Volume 300, number 2, 127–130 FEBS 10864 March 1992

Characterization of a prolyl endopeptidase from spinach thylakoids

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Received 10 January 1992; revised version received 12 February 1992

A prolyl endopeptidase (PEPase, EC 3.4.21.26) that specifically cleaves the 18-kDa protein of photosystem II was extracted from photosystem II membranes with 1 M NaCl. Proteolytic activity measured with artificial substrates was less than a quarter of that with the protein. Studies on inhibition of the proteolysis by an artificial substrate suggested that the protease recognizes the scissile prolyl bond. The protease was inhibited by $CuCl_2$, but not by diisopropyl fluorophosphate or *p*-chloromercuriphenylsulfonic acid. These findings suggest that the protease represents a new class of PEPase. The specificity of the enzyme is discussed in relation to the structure of the 18-kDa protein.

Prolyl endopeptidase; Photosystem II membrane; Membrane-bound protease; Destructive processing; Chloroplast protease; Spinach

1. INTRODUCTION

Intracellular proteases play an important role in the macroeconomics of living cells. Digestive enzymes, as in plant seeds for example, produce amino acids by digestion of storage proteins; processing enzymes produce mature functional proteins; and proteases involved in protein turnover eliminate overcrowding by degrading damaged or unnecessary proteins and recycling the degradation products. To regulate a particular function in a complex biological system, it would be convenient if there were proteases that could target specific proteins, and such proteases are exemplified by processing enzymes. In plants, proteolysis that is apparently specific to a particular species of protein has been reported in the case of certain degradative processes, such as the photodegradation of NADPH-protochlorophyllide oxidoreductase [1], the photodegradation of D1 protein of the photosystem II (PS II) reaction center [2], the degradation of unassembled small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [3], and the degradation of the apoprotein of the light-harvesting complex (LHC II) [4]. It is likely that plants can pay a relatively high cost for the construction and maintenance of the cellular machinery required for photosynthesis, and the housekeeping system of the chloroplast is expected to include a variety of specific proteases.

In this study, attention was directed to the specific proteolysis of the extrinsic 18-kDa protein of the PS II water-oxidation complex. The protease involved in the degradation has the characteristics of a prolyl endopeptidase (PEPase, EC 3.4.21.26) and seems to recognize some aspects of the higher-order structure of the substrate.

2. MATERIALS AND METHODS

N-α-Carbobenzoxy-L-lysyl-L-proline 4-methoxy-β-naphtylamide (ZKP-4MNA) and 4-methoxy-β-naphtylamine (4MNA) were purchased from Bachem (Bubendorf). *N*-Carbobenzoxy-glycyl-L-proline *p*-nitroanilide (ZGP-*p*NA) was from Seikagaku Kogyo (Tokyo). *N*-α-Carbobenzoxy-L-lysyl-L-proline (ZKP), which was synthesized by standard methods from *N*-α-carbobenzoxy-*N*-ε-*t*-butyloxycarbonyl-L-lysine and L-proline benzyl ester and purified to homogeneity by HPLC on an ODS column, was a gift from Dr. M. Tamaki, Tohoku University. Diisopropyl fluorophosphate (DFP) and *p*-chloromercuriphenylsulfonic acid (*p*CMPS) were obtained from Wako (Osaka) and Sigma (St Louis). *N*-Carbobenzoxy-L-thioprolyl thiazolidine (Z-TPT) was a gift from Prof. T. Yoshimoto, Nagasaki University. Antiserum to spinach D1 protein was a gift from Dr. M. Ikeuchi, Riken.

PS II membranes were prepared from spinach thylakoids as in [5], modifying the original procedure [6]. The membranes were suspended in 25 mM MES-NaOH (pH 6.5)/1 M NaCl at a chlorophyll concentration of 2 mg/ml. The suspension was centrifuged at 35,000 × *g* for 20 min. The NaCl extract obtained as the supernatant was stored at −80°C until use.

For proteolysis of the 18-kDa protein, the extract was dialyzed against 10 mM MES–NaOH (pH 6.0) at 7°C for 4 h, diluted 2-fold with the dialysis medium, and incubated at 37°C for 4 h. Dialysis was performed with Desalyzer 1 (Atto, Tokyo) [5] using the dialysis membrane, Spectra/Por 3 (Spectrum, Los Angeles). SDS-PAGE was carried out in the presence of 4 M urea [5]. Gel plates were stained with Coomassie blue R-250 [5].

Gel-filtration chromatography of the NaCl extract was performed with Superose 12 HR 10/30 (Pharmacia) as in [5].

Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; MES, 2-(*N*-morpholino)-ethanesulfonic acid; *p*CMPS, *p*-chloromercuriphenyl-sulfonic acid; PEPase, prolyl endopeptidase; PS II, photosystem II; SDS, sodium dodecylsulfate; ZGP-*p*NA, *N*-carbobenzoxy-glycyl-L-proline *p*-nitroanilide; ZKP-4MNA, *N*-α-carbobenzoxy-L-lysyl-L-proline 4-methoxy-β-naphthylamide; Z-TPT, *N*-carbobenzoxy-L-thioprolyl thiazolidine.

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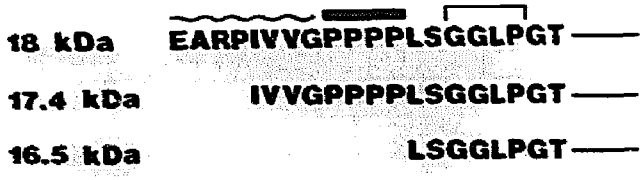

Fig. 1. N-Terminal amino-acid sequences of the 18-kDa protein and degradation products of 17.4 and 16.5 kDa. The sequences were determined in the previous studies [7,8]. Predicted secondary structures, a β-sheet from E1 to G8, a rigid helix from P8 to P12, and a β-turn from G15 to P18, are indicated above the sequence.

3. RESULTS AND DISCUSSION

Previous studies showed that when the NaCl extract was dialyzed against a low-salt buffer, the 18-kDa protein in the extract van degraded to a fragment of 16.5 kDa [7]. Moreover, when the proteolytic reaction was performed after removal of nucleic acids by hydrophobic chromatography, another degradation product of 17.4 kDa was produced in addition to the 16.5-kDa fragment [8]. The N-terminal amino acid sequences of the degradation products (Fig. 1) suggested that the proteolysis occurred at the carboxyl side of the 4th and 12th proline residues and the responsible protease may be a PEPase [7,8]. However, it has not been clarified whether the protease recognizes the proline residue at the -1 position or a higher-order structure of the substrate like processing enzymes. To elucidate this artificial substrates were tested, and a new reaction procedure introduced which consisted of the brief dialysis and subsequent incubation at the optimum temperature, as described in section 2 in place of the reaction by prolonged dialysis [5,8]. It was confirmed that the fundamental profile of the reaction by the new procedure was the same as that by the previous one. The proteolysis occurred over the range from pH 4–9, and low-salt conditions were required for the proteolysis [7]. These characteristics are indicative of ionic interactions between the protease and substrate. Since the isoelectric point of the 18-kDa protein is 9.5 [9], the protease should be negatively charged at those pH's at which it functions.

Two artificial substrates of PEPase, ZKP-4MNA and ZGP-*p*NA, were tested for cleavage by the extract. The extent of hydrolysis of ZKP-4MNA was very low (Table 1), but the activity was evident for at least 4 h. No hydrolysis of ZGP-*p*NA could be detected. On the other hand, the reaction in the absence of artificial substrate converted almost all of the 18-kDa protein to the 16.5-kDa fragment (see the lanes of control reactions of Figs. 2 and 3). This activity was calculated to be about 4 times that with ZKP-4MNA (Table 1). These results suggest that the 18-kDa protein is a better substrate than the artificial ones. It is noteworthy that ZKP-4MNA strongly inhibited the proteolysis of the protein, while the inhibitory action of ZGP-*p*NA was minimal

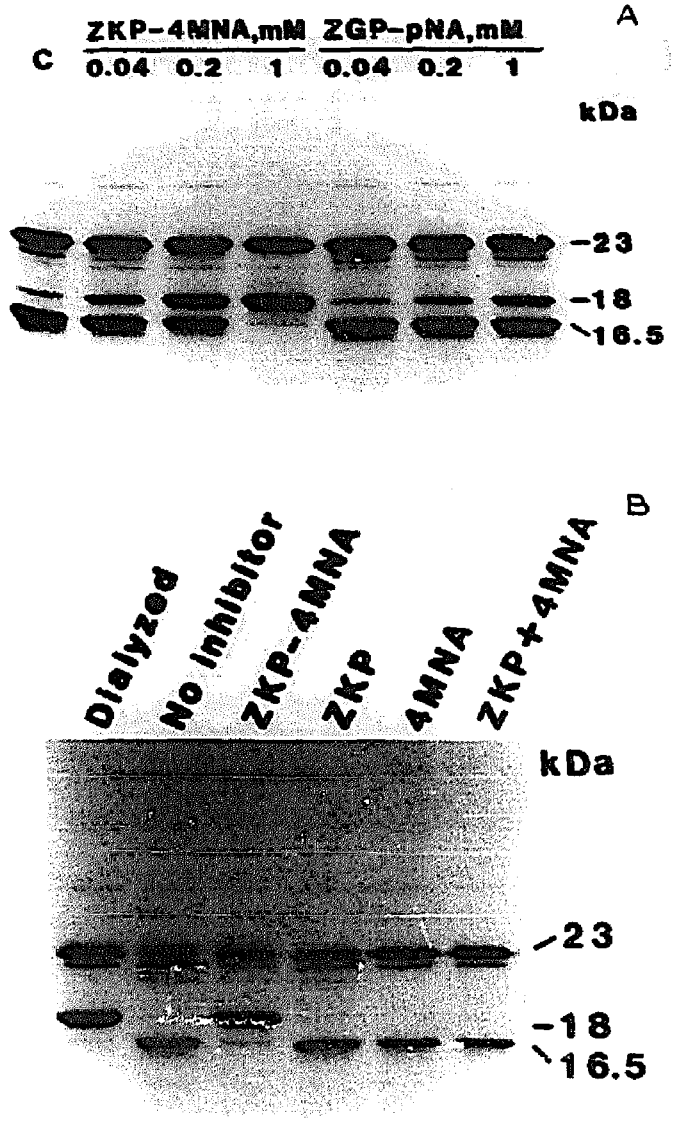

Fig. 2. Effects of (A) ZKP-4MNA and ZGP-*p*NA and of (B) compounds related to ZKP-4MNA on the degradation of the 18-kDa protein. The NaCl extract was subjected to the proteolytic reaction in the presence of the designated compounds. In the experiment of B, the concentration of each compound was 1 mM. 'Dialyzed' refers to a sample to which 2% SDS was added immediately after dialysis.

(Fig. 2A). To determine which aspects of the molecular structure of ZKP-4MNA are essential to the inhibition, the effects of ZKP and 4MNA, alone and in combination, were compared with those of ZKP-4MNA (Fig. 2B). It was clear that neither compound was inhibitory, and these results strongly suggest that the prolyl bond is essential for the inhibition. The difference between ZKP-4MNA and ZGP-*p*NA may be ascribable to the positive charge on the lysine residue of the former which would compete with the positively charged 18-kDa protein.

The PEPases reported to date are sensitive to DFP and *p*-chloro-mercuriphenyl compounds [14]. However, the proteolysis of the 18-kDa protein was not inhibited

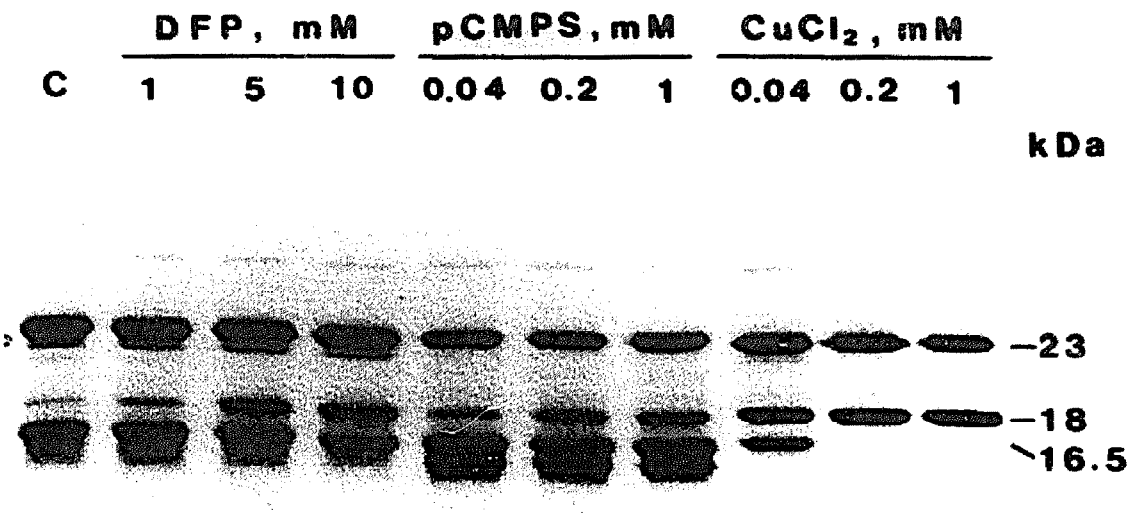

Fig. 3. Effects of DFP, *p*CMPS, and $CuCl_2$ on the degradation of the 18-kDa protein. The NaCl extract was subjected to the proteolytic reaction in the presence of the designated inhibitors. Lane C shows results of a control reaction.

to any significant extent by these reagents (Fig. 3). Furthermore, this activity was not inhibited by 2 μM Z-TPT, a specific inhibitor of PEPase, recently synthesized by Tsuru et al. [15]. These results indicate that the protease is different from previously identified PEPases. In a survey of inhibitors, $CuCl_2$ at 0.2 mM was found to be effective (Fig. 3). As for other metals, $ZnCl_2$ was inhibitory, but $MgCl_2$, $CaCl_2$ and $MnCl_2$ were not inhibitory at 1 mM. The protease was inactivated by ionic detergents (SDS and cetyl pyridinium chloride) but not by non-ionic detergents (Triton X-100 and Tween-20) at 0.1% (w/v). The activity was not affected at all by Mg-ATP, DTT, *N*-ethylmaleimide, E-64, 1,10-phenanthroline, EDTA, or phenylmethylsulfonyl fluoride at 1 mM. These results indicate that this protease is not identical to any proteases of chloroplast origin reported elsewhere [16–20].

Table 1

PEPase activity of the NaCl extract from PS II membranes

Substrate	Activity (nmol/ml extract/h)
18-kDa protein[b]	2.5
ZKP-4MNA[c]	0.6
ZGP-*p*NA[d]	0.0

The NaCl extract was dialyzed and diluted as described in section 2. The sample was incubated without additive, or with 0.56 mM ZKP-4MNA or 0.2 mM ZGP-*p*NA at 37°C for up to 4 h. The concentration of the 18-kDa protein in the reaction mixture was about 5μM[a].

[a] Calculated from the ratio of chlorophyll to 18-kDa protein of 220:1 (mol/mol) in the PS II membranes [10], and on the basis of 100% extraction of the 18-kDa protein by the NaCl wash [11].

[b] Estimated from the result that the 18-kDa protein in this sample was completely degraded within 4 h. The accumulation of the 16.5-kDa fragment during the reaction suggested that contribution of proteases other than the PEPase to the degradation of the 18-kDa protein is very little.

[c] Production of free 4MNA was measured according to Taylor et al. [12].

[d] Production of free *p*NA was measured according to Sarath et al. [13].

On gel-filtration chromatography of the extract, the proteolytic activity was eluted at a position that corresponded to a molecular weight of 34–45 kDa, being separated from the 18-kDa and 23-kDa proteins. This preparation showed no Coomassie blue-stainable polypeptides but more than ten polypeptides upon silver-staining. Polypeptides of the protease have not yet been identified. The partially purified PEPase could not degrade the purified preparations [7] of the 33-kDa Mn-stabilizing protein and the 23-kDa protein, and proteins extracted from the NaCl-treated PS II membranes with 1% (w/v) Triton X-100 that included antenna chlorophyll-binding proteins (CP47 and CP43) and LHC II. It was also shown by immunostaining that D1 protein was not degraded by the PEPase. The PEPase was also unable to degrade basic proteins from other origins, such as lysozyme (hen egg), chymotrypsinogen A (bovine pancreas) and cytochrome *c* (horse heart).

The accumulation of the 16.5-kDa fragment during the course of the reaction indicates that this fragment is hardly digested at all by the PEPase, even though it is supposed to contain 8 proline residues [7,21]. This observation can be explained in terms of the general inability of PEPase to digest (folded) proteins [14]. Prediction of the secondary structure [22] of the 18-kDa protein suggests that the scissile bond (Pro^{12}–Leu^{13}) is preceded by a β-sheet and a rigid helix formed by the 4 sequential proline residues and is followed by a β-turn (Fig. 1). This profile suggests that the N-terminal portion of the protein is free from folding and, thus, is susceptible to the protease. The results in Fig. 2 suggest that the PEPase recognizes the scissile prolyl bond. More-over, the inability of the PEPase to digest the

artificial substrates and other proteins strongly suggest that a higher-order structure of the substrate is required for the cleavage of the prolyl bond.

Acknowledgements: The author thanks Dr. M. Tamaki for ZKP, Prof. T. Yoshimoto for Z-TPT, and Dr. M. Ikeuchi for the antiserum to D1 protein.

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