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Limited proteolysis indicates that the structure of the photosystem II extrinsic 33 kDa protein is different among different plant species

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Introduction

The extrinsic 33 kDa protein is present in all oxygen-evolving organisms from cyanobacteria to higher plants, and is needed to maintain the function of the Mn cluster (Miyao and Murata 1984, Ono and Inoue 1984). The amino acid sequence of the 33 kDa protein showed a relatively high homology around 40-50% from cyanobacteria to higher plants. The structure of the 33 kDa protein has been, therefore, considered to be similar among all plant species. However, it has been reported that the cleavage sites of the 33 kDa protein by proteases are different between cyanobacteria and higher plants. The cyanobacterial 33 kDa protein was cleaved primarily at 156F and 190F by chymotrypsin (Motoki et al. 1998), while the higher plant 33 kDa protein was cleaved at 16Y by chymotrypsin, and at 18E by V8 protease (Eaton-Rye and Murata 1989). In order to investigate whether the structure of the 33 kDa protein has changed during evolution from prokaryote to eukaryote, we determined the cleavage-sites by chymotrypsin and V8 protease of the 33 kDa protein from a red alga, *Cyanidium caldarium*, and the cleavage-sites by V8 protease of the 33 kDa protein from a cyanobacterium, *Synechococcus elongatus*. The obtained results were compared with those reported previously for cyanobacteria and higher plants.

Materials and methods

Preparation. The red algal 33 kDa protein was purified from *C. caldarium* PSII particles as described previously (Enami et al. 1998). The recombinant 33 kDa protein of a cyanobacterium, *S. elongatus*, was prepared according to Motoki et al. (1998). These 33 kDa proteins were dialyzed against 40mM MES-NaOH (pH 6.5) and stored at –80°C before protease treatments.

Limited proteolysis. The 33 kDa protein was treated with α-chymotrypsin (VII from bovine pancreas, Sigma) or *S. aureus* V8 protease (XVII, Sigma) for 30 minutes or 2 hours at 30°C, in 50 mM MES-NaOH (pH 6.5) with the protein-to-enzyme weight ratio of 1000. The reaction was stopped by adding PMSF to a final concentration of 3.0 mM.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with a gradient gel containing 16-22% acrylamide and 7.5 M urea according to Ikeuchi and Inoue (1988). The samples were solubilized with 5% lithium lauryl sulfate and 75 mM dithiothreitol for 30 minutes prior to electrophoresis.

N-terminal sequence. Polypeptides resolved by SDS-PAGE were semi-dry-blotted at a constant current of 2.0 mA/cm^2 for 1 hour onto polyvinylidene difluoride membranes (Immobilon^{TM}-P; Millipore). The N-terminal amino acid sequences of the blotted peptides were analyzed with an Applied Biosystems 477A protein sequencer.

Results and discussion

Chymotrypsin treatments of the red algal 33 kDa protein produced four fragments at 22, 17, 10 and 7 kDa as analyzed by SDS-PAGE. These four fragments were named R1, R2, R3 and R4, and their N-terminal sequences were shown in Table 1. By comparing the original sequence of the 33 kDa protein, we determined that chymotrypsin cleaved the red algal 33 kDa protein at 159M and 192L.

When the red algal 33 kDa protein was treated with V8 protease, four peptide fragments appeared at 22 (R5), 20 (R6), 8 (R7), and 6 (R8) kDa on SDS-PAGE. Their N-terminal sequences were also shown in Table 1. The results indicate that the red algal 33 kDa protein was digested at 182E and 195E by V8 protease. In addition, V8 protease-treatment of the cyanobacterial 33 kDa protein produced two fragments with apparent molecular masses of 20 kDa (C1) and 7 kDa (C2); analysis of their N-terminal sequence indicated that the protein was cleaved at 181E (Table 1).

Table 1 N-terminal sequences of peptide fragments produced by proteolysis of the red algal and cyanobacterial 33 kDa protein with chymotrypsin and V8 protease

Protease	Fragments	N-terminal sequence	Protease	Fragments	N-terminal sequence
		1			1
Chymotrypsin	R1	LTSQD	V8-protease	R5	LTSQD
		1			1
	R2	LTSQD		R6	LTSQD
		160			183
	R3	FLDPK		R7	ADGAE
		193			196
	R4	RKEND		R8	NDKIF
				C1	1 AKQTL
				C2	182 LARAN

The cleavage sites of the 33 kDa protein from different species with chymotrypsin and V8 protease were summarized in Table 2. Chymotrypsin cleaved the spinach 33 kDa protein at 16Y (Eaton-Rye and Murata 1989), whereas it cleaved the cyanobacterial 33 kDa protein at 156F and 190F (Motoki et al. 1998). On the other hand, V8 protease cleaved the spinach 33 kDa protein at 18E (Eaton-Rye and Murata 1989) and the cyanobacterial 33 kDa protein at 181E (present study). The red algal 33 kDa protein was cleaved at 159M and 192L by chymotrypsin and at 182E and 195E by V8 protease. Since most of the amino acids around these cleavage sites were conserved among cyanobacterial, red algal and higher plant 33 kDa proteins (data not shown), the differences in the cleavage sites imply the difference of the structure of the 33 kDa protein among different plant species. The cleavage sites of the red algal 33 kDa protein is rather similar to those of the cyanobacterial 33 kDa protein. Thus, in terms of the protease-sensitive sites, we conclude that the structure of the red algal 33 kDa protein resembles that of the cyanobacterial one but not the higher plant one. We are planning to determine the cleavage sites of the 33 kDa protein from green algae and euglena, to elucidate the structural changes of the extrinsic 33 kDa protein during evolution of the oxygen-evolving PSII complex.

Table 2 Comparison of cleavage sites of the extrinsic 33 kDa protein by chymotrypsin and V8 protease among cyanobacterium, red alga and higher plant

Protease	Cyanobacterium (*S. elongates*)	Red Alga (*C. caldarium*)	Higher Plant (*Spinach*)
Chymotrypsin	156F, 190F*	159M, 192L	16Y**
V8 protease	181E	182E, 195E	18E**

* Motoki et al. (1998)

** Eaton-Rye and Murata (1989)

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