The 60-kDa precursor to the dithiothreitol-sensitive tetrameric protease of spinach thylakoids: structural similarities between the protease and polyphenol oxidase

Tomohiko Kuwabara*

Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Received 6 June 1995; revised version received 10 July 1995

Abstract **The 60-kDa precursor to the 39-kDa dithiothreitol-sensitive protease was purified from photosystem II membranes of spinach. When partially purified 60-kDa protein was stored at 4°C, the protein was degraded to fragments of 39 and 21 kDa. The 39-kDa fragment was suggested to be identical to the 39-kDa protease from effects of dithiothreitol on these polypeptides. The N-terminal amino acid sequences of the 60-kDa protein and the 39-kDa protease were the same, APILPDVEK-, suggesting that the latter was derived from the N-terminal portion of the former. Immunostaining with polyclonal antibodies against the 60-kDa protein indicated that the 60-kDa protein represents the species that occurs in the native thylakoids. These and other structural properties suggest that the protein might be identical to polyphenol oxidase.**

Key words: Dithiothreitol-sensitive tetrameric protease; Metallo-protease; Photosystem II; Polyphenol oxidase; Spinach thylakoid

1. Introduction

Intracellular levels of individual proteins are controlled by rates of both synthesis and degradation [1,2]. In plant chloroplasts, thylakoid proteins are at risk of injury as a consequence of an excess of light-induced free radicals [3]. Thus, proteolytic systems that specifically degrade damaged proteins must be required for maintenance of the architecture of the photosynthetic machinery. Furthermore, the degradation of proteins might play an essential role in adjusting the stoichiometry of subunits in supramolecular protein complexes, as well as in regulating the stoichiometry among different supramolecular complexes in response to environmental changes. However, little is known about the proteases involved in such degradative processes.

In a previous study [4], we purified a dithiothreitol-sensitive tetrameric protease from PS II membranes of spinach, monitoring the proteolytic activity that degraded the extrinsic 18-kDa protein of PS II. The protease exists as interconvertible 39-kDa monomers and 156-kDa tetramers. The protein has an intramolecular disulfide linkage and reduction of this linkage converts the protein from the active 39-kDa form to an inactive 41-kDa form. The protease was suggested to be a metallo-protease on the basis of the inhibition of its activity by 1,10-phenanthroline.

In the present study, the 60-kDa precursor to the 39-kDa protease was purified. Possible identification of the protein as a polyphenol oxidase is suggested from a comparison of the structural properties of the two proteins.

2. Materials and methods

2.1. Purification of the 60-kDa protein

PS II membranes prepared from spinach thylakoids [4] were suspended in 25 mM sodium phosphate (pH 6.5)/1 M NaCl at a chlorophyll concentration of 2 $mg^{-1} \cdot ml^{-1}$. The suspension was incubated at 0°C for 20 min in darkness and then centrifuged at 35,000 × *g* for 20 min. The supernatant was designated the NaCl extract and was stored frozen at −80°C until use. The extract (13 ml) was loaded on a column of Superdex 200 (2.6 cm i.d. × 60 cm; Pharmacia) that had been equilibrated with 20 mM sodium phosphate (pH 6.5)/0.3 M NaCl (buffer A). Elution was performed with the same buffer at a flow rate of 2 $ml \cdot min^{-1}$ and fractions of 4.6 ml were collected. Fractions containing the proteolytic activity (see below) were pooled and loaded on a column of Chelating Superose (1 cm i.d. × 2 cm; Pharmacia) that had been charged with Ni^{2+} ions, washed with buffer A that contained 1 M NH_4Cl and then equilibrated with buffer A. After loading, the column was washed sequentially with a 5-ml linear gradient of 0–1 M NH_4Cl in buffer A, 5 ml of buffer A that contained 1 M NH_4Cl, a 5-ml linear gradient of 1–0 M NH_4Cl in buffer A, and 5 ml of buffer A, in that order. The 60-kDa protein was finally eluted with buffer A that contained 35 mM imidazole at a flow rate of 1 $ml \cdot min^{-1}$ and fractions of 0.5 ml were collected.

2.2. Proteolytic reaction

The substrate for the proteolytic reaction was the extrinsic 23-kDa protein that had been purified from PS II membranes of spinach [5]. Preparations of protease and substrate were dialyzed separately against 10 mM HEPES-NaOH (pH 7.0) at 7°C for 4 h [6]. They were then combined and incubated at 37°C for 7–12 h. The effects of pH and protease inhibitors were studied as described previously [4].

2.3. Other procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on 12% polyacrylamide gels [4], unless otherwise stated.

Protein concentrations were estimated and amino-terminal amino acid sequences were analyzed, as described previously [5].

Polyclonal antibodies were raised against the 60-kDa protein in rabbits, with the purified 60-kDa protein as antigen, by the standard method, as described previously [7].

3. Results

In a previous study [4], we purified a DTT-sensitive protease of 39 kDa, which could be converted reversibly to a tetramer of 156 kDa. The 39-kDa protease was characteristic in that its oxidized and reduced forms appeared at 39 kDa and 41 kDa, respectively, upon SDS-PAGE [4]. Further studies revealed

*Corresponding author. Fax (81) (298) 53 6614.

Abbreviations: Buffer A, 20 mM sodium phosphate (pH 6.5)/0.3 M NaCl; DTT, dithiothreitol; PPO, polyphenol oxidase; PS, photosystem.

SSDI 0014-5793(95)00911-6

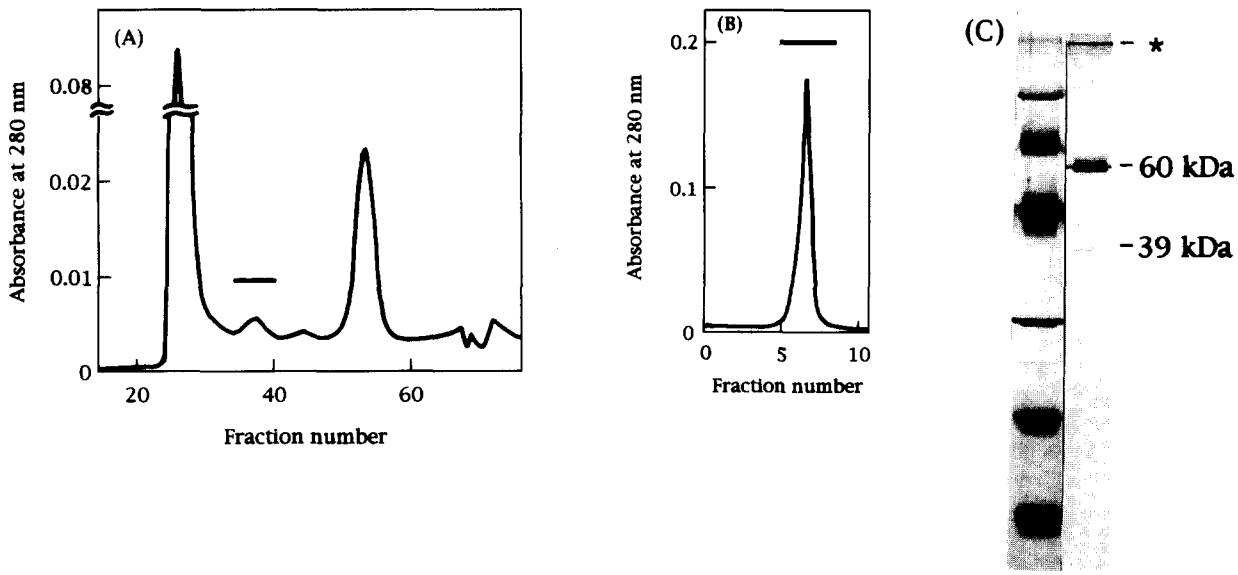

Fig. 1. (A) Gel-filtration chromatography of an NaCl extract on Superdex 200. Fractions were analyzed for the presence of the 60-kDa protein and for proteolytic activity that degraded the 23-kDa protein to a 20-kDa fragment. Fractions 35–40, indicated by a bar, were pooled as the Superdex preparation. (B) Chromatography of the Superdex preparation on Chelating Superose. The figure shows the profile of elution with buffer A plus 35 mM imidazole. The peak fraction, indicated by a bar, was pooled as the purified 60-kDa protein. (C) SDS-PAGE of the purified preparation of the 60-kDa protein (right lane). An asterisk indicates a band of aggregates. The left lane shows mobilities of molecular mass markers (from the top: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa). Note that the 60-kDa protein was neither reduced nor heated, while the markers were reduced with 2 mM DTT and heated at 95°C for 1 min prior to SDS-PAGE. Proteins were visualized by silver-staining.

that the 39-kDa protein had been generated from a 60-kDa protein under low-salt conditions, which were necessary to perform anion-exchange chromatography in the previous study [4]. Therefore, I developed a method for the purification of the 60-kDa protein in one day using buffers that contained 0.3 M NaCl (Fig. 1). By this method, the 60-kDa protein was copurified with the proteolytic activity that degraded the extrinsic 23-kDa protein of PS II to a 20-kDa fragment (see Fig. 4). In the final preparation of the experiment of Fig. 1, 25 μg proteins were recovered from 13 ml of NaCl extract. The preparation yielded a band of a 60-kDa protein, as well as a faint band of a 39-kDa polypeptide and a band of aggregates, when the sample was not reduced prior to SDS-PAGE (Fig. 1C). When reduced with 5 mM DTT, the 60-kDa protein and the 39-kDa polypeptide shifted to the positions of 62 and 41 kDa, respectively, and the band of aggregates disappeared (data not shown, see Fig. 3 and [4] for the 62-kDa and the 41-kDa species, respectively). The effects of DTT suggest that (i) the 60-kDa protein has an intramolecular disulfide linkage like the 39-kDa protease [4], (ii) the 39-kDa polypeptide may be identical to the 39-kDa protease, and (iii) the band of aggregates was generated by formation of an intermolecular disulfide linkage between 60-kDa proteins.

When the Superdex preparation was stored at 4°C for days, the 60-kDa protein decreased and polypeptides of 39 and 21 kDa accumulated, although these polypeptides also decreased by prolonged storage (Fig. 2). This result suggests that the 60-kDa protein was first degraded to the 39- and 21-kDa fragments and then to smaller fragments. When the samples of Fig. 2 were reduced with DTT prior to SDS-PAGE, the 60-kDa protein and the 39-kDa fragment changed their mobility, as those in the purified preparation did, while the mobility of the 21-kDa fragment was unchanged (data not shown). Thus, it is likely that the 39-kDa protease, which is supposed to be identical to the 39-kDa fragment, was generated by degradation of the 60-kDa protein. When the purified preparation was similarly stored, the 60-kDa protein was degraded but no fragments were accumulated during the course of the storage (data not

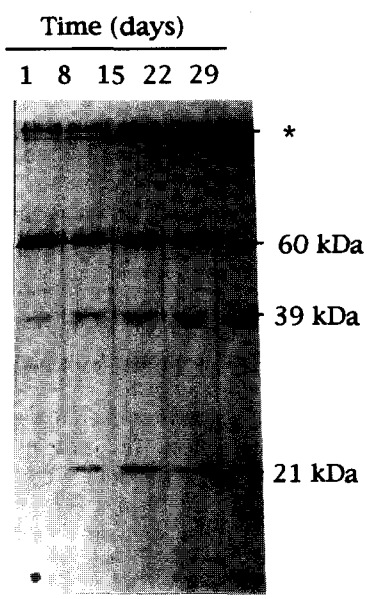

Fig. 2. Degradation of the 60-kDa protein in the Superdex preparation during storage. The Superdex preparation was stored in buffer A at 4°C for designated periods and subjected to electrophoresis without reduction. Proteins were visualized by silver-staining.

shown). This observation suggests that some factor(s) in the Superdex preparation facilitated the degradation of the 60-kDa protein to the 39- and 21-kDa fragments and/or retarded the degradation of the two fragments.

The N-terminal amino acid sequences of the 60-kDa protein and the 39-kDa protease, which had been prepared by the previously described method [4], were found to be the same, namely, APILPDVEK-. These results suggest that the 39-kDa protease was derived from the N-terminal portion of the 60-kDa protein.

A fresh NaCl extract was examined by Western blotting with polyclonal antibodies against the 60-kDa protein to know whether a larger protein species related to the 60-kDa protein might exist. When the non-reduced and reduced samples were examined, only the 60-kDa and the 62-kDa polypeptides were immunostained, respectively (Fig. 3). This result confirms that the immunostained polypeptide is the 60-kDa protein and indicates that the 60-kDa protein represents the original species in the thylakoids. With the anti-60-kDa protein antibodies, the 39- and 21-kDa fragments, which were generated during storage of the Superdex preparation (Fig. 2), were also immunostained (data not shown), supporting that these fragments were derived from the 60-kDa protein.

Fig. 4 shows the effects of DTT on the proteolytic cleavage of the 23-kDa protein by the 60-kDa protein. The 23-kDa protein was degraded to a fragment of 20 kDa in the absence of DTT. When DTT at 0.2 to 1.0 mM was present, degradation was suppressed but a smeared band was generated above the band of the 23-kDa protein. In the presence of DTT at 5–25 mM, neither degradation nor the smeared band was apparent. These results suggest that the smeared band represents an intermediate in the proteolytic reaction or that the partially reduced protease acted on the substrate differently from the non-reduced protease. A similar smeared band was observed previously after the reaction of the 39-kDa protease with the 18-kDa protein from spinach [4].

The effects of pH and protease inhibitors on the proteolysis were also examined, and they were found to be similar to those observed with the 39-kDa protease [4]; proteolysis occurred from pH 5 to pH 10 and was inhibited by 1,10-phenanthroline but not by trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride (data not shown). These results suggest that the 60-kDa protein had proteolytic activity similar to that of the 39-kDa protease. Alternatively, the activity was due to the

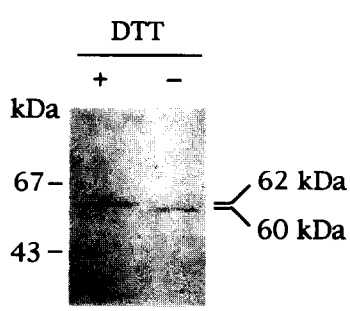

Fig. 3. The effect of DTT on the electrophoretic mobility of the 60-kDa protein. An NaCl extract, either reduced with 5 mM DTT (+) or non-reduced (−), was subjected to SDS-PAGE, Western blotted and immunostained with polyclonal antibodies against the 60-kDa protein and peroxidase-conjugated second antibody. The positions of molecular mass markers are shown on the left.

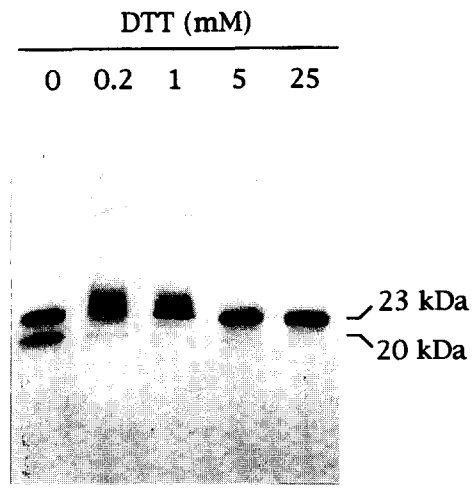

Fig. 4. Effects of DTT on the degradation of the 23-kDa protein by the 60-kDa protein. The proteolytic reaction was performed with the purified preparation of 60-kDa protein equivalent to 0.1 μg protein and 2 μg of 23-kDa protein in the presence of designated concentrations of DTT. Then, all samples were reduced with 2 mM DTT and subjected to SDS-PAGE on a 15% polyacrylamide gel in the presence of 4 M urea [4]. Proteins were visualized by staining with Coomassie brilliant blue.

39-kDa protease, generated from the 60-kDa protein during the reaction.

4. Discussion

In the present study, a 60-kDa protein was purified as a protease that degraded the extrinsic 23-kDa protein of PS II. It was necessary to use buffers that contained 0.3 M NaCl and to complete the purification within one day in order to minimize degradation of the 60-kDa protein during the course of the purification.

The peak that corresponded to the 60-kDa protein during elution from Superdex 200 was often but not always conspicuous [8]. This observation suggests that the amount of the protein that had been bound to the PS II membranes varied from preparation to preparation, and that some of the protein might sometimes be lost during preparation of the PS II membranes. This interpretation is supported by the recent finding that the immunostainable 60-kDa polypeptide is present at a rather constant level in the intact thylakoids (Masuda and Kuwabara, unpublished).

A homology search indicated that the N-terminal amino acid sequence of the 60-kDa protein, APILPDVEK-, is identical to that of the 64-kDa kinase from spinach that phosphorylated the light-harvesting chlorophyll *a/b*-protein complex [9]. However, it was suggested recently that the sequence could have been derived from polyphenol oxidase (PPO) that had not been separated from the kinase on SDS-PAGE (I. Ohad, unpublished). The mature PPOs from other plants have similar N-terminal sequences [10–13]. Thus, the 60-kDa protein is likely to be identical to PPO. The molecular mass of PPO was thought initially to be 40–43 kDa [14]. However, Lanker et al. [15] demonstrated the presence of a 60/63-kDa polypeptide (doublet band) that was cross-reactive with antibodies against PPO but was inactive in terms of PPO activity. Robinson and Dry [16] indicated that the native mass of the mature PPO protein of

Vicia faba was 58/60 kDa and that it was easily degraded to polypeptides of 41/43 kDa during purification, with removal of a C-terminal moiety. Golbeck and Cammarata [17] revealed that the 42.5-kDa PPO of spinach, which was probably the product of degradation of the mature PPO protein, formed a tetramer of 158 kDa that was reversibly convertible into the monomers. These features are very similar to those of the 60-kDa protein and the 39-kDa protease [4]. Although the 60-kDa protein did not yield a doublet band on SDS-PAGE, it behaved like a 62-kDa protein upon reduction. Thus, it is likely that the doublet bands of PPO could result from a mixed population of reduced and non-reduced protein molecules. Recently, we revealed that the anti-60-kDa protein IgG specifically inhibited a PPO activity of spinach thylakoids that was measured with DL-β-(3,4-dihydroxyphenyl)alanine as substrate in the presence of an activator, linolenic acid [17] (Masuda and Kuwabara, unpublished). This finding supports the identification of the 60-kDa protein as PPO.

Cytochemical and immunocytochemical studies of PPO have revealed that the protein is localized in various types of plastid, and is associated with thylakoid membranes in chloroplasts [14]. Cloning of cDNAs and genes yielded deduced amino acid sequences that indicated that the protein is translated as a precursor with a transit sequence that would direct the precursor into the thylakoid lumen [10–13]. Nevertheless, the physiological function of this protein remains to be established [14] because there is no reason to believe that *o*-diphenol substrates of PPO would be present in the thylakoid lumen. In this context, it is noteworthy that the protein is involved in the architecture of PS II [18,19]. Given the identity of the 60-kDa protein to PPO, it is possible that the protein functions as a protease for degradation of PS II proteins, especially when proteins of PS II are damaged and/or liberated from the membranes. It seems also possible that PPO activity could be correlated with the proteolytic activity: proteolysis could be initiated by oxidative modification of amino acid residues.

Acknowledgements: The author thanks K. Suzuki for conducting preliminary experiments. This research was supported by a Grant-in-Aid for Cooperative Research (no. 04304004) from the Ministry of Education, Science and Culture of Japan and by the University of Tsukuba Project Research Fund.

References

[1] Hershko, A. and Ciechanover, A. (1982) Annu. Rev. Biochem. 51, 335–364.
[2] Vierstra, R.D. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 385–410.
[3] Barber, J. and Andersson, B. (1992) Trends Biochem. Sci. 17, 61–66.
[4] Kuwabara, T. and Hashimoto, Y. (1990) Plant Cell Physiol. 31, 581–589.
[5] Kuwabara, T. and Suzuki, K. (1994) Plant Cell Physiol. 35, 665–675.
[6] Kuwabara, T. (1992) FEBS Lett. 300, 127–130.
[7] Murata, N., Miyao, M., Omata, T., Matsunami, H. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.
[8] Kuwabara, T. and Suzuki, K. (1995) Plant Cell Physiol. 36, 495–504.
[9] Gal, A., Herrmann, R.G., Lottspeich, F. and Ohad, I. (1992) FEBS Lett. 298, 33–35.
[10] Shahar, T., Hennig, N., Gutfinger, T., Hareven, D. and Lifschitz, E. (1992) Plant Cell 4, 135–147.
[11] Cary, J.W., Lax, A.R. and Flurkey, W.H. (1992) Plant Mol. Biol. 20, 245–253.
[12] Hunt, M.D., Eannetta, N.T., Yu, H., Newman, S.M. and Steffens, J.C. (1993) Plant Mol. Biol. 21, 59–68.
[13] Newman, S.M., Eannetta, N.T., Yu, H., Prince, J.P., Carmen de Vicente, M., Tanksley, S.D. and Steffens, J.C. (1993) Plant Mol. Biol. 21, 1035–1051.
[14] Vaughn, K.C., Lax, A.R. and Duke, S.O. (1988) Physiol. Plant. 72, 659–665.
[15] Lanker, T., Flurkey, W.H. and Hughes, J.P. (1988) Phytochemistry 27, 3731–3734.
[16] Robinson, S.P. and Dry, I.B. (1992) Plant Physiol. 99, 317–323.
[17] Golbeck, J.H. and Cammarata, K.V. (1981) Plant Physiol. 67, 977–984.
[18] Vaughn, K.C. and Duke, S.O. (1981) Protoplasma 108, 319–327.
[19] Lax, A.R. and Vaughn, K.C. (1991) Plant Physiol. 96, 26–31.