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Expression of an Endopeptidase (EP-C1) in *Phaseolus vulgaris* Plants[1]

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Endopeptidase activity increases continually in pods of maturing fruits of French bean (*Phaseolus vulgaris* L. cv Goldstar) plants and is thought to participate in the protein mobilization in pods during the development of seeds (M. Endo, T. Minamikawa, D. Yamauchi, W. Mitsuhashi [1987] J Exp Bot 38: 1988–1995). In the present studies, one of the major endopeptidase forms, designated EP-C1, was purified as a 34-kD polypeptide from pods of maturing French bean fruits. EP-C1 was found to be immunologically distinguished from other forms in extracts from pods, but homologous to SH-EP, the major cysteine endopeptidase expressed in cotyledons of germinating *Vigna mungo* seeds (W. Mitsuhashi, T. Minamikawa [1989] Plant Physiol 89: 274–279). The level of endopeptidase that reacted with the antiserum to EP-C1 increased in pods as the fruit maturation proceeded. EP-C1 was also immunologically detected in stems of French bean plants bearing fruits of later maturation stages. Protein immunoblotting showed that a 34-kD polypeptide corresponding to EP-C1 in molecular mass occurred in extracts from 7- to 9-d cotyledons of germinating French bean seeds. In addition, two other polypeptides with slightly higher molecular masses were observed in extracts from 3- to 5-d cotyledons. We suggest that these two polypeptides are intermediates involved in posttranslational processing of EP-C1. RNA blot hybridization with EP-C1 cDNA as a probe showed that EP-C1 mRNA occurred in pods of fruits at later maturing stages and also in cotyledons of 3- to 7-d germinating seeds.

Legume pods not only protect the seeds therein but are also metabolically active tissues storing nutritional reserves for seed growth (Flinn et al., 1970; Oliker et al., 1978; Bewley and Black, 1985; Peoples et al., 1985; Endo et al., 1987). We reported evidence that, during the maturation of French bean (*Phaseolus vulgaris*) plants, a proportion of nitrogenous nutrients are temporally stored in pods as proteins and soluble amino nitrogens and are subsequently used for seed growth (Endo et al., 1987). We also observed a continual increase of endopeptidase activity, but not of amino- or carboxypeptidases, throughout the development and subsequent senescence of pods. The endopeptidase activity was thought to participate in the protein mobilization in pods during fruit maturation. Ion-exchange chromatography of enzyme extracts from pods at late stages of fruit maturation revealed three forms of endopeptidase (Endo et al., 1987).

In this work, we show the occurrence of at least five forms of endopeptidase by column chromatography of the enzyme extracts from French bean pods followed by the activity staining of the enzyme after nondenaturing PAGE. We purified one of the major forms, designated EP-C1, and, using antibody raised against this enzyme, we examined changes in amounts of the enzyme during fruit maturation. The enzyme protein and its activity were also detected in cotyledons and other organs of seedlings. EP-C1 was found to be immunologically identical to SH-EP, the major Cys endopeptidase expressed in cotyledons of germinating *Vigna mungo* seeds (Mitsuhashi et al., 1986; Akasofu et al., 1989; Mitsuhashi and Minamikawa, 1989), although there was a small difference in molecular mass between them. In fact, the amino acid sequence deduced from EP-C1 cDNA, which we reported separately (Tanaka et al., 1991), had a high degree of homology (94%) with that from SH-EP cDNA (Akasofu et al., 1989).

MATERIALS AND METHODS

Plant Materials

French bean (*Phaseolus vulgaris* L. cv Goldstar) plants were grown at the experimental farm of Tokyo Metropolitan University from May 1989 to August 1991, and, at desired developmental stages, maturing fruits were harvested and stored at −20°C until use. Fruits were harvested only once from a plant to avoid the possible influence of wounding due to depodding. We have defined five stages of French bean pod development: I (0–7 DAF), II (8–12 DAF), III (13–16 DAF), IV (17–21 DAF), and V (22–27 DAF) (Endo et al., 1987). Analysis was conducted using pods at stages II to V. For experiments with seedlings, seeds were germinated on wet vermiculite in the dark at 27°C. Green leaves and stems were collected from plants grown in a phytotron with 14 h of light (30,000 lux) and 10 h of darkness.

Endopeptidase Assay

Endopeptidase was assayed using azoalbumin as the substrate essentially as described by Endo et al. (1987). The reaction mixture, consisting of 0.3 mL of an enzyme solution containing 5 mM 2-ME and 0.1 mL of 1% azoalbumin solution in 0.1 M sodium acetate buffer (pH 5.4) in a plastic microtube, was incubated for 60 to 90 min at 30°C. The reaction was terminated by the addition of 0.4 mL of cold 10% TCA solution. The mixture was left for at least 30 min at 0 to 4°C, centrifuged, and the A_{366} of the supernatant solution was recorded against the zero-time control in a Hitachi spectro-

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Abbreviations: 2-ME, 2-mercaptoethanol; PG-plate, gelatin-containing polyacrylamide gel plate.

photometer-data processor 150–20. One unit of activity is defined as the amount of enzyme that gives an increase in 1.0 *A* unit/h under the described assay conditions.

For the assay with crude enzyme extracts from cotyledons at various germination stages, 10 pairs of cotyledons were each homogenized with 30 mL of 0.2 M sodium acetate buffer (pH 5.4) containing 5 mM 2-ME in a chilled mortar and pestle. The homogenate was centrifuged at 30,000*g* for 15 min, and the supernatant solution was used for the assay. Pods from maturing fruits were also homogenized with the buffer at w/v ratios similar to those described above. Roots, axes, and green leaves were powdered with cold acetone, dried powder was homogenized with the buffer (Endo et al., 1987), and the enzymic activities in the supernatant solution were measured as described above. Protein contents were measured by the method of Bradford (1976) using BSA as a standard.

Activity Staining of Endopeptidase on Polyacrylamide Gels

Endopeptidase activity on gels was detected by using PG-plates (Mitsuhashi et al., 1986). After the electrophoresis of enzyme solutions in a 2-mm-thick nondenaturing 9% (w/v) polyacrylamide gel (pH 9.5) at 4°C, the gel was immersed in 50 mM sodium acetate buffer (pH 5.4) containing 10 mM 2-ME for 5 to 10 min at room temperature. The gel was then placed on a PG-plate and incubated at 30°C for 120 min. The plate was stained with 1% (w/v) amido black and destained with 7.5% (v/v) acetic acid solution.

Purification of EP-C1

All manipulations were conducted at 0 to 4°C. Acetone-dried powder (10.0 g) prepared from pods (140 g) at stage V was homogenized with 300 mL of 50 mM potassium phosphate buffer (pH 7.2) containing 10 mM 2-ME in a mortar and pestle. The homogenate was squeezed through a single layer of nylon cloth and centrifuged at 27,000*g* for 20 min. Solid ammonium sulfate was added to the supernatant solution to 70% saturation and the precipitated protein was dialyzed overnight against the above buffer. After centrifugation, the dialyzed solution (50 mg of protein) was applied to a column (2 × 15 cm) of DEAE-cellulose (Whatman DE-52) that had been preequilibrated with the same buffer. The loaded column was first washed with the buffer and subsequently eluted with a linear gradient (150 mL/150 mL) of 0 to 0.6 M KCl in the buffer. The eluate was collected in 5.5-mL fractions at a flow rate of 25 mL/h. Three peaks (A, B, and C) of endopeptidase activity were detected: active fractions in each peak were concentrated by Centriprep-10 (Amicon). A portion (3.5 mg of protein) of the concentrated solution of endopeptidase C was then loaded onto a column (1.5 × 75 cm) of Sephacryl S-200 SF (Pharmacia Fine Chemicals), which had been preequilibrated with 50 mM sodium acetate buffer (pH 5.4) containing 10 mM 2-ME and 0.2 M KCl. The column was eluted with the same buffer at a flow rate of 18 mL/h, and 5.5-mL fractions were collected. The active fractions were combined, concentrated to about 1 mL (0.1 mg of protein) as above, and electrophoresed in a 2-mm-thick 9% acrylamide slab (pH 9.5) at 4°C. Narrow strips of the gel were stained for the endopeptidase activity by the PG-plate method (Mitsuhashi et al., 1986) as well as for protein with Coomassie brilliant blue R. Three activity-stained bands (EP-C1, C2, and C3) were detected on the strips. The gel band corresponding to EP-C1, the major 34-kD endopeptidase, was cut from the gel plate.

Preparation of Antiserum and Protein Immunoblotting

The fragment of gel containing the purified EP-C1 was homogenized with 1 mL of 20 mM sodium phosphate buffer (pH 7.2) containing 0.15 N NaCl in a mortar and pestle. This homogenate was emulsified with Freund's complete adjuvant and injected subcutaneously into a rabbit at intervals of 10 to 14 d for a total of nine injections. Crude immunoglobulin G fraction was separated from the serum (40 mL) by ammonium sulfate precipitation at 40% saturation and was dialyzed against the above buffer. The dialyzed fraction was diluted to 40 mL with the buffer and used as the antiserum.

The immunospecificity of the antiserum to the endopeptidase was examined by the immunoblot method. After SDS-PAGE on 1-mm-thick 12.5% gels, the immunoblotting was conducted as described previously (Yamauchi and Minamikawa, 1987) except that the blocking solution contained 3% skim milk instead of gelatin. Protein bands were visualized by Vectastain ABC kit (Vector Laboratories, Burlingame, CA). To test the immunoadsorption of enzymic activity by the antiserum, 10 μL of the active fraction C (30 μg of protein) obtained from the column of DEAE-cellulose was mixed with 100 μL of the antiserum that had been bound to protein A-Sepharose CL-4B (30 mg; Pharmacia Fine Chemicals) in 50 mM Tris-HCl buffer (pH 7.8) containing 0.9% NaCl, and was incubated for 1 h at 30°C. The mixture was centrifuged and the residual enzymic activity in the supernatant was detected by the PG-plate method after nondenaturing PAGE.

RNA Blot Hybridization

Total RNA fractions from cotyledons were prepared by the SDS-phenol method essentially as described previously (Suzuki and Minamikawa, 1983). Total RNA fractions from pods were prepared by the guanidium thiocyanate method followed by centrifugation in a cesium chloride solution (Sambrook et al., 1989). Poly(A^+) RNAs were separated using messenger-activated paper (Takara Shuzo) (Wreschner and Herzberg, 1984). The glyoxalated RNAs were separated by electrophoresis on 1.4% agarose gels and hybridized at 55°C with the ^{32}P-labeled cDNA insert of pPPP130 (Tanaka et al., 1991) after the method of Thomas (1980), except that a Hybond nylon membrane (Amersham) was used instead of a nitrocellulose sheet. ^{32}P-labeled EP-C1 cDNA, PPP130, probe was generated using a random primer labeling kit (Boehringer Mannheim).

RESULTS

Purification of EP-C1

Chromatography on a column of DEAE-cellulose separated the crude preparation of enzyme into three active fractions (A, B, and C). When subjected to nondenaturing PAGE, enzyme C was found to consist of three forms (EP-C1, -C2,

and -C3) (Fig. 1). Further chromatography of enzyme C on a column of Sephacryl S-200 gave a single peak of endopeptidase (Fig. 2A). This gel filtration procedure removed the bulk of other proteins from enzyme C, and, when examined by nondenaturing PAGE, five protein bands appeared on the gel, three of which corresponded to the EP-C1, -C2, and -C3 bands observed by the activity staining (Fig. 2B). Thus, three protein bands were excised separately from the gel plate. Each of the proteins, EP-C1, -C2, and -C3, in excised gels gave a single polypeptide band as revealed by SDS-PAGE (Fig. 2C); molecular masses were estimated to be 34, 23, and 30 kD, respectively. The results were confirmed by two-dimensional PAGE (nondenaturing PAGE and SDS-PAGE) of enzyme C obtained from the gel-filtration column (data not shown).

Nondenaturing PAGE of an active fraction C obtained from the DEAE-cellulose column followed by activity staining of endopeptidase activity by the PG-plate method showed that EP-C1, -C2, and -C3 were highly sensitive to Cys protease inhibitors, 5 μM leupeptin, 5 μM antipain, and 10 μM E-64 (Fig. 3). These three enzymes had little sensitivity to a Ser protease inhibitor, PMSF, at a 2-mM concentration (data not shown).

Specificity of Antiserum to EP-C1

Immunoblotting after SDS-PAGE of a crude extract (30 μg of protein) from pods at stage V showed that the antiserum reacted strongly only with the 34-kD polypeptide corresponding to EP-C1 (Fig. 4A). The antiserum also appeared to adsorb only the activity of EP-C1, and not activity of EP-C2 or -C3, in the presence of protein A-Sepharose CL-4B (Fig. 4B). The results indicated that the antiserum was specific to EP-C1 and reacted with neither EP-C2 nor -C3.

Changes in Levels of EP-C1 in Pods during Fruit Maturation

Endopeptidase activity in pods increases throughout development and maturation of French bean fruits, with the highest activity in extracts from pods at stage V despite the progress of senescence (Endo et al., 1987). Protein immunoblot analysis showed that levels of endopeptidase that reacted with the antiserum to EP-C1 also increased in pods as the fruit maturation proceeded from stages II to V (Fig. 5A). Although the total endopeptidase activity in pods was not necessarily attributable exclusively to levels of the enzyme that reacted with the antiserum to EP-C1, the change in levels of EP-C1 corresponded well with the change in the endopeptidase activity during fruit maturation.

The protein immunoblotting revealed that the protein that reacted with the antiserum to EP-C1 was present in stems of French bean plants bearing the stage V fruits, but this was not detectable in green leaves of the same plants or in immature seeds in pods at stages III to V (Fig. 5B).

Immunological Homology between EP-C1 and SH-EP

We previously reported the synthesis and expression of a Cys endopeptidase (SH-EP) in cotyledons of germinating *Vigna mungo* seeds (Mitsuhashi et al., 1986, Mitsuhashi and Minamikawa, 1989). SH-EP acts on the degradation of seed

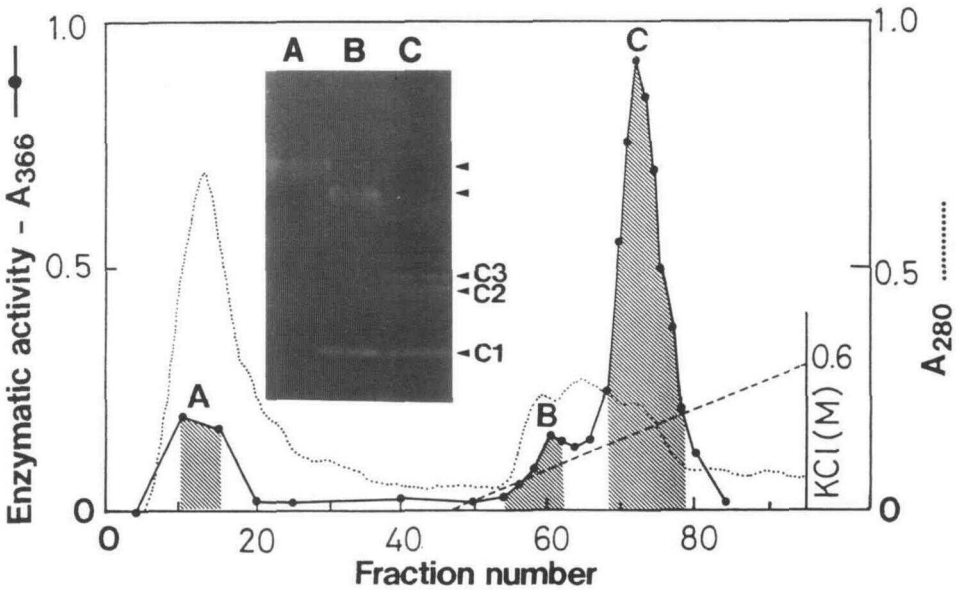

Figure 1. DEAE-cellulose column chromatography of endopeptidase. A 70% saturated ammonium sulfate fraction of crude enzyme extracts from pods of stage V was applied to the column (2 × 15 cm), which had been preequilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 10 mM 2-ME. The column was eluted with a linear gradient of 0 to 0.6 M KCl in the same buffer. Endopeptidase was assayed as described in "Materials and Methods." Inset, PAGE patterns of endopeptidase activity in active fractions A, B, and C from the DEAE-cellulose column. Three active fractions (30 μL each) were separated by nondenaturing PAGE, and activity bands (indicated by arrows) were detected by the PG-plate method.

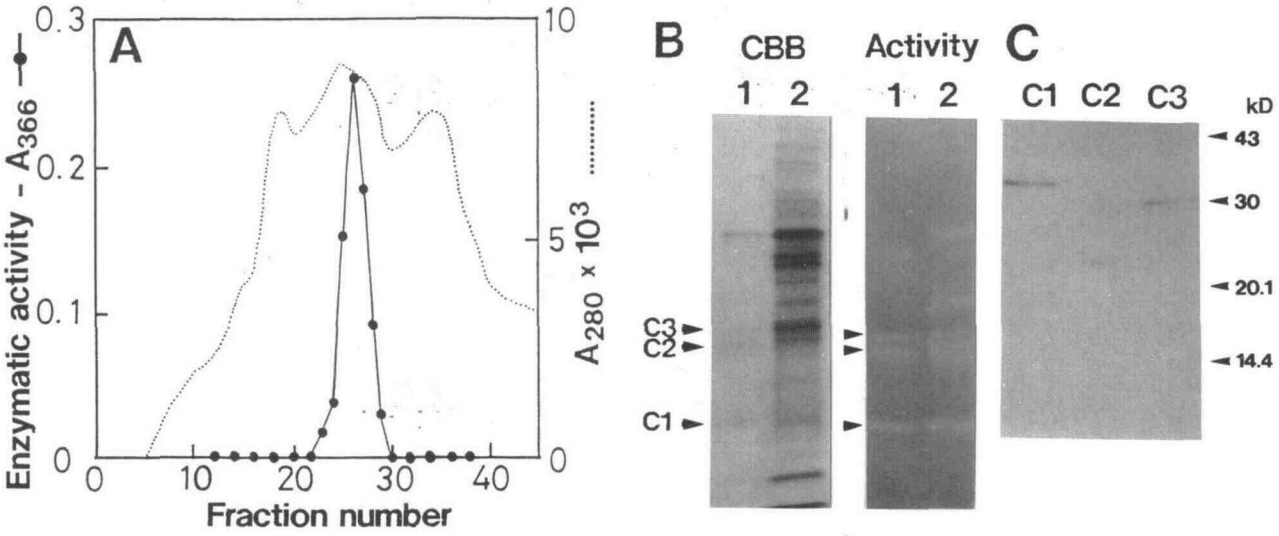

Figure 2. Separation of endopeptidase in active fraction C by gel filtration chromatography (A) and PAGE (B). A, Active fraction C obtained from the DEAE-cellulose column was concentrated and applied to a Sephacryl S-200 column and eluted with 50 mM sodium acetate buffer (pH 5.4) containing 0.2 M KCl and 10 mM 2-ME. The eluted fractions were assayed for endopeptidase activity as described in "Materials and Methods." B, The active fraction obtained from the gel filtration column was concentrated and separated by PAGE (lane 1). Concentrated active fraction C from the DEAE-cellulose column (Fig. 1) was loaded on lane 2. After gel electrophoresis, the gels were stained with Coomassie brilliant blue R (CBB) or activity stained by the PG-plate method. The bands corresponding to EP-C1, -C2, and -C3 are indicated by arrowheads. C, The active fraction obtained from the Sephacryl S-200 column was concentrated, separated by PAGE, and the protein bands corresponding to EP-C1, -C2, and -C3 were separately excised from the gel plate. The proteins in the excised gels were then analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue R.

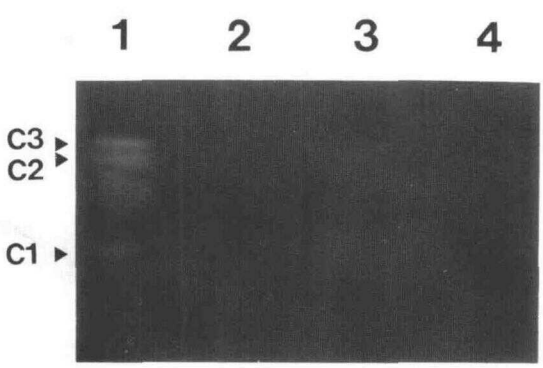

Figure 3. Effects of inhibitors on endopeptidase activities. An active fraction C (10 μg of protein) obtained from the DEAE-cellulose column (cf. Fig. 1) was concentrated by Centriprep-10 and separated by nondenaturing PAGE. After the electrophoresis, the gels were subsequently incubated in 50 mM sodium acetate buffer (pH 5.4) containing 10 mM 2-ME in the absence (lane 1) or presence of inhibitors, 5 μM leupeptin (lane 2), 5 μM antipain (lane 3), and 10 μM E-64 (lane 4). The activity-stained bands of the endopeptidases indicated by the arrowheads were detected by the PG-plate method.

globulin in combination with the other major enzyme, a Ser endopeptidase, and was purified as a 33-kD polypeptide. Therefore, we examined the immunological relationship between SH-EP and EP-C1 by protein immunoblotting after SDS-PAGE using crude extracts from pods of maturing fruits and cotyledons of germinating seeds of both *V. mungo* and French bean. Results indicated that antiserum to SH-EP reacted not only with a 33-kD enzyme from *V. mungo* pods,

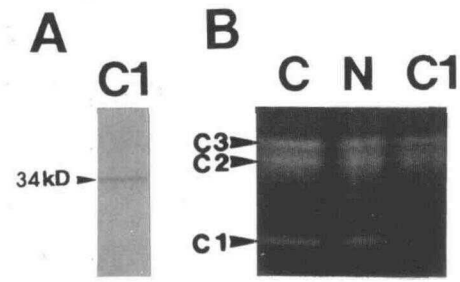

Figure 4. Specificity of the antiserum raised against EP-C1. A, Crude extract (30 μg of protein) from pods of stage V was analyzed by SDS-PAGE followed by immunoblotting using the antiserum to EP-C1. B, The active fraction C (30 μg of protein) from the DEAE-cellulose column was adsorbed by a nonimmune serum (lane N), and the antiserum to EP-C1 (lane C1). A nonadsorbed sample was loaded onto lane C. The active bands of endopeptidase were separated by PAGE and detected by the PG-plate method.

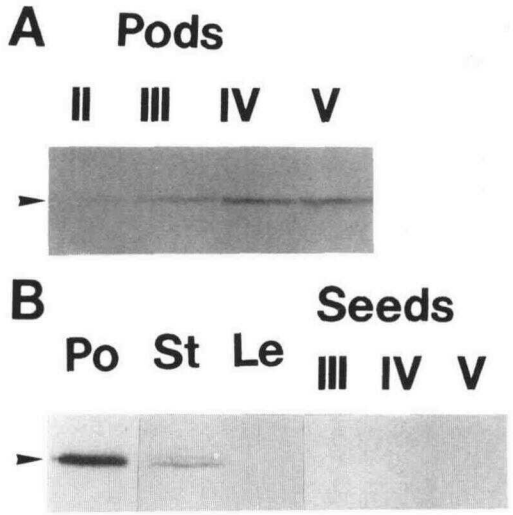

Figure 5. Distribution of EP-C1 in various organs of French bean plants bearing maturing fruits. A, Crude extracts (30 μg of protein) prepared from pods of stages II to V were analyzed by SDS-PAGE followed by immunoblotting using the antiserum to EP-C1. B, Crude extracts (30 μg of protein) prepared from pods (Po), leaves (Le), and stems (St) of plants bearing fruits of stage V and developing seeds collected from fruits of stages III to V were analyzed by SDS-PAGE and immunoblotting as described above.

but also with EP-C1 from both pods and cotyledons of French bean plants (Fig. 6). However, bands corresponding to EP-C1 from these organs of French bean showed a molecular mass of 34 kD, approximately 1 kD larger than that of SH-EP from *V. mungo*. The antiserum to SH-EP did not react with the endopeptidases other than EP-C1 in extracts from French bean pods. Almost identical results were observed when the antiserum to EP-C1 was used in the immunoblotting.

Distribution of Endopeptidase in Various Organs of Seedlings

Little or no endopeptidase activities were detected in quiescent seeds of French beans. The enzymic activity became detectable at 3 d postimbibition, reached a maximum at 7 to 9 d, and decreased thereafter in cotyledons of dark-grown seedlings (Fig. 7A). The developmental pattern of the enzymic activity coincided with the decrease in fresh weight of cotyledons during germination, suggesting the contribution of the endopeptidase in mobilization of reserve proteins in seeds. Immunoblot analysis revealed that a 34-kD polypeptide corresponding to EP-C1 in molecular mass occurred in extracts from 7-, 9-, and 11-d cotyledons. However, two polypeptides with approximate molecular masses of 34.5 and 35.5 kD were detected in extracts from 3-d cotyledons, and two close polypeptide bands of 34 and 34.5 kD were detected in extracts from 5-d cotyledons. The intensities of bands stained by immunoblotting were found to be inconsistent with the enzymic activities when compared with intensities and activities for 7- to 11-d cotyledons (Fig. 7A). Endopeptidase activities were also detected in other organs of seedlings. When compared with cotyledons, the enzymic activities were very low in axes of dark-grown seedlings and still lower in roots of seedlings and young green leaves (Fig. 7B). Only the band of 34-kD polypeptide was detected in extracts from cotyledons and roots by immunoblotting. However, the band corresponding to the 33-kD polypeptide of SH-EP and the faint band of the 34-kD polypeptide were observed in extracts from axes and green leaves.

Occurrence of EP-C1 mRNA in Pods and Other Organs

RNA blot hybridization with EP-C1 cDNA as a probe showed that EP-C1 mRNA occurred in pods of maturing fruits at stages III to V but not at stage II, the maximum level being at stage IV (Fig. 8A). The EP-C1 mRNA was not detected in cotyledons of immature seeds in fruits at stage V and of quiescent mature seeds (0-d seeds), but it became detectable in 3- and 7-d cotyledons (Fig. 8B). EP-C1 mRNA migrated to the same position as 1.3-kb SH-EP mRNA.

DISCUSSION

Pods of maturing French bean fruits started to increase in fresh weight at 7 DAF, reached a peak at 16 to 18 DAF, and turned to senescence thereafter, whereas seeds began to grow at 14 DAF and continued to increase in fresh weight during the developmental period up to 27 DAF (Endo et al., 1987). Endopeptidase activity in pods increased throughout the maturation of fruits, and ion-exchange chromatography of enzyme extracts from pods at stage V (22–27 DAF) revealed three forms (Endo et al., 1987). These forms were thought to play a leading role in the protein mobilization in pods. In the present studies with column chromatography and nondenaturing PAGE analysis, we detected at least five forms of endopeptidase in extracts of pods at stage V (Fig. 1).

EP-C1, one of the major endopeptidase forms occurring in French bean pods, differed in molecular mass from other forms, EP-C2 and -C3, and the antiserum to EP-C1 did not react with the other forms (Fig. 2), although these were all shown to be sensitive to the Cys protease inhibitors (Fig. 3). However, EP-C1 had immunological homology with SH-EP, the Cys endopeptidase acting on storage globulin in germi-

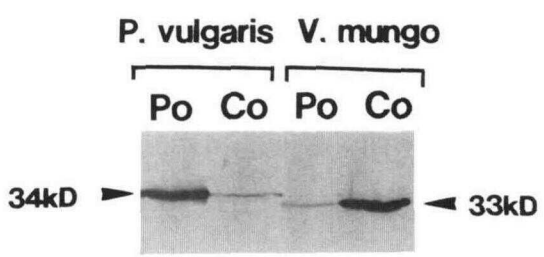

Figure 6. Immunological homology between EP-C1 of French bean plants and SH-EP of *V. mungo*. Crude extracts were prepared from pods (Po) and cotyledons (Co) of both French bean plants and *V. mungo* and were analyzed by SDS-PAGE followed by immunoblotting using the antiserum to SH-EP. The amount of protein loaded was 20 μg each for pods and 15 μg each for cotyledons. The arrowheads point to 34-kD EP-C1 (left) and 33-kD SH-EP (right).

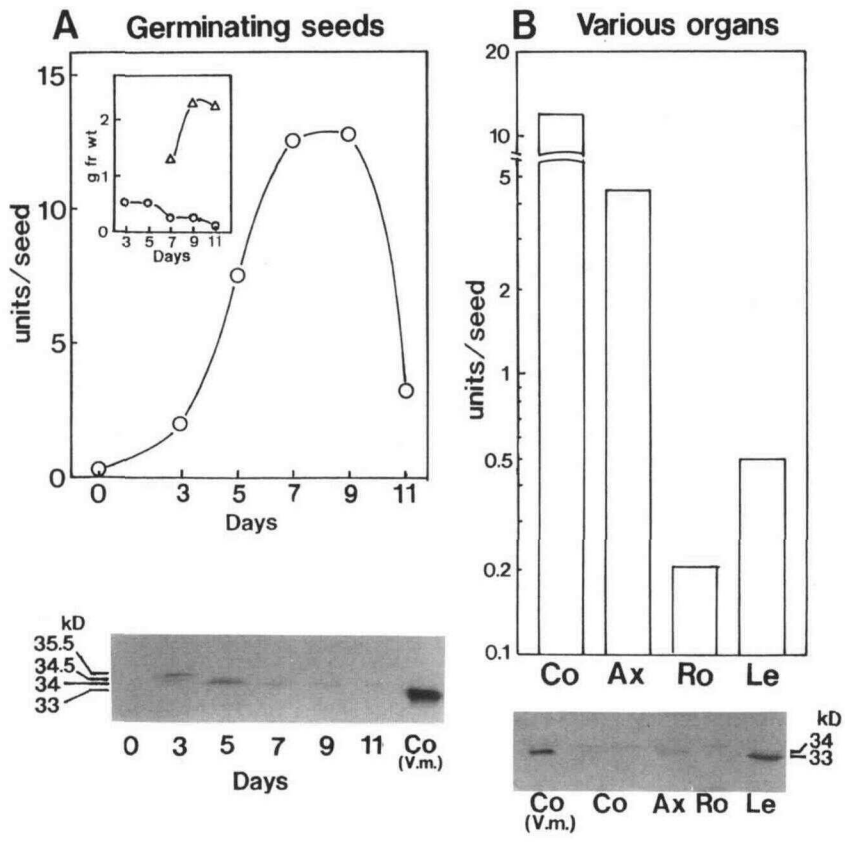

Figure 7. Activities and immunoblot patterns of endopeptidase in cotyledons (A) and other organs (B) of French bean seedlings. A, Enzymic activities in extracts from 0- to 11-d cotyledons were measured as described in "Materials and Methods." After SDS-PAGE of the extracts (20 μL each), bands of the enzyme were visualized by immunoblotting using the antiserum to SH-EP. Co (V.m.), An extract from 4-d cotyledons of *V. mungo*. B, Extracts from 9-d cotyledons (Co), 9-d axes (Ax), 9-d roots (Ro), and 11-d green leaves (Le) were analyzed by SDS-PAGE followed by immunoblotting. To concentrate the extracts from acetone-dried powder of axes, roots, and leaves, each extract was dialyzed against distilled water, lyophilized, and dissolved in a small volume of the SDS-PAGE sample buffer; then a solution corresponding to the extract from a seedling was loaded onto a lane of PAGE. The extract from cotyledons was electrophoresed as described above.

nating *V. mungo* seeds (Fig. 6). In fact, our recent results indicated 96% homology in the amino acid sequence of the putative mature enzyme regions between EP-C1 and SH-EP (Akasofu et al., 1989; Tanaka et al., 1991), although there was a small difference in molecular mass between the two (Fig. 6). The amino acid sequence of EP-C1 also exhibited high degrees of homology with those of other Cys endopeptidases reported to date, such as barley EP-B (60%) (Koehler and Ho, 1990), barley aleurain (43%) (Rogers et al., 1985), papain (46%) (Cohen et al., 1986), and actinidin (52%) (Podivinsky et al., 1989). Therefore, we postulate that EP-C1 of French bean plants is a Cys endopeptidase of a type closely related to SH-EP, which is mainly expressed in cotyledons of germinating *V. mungo* seeds.

SDS-PAGE immunoblotting and RNA blot hybridization indicated that the amount of EP-C1 mRNA in pods of stages II to V also coincided with amounts of 34-kD EP-C1 (Fig. 8), suggesting the regulation of EP-C1 expression in pods at the transcriptional level. The endopeptidase that reacted with the antiserum to EP-C1 or SH-EP was detected not only in pods, but also in cotyledons and other organs of seedlings (Fig. 7). With regard to molecular mass, a 34-kD enzyme corresponding to EP-C1 was detected in cotyledons and roots and a 33-kD enzyme corresponding to SH-EP was detected mainly in axes and young leaves (Fig. 7). This suggests that EP-C1 or related endopeptidases are distributed in organs of different growth stages. A disparity in molecular mass between immunologically homologous endopeptidases detected in different organs was also observed in *V. mungo* seedlings (Yamauchi et al., 1992). Whether or not these endopeptidase forms are directed by the same gene remains an open question, although the genomic DNA gel blot analysis has suggested that there is a single copy of SH-EP in *V. mungo* (Yamauchi et al., 1992).

In addition to pods of maturing fruits, high endopeptidase activities with a maximum at 7 to 9 d were observed in cotyledons of germinating bean seeds. Intensities of the EP-C1 bands visualized by the immunoblotting, however, did not always correspond to the enzymic activities, and two cross-reacting antigens with molecular masses higher than EP-C1 were observed in extracts from cotyledons at 3 and 5 d (Fig. 7). We postulate that these polypeptides with tentatively estimated molecular masses of 34.5 and 35.5 kD are inactive precursors of the 34-kD mature EP-C1, since enzyme extracts from 3- and 5-d cotyledons exhibited lower enzymic activities than those for 7- and 9-d cotyledons.

Unlike EP-C1, we have not observed cross-reacting antigens of postulated precursors of SH-EP in our time-course study of the endopeptidase in cotyledons of germinating *V. mungo* seeds (Mitsuhashi and Minamikawa, 1989). However, when enzyme extracts from 3-d cotyledons of *V. mungo* seeds were incubated at 5°C for 3 to 25 h, several polypeptides with molecular masses in a range of 36 to 43 kD were immunologically detected after nondenaturing PAGE. We have proposed possible steps of the posttranslational processing of SH-EP in which the enzyme is first synthesized as the 45-kD precursor with a 2-kD signal peptide being cleaved to the 43-kD polypeptide, which is further cleaved to give

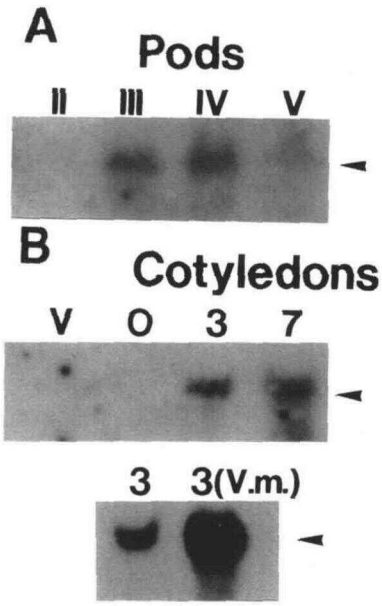

Figure 8. RNA blot hybridization showing the presence of EP-C1 mRNA in both pods of maturing fruits and cotyledons of germinating seeds. A, Poly(A$^+$) RNA (5 μg each) from pods at stages II to V were analyzed by RNA blotting with PPP130 cDNA as a probe. The arrowhead points to EP-C1 mRNA. B, Total RNAs (15 μg each) prepared from cotyledons of immature seeds collected from fruits of stage V (lane V) and cotyledons of 0-, 3-, and 7-d germinating seeds (lanes 0, 3, and 7, respectively) were analyzed as above. Poly(A$^+$) RNA (3 μg) from 3-d cotyledons of germinating *V. mungo* (V.m.) seeds was analyzed together with 3-d cotyledons of germinating French bean seeds as above. The arrowheads point to both EP-C1 mRNA and SH-EP mRNA.

the 33-kD mature enzyme through 39- and 36-kD intermediates (Mitsuhashi and Minamikawa, 1989).

Other evidence for detection of plant endopeptidase precursors has been reported by Koehler and Ho (1990). They investigated the posttranslational processing of EP-B and found that prepro-EP-B of 42.5 kD is processed in a multistep fashion to produce the mature EP-B of 30 kD through several intermediates with molecular masses in a range of 37 to 41 kD. EP-A, another major Cys protease with a molecular mass of 37 kD in barley aleurone layers, also appeared to be formed from the 47.5-kD precursor through 45- and 44-kD intermediates (Koehler and Ho, 1990).

Recently, Kalinski et al. (1992) reported a soybean vacuolar protein (P34) related to Cys proteases. This 34-kD protein is synthesized as a 47-kD glycosylated pro-form during seed maturation, and the precursor is processed to mature P34 in a single step. Provided that the two polypeptides detected in cotyledonary extracts of French bean seedlings by SDS-PAGE immunoblotting are intermediates involved in posttranslational processing of EP-C1, the cotyledons may provide a useful experimental system to investigate the processing mechanism of plant Cys endopeptidases. It is also of interest to examine whether the mature EP-C1 is synthesized in pods of maturing French bean fruits through the same posttranslational processing that occurs in seedlings.

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