor protein.

Protein, D1; Enzyme, processing; Gene, *psb*A; Photosystem II; Reaction center; Oligopeptide, synthetic

1. INTRODUCTION

It is generally believed that the D1 and D2 proteins constitute the reaction center of photosystem II (PS II) in a similar manner to the L and M subunits forming the purple bacterial reaction center [1–4]. Unexpectedly, however, the D1 protein is recognized as being one of the most unstable proteins in thylakoid membranes in the light and is known to be rapidly recovered through light-regulated de novo synthesis [5,6]. The protein is synthesized on thylakoid-bound ribosomes as a precursor 1–2 kDa greater in size than that of the mature form [7,8]. The maturation process of the newly synthesized precursor protein is predicted to occur through a COOH-terminal (C-terminal) cleavage at Ala-344 on the amino acid sequence deduced from the spinach *psb*A gene [9–11]. The processing seems to be essential for assembly of the catalytic center for water cleavage, but not for the primary photochemistry of PS II [12,13]. The enzyme involved in the processing has recently been solubilized from a wild-type strain of *Scenedesmus obliquus* [13] and spinach [14]. The spinach enzyme, which has a molecular mass of about 34 kDa [14], has recently been highly purified and characterized [15].

Here, a synthetic C-terminal oligopeptide of D1 protein deduced from the spinach *psb*A gene was subjected to proteolytic cleavage by the purified processing enzyme. It was confirmed that the cleavage takes place exactly at the C-side of Ala-344 as predicted from previous studies [10,11]. It was also concluded that the oligopeptide consisting of 29 amino acids of the C-terminal region of D1 precursor protein is sufficient for recognition as substrate by the enzyme.

Correspondence address: K. Satoh, Department of Biology, Faculty of Science, Okayama University, Okayama 700, Japan

Abbreviations: HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PS, photosystem

2. MATERIALS AND METHODS

2.1. *Enzyme and substrate*

Processing enzyme of D1 protein was purified from Triton

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X-100 extracts of spinach thylakoids as in [14,15]. An oligopeptide corresponding to the C-terminal 29-amino-acid sequence deduced from the spinach *psb*A gene was synthesized on an Applied Biosystems model 430 A peptide synthesizer and purified by preparative HPLC using a reverse-phase column (Applied Biochem, Aquapore RP-300).

2.2. *Enzyme assay*

The standard assay mixture (30 μl) contained 8 nmol substrate and 27.5 μg enzyme protein in 50 mM Tris-HCl (pH 7.2) buffer containing 0.05% Triton X-100. Incubation was carried out at 27°C for definite time intervals. The reaction was stopped by the addition of 18% (w/v) trichloroacetic acid to a final concentration of 3%. The trichloroacetic acid-treated mixture was allowed to stand for 30 min at room temperature and then centrifuged at 20000 × *g* for 20 min, the resultant supernatant being subjected to HPLC analysis.

2.3. *HPLC analysis*

A Shimadzu (Kyoto) model LC-6A liquid chromatograph equipped with a dual-wavelength UV-VIS detector (SPD-6AV) and an analyzer (C-R2AX) was employed for analysis. A reverse-phase pre-packed, stainless-steel column (4.6 × 250 mm, Shin-Pack CLC-C8M) equipped with a guard column (Shin-Pack G-C8(4)) was used at a flow rate of 1.0 ml/min at 23.5–24.0°C. To 30 μl of trichloroacetic acid extracts, 150 μl of 0.1% (v/v) TFA was added, the mixture was passed through a Millipore filter (pore size, 0.45 μm), and then an aliquot (150 μl) was applied to the column. Two solutions were used for elution; solution A, 0.1% (v/v) TFA; solution B, 0.1% (v/v) TFA in 70% (v/v) acetonitrile. Elution was conducted according to the following regime: (i) 0–1 min, solution A; (ii) 1–30 min, increasing linear gradient from solution A to solution B; (iii) 30–31 min, solution B; (4) decreasing linear gradient from solution B to solution A. Elution was monitored at 220 nm at a full scale of 0.32 (*A* unit).

2.4. *Amino acid sequence analysis*

The eluate from HPLC was fractionated into test tubes and freeze dried after evaporating the buffer solution with vacuum pump. Freeze-dried samples were dissolved in water and applied to a gas-phase protein sequencer (Applied Biosystems model 470A).

3. RESULTS AND DISCUSSION

In the experiment shown in fig.1, the purified processing enzyme of D1 protein was incubated with a synthetic oligopeptide consisting of the C-terminal 29 amino acids of D1 precursor protein deduced from the spinach *psb*A gene. In the control experiment (incubation time, 0 h), where the enzyme was incubated with the substrate oligopeptide in a solution containing 3% trichloroacetic acid, two prominent peaks, in addition to some minor peaks, were observed on the chromatogram from reverse-phase HPLC. The large peak with a retention time of about 10 min is ascribable to trichloroacetic acid in the extracts, whereas the peak at about 20 min (S) was identified as being the substrate oligopeptide based on the amino acid sequence analysis. After 2 h incubation, two additional components appeared on the chromatogram, a larger (P-1) and a smaller (P-2) peak, the ratio of the two peak areas being estimated to be about 3 (P-1/P-2). The amounts of both components increased further with longer times of incubation, the ratio between them remaining almost constant, concomitant with a decrease (not shown) in the amount of substrate (S). The time course of the decrease in substrate is shown in fig.2 (triangles), where the ratios (lines A,B) of the substrate decrement to the increment of the two components (P-1 and P-2, respectively) are also presented. The constant ratio for both components produced to the decrement in substrate over a wide range of reaction times strongly suggests that both components are the degradation products of the substrate. The activity depended linearly on the amount of enzyme solution added to the reaction mixture under the experimental conditions used. The activity was completely abolished by heat treatment (70°C, 3 min) of the enzyme solution, in a manner similar to that of the processing activity for the native precursor of D1 protein [14]. The enzyme activity was relatively stable at room temperature as shown by the results in fig.1.

The result of amino acid sequence analysis of the two enzymatic products is shown in fig.3. The major product (P-1) consisted of a polypeptide with the sequence: Ala-Ile-Glu-Ala-Pro-Ser-Thr-Asn-Gly. This sequence was found to be identical with the 9 C-terminal amino acids of D1 precursor protein deduced from the spinach *psb*A gene and thus the site of cleavage of the oligopeptide by this enzyme is identified as being the C-side of Ala-344. This is in accordance with the prediction based on sequence analysis of the mature protein [10,11]. Conversely, this result proves that the purified enzyme is really a processing enzyme responsible for maturation of D1 precursor protein.

The NH_2-terminal region of the second product (P-2) was found to be identical with that of the substrate. However, the sequence extended only as far as the first 10 amino acids, the subsequent 10-amino-acid region thus being missing. We could detect no component which increased with incubation time other than the two described here on the

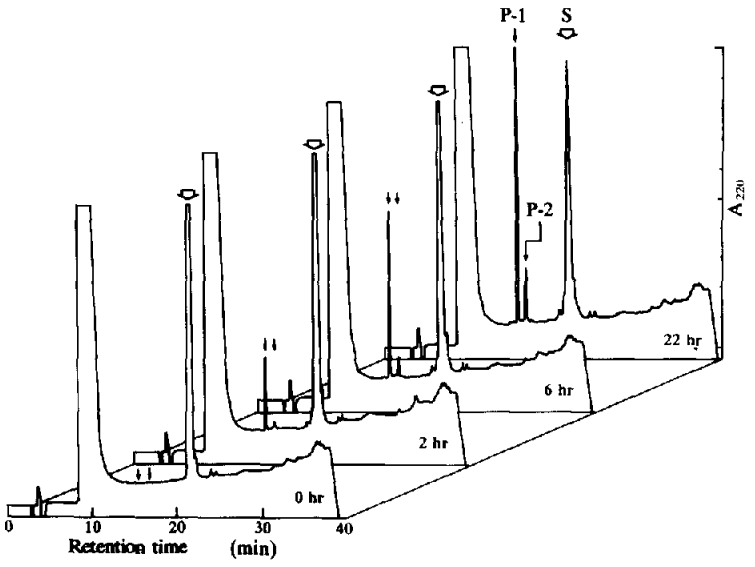

Fig.1. HPLC monitoring of the cleavage of a synthetic oligopeptide by the purified processing enzyme of D1 protein. Oligopeptide was incubated with the purified enzyme for 0, 2, 6 and 22 h and at 27°C. The samples were then treated with 3% trichloroacetic acid and centrifuged. The resultant supernatant was then applied to a reverse-phase column. Two peptide peaks, indicated P-1 and P-2, appeared and increased during the course of incubation. The large arrowhead (S) indicates the original oligopeptide which was used as substrate. See text for further explanation.

chromatogram in this experiment. The possibility exists that the additional cleavage at the C-side of Arg-344 is due to a contaminating trypsin-type protease in the enzyme solution. However, this does not appear to be the case, since when in vitro translated D1 precursor protein was incubated with our enzyme solution, the product we were able to detect was only the mature-sized protein [14].

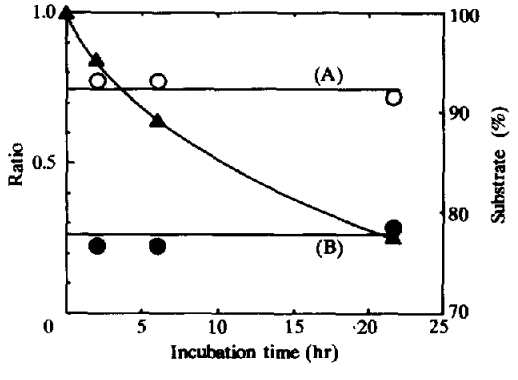

Fig.2. Time course of decrease in amount of substrate as expressed by percentage of the initial value (substrate (%)) and its ratios to the amounts of P-1 (A) or P-2 (B), respectively. Average of three independent experiments.

Thus, it may more likely to assume that the processing enzyme has the capacity to cleave at an additional site, but that the cleavage is prevented under in situ conditions where the substrate is packed in a more orderly fashion in the hydrophobic environment of thylakoid membranes.

An ambiguity in the analyses is that the peptide fragment between P-1 and P-2 was missing from the chromatogram. This may be due either to further degradation of the fragment into smaller pieces or to association or adsorption of the fragment onto the enzyme or somewhere else, thus being lost from the trichloroacetic acid extracts. The relatively high abundance of hydrophobic residues

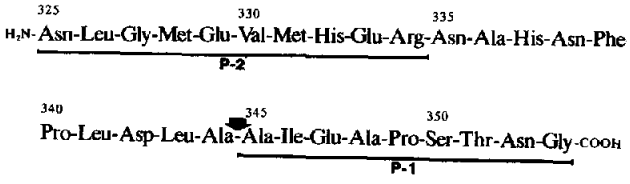

Fig.3. Amino acid sequence of the two breakdown products (P-1 and P-2) obtained after cleavage of oligopeptide by the purified processing enzyme of D1 protein. The arrowhead indicates predicted cleavage site.

in this fragment may be relevant to this phenomenon. In general, the yield of peptide in this kind of analysis seems to be largely dependent on the type of peptides examined. This may be the cause of the lower yield of P-2 peptide vs that of P-1 in spite of the almost identical value of the absorption at 220 nm due to the peptide bond [16] expected for both fragments. The yield of substrate is also less than that of P-1 (the decrement in substrate should be about 3-fold greater than the content of P-1 produced, provided the absorption is proportional to the amount of peptide bonds).

Despite the ambiguities discussed above, we may at least conclude that the C-terminal 29-amino-acid sequence of D1 precursor protein is sufficient as the recognition domain for the processing enzyme to effect correct cleavage, and that the processing site is conclusively at the C-side of Ala-344 in accordance with previous predictions [10,11]. Although further analysis is evidently needed, this study has opened the way to elucidate the recognition site of the processing enzyme and to develop a more sensitive assay system by using synthetic peptides.

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