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A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms

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Proprotein precursors of vacuolar components are transported from the endoplasmic reticulum into vacuoles, where they are proteolytically processed into their mature forms. However, the processing mechanism in plant vacuoles is very obscure. Characterization of a purified processing enzyme is required to determine whether a single enzyme is responsible for processing many vacuolar proteins with a large variability of molecular structure. If this is true, how can it recognize the numerous varieties of processing sites? We have now purified a processing enzyme (M_r=37 000) from castor bean seeds. Our results show that the purified enzyme can process 3 different proproteins isolated from either the endoplasmic reticulum or transport vesicles in cotyledon cells to produce the mature forms of these proteins which are found at different suborganellar locations in the vacuole: the 2S protein found in the soluble matrix, the 11S globulin found in the insoluble crystalloid and the 51 kDa protein associated with the membrane. Thus a single vacuolar processing enzyme is capable of converting several proprotein precursors into their respective mature forms.

Processing enzyme; Vacuole; Dense vesicle; Protein body; Proprotein precursor; Seed protein; Castor bean, *Ricinus communis*; Pumpkin, *Cucurbita* sp.

1. INTRODUCTION

Most proprotein precursors of vacuolar proteins in plants are post-translationally processed into their mature forms in the vacuole [1–3]. However, only two investigations of crude vacuolar processing enzymes have been made [4,5], so the mechanism of the process is not clear. An activity which converts proglobulin to mature 11S globulin, a major seed protein, accumulates in the matrix of vacuoles in developing pumpkin cotyledons, but cannot be detected in the dry seeds [4]. Another activity which converts proricin into ricin is present in both developing and dry castor bean seeds [5]. We have purified a processing activity from protein bodies, specialized vacuoles present in dry seeds [6,7], of castor bean. To demonstrate that the purified vacuolar processing enzyme has an ability to cleave the authentic proprotein precursors which are synthesized on rough endoplasmic reticulum (ER) and transported to the vacuoles via dense vesicles, we have isolated the dense vesicles from developing pumpkin cotyledons and have also prepared ER fraction from the pulse-labeled cotyledons. The results clearly show that the purified processing enzyme can process 3 different precursor proteins accumulated in the isolated dense vesicles to produce their mature forms as well as 2 labeled precursors in the ER fraction.

Abbreviations: ConA, concanavalin A; ER, endoplasmic reticulum; HPLC, high-pressure liquid chromatography

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2. MATERIALS AND METHODS

2.1. *Materials*

Castor bean (*Ricinus communis*) seeds were obtained from Itoh Seiyu Co., Japan. Seeds of pumpkin (*Cucurbita* sp., cv Kurokawa Amakuri Nankin) were grown during summer season of 1991. The cotyledons of seeds freshly harvested at 17–23 days after anthesis were used for experimentation.

2.2. *Decapeptide synthesis*

The decapeptide substrate for the vacuolar processing enzyme was synthesized with a peptide synthesizer (Applied Biosystems Model 430A). Its sequence was derived from the sequence around the processing site of the proglobulin [8], which is a proprotein precursor of 11S globulin, a major protein in pumpkin seed.

2.3. *Determination of the cleavage site on the decapeptide by a vacuolar processing enzyme*

The cleavage reaction mixture contained 12 nmol of the decapeptide and the processing enzyme (1 μl of protein body matrix, prepared as described below) in 20 μl of 20 mM sodium acetate buffer, pH 5.5 and 5 mM dithiothreitol. EDTA is required to detect full enzyme activity, however, this experiment was conducted in the absence of EDTA since it co-elutes with the N-terminal pentapeptide on high-pressure liquid chromatography (HPLC), making subsequent analysis difficult. The products of the reaction were applied to HPLC (Chemcosorb 5-ODS-H column) before and after incubation for 3 h at 37°C. The HPLC system consisted of an LKB 2152 controller, an LKB 2150 pump, an LKB 2151 variable wave length monitor and an LKB 2211 SuperRac fraction collector. The amino acid composition of each peak fraction was determined using a Hitachi 835 Amino Acid Analyzer.

2.4. *Assay for vacuolar processing enzyme activity*

A reaction mixture containing the enzyme fraction and 5 nmol of the decapeptide substrate in 10 μl of 0.1 M sodium acetate, pH 5.5, 0.1 M dithiothreitol and 0.1 mM EDTA was incubated for 1 to 30 min at 37°C, followed by HPLC analysis. One unit was defined as 1 μmol C-terminal pentapeptide produced per min.

2.5. *Preparation of the protein body matrix fraction*

Protein bodies were isolated from dry castor bean seeds (200 g) as described [9] and were lysed by adding 20 ml of 10 mM Tris-MES, pH 6.4, 5 mM EDTA and 0.1 M sucrose. After sonication the homogenate was centrifuged (250 × *g*, 15 min) to remove the crystalloid fraction. Five ml of 5 M NaCl was added to the supernatant and centrifuged (100 000×*g*, 1 h; Beckman 45Ti rotor) to eliminate the membranes. The resulting supernatant is designated the protein body matrix.

2.6. *Purification of the vacuolar processing enzyme*

The protein body matrix was sequentially subjected to 80% saturated ammonium sulfate precipitation, suspending in a buffer of 15 mM sodium citrate, pH 7.0 and 0.15 N NaCl, centrifugation to remove insoluble proteins, concanavalin A (ConA)-Sepharose chromatography, dialysis against 25 mM sodium acetate, pH 5.5, and 5 mM EDTA and MonoS (Pharmacia LKB Biotechnology) chromatography using a 0–1 M NaCl gradient twice. Protein content was analyzed uzing a Bio-Rad Protein Assay Kit.

2.7. *Isolation of dense vesicles*

Dense vesicles were isolated from developing pumpkin cotyledons. The homogenate of cotyledon (3 g) with 20 ml of 20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA and 0.3 M mannitol was filtered and centrifuged (3000×*g*, 15 min), then the supernatant was centrifuged (8000×*g*, 20 min). The pellet was suspended in 1 ml of 10 mM HEPES-KOH, pH 7.2, 1 mM EDTA and 0.3 M mannitol. This was layered on 28% Percoll (Pharmacia LKB Biotechnology) followed by centrifugation (40 000×*g*, 30 min). The resulting vesicle fraction was washed in the above HEPES-KOH buffer. The isolated dense vesicles were subjected to SDS-PAGE and the protein components transferred to GVHP membrane (Millipore) were identified by both amino acid sequence analysis using an Applied Biosystems Model 470A Peptide Sequencer and immunostaining with each specific antiserum against 11S globulin, pro2S protein and 51 kDa protein.

2.8. *In vitro processing of proprotein precursors present in the isolated dense vesicles*

The isolated vesicles (10 μg protein) were incubated with the purified processing enzyme (60 ng) in 20 μl of 0.1 M sodium acetate, pH 5.5, 0.1 M dithiothreitol and 0.1 mM EDTA. After incubation for 0, 5 and 30 min at 37°C, each reaction mixture was subjected to SDS-PAGE (12.5% acrylamide) and the proteins were stained with Coomassie blue R-250.

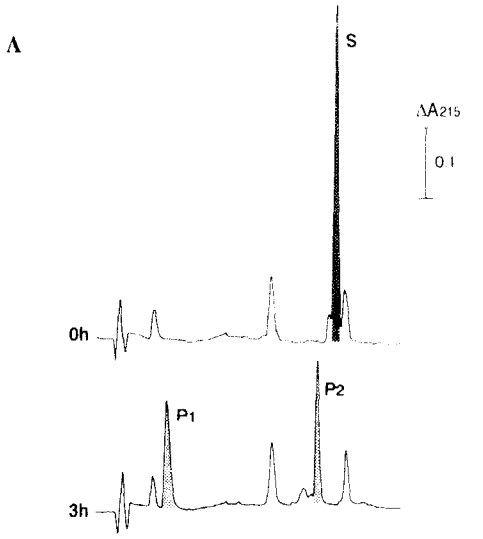
2.9. *In vitro processing of proprotein precursors located in the isolated ER fraction*

Developing pumpkin cotyledons were pulse-labeled with [^{35}S]methionine and the ER and the vacuole fractions were isolated as described [4]. The reaction mixture was the same as that used for assaying cleavage of dense vesicle proteins, except that 120 ng of purified enzyme was used. The reactions were incubated for 0, 5 and 30 min with or without *N*-ethylmaleimide at 37°C then subjected to SDS-PAGE (16.5% acrylamide) using a Tris-tricine buffer system [10] followed by fluorography.

3. RESULTS AND DISCUSSION

To assay the processing activity during purification, we used a chemically synthesized decapeptide (Fig. 1C) which represents the sequence at the cleavage site of proglobulin [8]. After incubation with the protein body matrix isolated from castor bean seeds, the decapeptide was split into two pentapeptide fragments (Fig. 1A). Amino acid composition analysis of the resulting peptides revealed that a single cleavage had occurred between the Asn and Gly residues (Fig. 1B,C) as expected from the in vivo processing of proglobulin. This cleavage was completed after 3 h of incubation (Fig. 1A) and no further degradation of the pentapeptides was observed (data not shown), suggesting that only a single activity in the protein body matrix can degrade this decapeptide.

This protealytic activity is extremely unstable and easily inactivated after freeze–thawing, so a simple, rapid purification was developed. The matrix fraction was sequentially subjected to ammonium sulfate precipitation to concentrate the activity, dialysis and centrifugation to remove the insoluble 11S globulin, ConA-



B

Amino acid	S	P1	P2
Asp	0.92 (1)	1.00 (1)	n.d.
Thr	0.92 (1)	n.d.	0.69 (1)
Ser	1.73 (2)	1.78 (2)	n.d.
Glu	3.95 (4)	2.06 (2)	1.99 (2)
Gly	1.00 (1)	n.d.	1.03 (1)
Leu	1.00 (1)	n.d.	1.00 (1)

C

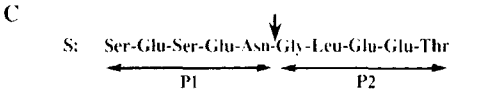


Fig. 1. Cleavage of a synthetic decapeptide at the peptide bond located on the C-terminal side of an asparagine residue by a vacuolar processing enzyme. The sequence of the decapeptide as shown in (C) was derived from the sequence around the processing site of the proglobulin which is a proprotein precursor of 11S globulin [8]. (A) HPLC elution profiles of the decapeptide substrate (S), an the two cleavage products (P_1 and P_2) after incubation with a vacuolar processing enzyme for 3 h. (B) The amino acid compositions (based on 1 mol of leucine or aspartic acid) of S, P_1 and P_2. The values in parentheses are the estimated unit content of each amino acid. n.d.=not detected. (C) Amino acid sequence of the synthetic decapeptide. The arrow indicates the site of proteolytic cleavage by the processing enzyme.

Sepharose chromatography to remove glycoproteins such as ricin, and MonoS chromatography twice. The final preparation of this processing activity contained a homogeneous band of M_r 37 000 on SDS-PAGE with Coomassie staining (Fig. 2). Although the purified enzyme transferred from SDS-gel to GVHP membrane was applied to a Peptide Sequencer, the N-terminal amino acid was not detected. Specific activity of the purified vacuolar processing enzyme was determined to be 1.9 unit per mg protein.

The purified enzyme did not bind to ConA-Sepharose, indicating that it has no high mannose oligosaccharide. The optimum pH of the purified enzyme is the same as that of the vacuolar interior, about 5.5. The processing activity is highly sensitive to thiol reagents; 80% of the activity was lost in the presence of 2 mM *N*-ethylmaleimide. The activity was also reduced 77% by 2 mM $CuCl_2$, but was unaffected by peptidyl proteinase inhibitors, 2 mg/ml pepstatin and 0.1 mg/ml cystatin. The activity was enhanced 2.5-fold by 0.1 M dithiothreitol. These data correspond to the characteristics reported for the proglobulin processing enzyme [4] and indicate that the enzyme is a thiol type endopeptidase.

Newly-synthesized proprotein precursors on rough ER are transported to the vesicles and then targeted to the vacuoles where they are proteolytically processed into their mature forms. To examine an ability of the purified enzyme to cleave the authentic proprotein pre-

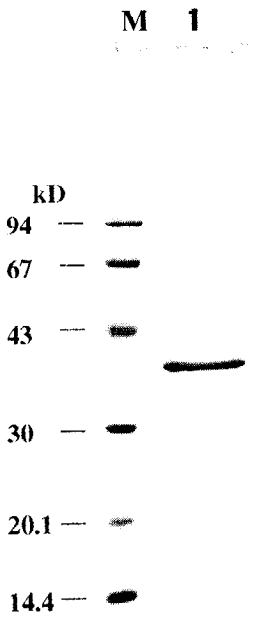


Fig. 2. Vacuolar processing enzyme purified from castor bean seeds. The processing enzyme was purified as described in Section 2 and the final preparation (14 μg) was subjected to SDS-PAGE followed by staining with Coomassie blue R-250 (lane 1). M, marker proteins; phophorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

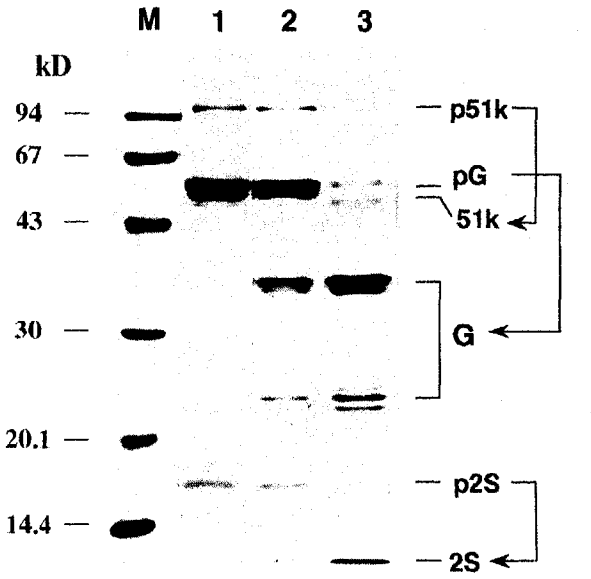


Fig. 3. In vitro proteolytic processing of proproteins located in the isolated dense vesicles. The isolated dense vesicles contained 3 different proprotein precursors (lane 1). After incubation with the purified vacuolar processing enzyme for 5 (lane 2) and 30 min (lane 3) at 37°C, each reaction mixture was subjected to SDS-PAGE (12.5% acrylamide) and the proteins were stained with Coomassie blue. Conversion of the following proproteins to the mature forms are shown: pro 51 kDa protein (p51k) to 51 kDa protein (51k), proglobulin, (pG) to 11S globulin (G) and pro2S protein (p2S) to 2S protein (2S).

cursors located in both ER and dense vesicles, we have isolated these organelles. Dense vesicles isolated from developing pumpkin cotyledons have sufficient amounts of proglobulin, pro51 kDa protein and pro2S protein to be detected by SDS-PAGE with Coomassie staining (Fig. 3, lane 1). The purified processing enzyme also cleaved not only the proglobulin but also the pro51 kDa protein and the pro2S protein to produce a mature protein of the correct size in each case (Fig. 3, lanes 2,3). The identity of each precursor and its corresponding mature form was confirmed by protein sequence analysis and immunoblot analysis (data not shown). It is noted that these mature proteins are found at different locations in the vacuole: the 2S protein is found in the soluble vacuolar matrix, the 11S globulin found in the insoluble crystalloid and the 51 kDa protein associated with the vacuole membrane.

We also examined proproteins located in ER isolated from pulse-labeled developing pumpkin cotyledons. In the ER labeled proglobulin and pro2S protein were visualized by fluorography but the pro51 kDa protein was not (Fig. 4, lane 1). During incubation of the labeled preparations with the processing enzyme the radioactivity in the proproteins decreased with a concomitant increase in the radioactivity found in the mature globulin and 2S protein (Fig. 4, lanes 2,3). Vacuoles isolated from pulse-labeled developing cotyledons contain both the precursors and mature forms of 11S globulin and the 2S protein and these were used as size

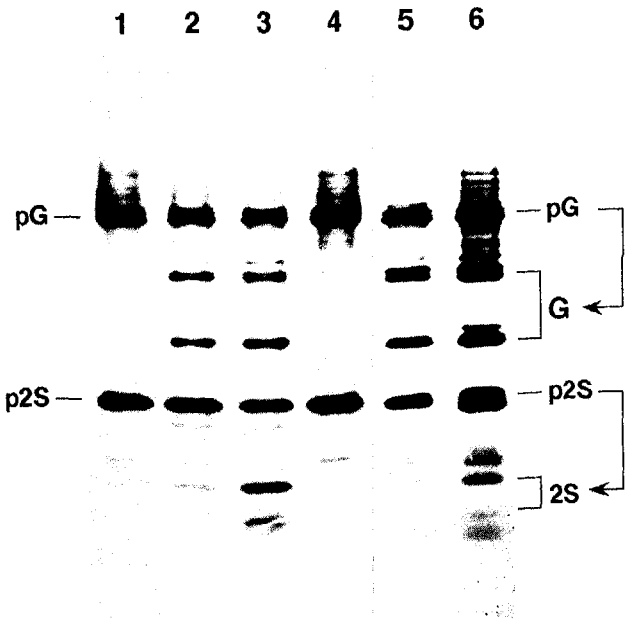


Fig. 4. In vitro proteolytic processing of proproteins from the ER labeled with ^{35}S. The ER fraction containes 2 kinds of the labeled proproteins (lane 1). The reactions with the purified vacuolar processing enzyme were incubated for 5 (lane 2), 30 min (lane 3) and 30 min in the presence of *N*-ethylmaleimide (lane 4) at 37°C then subjected to SDS-PAGE (16.5% acrylamide) using a Tris-tricine buffer system [10] followed by fluorography. Conversion of the following proproteins to the mature forms are shown: proglobulin (pG) to 11S globulin (G) and pro2S protein (p2S) to 2S protein (2S). Vacuole fraction (1 or 3 μl) containing both the precursors and mature forms of 11S globulin and the 2S protein was also shown in lane 5 of 6, respectively.

markers in the fluorograph (Fig. 4, lanes 5,6). In this gel system, using Tris-tricine as a cathode buffer and 16.5% acrylamide [10], the two polypeptides which comprise the mature 2S protein were separated. The reducing agent, dithiothreitol, splits the intrachain disulfide bonds in the proglobulin and the pro2S protein, but this appeared to have no effect on the processing of these precursors. In the presence of 2 mM *N*-ethylmaleimide, the processing of both precursors was inhibited (Fig. 4, lane 4), suggesting that a single thiol endopeptidase plays a role in this processing.

Overall, the results of this work suggest that the enzyme we have purified is the authentic processing enzyme of vacuoles and is responsible for the conversion of several vacuolar precursors into their mature forms, despite the fact that these proteins are found in different suborganellar locations. A cDNA clone of the 2S protein was isolated and used to determine the primary sequence of the precursor. The N-terminal sequence of the mature 2S protein was also determined. These sequences reveal that proteolytic cleavage occurred on the C-terminal side of an asparagine residue, as was the case in the cleavage of the proglobulin (Fig. 5).

These results raise the question of how a single processing enzyme can recognize the numerous variety of cleavage sites. Fig. 5 shows the sequences around the processing sites of the proprotein precursors to vacuolar proteins from several origins including seeds, mature leaf and yeast; the pumpkin 2S protein, pumpkin 11S globulin [8], pea vicilin [11], castor bean ricin [12], jackbean ConA [13-15], tomato proteinase inhibitor [16] and yeast carboxypeptidase Y [17]. Although processing of each of these proteins occurs on the C-terminal side of an asparagine residue, there is no homology in the primary or secondary structures of these sequences. Yeast proteinase A cleaving precursor of carboxypeptidase Y is a glycoprotein and the activity is inhibited by pepstatin [18–20], showing different characteristics from those of the vacuolar processing enzyme of castor bean. Biosynthesis of ConA is well analyzed from the stucture of its precursor [13,14]. It has been suggested that four proteolytic processing events, one of which involves a transpeptidation reaction, are carried

Pumpkin 2S Protein	26 TITTVEVEEN*RQGREERCRQ 45 65 DVLQMRGIEN*PWRREGGSFD 84
Pumpkin 11S Globulin [8]	266 YIESESESEN*GLEETICTLR 285
Pea Vicilin [11]	312 QRNENQGKEN*DKEEEQEEET 331
Castor bean Ricin [12]	294 LIRPVVPNFN*ADVCMDPEPI 313
Jackbean Concanavarin A [13-15]	139 RLLGLFPDAN*VIRNSTTIDFN*AAYN*ADTIVAVELD 173 272 WSFTSKLKSN*EIPDIATVV-COOH 290
Tomato Proteinase Inhibitor [16]	33 IELLKEFDSN*LMCEGKQMWP 52
Yeast Carboxypeptidase Y [17]	102 AIENYQLRVN*KIKDPKILGI 121

Fig. 5. The sequences around proteolytic processing site of precursors of vacuolar proteins from seeds, mature leas and yeast. The primary sequence of precursor protein for pumpkin 2S protein was deduced from the nucleotide sequence of its cDNA clone which we have isolated. The N-terminal sequence of the mature 2S protein was also determined. Post-translational processing site(s) of each precursor is indicated by an asterisk (*). Sequences are numbered relative to the start methionine of each precursor.

out by an asparagine endopeptidase on the C-terminal side of the asparagine residue which is exposed on the surface of the ConA precursor [15]. A similar processing enzyme to that we have isolated appears to function on the ConA precursor. Although so far processing enzymes specific to each proprotein precursor have been thought to function in vacuoles, this work suggests that a single processing enzyme cleaves the peptide bond on the C-terminal side of an exposed asparagine residue of various proproteins and the proteolytic processing is a universal event in vacuoles of different organisms.

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