Multiple Functional Proteins Are Produced by Cleaving Asn-Gln Bonds of a Single Precursor by Vacular Processing Enzyme*

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Precursor-accumulating vesicles mediate transport of the precursors of seed proteins to protein storage vacuoles in maturing pumpkin seeds. We isolated the precursor-accumulating vesicles and characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97,310 Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CXXC motifs, a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Both immunocytochemistry and immunoblots with anti-PV100 antibodies showed that <10-kDa proteins and the 50-kDa vicilin-like protein were accumulated in the vacuoles. To identify the mature proteins derived from PV100, soluble proteins of the vacuoles were separated, and their molecular structures were determined. Mass spectrometry and peptide sequencing showed that two Cys-rich peptides, three Arg/Glu-rich peptides, and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all of these proteins had a pyroglutamate at their NH2 termini. To clarify the cleavage mechanism, in vitro processing of PV100 was performed with purified vacuolar processing enzyme (VPE). Taken together, these results suggested that VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins. It is likely that the Asn-Gln stretch is not only provide cleavage sites for VPE but also produce aminopeptidase-resistant proteins. We also found that the Cys-rich peptide functions as a trypsin inhibitor. Our findings suggested that PV100 is converted into different functional proteins, such as a proteinase inhibitor and a storage protein, in the vacuoles of seed cells.

In higher plants, proprotein precursors of most seed proteins are synthesized on the rough endoplasmic reticulum and are then transported to protein storage vacuoles in maturing seed cells (1–3). We have shown that the vesicles with a density of 1.24 g/cm3 mediate the delivery of proprotein precursors of seed proteins to the vacuoles (4–6). We have succeeded in isolating a unique mechanism for vacuolar processing at Asn-Gln cas-

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‡‡The abbreviations used are: PAC, precursor-accumulating; PV100, a 100-kDa component of PAC vesicles; VPE, vacuolar processing enzyme; BAPA, a-N-benzoyl-Arg-Arg-p-nitroanilide HCl; bp, base pair; PAGE, polyacylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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sets in the precursor sequence to produce aminopeptidase-resistant proteins in the plant vacuoles.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Pumpkin (*Cucurbita maxima* cv. Kurokawa Amakuri Nankan) seeds were purchased from Aisian Shubyo Seed Co. (Nagoya, Japan). For isolation of PAC vesicles and immunocytochemical analysis, pumpkin seeds were planted in the farm of Nagoya University during the summer season, and cotyledons of the maturing seeds, freshly harvested 22–28 days after pollination, were collected.

**Isolation of PAC Vesicles**—PAC vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation essentially as described previously (7). The cotyledons (15 g) were homogenized in a solution (7 ml/g fresh weight of cotyledons) of 20 mM sodium pyrophosphate (pH 7.5), 1 mM EDTA, and 0.3 M mannitol with an ice-chilled mortar and pestle, and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 3000 rpm for 15 min and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 3000 rpm for 15 min. The supernatant was centrifuged again at 40,000 rpm for 30 min at 4 °C. The remaining fraction was centrifuged again in a self-generated Percoll gradient. The resulting vesicle fraction was washed in the above-described Hepes-KOH buffer and used for immunoelectron microscopy and immunoblot analysis.

**Isolation of Protein Storage Vacuoles and Purification of Proteins**

*Derived from PV100*—Protein storage vacuoles (protein bodies) were isolated from dry pumpkin seeds (50 g) by a nonaqueous isolation method, as described previously (17). Isolated protein storage vacuoles were burst in 100 ml of 10 mM Tris-MES (pH 6.5), 0.1 M sucrose, 1 mM EDTA; sonicated; and then centrifuged at 100,000 × g for 1 h at 4 °C to remove insoluble proteins and membranes as a pellet, as described previously (18). Ammonium sulfate was added to the supernatant solution to a concentration of 30% saturation. The mixture was incubated for 1 h at 4 °C after sonification, then centrifuged at 100,000 × g for 1 h at 4 °C. The ammonium sulfate concentration of the supernatant was then increased to 100% saturation, and the incubation and centrifugation steps were repeated. The precipitate was suspended in 4 ml of a solution of 25 mM sodium acetate (pH 5.5) and 5 mM EDTA and then applied to an Econo-Pac10 DG column (Bio-Rad) to remove ammonium sulfate. The preparation was used as the matrix fraction of the protein storage vacuoles.

The matrix fraction was found to contain a large amount of the 4–6-kDa proteins and the 50-kDa protein that were derived from vacuoles.

**Degradation**—To determine the exact molecular mass of the 4–6-kDa proteins of the protein storage vacuoles, each fraction that was isolated on a SMART system as described above was applied to an API 300 triple quadrupole mass spectrometer (PE SCIEX, Foster City, CA) in positive ion detection mode, equipped with an ion spray interface. Samples were dissolved in 0.1% formic acid and 50% acetonitrile and then delivered at 3 μl/min. The sprayer was held at a potential of 4.5 kV. Orbitif potential was maintained at 25 V.

**Assay of Trypsin Inhibitory Activity**—Trypsin inhibitory activity was assayed essentially as described by Cechova (22). α-N-Benzoyl-L-arginine-p-nitroanilide HCl (BAPA) was used as a substrate of trypsin. One of the PV100-derived small proteins, C2 peptide, purified by a reverse phase chromatography, was dissolved in 0.1 M Tris-HCl (pH 8.0) and 25 mM CaCl₂. After preincubation of the C2 peptide (0–2.4 nmol) with 10 μl of trypsin (Sigma) in a 676 μl of 0.1 M Tris-HCl (pH 8.0) and 25 mM CaCl₂ at room temperature for 30 min, 33 μl of a 20 mM substrate BAPA solution was added to start the reaction. The reaction mixture was incubated at room temperature for 30 min. Then, 1 ml of 5% trichloroacetic acid was added to stop the reaction. The residual enzyme activity was measured at 405 nm. The amount of the C2 peptide was estimated from the absorbance at 280 nm, and the molar absorption coefficient at 280 nm of the C2 peptide was computed.

**Isolation of cDNA for PV100 and Determination of Nucleotide Sequence**—A cDNA library in pBluescript II SK+ (Stratagene, La Jolla, CA) was constructed with the poly(A) RNA from maturing pumpkin seed (20). A set of PCR primers was designed on the 5′ region of PV100 cDNA using a 5′-Full RACE Core Set (TaKaRa, Tokyo, Japan). Two identical clones were amplified, and the nucleotide sequence was overlapped with the isolated cDNA sequence that lacked an initiation codon. Subsequently, we amplified DNAs covering the 5′ region of PV100 cDNA using a 5′-Full RACE Core Set (TaKaRa, Tokyo, Japan). Two identical clones were amplified, and the nucleotide sequence was overlapped with the isolated cDNA sequence that lacked an initiation codon.

**DNA sequencing** was performed with a DNA sequencer (model 377, Applied Biosystems Inc.) and a 21M13 forward and M13 reverse fluorescent primers in accordance with the manufacturer’s directions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (Gene Works, IntelliGenetics, Mountain View, CA). The hydrophobicity profile of the amino acid sequences was comp-
NH₂-terminal sequences of PV100-derived mature proteins that had been digested by pyroglutamate aminopeptidase (see Figs. 6 and 8), and a large box Arg/Glu-rich domain (enclosed in the triangle, represent posttranslational processing sites to produce multiple seed proteins, each with a pyroglutamate at its NH₂ terminus. The nucleotide sequence has been submitted to the DNA Data Bank of Japan and GenBank™ with the accession number AB019195.

Preparation of Specific Antisera—The isolated PAC vesicles were subjected to SDS-PAGE on a 12.5% polyacrylamide gel with subsequent staining with Coomassie Blue. The band corresponding to the PV100 protein with a molecular mass of 100 kDa was cut out from the gel and gently shaken in phosphate-buffered saline for several hours. The gel was emulsified with complete Freund’s adjuvant and injected subcutaneously into a rabbit. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. One week after the booster injections, blood was drawn, and the antisera was prepared.

Immunoblot Analysis—Both the PAC vesicles and protein storage vacuoles were subjected to SDS-PAGE followed by either Coomassie Blue staining or immunoblotting. The purified 4–6-kDa proteins of the protein storage vacuoles were also subjected to immunoblot analysis. The immunoblot was performed essentially as described previously (18). The separated proteins on gels were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm) (Nihon Millipore Ltd., Tokyo, Japan). The membrane blot was incubated overnight with anti-PV100 antibodies that were diluted 2000-fold in a solution of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween 20, and 3% (w/v) skim milk. Alkaline phosphatase-conjugated antibodies (Cappel, West Chester, PA) and horseradish peroxidase-conjugated antibodies (Amer sham Pharmacia Biotech) that were raised in goats against rabbit IgG were diluted 2000-fold and used as second antibodies.

Immunoelectron Microscopy—Maturing pumpkin seeds were freshly harvested. The cotyledons were vacuum-infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.16 M sucrose in 0.5 M cacodylate buffer (pH 7.4). The tissues were then cut into slices of less than 1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative. The isolated PAC vesicles were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 0.3 M mannitol, 1 mM EDTA, and 10 mM Hepes-KOH (pH 7.2) for 1 h at 4 °C. The samples were dehydrated in a graded dimethylformamide series at -20 °C and embedded in LR white resin (London Resin Co. Ltd., Basingstoke, Hampshire, UK). Immunogold labeling procedures were essentially the same as those described previously (26), except for the use of the anti-PV100 antibodies that were diluted 1000-fold in blocking solution (1% bovine serum albumin in phosphate-buffered saline). Protein A-gold (15 nm) (Amer sham Pharmacia Biotech) was diluted 40-fold and used. The ultrathin sections were examined with a transmission microscope (model 1200EX) (JEOL, Tokyo, Japan) at 80 kV.

RESULTS

PV100 Is a 100-kDa Protein Component of PAC Vesicles—We have shown that the PAC vesicles are responsible for the intracellular transport of precursors of major seed proteins, including 11S globulin and 2S albumin, to protein storage vacuoles in maturing pumpkin seeds (4, 6–8). The PAC vesicles were highly purified from cotyledons of maturing pumpkin seeds. Electron microscopy revealed that each PAC vesicle contained an electron-dense core with a diameter of 300–500 nm and that the isolated vesicles were barely contaminated by other cellular components (Fig. 1B). Fig. 1A (lane 1) shows the protein components of the vesicles that were separated on an SDS-gel with Coomassie Blue staining. Three major proteins were found in the PAC vesicle fraction. Two of them have been shown to correspond to proglobulin, a proprotein precursor of 11S globulin (4), and to pro2S albumin, a proprotein precursor of 2S albumin (6), as indicated by pG and p2S in Fig. 1A (lane 1), respectively. The third component of the PAC vesicles, with

**FIG. 1.** PV100 is one of the major proteins in the PAC vesicles from maturing pumpkin seeds. A, isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (lane 1) or immunoblot with anti-PV100 antibodies (lane 2). pG and p2S represent proprotein precursors of 11S globulin and 2S albumin, respectively. The molecular mass of each marker protein is given on the left in kDa. PAC vesicles were isolated from the cotyledons at the middle stage of seed maturation of pumpkin. B, immunogold labeling of the isolated PAC vesicles with anti-PV100 antibodies. Bar, 500 nm.

**FIG. 2.** Deduced amino acid sequence from a cDNA that encodes PV100. Isolated cDNA encodes a 97,310-Da protein of 810 amino acids, which consists of a hydrophobic signal peptide followed by the PV100 sequence. The NH₂-terminal sequence and two internal sequences of PV100 that were determined are indicated by double underlining. An open triangle indicates a cleavage site of a signal peptide. The PV100 sequence was divided into three domains: an 11-kDa Cys-rich domain (indicated by light shading) with four CXXXC motifs (enclosed in the small boxes), a 34-kDa Arg/Glu-rich domain (enclosed in the large box), and a 50-kDa vicilin-like domain (indicated by light shading). The arrows indicate the determined NH₂-terminal sequences of PV100-derived mature proteins that had been digested by pyroglutamate aminopeptidase (see Figs. 6 and 8), and a dotted line indicates the NH₂-terminal sequence of the vicilin-like protein from dry seeds. Boldfaced NQ (Asn-Gln) stretches, marked with a closed triangle, represent posttranslational processing sites to produce multiple seed proteins, each with a pyroglutamate at its NH₂ terminus. The nucleotide sequence has been submitted to the DNA Data Bank of Japan and GenBank™ with the accession number AB019195.
Multiple Functional Proteins Derived from a Single Precursor

The PV100 sequence was composed of three domains: an 11-kDa Cys-rich domain, a 34-kDa Arg/Glu-rich domain, and a 50-kDa vicilin-like domain, as shown in Fig. 2. The Cys-rich domain contained four CXXX motifs (Fig. 2, boxes) of two Cys residues separated by three other amino acids. The 50-kDa domain (Fig. 2, light shading) exhibited a 30–35% identity in amino acids to the vicilin homologs, pea vicilin (28), soybean vicilin (29), and jack bean vicilin (30). Precursors of vicilin homologs of pea, soybean, and jack bean are composed of a signal peptide followed by a vicilin domain, whereas the amino acid sequences around the signal peptide are consistent with that predicted by application of the rules of Von Heijne (27).

The Arg/Glu-rich domain of PV100 is composed of 37 mol % Arg and 27 mol % Glu, as shown in Fig. 2 (large box). It should be noted that this domain is unique to pumpkin PV100. A homology plot of PV100 shows that six homologous repeats are found in the Arg/Glu-rich domain, as shown in Fig. 3A. An alignment revealed that the six homologous repeats were separated by Asn-Gln (Glu) sequences (Fig. 3B). The six repeats rich in Arg and Glu were designated RE1–RE6 in order from the NH2 terminus. They show a sequence homology to pumpkin basic peptide, where the second and third possible amino acids are also shown, as reported by Naisbitt et al. (33). Both peptides are composed of 36 amino acids, as indicated on the right side of each sequence. Boxes enclose identical amino acids, and shading indicates homologous amino acids.

The circular homology plot of PV100 and an amino acid alignment of the six homologous repeats in the Arg/Glu-rich domain. A, a homology plot was performed with the PAM-250 algorithm (25) with a window of 10 residues. Each pair of windows that exhibited more than 35% identity in amino acids is indicated by a dot in the matrix. Six homologous repeats were found in the Arg/Glu-rich domain. B, Asn-Glu/Glu bonds separate the Arg/Glu-rich domain into six repeats (see Fig. 9). The six Arg/Glu-rich repeats that were designated RE1 to RE6 in order from the NH2 terminus were aligned. Numbers on the right side of each sequence refer to the positions of the amino acids starting from the initiation Met. C, the mature RE3 peptide (see Fig. 6B) was aligned with the sequence of pumpkin basic peptide, where the second and third possible amino acids are also shown, as reported by Naisbitt et al. (33). Both peptides are composed of 36 amino acids, as indicated on the right side of each sequence. Boxes enclose identical amino acids, and shading indicates homologous amino acids.

For immunochemical characterization of PV100, polyclonal antibodies were raised against the PV100 protein. An immunoblot of the PAC vesicles with the anti-PV100 antibodies showed that the antibodies specifically recognized PV100 on the blot, as shown in Fig. 1A (lane 2). Immunoelectron microscopy of the isolated PAC vesicles with the anti-PV100 antibodies shows that gold particles are inside the PAC vesicles (Fig. 1B). These results indicate that PV100 is localized in the PAC vesicles together with proproteins of the major seed proteins, 11S globulin and 2S albumin.

PV100 Is Composed of Three Domains—The next issue to be solved was the molecular structure of PV100 to clarify the manner of posttranslational cleavage. We determined the amino acid sequences of the NH2 terminus and two internal fragments of PV100 and then isolated a cDNA with a 4.3-kb insert from the library of maturing pumpkin seeds based on the amino acid sequences. The cDNA encoded a 97,310-Da protein precursor of cacao vicilin (31) and upland cotton (32) have six and four CXXX motifs, respectively, preceding a signal peptide and then are converted into their respective mature forms (4, 6). This raises the question of whether PV100 is also incorporated into the protein storage vacuoles. Immunocytochemistry of the maturing pumpkin seeds with the anti-PV100 antibodies answered this question. Electron-dense PAC vesicles with diameters of 300–500 nm and protein storage vacuoles composed of crystalloids of 11S globulin and the matrix were observed in the cells, as shown in Fig. 4A. Gold particles can be seen distributed in the vacuolar matrix region, the PAC vesicles, and the rough endoplasmic reticulum. In contrast, none of the vacuolar crystalloid, the lipid bodies, the mitochondria, or the cell wall was labeled with gold particles. These results suggested that PV100, which is synthesized on rough endoplasmic reticulum, is transported to PAC vesicles and then to protein storage vacuoles.

The molecular structure of PV100 implies that multiple seed proteins are derived from PV100. The next question is whether PV100 is processed to make such seed proteins in the protein
storage vacuole. To answer this question, the protein storage vacuoles were isolated from dry pumpkin seeds and subjected to SDS-PAGE followed by immunoblot analysis, as shown in Fig. 4B. The vacuoles contained a large amount of 11S globulin (Fig. 4B, lane 1). On the immunoblot with anti-PV100 antibodies, two bands, corresponding to a 50-kDa protein and a ~6-kDa C2 peptide (C), were detected on the blot. The determined NH$_2$-terminal sequence of the 50-kDa vicilin-like protein is shown. G represents the 11S globulin. The molecular mass of each marker protein is given on the left in kDa.

To identify the <10-kDa small protein(s), soluble proteins of the protein storage vacuoles were separated by HPLC, as shown in Fig. 5A. Each peak fraction of the HPLC was subjected to both mass spectrometry and automatic Edman degradation (Fig. 6). Fractions 37 and 41 contained the known C. maxima trypsin inhibitor (34), which is not related to PV100. The NH$_2$ termini of all proteins of fractions 12, 14, 17, 43, and 45 were blocked. When digested by pyroglutamate aminopeptidase, each protein gave an NH$_2$-terminal amino acid sequence that was consistent with the sequence starting from the second residues of the respective small protein derived from PV100, as indicated in Figs. 2 and 6B (arrow). Fractions 12, 14, and 17 corresponded to RE4, RE3, and RE5 of the Arg/Glu-rich domain, respectively, and fractions 43 and 45 corresponded to the latter half (C2) of the Cys-rich domain (Fig. 6). Interestingly, all of these small proteins had a pyroglutamate at their NH$_2$ termini. It should be noted that an Asn residue always preceded all Gln residues to be converted into a pyroglutamate, as indicated in Fig. 2 (boldfaced NQ in the PV100 sequence) (discussed below).

The observed molecular masses of fractions 12, 14, and 17 showed good agreement with the theoretical masses of sequence d of RE4, sequence c of RE3, and sequence e of RE5, respectively, each of which has a pyroglutamate at the NH$_2$ terminus and an Asp residue at the COOH terminus (Fig. 6). Thus, two steps of processing might be required to produce the mature forms of RE peptides from PV100: the first is cleavage at Asn-Gln bonds of PV100, and the second is trimming 2 or 5 amino acids off at the COOH termini of RE intermediates. All of the mature peptides of the Arg/Glu-rich RE3, RE4, and RE5 found in seeds are basic ones with estimated pIs of 11.90, 11.54, and 10.20, respectively. This is in contrast to the neutral pIs of RE intermediates before trimming their COOH-terminal few amino acids.

The observed molecular masses of fractions 43 and 45 also showed good agreement with the theoretical masses of sequences a and b of C2 from the Cys-rich domain, respectively, indicating that each sequence has a pyroglutamate at the NH$_2$ terminus and two intramolecular disulfide bonds (Fig. 6). The

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**Fig. 4.** PV100-derived proteins are localized in protein storage vacuoles in pumpkin seeds. A, immunoelectron micrograph of maturing pumpkin seeds after staining with anti-PV100 antibodies. Gold particles were distributed in the PAC vesicles (PV), the matrix region (VM) of protein storage vacuoles and ER. VC, vacuolar crystallloid composed of 11S globulin; Mt, mitochondrion; LB, lipid body; CW, cell wall. Bar, 1 μm. B, isolated protein storage vacuoles (protein bodies) from dry pumpkin seeds were subjected to SDS-PAGE followed by staining with Coomassie Blue (lane 1) or immunoblot with anti-PV100 antibodies (lane 2). PV100-derived proteins, the 50-kDa vicilin-like protein (V) and a ~6-kDa C2 peptide (C) were detected on the blot. The determined NH$_2$-terminal sequence of the 50-kDa vicilin-like protein is shown. G represents the 11S globulin. The molecular mass of each marker protein is given on the left in kDa.

**Fig. 5.** An HPLC profile of PV100-derived peptides from the protein storage vacuoles. A, soluble fraction of the protein storage vacuoles that contained the PV100-derived peptides was applied to a reverse phase column. Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile. Chromatography was monitored in terms of absorbance at 214 nm. B, immunoblot analysis of each peak fraction with anti-PV100 antibodies.
disulfide bridges are deduced from the data of buckwheat trypsin inhibitor, an allergenic protein, that exhibits a similar characteristic to the C2 peptide and has two CXXXC motifs and two disulfide bridges (35).

To explore function of the C2 peptide, we examined trypsin inhibitory activity of the C2 peptide using BAPA as a substrate of trypsin. The C2 peptide was highly purified. Mass spectrometry showed that the final preparation of the C2 peptide used for the assay was not contaminated by C. maxima trypsin inhibitor. We found that the C2 peptide had an inhibitory activity against trypsin, as shown in Fig. 7. Ten μg of trypsin was completely inhibited by 1.2 nmol of C2 peptide. The C2 peptide of 49 amino acids exhibits an 18% identity in amino acids to buckwheat trypsin inhibitor of 51 amino acids. In contrast to the low identity between the two sequences, they have a similar characteristic in the presence of two CXXXC motifs in their sequences. The reactive site of buckwheat trypsin inhibitor for trypsin was reported to be Arg\textsuperscript{19}, between the two CXXXC motifs (35). The C2 peptide conserves Arg\textsuperscript{21} between the two CXXXC motifs (Fig. 6B), and the residue might be the reactive site for trypsin (discussed below).

None of RE1, RE2, RE6, or the former half (C1) of the Cys-rich domain was detected in protein storage vacuoles. They might be degraded in the vacuoles during seed maturation (discussed below). These results indicated that −6-kDa C2, −5-kDa RE3, −4-kDa RE4, and −5-kDa RE5 are accumulated in the protein storage vacuoles. Fig. 5B shows an immunoblot of RE3 (fraction 14), RE4 (fraction 12), RE5 (fraction 17), and C2 (fractions 43 and 45) with anti-PV100 antibodies. Surprisingly, the polyclonal antibodies recognized C2 peptide efficiently, but no RE peptides at all appeared on the blot. These results suggested that the antigenicity of the Cys-rich peptides was much higher than that of the extremely hydrophilic Arg/Glu-rich peptides. On the immunoblot of protein storage vacuoles, the signal corresponding to <10 kDa might be caused by C2 peptide (Fig. 4B). The intensity of the signal was much higher than that of the 50-kDa vicilin-like protein. It seems likely that such CXXXC motifs cause allergy to animals as buckwheat trypsin inhibitor does (35).

**VPE Mediates the Conversion of PV100 into Multiple Seed Proteins**—We previously showed that VPE is involved in maturation of various seed proteins in the protein storage vacuoles by cleaving a peptide bond on the carbonyl side of Asn residues (6, 10, 15). This raised the question of whether VPE mediates the proteolytic processing of PV100. To answer this question, we performed an *in vitro* processing of PV100 by the purified VPE from castor bean seeds. We used proproteins in the isolated PAC vesicles as substrates, including PV100, proglobulin, and pro2S albumin, as shown in Fig. 1A (lane 1). After incubation of these proteins with the purified VPE, the amount of PV100 decreased in association with the increase of the amount of a 50-kDa protein and <10-kDa proteins, as shown in Fig. 8. The <10-kDa proteins contained not only PV100-derived small proteins but also 2S albumin, composed of 3.8- and 8.0-kDa subunits, which was produced from pro2S albumin. This indicated that VPE was involved in the conversion of PV100 into the 50-kDa protein and the <10-kDa proteins.

The 50-kDa protein was subjected to automatic Edman degradation after digestion by pyroglutamate aminopeptidase (Fig. 8). The determined NH\textsubscript{2}-terminal sequence, <QVAIR-
RTEQEQLSNAMY, was found in the sequence of PV100, as in Fig. 2 (arrow). The NH₂-terminal sequence determined after in vitro processing was consistent with that of the 50-kDa protein accumulated in maturing seeds (data not shown). This suggests that processing similar to the in vitro processing of PV100 occurs by endogenous VPE during seed maturation. The result indicated that VPE mediated the production of the 50-kDa vicilin-like protein by cleaving an Asn375-Gln376 bond of PV100. The cleavage was consistent with the substrate specificity of VPE toward Asn residues. Further degradation to remove NH₂-terminal three amino acids must occur at the later stage of seed maturation and produce a mature 50-kDa vicilin-like protein with the NH₂-terminal sequence, IRRTEQEQLSNAMYYFQ (Figs. 2 and 4B). We previously reported that protein storage vacuoles accumulated not only VPE but also aspartic proteinase (36). It seems likely that such aspartic proteinase might be involved in the proteolytic trimming.

It should be noted that most processing occurs at Asn-Gln bonds in the hydrophilic region of PV100, and all of the mature proteins, the 50-kDa protein, and C2 and RE peptides have a pyroglutamate at their NH₂ termini, as shown in Fig. 9. Similar VPE-mediated processing of PV100 might occur to produce the multiple seed proteins (discussed below).

**DISCUSSION**

**PV100 Is a Unique Precursor to Multiple Functional Proteins**—The present study demonstrates that PV100 is not only a precursor of vicilin storage protein but also a precursor of the Arg/Glu-rich RE peptides and a precursor of the Cys-rich C2 peptide that acts as a trypsin inhibitor. PV100 is a unique precursor for multiple seed proteins with different functions.

The C2 peptide was shown to have trypsin inhibitory activity. However, the sequence of the C2 peptide has no homology to known trypsin inhibitors, including members of squash trypsin inhibitor family (34), except for buckwheat trypsin inhibitor, showing a 18% identity to the C2 peptide. Interestingly, despite such low homology in primary structure, the higher structure of the C2 peptide might be analogous to that of buckwheat inhibitor. It has been shown that the buckwheat trypsin inhibitor forms a hairpin structure, in which two CXXC motifs are linked by two disulfide bonds, and that Arg⁹, between the two CXXC motifs, is the reactive site for trypsin (35). Similarly, all four Cys residues of the C2 peptide formed two disulfide bonds, and Arg⁴ is found between the two CXXC motifs (Fig. 6B). The result suggests that the mature C2 peptide and buckwheat inhibitor belong to a novel family of trypsin inhibitors. These inhibitors might play a role in protecting the seeds from animals.

Among the Arg/Glu-rich RE peptides, the mature RE3 with the highest pI value (pI 11.90) shows the highest content in pumpkin seeds. We compared the RE3 composed of 36 amino acids with the pumpkin basic peptide that has been shown to be toxic to mouse B-16 cells (33) (Fig. 3C). The cytotoxic basic peptide was composed of 36 amino acids, and the probable amino acid sequence of the peptide was reported by Naisbitt et al. (33). Both sequences are identical to each other, except for two residues. It is likely that the mature RE3 accumulated in the vacuoles of pumpkin seeds might be identical to the cytotoxic basic peptide that was characterized by Naisbitt et al. (33). This suggests that the mature RE3 might function as a toxin to prevent animals from eating the seeds.

Most vacuolar proteins are synthesized as a propeptide precursor on the rough endoplasmic reticulum and are then transported to vacuoles. The vacuolar targeting signals have been shown to be present in the propeptides of some vacuolar proteins, including barley aleurain (37), barley lectin (38), sweet potato sporamin (39), and tobacco chitinase (40). It has been thought that the propeptides are cleaved off and degraded after...
arrival of the proproteins at the vacuoles. However, the possibility cannot be excluded that the propeptides exhibit some functions in the vacuoles after being removed from the precursor proteins, as the 4–6-kDa RE and C2 peptides are accumulated to act as functional proteins in the vacuoles.

VPE-mediated Cleavage at Asn-Gln Bonds of PV100 to Produce Multiple Seed Proteins with a Pyroglutamate at Their NH$_2$ Termi—Fig. 9 shows the hydrophobicity plot of PV100 and a hypothetical mechanism for vacuolar processing of PV100 to produce C2 peptide, RE peptides, and a vicilin-like protein. PV100 contains nine Asn-Gln bonds in the sequence. All six Asn-Gln bonds to be cleaved are located in the hydrophilic region of the PV100 sequence, whereas the other three non-cleavable Asn-Gln bonds are found in the hydrophobic region of the vicilin-like domain. The result is consistent with our previous data showing that VPE recognizes Asn residues that are located in the hydrophilic region and are exposed on the surface of precursor molecules (6).

We previously reported that one subunit of pumpkin 11S globulin has a pyroglutamate at the NH$_2$ terminus (41). NH$_2$-terminal sequencing of the proglobulin in the isolated PAC tidases, if they were produced by cleaving an Asp 118-Glu119 bond and an Asn 161-Glu162 bond by VPE, respectively. Further proteolysis for COOH-terminal trimming at the Asp residues must occur to make the final mature forms of the RE3, RE4, and RE5 peptides. However, both RE1 and RE2, with a Glu residue at each NH$_2$ terminus, could be sensitive to aminopeptidases if they were produced by cleaving an Asp$^{118}$-Glu$^{119}$ bond and an Asn$^{161}$-Glu$^{162}$ bond by VPE, respectively.

Further proteolysis for COOH-terminal trimming at the Asp residues must occur to make the final mature forms of the RE3, RE4, and RE5 peptides (Fig. 6). Recently, we have found that VPE also cleaves a peptide bond at carboxyl side of Asp, although the activity toward Asp is less than that toward Asn (data not shown). The finding is consistent with the report that the VPE homolog of hETCH has a substrate specificity toward both Asn and Asp residues (42). Therefore, it seems likely that the COOH-terminal trimming of RE3, RE4, and RE5 peptides is also mediated by VPE.

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REFERENCES
1. Akazawa, T., and Hara-Nishimura, I. (1985) Annu. Rev. Plant Physiol. 36, 441–472
2. Christie, M. J. (1991) Ann. Rev. Plant Physiol. 42, 21–53
3. Okita, T. W., and Rogers, J. C. (1996) Annu. Rev. Plant Physiol. 47, 327–350
4. Hara-Nishimura, I., Nishimura, M., and Akazawa, T. (1985) Plant Physiol. 77, 274–752
5. Fukasawa, T., Hara-Nishimura, I., and Nishimura, M. (1988) Plant Cell Physiol. 29, 339–345
6. Hara-Nishimura, I., Takeuchi, Y., Inoue, K., and Nishimura, M. (1993) Plant J. 4, 793–800
7. Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y., and Nishimura, M. (1998) Plant Cell 10, 825–836
8. Shimada, T., Kurayayagi, M., Nishimura, M., and Hara-Nishimura, I. (1997) Plant Cell Physiol. 38, 1414–1420
9. Hara-Nishimura, I., and Nishimura, M. (1987) Plant Physiol. 85, 440–445
10. Hara-Nishimura, I., Shimada, T., Hiraizumi, N., and Nishimura, M. (1995) J. Plant Physiol. 145, 632–640
11. Hara-Nishimura, I., Inoue, K., and Nishimura, M. (1991) FEBS Lett. 294, 89–93
12. Hara-Nishimura, I., Takeuchi, Y., and Nishimura, M. (1993) Plant Cell 5, 1651–1659
13. Kinoshita, T., Nishimura, M., and Hara-Nishimura, I. (1995) Plant Mol. Biol. 29, 81–89
14. Kinoshita, T., Nishimura, M., and Hara-Nishimura, I. (1995) Plant Cell Physiol. 36, 1555–1562
15. Hara-Nishimura, I., Kinoshita, T., Hiraizumi, N., and Nishimura, M. (1998) J. Plant Physiol. 152, 668–674
16. Atkinson, A. H., Heath, R. L., Simpson, R. J., Clarke, A. E., and Anderson, M. A. (1988) Plant Physiol. 93, 235–243
17. Hara-Nishimura, I., Nishimura, M., Matsuoka, H., and Akazawa, T. (1982) Plant Physiol. 70, 699–703
18. Inoue, K., Motozaki, A., Takeuchi, Y., Nishimura, M., and Hara-Nishimura, I. (1995) Plant Cell 7, 235–243
19. Inoue, K., Takeuchi, Y., Nishimura, M., and Hara-Nishimura, I. (1995) Plant Mol. Biol. 28, 1089–1101
20. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 103–107
21. Schagger, H., and Jagow, G. V. (1987) Anal. Biochem. 166, 368–379
22. Cechova, D. (1976) Methods Enzymol. 45, 806–813
23. Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I., and Nishimura, M. (1991) Eur. J. Biochem. 197, 331–336
24. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
25. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448
26. Nishimura, M., Takeuchi, Y., De Bellis, L., and Hara-Nishimura, I. (1993) Protoplasma 175, 131–137
27. von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690
28. Watson, M. D., Lambert, N., Delauney, A., Yarwood, J. N., Croy, R. R., Gatehouse, J. A., Wright, D. J., and Boulter, D. (1988) Biochem. 251, 857–864
29. Harada, J. J., Barker, S. J., and Goldberg, R. B. (1989) Plant Cell 1, 415–425
30. Ng, J. D., Ko, T.-P., and McPherson, A. (1992) Plant Physiol. 101, 713–728
31. McHenry, L., and Frix, P. J. (1992) Plant Mol. Biol. 18, 1173–1176
32. Chian, C. A., Pyle, J. B., Leegocki, A. B., and Dure, L. (1986) Plant Mol. Biol. 3, 475–489
33. Naisbit, G. H., Lu, M., Gray, W. R., and Vernon, L. P. (1988) Plant Physiol. 88, 770–773
34. Wietrzk, M., Otlewski, J., Cook, J., Parks, K., Leluk, J., Wilimowska-Pele, A., Polanowski, A., Wilske, T., and Laskowski, J. M. (1985) Biochem. Biophys. Res. Commun. 126, 646–652
35. Park, S., Abe, K., Kimura, M., Utsui, A., and Yamasaki, N. (1997) FEBS Lett. 400, 103–107
36. Hara-Nishimura, I., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (1997) Eur. J. Biochem. 246, 133–141
37. Bednarek, S. Y., and Raikhel, N. V. (1991) Plant Physiol. 95, 307–318
38. Bednarek, S. Y., and Raikhel, N. V. (1991) Plant Physiol. 95, 307–318
39. Matsuoka, K., and Nakamura, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 834–838
40. Neuhauß, J.-M., Sticher, L., Meins, F. J., and Boller, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10362–10366
41. Ohmiya, M., Hara, I., and Matsubara, H. (1980) Plant Cell Physiol. 21, 157–167
42. Becker, C., Shutov, A. D., Nong, V. H., Senyuk, V. I., Jung, R., Horstmann, C., Fischer, J., Nielsen, N. C., and Münz, K. (1995) Eur. J. Biochem. 228, 456–462
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