Vacuolar processing enzyme is self-catalytically activated by sequential removal of the C-terminal and N-terminal propeptides

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Abstract A vacuolar processing enzyme (VPE) responsible for maturation of various vacuolar proteins is synthesized as an inactive precursor. To clarify how to convert the VPE precursor into the active enzyme, we expressed point mutated VPE precursors of castor bean in the *pep4* strain of *Saccharomyces cerevisiae*. A VPE with a substitution of the active site Cys with Gly showed no ability to convert itself into the mature form, although a wild VPE had the ability. The mutated VPE was converted by the action of the VPE that had been purified from castor bean. Substitution of the conserved Asp-Asp at the putative cleavage site of the C-terminal propeptide with Gly-Gly abolished both the conversion into the mature form and the activation of the mutated VPE. In vitro assay with synthetic peptides demonstrated that a VPE exhibited activity towards Asp residues and that a VPE cleaved an Asp-Gln bond to remove the N-terminal propeptide. Taken together, the results indicate that the VPE is self-catalytically maturated to be converted into the active enzyme by removal of the C-terminal propeptide and subsequent removal of the N-terminal one.

Key words: Asparaginyl endopeptidase; Castor bean; Legumain homologue; Proprotein processing; Vacuolar processing enzyme; Vacuole

1. Introduction

In higher plants, proprotein precursors of various vacuolar proteins are converted post-translationally to their respective mature forms by the action of vacuolar processing enzymes (VPEs) [1–7]. The molecular structure of the enzyme was originally reported for castor bean VPE [3]. VPE homologues have been found in plants (soybean [7], Jack bean [8], *Arabidopsis* [5,6], vetch [9] and citrus [10]) and animals (*Schistosoma mansoni* [11], human [12] and mouse [13]). They are cysteine proteinases with a specificity towards asparaginyl bonds and exhibit no identity to cysteine proteinases in the papain family [14]. A VPE precursor is composed of a signal peptide, an N-terminal propeptide, the mature VPE domain and a C-terminal propeptide, as described before [2,3]. Previously we reported that a yeast (*Saccharomyces cerevisiae*) transformant expressing a VPE precursor of castor bean accumulates the mature form in the vacuoles [15]. The mature protein had a vacuolar processing activity, in contrast the precursor had no activity. Analysis of mutants having no activity suggested that the conversion of the proprotein precursor of VPE into an active form might be mediated self-catalytically [15]. We show here that sequential removal of C-terminal and N-terminal propeptides occurs self-catalytically to produce active VPE of castor bean.

2. Materials and methods

2.1. Construction of plasmids, yeast strains, culture conditions and transformation

We constructed the plasmid pYES2-*ppVPE* in which a cDNA encoding a precursor to castor bean VPE was flanked with the *GAL1* promoter and *CYC1* termination sequences of the yeast expression vector, pYES2 (Invitrogen, USA) and expressed the VPE in yeast *pep4* strains (YW7-6D; *MAT*a *leu2 ura3-52 pep4-3*) of *S. cerevisiae*, as described previously [15].

2.2. Site-directed mutagenesis of VPE

To mutagenize castor bean VPE, we used a QuikChange Site-directed Mutagenesis Kit (Stratagene, USA) in accordance with the manufacturer's directions. Three sense oligonucleotide primers (5′-GCAATATGGCAACCAAGGCATCAAAGCAGATAAGC-3′, 5′-CAAGAGATCAGAAAATGGGGGGGAGAAGAAGAAAGAAA-TC-3′ and 5′-GTCGCCTCTCGTAGGTGGCTGGGGCTGCTTAA-AATC-3′) and three antisense oligonucleotide primers (5′-GCTTAT-CTGCTTTGATGCCTTGGTTGCCATATTGC-3′, 5′-GATTTCTT-TCTTCTTCTCCCCCCATTTTCTGATCTCTTG-3′ and 5′-GAT-TTTAAGCAGCCCCAGCCACCTACGAGAGGCGAC-3′) were used to introduce mutations into the VPE gene. Each of the residues Ser-322, Ser-376, Asp-429 and Asp-430 was substituted with a Gly residue. The nucleotide sequences of the mutant VPEs were subsequently determined with an automatic DNA sequencer (model 377A, Perkin Elmer/Applied Biosystems, USA) to confirm the respective mutation.

2.3. Preparation of yeast cell lysates and immunoblot analysis

Whole cell lysates were prepared from SC (synthetic medium)-galactose-grown transformant cells, as described previously [16]. Aliquots of the lysates were subjected to SDS-PAGE and subsequent immunoblot analysis. Immunoblot analysis was performed essentially as described previously [15,17].

2.4. Purification of a VPE and in vitro processing of proVPE

We purified a VPE from the soluble matrix fraction of protein-storage vacuoles of castor bean, as described previously [1]. VPE precursor in the transformant of a mutant (C222G [15]) was used as substrate for in vitro processing. The vacuolar processing enzyme (0.6 mU) was mixed with the extract of the transformant in 20 μl of 0.1 M sodium acetate (pH 5.5), 0.1 M dithiothreitol and 0.1 mM EDTA with or without *N*-ethylmaleimide (NEM). The reaction products were incubated for 0, 30 and 120 min at 37°C. The reaction products were subjected to SDS-PAGE on a 12.5% acrylamide gel and subsequent immunoblot analysis.

2.5. Isolation and suborganellar fractionation of protein bodies from dry seeds of castor bean

Protein bodies were prepared from dry seeds of castor bean by the glycerol-isolation method, as described previously [4,18]. After lysis of the protein bodies by addition of a hypotonic buffer solution of 10 mM Tris-HCl (pH 7.5), the homogenate was centrifuged at 100 000 × *g* for 20 min at 4°C. We used the supernatant fraction as

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vacuolar matrix of castor bean for immunoblot analysis with VPE-specific antibodies.

2.6. Assay for vacuolar processing activity

The activity of VPE was assayed essentially as described previously [1]. A chemically synthesized decapeptide, Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr, was used as the substrate [19]. The reaction mixture contained 9 nmol of the decapeptide and the crude extract in 10 μl of 50 mM sodium acetate (pH 5.5), 50 mM dithiothreitol and 0.1 mM EDTA. The mixture was incubated for 0.5 h at 37°C and the products of the reaction were subjected to analytical capillary electrophoresis (HP^{3D} Capillary Electrophoresis System, Hewlett-Packard, Germany) at 25°C and 30 kV in 20 mM sodium borate buffer (pH 9.2). Electrophoresis was monitored in terms of absorbance at 200 nm. The VPE cleaves only the peptide bond on the C-terminal side of the Asn residue of the substrate decapeptide to generate an N-terminal pentapeptide (P1) and a C-terminal pentapeptide (P2). One unit of activity was defined as the amount that liberated 1 μmol of pentapeptide P1 per minute under these conditions.

2.7. In vitro processing and determination of the cleavage sites of a substrate peptide by the VPE

Four peptides were chemically synthesized with a peptide synthesizer (model 430A, Perkin Elmer/Applied Biosystems, USA). The cleavage sites of a peptide derived from the proprotein precursor of 2S albumin in *Arabidopsis* were determined essentially as described previously [20]. The products of the reaction were characterized by a protein sequencer (model 494, Perkin Elmer/Applied Biosystems, USA) and mass spectrometry (Sciex API-300, Perkin Elmer, USA).

3. Results and discussion

3.1. Conversion of a proVPE to the mature form occurs self-catalytically

Previously we proposed that Cys-83, His-180 and Cys-222 residues of castor bean enzyme are essential for proteolytic activity and suggested that the conversion of an inactive precursor into an active VPE might be mediated self-catalytically [15]. To clarify whether the conversion occurs self-catalytically, we expressed a mutant VPE precursor, proVPE (C222G), of castor bean that exhibited no processing activity in yeast (*S. cerevisiae*) and used it as a substrate for an in vitro processing assay. The transformant accumulated only the VPE precursor, proVPE (C222G), but not the mature protein, mVPE (Fig. 1, lane 1). After the proVPE (C222G) was incubated without any enzyme, no conversion was observed (Fig. 1, lane 2). During 30- and 120-min incubations of proVPE (C222G) with a purified 37-kDa VPE from castor bean (cbVPE), the amount of proVPE (C222G) decreased concomitantly with an increase in the amount of the mVPE (C222G) with the same mobility of the wild mVPE on an SDS-gel (Fig. 1, lanes 3, 4 and 6). It has been shown that the VPE expressed in yeast is glycosylated, but the purified cbVPE from the plants is not [15] (discussed below). This caused the different mobility of the mVPE from the transformant (mVPEsc) from that of the purified cbVPE on the SDS-gel (Fig. 1). The conversion of the proVPE (C222G) into the mVPE (C222G) was inhibited in the presence of 2 mM NEM (Fig. 1, lane 5). It has been shown that VPE is inhibited by thiol reagents, such as NEM, *p*-mercuribenzoate and monoiodic acetic acid [1,20,21]. The result indicated that an exogenous VPE converted the precursor of the inactive VPE (C222G) into the mature form. Taken together, these findings indicated that the proVPE is self-catalytically converted into the mVPE. This was also supported by the evidence that the conversion of proVPE into the mature form was detected in the yeast transformant with the wild VPE (Fig. 1, lane 6), although the conversion was not detected in the transformants with the inactive VPEs (C222G, H180G and C83G) [15]. This raises the question: where is the processing site of the VPE precursor?

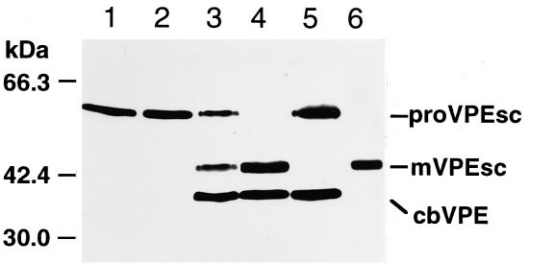

Fig. 1. In vitro conversion of inactive VPE precursor into the mature form by a purified VPE from castor bean. Inactive mutant VPE (C222G) of castor bean was expressed in *S. cerevisiae* (*pep4* strain). The transformants accumulated the VPE precursor (proVPEsc, lane 1), in contrast to the accumulation of the mature VPE (mVPEsc) in the transformants with the wild VPE gene (lane 6). The proVPEsc in the transformants was incubated in the absence (lane 2) or presence (lanes 3–5) of 0.6 mU of purified VPE (cbVPE) from castor bean at 37°C for 0.5 h (lane 3) and 2 h (lanes 2, 4 and 5). In lane 5, the extract was incubated with 2 mM NEM, an inhibitor of VPE. The products of the reaction were subjected to SDS-PAGE and subsequent immunoblot analysis with VPE-specific antibodies. Both proVPEsc and mVPEsc are glycosylated, but purified VPE from castor bean is not (see Fig. 4). The molecular mass of each marker protein is given on the left in kDa.

3.2. Removal of the N-terminal propeptide at di-aspartic acid site occurs in a self-catalytic manner

To answer the preceding question, we compared the sequences around the predicted N-terminal and C-terminal processing sites among six VPE homologues in Figs. 2A and 4A, respectively. The N-terminal amino acid of the castor bean enzyme was blocked, but that of Jack bean is reported to be a Glu residue at position 36 [22] (Fig. 2A, closed circle). The cleavage of the N-terminal propeptide of the Jack bean enzyme occurs after the Asp residue at position 35 (Fig. 2A). The respective Asp residues are conserved in these VPE homologues (Fig. 2A). This suggests that the N-terminal propeptide of castor bean enzyme might be cleaved at Asp-57. To examine whether Asp-57 is the processing site, we synthesized a peptide composed of 15 amino acids from Ala-48 to Arg-62 of castor bean VPE (Fig. 2A, underlined sequence, Fig. 2C). During incubation of the peptide with the VPE, the peptide was hydrolyzed to produce mainly two peptide fragments (Fig. 2B). This revealed that the enzyme cleaved the propeptide at a site between Asp-10 and Gln-11 (Fig. 2B,C). Two minor fragments, which were composed of Ala-1–Asp-8 and Ala-1–Asp-9, were also detected (data not shown). This suggests that the removal of the N-terminal propeptide of the castor bean VPE can occur self-catalytically at Asp-57 (Fig. 2A).

It has been shown that VPE homologues have a specificity towards asparaginyl bonds [14]. To examine whether VPE can cleave a peptide bond at the C-terminal side of an Asp residue, we synthesized peptides containing Asn and Asp residues to use as substrates of castor bean VPE (Fig. 3). VPE cleaved a peptide SESENGLEET at the Asn residue (Fig. 3), as described previously [1]. The peptide was derived from the sequence around the processing site of a major seed protein, 11S

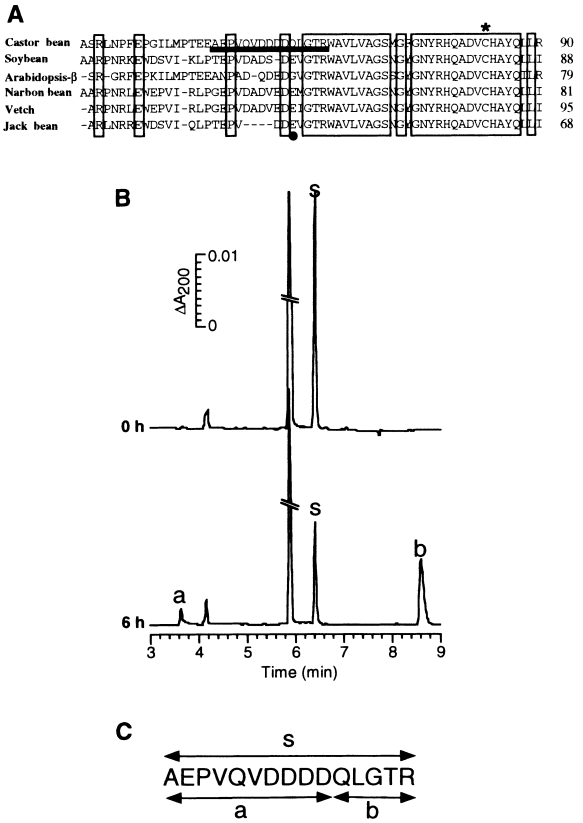

Fig. 2. Self-catalytic cleavage of the N-terminal propeptide of castor bean VPE precursor at Asp-57. A: Comparison of N-terminal sequences among VPE homologues (castor bean [3], soybean [7], *Arabidopsis* β [6], Jack bean [8], narbon bean (accession number Z99174) and vetch (accession number AJ007743)). Conserved amino acids are boxed. An asterisk indicates Cys in the potential active site of VPEs and a dot indicates the N-terminal amino acid of Jack bean enzyme. A synthetic peptide used in this study is underlined. B: Capillary electrophoretic pattern of the substrate peptide (s, underlined in A) and two cleaved products (a and b) produced by the purified VPE from castor bean, before incubation (top, 0 h) and after 6 h of incubation (bottom). The peak at 5.9 min is dithiothreitol. C: Sequences of the two cleaved products (a and b in B) were determined by mass spectrometry and peptide sequence analysis.

globulin [19]. In one of the peptides (SESEDGLEET), an Asn residue in the original sequence was substituted with an Asp residue. In the other peptide (SESADGLEET), a Glu-Asn sequence in the original sequence was substituted with an Ala-Asp sequence. As shown by the relative activities in Fig. 3, castor bean VPE was able to cleave both of the synthetic peptides on the C-terminal side of the Asp residue. We also examined a peptide PSLDDEFDLEDDIENPQG, which was derived from the sequence around the processing site of another major seed protein, *Arabidopsis* pro 2S albumin. VPE cleaved the peptide at two Asp residues, as shown in Fig. 3. The result is consistent with the report that a VPE homologue, vetch proteinase B, acts towards Asp as well as Asn [9]. Some other vacuolar proteins have been reported to be cleaved at the Asp residue to form the respective mature proteins [23–26]. This suggests that VPEs might be involved in the processing of plant vacuolar proteins after an Asp residue as well as after an Asn residue to produce the mature proteins in plant vacuoles.

3.3. Removal of the C-terminal propeptide at di-aspartic acid site is required for the activation of VPE

The molecular mass of castor bean VPE suggests that the cleavage of the C-terminal propeptide might occur at the Asp-429–Asp-430 [15] (indicated by a closed triangle in Fig. 4A). The Asp-Asp sequence was conserved among six VPE homologues (Fig. 4A). To examine whether the di-aspartic acid site is the processing site of the C-terminal propeptide, we constructed a mutant VPE (D429G/D430G) in which Asp-429 and Asp-430 were substituted with Gly residues. The transformant with the mutant accumulated the precursor of VPE, but not the mature form (Fig. 4B, lane 2). On the other hand, the transformant with the wild VPE accumulated both the precursor and the mature protein (Fig. 4B, lane 1). The result indicated that the removal of the C-terminal propeptide at the di-aspartic acids causes the removal of the N-terminal propeptide. It should be noted that an intermediate VPE with the C-terminal propeptide but no N-terminal propeptide was not detected in the transformant with the mutant (Fig. 4B, lane 2).

The next issue to be solved was the activation mechanism of VPE in association with the conversion of the proVPE into the mature VPE. We used the processing of the precursor of carboxypeptidase Y (CPY) as a marker of VPE activity in vivo. The precursor of CPY, which is one of the yeast vacuolar proteinases, is known to be processed at a peptide bond on the C-terminal side of the Asn residue into the mature form [27]. Previously we reported that the active VPE expressed in the *pep4* transformant was responsible for the conversion of proCPY into mCPY, instead of the *PEP4* gene product, proteinase A [15]. As shown in Fig. 4B, a wild VPE mediated the conversion of proCPY to mature form (lane 5). The VPE (D429G/D430G) did not mediate the conversion (Fig. 4B, lane 6). The result indicated that VPE (D429G/D430G) has no VPE activity. Taken together, these findings suggest that the activation of VPE is caused by the sequential processing of the VPE precursor. The C-terminal propeptide was removed from the precursor and then the N-terminal propeptide was removed to produce the active mature VPE. Therefore,

Substrates	Relative activity
SESENGLEET	1.00
SESEDGLEET	0.37
SESADGLEET	0.65
PSLDDEFDLEDDIENPQG	Yield
a	41 %
b	12 %

Fig. 3. Substrate specificity of VPE towards Asn and Asp. SESENGLEET, sequence derived from that around the VPE-dependent processing site of pumpkin 11S globulin [19]. SESEDGLEET, sequence in which Asn in the original sequence was substituted with Asp. SESADGLEET, sequence in which Glu-Asn in the original sequence was substituted with Ala-Asp. These peptides were incubated with purified VPE from castor bean and the relative activity on each peptide was determined. Cleavage sites are indicated by closed triangles. PSLDDEFDLEDDIENPQG, an internal propeptide sequence of *Arabidopsis* 2S albumin [20], was cleaved at two sites by the purified VPE. Yield of two products is shown.

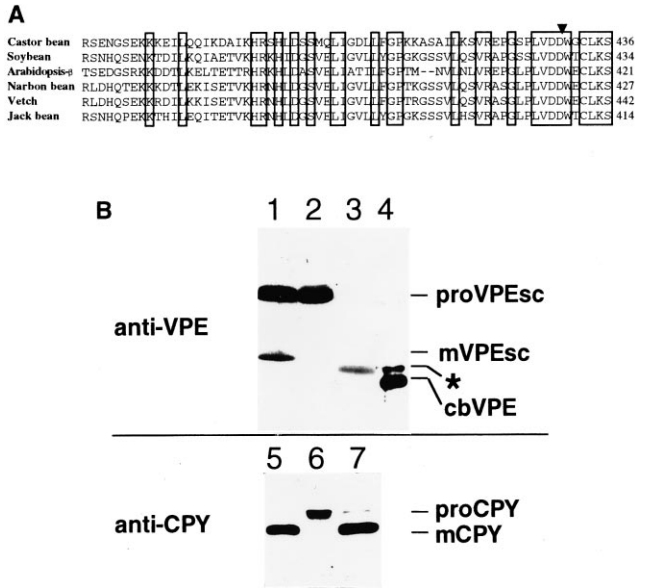

Fig. 4. In vivo conversion of the VPE precursor of castor bean into the mature form occurred correctly at the di-aspartic acid site to activate the enzyme. A: Comparison of the sequences around the putative processing site (indicated by a closed triangle) to remove the C-terminal propeptide among VPE homologues. The conserved amino acids are boxed. B: Transformant cells with each of wild VPE of castor bean (lanes 1 and 5) and the two mutated VPEs (lanes 2 and 6; D429G/D430G, lanes 3 and 7; S322G/S376G) and the isolated vacuoles from castor bean (lane 4) were subjected to SDS-PAGE and subsequent immunoblot analysis with specific antibodies against either VPE (anti-VPE; lanes 1–4) or carboxypeptidase Y (anti-CPY; lanes 5–7). An asterisk indicates a 40-kDa intermediate VPE. proVPEsc, expressed VPE precursor; mVPEsc, expressed mature VPE; proCPY, CPY precursor; mCPY, mature CPY; cbVPE, VPE in the vacuoles isolated from castor bean.

the processing of the C-terminal propeptide at the Asp-429–Asp-430 site is required for the activation of the VPE. The C-terminal propeptide of VPEs might act as an inhibitor that regulates processing activity. Coulombe et al. [28] reported that the large N-terminal propeptide of procathepsin L plays a role as a potent inhibitor of proteolytic activity. It has been shown that mutations at the cleavage site interfere with the autocatalytic processing of mammalian 20S proteasome [29] and the HIV-1 protease [30].

We previously showed that the VPE was glycosylated in yeast transformants, in contrast VPE was not glycosylated in plants [15]. To compare the molecular mass of the expressed mVPE with that of the plant mVPE, we expressed a mutant VPE (S322G/S376G) lacking all possible glycosylation sites, in which Ser-322 and Ser-376 were substituted with Gly residues. The mVPE (S322G/S376G) shows the same mobility as the 40-kDa VPE from castor bean (indicated by asterisks in Fig. 4B, lanes 3 and 4) and the processing activity to convert the proCPY into the CPY (Fig. 4B, lane 7). The molecular mass corresponded to that of the expressed wild mVPE that had been deglycosylated by *N*-glycosidase F [15]. We previously reported that further incubation of the deglycosylated product gave a 37-kDa VPE, whose molecular mass corresponded with that of the 37-kDa VPE from castor bean (indicated by cbVPE in Fig. 4B, lane 4) [15]. The trimming of a 40-kDa intermediate to produce the 37-kDa VPE was also observed in castor bean [4].

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