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Separation and Characterization of Two Endopeptidases from Cotyledons of Germinating *Vigna mungo* Seeds[1]

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ABSTRACT

Two major endopeptidases were present in cotyledons of germinating *Vigna mungo* seeds, as detected by the zymogram after polyacrylamide gel electrophoresis. They were not detectable in cotyledons of dry seeds, but their intensities on the zymogram increased during germination. During incubation of detached cotyledons, however, the activities showed only a slight increase for 5 days. These two endopeptidases could be separated by Sephacryl S-200 column chromatography. One of them was found to be a serine-endopeptidase as judged by phenylmethylsulfonyl-fluoride and diisopropyl fluorophosphate inhibition. The other was a sulfhydryl-endopeptidase because of its dependency on 2-mercaptoethanol and inhibition by leupeptin, chymostatin, and antipain. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis indicatd that the two endopeptidases digested the *Vigna mungo* seed globulin subunits at different rates. The serine enzyme digested the 56 kilodalton subunit at first, but the sulfhydryl enzyme digested the 54 kilodalton peptide more efficiently than the 56 kilodalton peptide. The pattern of digestion of globulin by the combination of the serine- and sulfhydryl-endopeptidases was similar to that using crude enzyme extracts.

Proteases are classified into endopeptidases and exopeptidases. Endopeptidases are further divided into serine-, sulfhydryl-, metal-, and acid-endopeptidases, based on mechanistic considerations (24). Many endopeptidases have been found in various plant tissues, especially in fruits and seeds. Papain and blomelain are well known fruit sulfhydryl endopeptidases (1, 19). A number of reports (6, 9, 10, 12, 23–29) have suggested that, in germinating seeds, sulfhydryl- and acid-endopeptidases are most responsible for the degradation of seed storage proteins.

We reported previously that endopeptidase activity increased in *Vigna mungo* cotyledons during germination and that by the removal of embryonic axis the increase was delayed and the mobilization of storage proteins depressed (16, 17). Thus, we forecast that the endopeptidase played a major role in the degradation of storage proteins in *V. mungo* cotyledons during germination, and examined the possibility that storage proteins are hydrolyzed by a cooperative action of more than one type of endopeptidase. In this paper, we have elucidated that at least two different types of endopeptidase are present in cotyledons of *V. mungo* seedlings. We separated them, characterized their enzymic properties, and examined them for digestion patterns of seed globulin by gel electrophoresis.

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MATERIALS AND METHODS

Plant Materials and Enzyme Preparation. *Vigna mungo* seeds were germinated on layers of wet filter paper at 26 to 28°C in the dark. For experiments with detached cotyledons, cotyledons excised from dry seeds were allowed to imbibe water for 6 h, they were then surface-sterilized in 1% NaOCl for 5 min, rinsed with sterile H_2O, and incubated in a Petri dish at 26 to 28°C as described previously (17). Cotyledons were harvested at desired stages of germination or incubation, and stored at −20°C until use. Cotyledons were homogenized with 50 mM Tris-HCl buffer (pH 7.4), containing 10 mM 2-ME[2] in a cold mortar and pestle and centrifuged at 25,000*g* for 20 min. The solution was dialyzed overnight against 50 mM sodium acetate buffer (pH 5.4), containing 10 mM 2-ME and centrifuged at 27,000*g* for 20 min. The clarified solution was used as a crude enzyme solution.

Separation of Two Endopeptidases. Cotyledons (8 g) from 4-d-old seedlings were blended in liquid N_2. The frozen powder was suspended in 16 ml of 50 mM Tris-HCl buffer (pH 7.4), containing 10 mM 2-ME, and centrifuged at 25,000*g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to give 80% saturation and the solution was centrifuged at 25,000*g* for 20 min. The precipitate was suspended in about 3 ml of 50 mM sodium acetate buffer (pH 5.4), containing 10 mM 2-ME, and dialyzed against the same buffer overnight. The dialyzed solution was clarified by centrifugation and 2 ml of the supernatant was mixed with 0.2 ml of Blue-dextran (4 mg/ml), 0.2 ml of DNP-alanine (2 mg/ml) and 0.4 ml of 80% (w/v) sucrose. The mixed solution was passed through a Sephacryl S-200 column (1.5 × 75 cm) preequilibrated with 50 mM sodium acetate buffer (pH 5.4), containing 10 mM 2-ME and 0.2 M KCl. The eluate was collected in 2.0 ml fractions at a flow rate of 10.2 ml/h. The contents of tubes 51 to 56 were combined to produce active fraction I and the contents of tubes 66 to 71 corresponding to fracton II were similarly combined.

Protease Assay. Hemoglobin, azocasein and gelatin were used as substrates for the measurement of endopeptidases activity. When hemoglobin was used, 0.2 ml of 1% (w/v) hemoglobin dissolved in 0.1 M sodium acetate buffer (pH 5.4) and 0.2 ml of enzyme solution were mixed. The mixture was incubated at 37°C for 50 min, and the reaction was stopped by the addition of 0.4 ml of 20% (w/v) TCA solution. The precipitated proteins were removed by centrifugation, and free α-amino N was determined using the ninhydrin color reaction (30). When azocasein was used, 150 μl of 2% (w/v) azocasein was added to the enzyme

[2] Abbreviations: 2-ME, 2-mercaptoethanol; AAPase, alanine-aminopeptidase; BAPAase, α-benzoyl-DL-arginine-*p*-nitroanilide hydrolyzing enzyme; BPB, bromophenol blue; CBPA, *N*-carbo-benzoxyl-L-phenylalanyl-L-alanine; DFP, diisopropyl fluorophosphate; I-AA, iodoacetic acid; LAPase, leucine-aminopeptidase; pCMB, *p*-chloromercuribenzoic acid; PG-plate, gelatin containing polyacrylamide plate; PMSF, phenylmethylsulfonylfluoride; STI, soybean trypsin inhibitor.

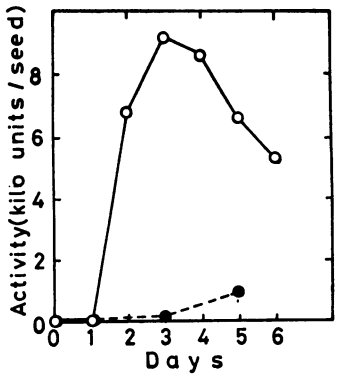

FIG. 1. Changes in endopeptidase (gelatin hydrolytic) activity of attached and detached cotyledons. Activity was measured using crude extracts prepared from attached cotyledons (O——O) and from detached and incubated cotyledons (●- - -●) by the gelatin containing polyacrylamide gel plate method (18). One unit of activity was defined as reduction rate of optical density at 525 nm/h under the assay conditions.

solution (150 μl) and incubated at 30°C for 40 min. After terminating the reaction with 5% (w/v) TCA (700 μl), the *A* at 366 nm was measured as described previously (17). Endopeptidase activity on gelatin was assayed by the PG-plate method (18). PG solution consists of 1% (w/v) gelatin, 7.5% (w/v) acrylamide, 0.18% (w/v) *N*, *N'*-methylene bis (acrylamide), 0.1% (w/v) ammonium persulfate, and 0.058% (v/v) *N*, *N*, *N'*,*N'*-tetramethylethylenediamine. This solution was spread on a glass plate, double layers of polyvinyl chloride tape were applied at the four corners, and covered with another glass plate. This layer was polymerized at 60°C for about 15 min. After the upper glass was removed carefully, the enzyme solution (10 μl) was spotted on the PG-plate surface and incubated at 30°C for 40 min. The plate was stained with 1% (w/v) Amido Black and destained. Absorbance of the clear zone was measured at 525 nm by a densitometer to estimate the enzyme activities. Activities of carboxypeptidase and aminopeptidase (LAPase and AAPase) were determined with CBPA and L-leucine-nitroanilide or L-alanine-nitroanilide as substrates, respectively (17).

For the detection of endopeptidase activity on the electrophoretogram, after the electrophoresis with Davis' system using 9% (w/v) polyacrylamide gel with 2 mm thickness, the gel was immersed in 1 M sodium acetate buffer (pH 5.4) containing 10 mM 2-ME for 5 to 10 min at room temperature. After the excess solution on the gel surface was removed by the lens paper, and the gel 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 (18).

Digestion of Seed Globulin *in Vitro*. Seed globulin was prepared from 1-d-imbibed *V. mungo* seeds according to the method of Basha and Beevers (2). The globulin (1.5 mg) was dissolved in 1 ml of 0.1 M NaCl and centrifuged at 27,000*g* for 20 min, and the clear supernatant was used as a substrate solution. Globulin solution (100 μl) was mixed with 100 μl of enzyme solution which was obtained from a Sephacryl S-200 column as described above. After an appropriate time of incubation at 30°C, the reaction was stopped by the addition of SDS sample buffer consisting of 25 mM Tris-HCl buffer (pH 6.8), 4% (w/v) SDS, 10% (v/v) 2-ME, 20% (v/v) glycerol, and a small amount of BPB, and the mixture was boiled for 2 min. Digested products were analyzed by SDS-PAGE (14).

Silver Staining. Proteins on a gel were stained after the method of Oakley *et al.* (22) with some modifications. For preparing the staining solution, one volume of 19.4% (w/v) $AgNO_3$ and 5.25 volumes of 0.36% (w/v) NaOH were mixed just before use. Conc. NH_4OH was added dropwise to the mixture until brown precipitates disappeared, and the solution was made up to 25 volumes. After electrophoresis, the gel was immersed twice in methanol:acetic acid:water (5:1:5, v/v/v) for 1 h each with gentle shaking, and washed with distilled H_2O for 30 min. The fixed gel was immersed in the silver staining solution with shaking for 20 min and rinsed with distilled H_2O for 4 min. The silver-treated gel was developed with 0.005% (w/v) citrate solution

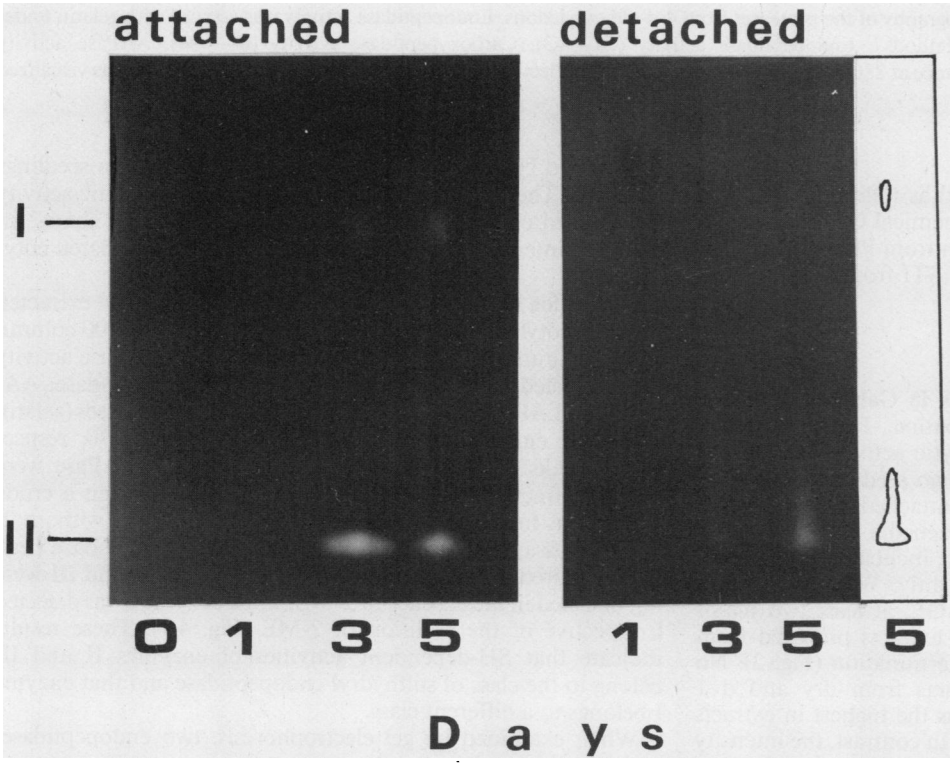

FIG. 2. Polyacrylamide slab gel electrophoresis pattern of endopeptidase activity in attached and detached cotyledons. Fifteen pairs each of the cotyledons were collected at indicated stages of germination and incubation. Dialyzed enzyme solutions were charged on a 9% polyacrylamide gel. For details, see "Materials and Methods".

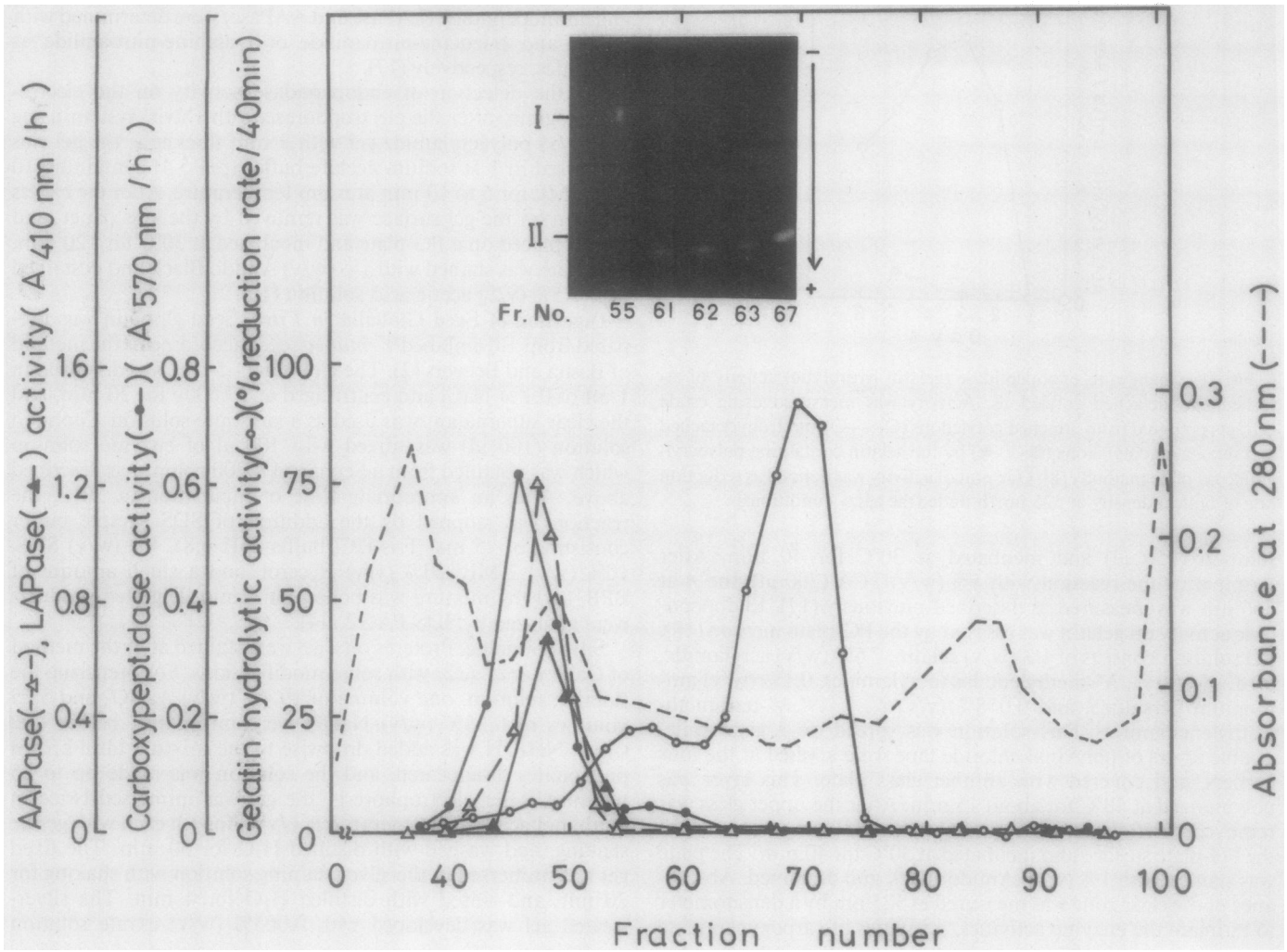

FIG. 3. Sephacryl S-200 column chromatography of the proteases from 4-d-old cotyledons. Endopeptidase activity was assayed with gelatin under the conditions given in "Materials and Methods". Endopeptidase activity (○——○), Carboxypeptidase activity (●——●), AAPase activity (△- - -△), LAPase activity (▲——▲). Absorbance at 280 nm (- - -). Polyacrylamide gel electrophoresis of eluted endopeptidase activity was visualized as for Figure 2.

containing 0.019% (v/v) formaldehyde.

Chemicals. Chemicals were purchased as follows: Azocasein, CBPA, PMSF, and DFP from Sigma Chemical Co. Ltd, pepstatin, antipain, leupeptin, and chymostatin from Protein Research Foundation (Minoh-shi, Osaka, Japan), STI from Worthington Biochem Co. Ltd. (Freehold, NJ).

RESULTS

Quantitative and Qualitative Changes in Gelatin Hydrolytic Activity during Germination or Incubation. Figure 1 shows changes in the levels of gelatin hydrolytic activity in attached and detached cotyledons of *Vigna mungo* seeds during germination or incubation. The activity in attached cotyledons increased rapidly after a lag period and reached a maximum level at d 3, then declined. In detached and incubated cotyledons, however, the activity increased very slightly. When the gelatin hydrolytic activity was analyzed by PAGE, at least two major bands of the activity were detected in extracts prepared from cotyledons at the 3 and 5 d stages of germination (Fig. 2). No bands were observed on gels of extracts from dry and d 1 cotyledons. The intensity of band II was the highest in extracts from d 3 and lower in extracts from d 5. In contrast, the intensity of the band I increased as extracts were prepared from seedlings up to d 5. There was only a small increase in proteolytic activity in detached cotyledons as indicated by only a slight change in the band intensity from extracts prepared from incubated cotyledons.

Separation of Two Endopeptidases. A crude enzyme extracted from d 4 cotyledons was fractionated by Sephacryl S-200 column chromatography (Fig. 3). At least five peaks of protease activity were detected; endopeptidases I and II, carboxypeptidase, AAPase, and LAPase. Two peaks (I and II) of endopeptidases (gelatin hydrolytic enzymes) appeared at fractions 55 and 70, respectively. Peaks of carboxypeptidase, AAPase, and LAPase were eluted at fractions 45, 47, and 48, respectively. When a crude extract was fractionated by column chromatography without 2-ME and assayed with added 2-ME, a new endopeptidase peak (III) appeared at fraction 80 (Fig. 4A). Enzymes II and III were not detected in the absence of 2-ME, while enzyme I was detected irrespective of the addition of 2-ME (Fig. 4A). These results indicate that SH-dependent activities of enzymes II and III belong to the class of sulfhydryl endopeptidase and that enzyme I belongs to a different class.

When examined by gel electrophoresis, two endopeptidases

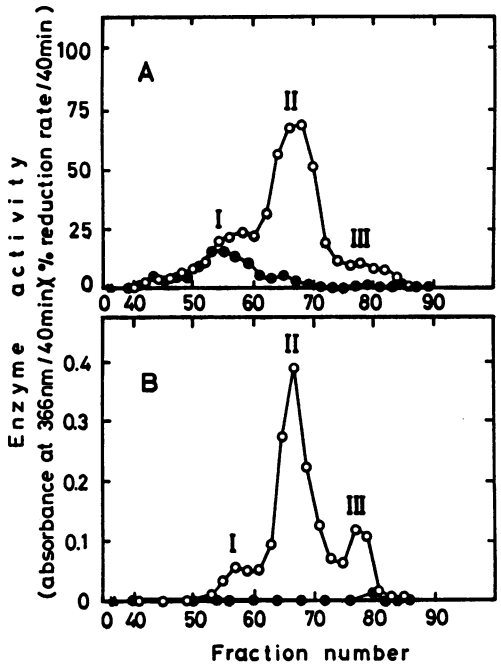

FIG. 4. Sephacryl S-200 column chromatography of the gelatin and azocasein hydrolytic activities from *V. mungo* cotyledons in the absence of 2-ME. Crude enzyme solution was applied to a Sephacryl S-200 column, and eluted with 2-ME free sodium acetate buffer. The eluted fractions were assayed using gelatin (A) or azocasein (B) as the substrate with (O) and without added 10 mM 2-ME (●). Details of assay procedures are described in "Materials and Methods".

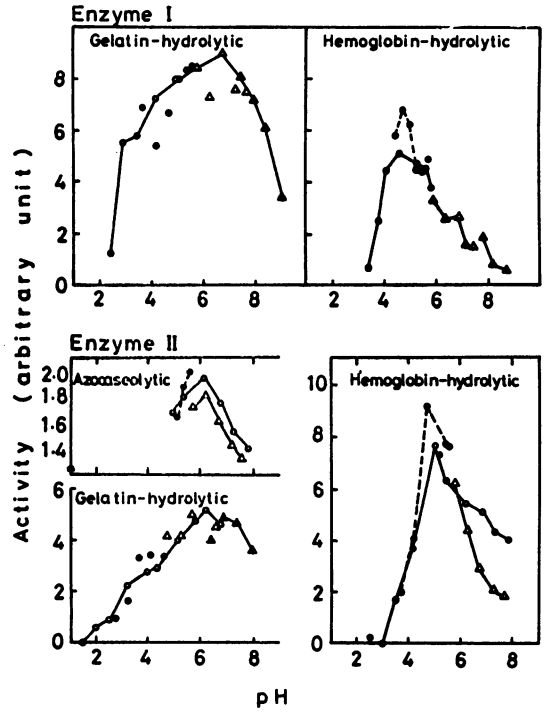

FIG. 5. pH-dependency of enzyme I and II. Enzyme I or II fractionated by a Sephacryl S-200 column was assayed after preincubation for 20 min in a buffer solution. Buffer solutions used were 0.1 M citrate phosphate (O——O), sodium acetate (●- - -●), sodium phosphate (Δ——Δ), and Tris-HCl (▲——▲). Assay methods are described in "Materials and Methods".

Table I. *Effects of Various Inhibitors on Endopeptidase Activities*

Enzyme I was assayed with 1% hemoglobin as the substrate in the absence of 2-ME, and enzyme II with azocasein in the presence of 2-ME. The enzyme in 2 ml of the sodium acetate buffer (pH 5.4) was preincubated with an inhibitor for 30 min before the addition of substrate under the conditions described in "Materials and Methods.

Addition	Concentration[a]	Relative Activity	
		I	II
		%	
None	—[b]	100	100
STI	500 μg/ml	120	108
Pepstatin	11 μg/ml	98.5	104
Chymostatin	2 μg/ml	86.6	2.8
Leupeptin	1 μg/ml	93.4	8.3
Antipain	1 μg/ml	105	10.3
DFP	1 mM	11.3	107
PMSF	2 mM	19.4	84.9
EDTA	1 mM	74.0	104
pCMB	1 mM	60.7	—[b]
I-AA	30 mM	94.6	—[b]

[a] Concentration in the preincubation mixture. [b] Not examined.

were found to be separated from each other, and enzymes I and II corresponded to bands I and II, respectively (Fig. 3). In the present study, enzyme III was not examined further because of its small amount and low activity.

Substrate Specificities of Endopeptidases. Figure 4 shows the comparison of gelatin hydrolytic and azocaseolytic activities eluted from the Sephacryl S-200 column in the absence of 2-ME. When 2-ME was added to the assay mixture, three peaks (I, II, and III) appeared against both substrates. In the absence of 2-ME, only peak I was detected against gelatin as the substrate (Fig. 4A), while all three peaks were completely abolished with azocasein (Fig. 4B). Enzymes II and III digested both azocasein and gelatin in the presence of 2-ME, and enzyme I digested gelatin, but not azocasein in the absence of 2-ME. However, when enzyme I coexisted with enzyme II, it cleaved azocasein to some extent (Fig. 4B). Hemoglobin was also well digested by these three enzymes (data not shown).

pH Optima of Endopeptidases. Enzyme I exhibited a sharp pH-activity curve with an optimum at pH 4.7 when hemoglobin was used as a substrate, whereas its gelatin-hydrolyzing activity showed a broad pH profile with a high activity range between pH 5.0 and 8.0 (Fig. 5). Since enzyme I hardly hydrolyzes azocasein as shown above, the pH optimum of this enzyme was not investigated against this substrate. On the other hand, Figure 5 shows a much broader pH range of the enzyme II activity against gelatin, and this enzyme had optima around pH 4.8 and 6.2 against hemoglobin and azocasein, respectively.

Effects of Inhibitors on Endopeptidase Activity. The effects of various inhibitors on endopeptidases I and II are summarized in Table I. Although 40% inhibition of enzyme I was observed by adding excess pCMB, the activity was not inhibited by I-AA and was not activated by 2-ME (Fig. 4A). The enzyme I activity was hardly inhibited by STI, leupeptin, and chymostatin, all of which are inhibitors of trypsin and chymotrypsin. In contrast, PMSF and DFP, typical inhibitors of serine-endopeptidase, strongly inhibited the enzyme I activity. Enzyme II had SH-dependency as described above, and showed high sensitivity to chymostatin, leupeptin, and antipain. pCMB and I-AA were not examined for enzyme II because the activity of this enzyme was very low in the absence of 2-ME. Both enzymes were insensitive to inhibition by pepstatin and EDTA.

***In Vitro* Digestion of Seed Globulin.** The ability of two endopeptidases (I, II) to hydrolyze seed storage proteins was examined

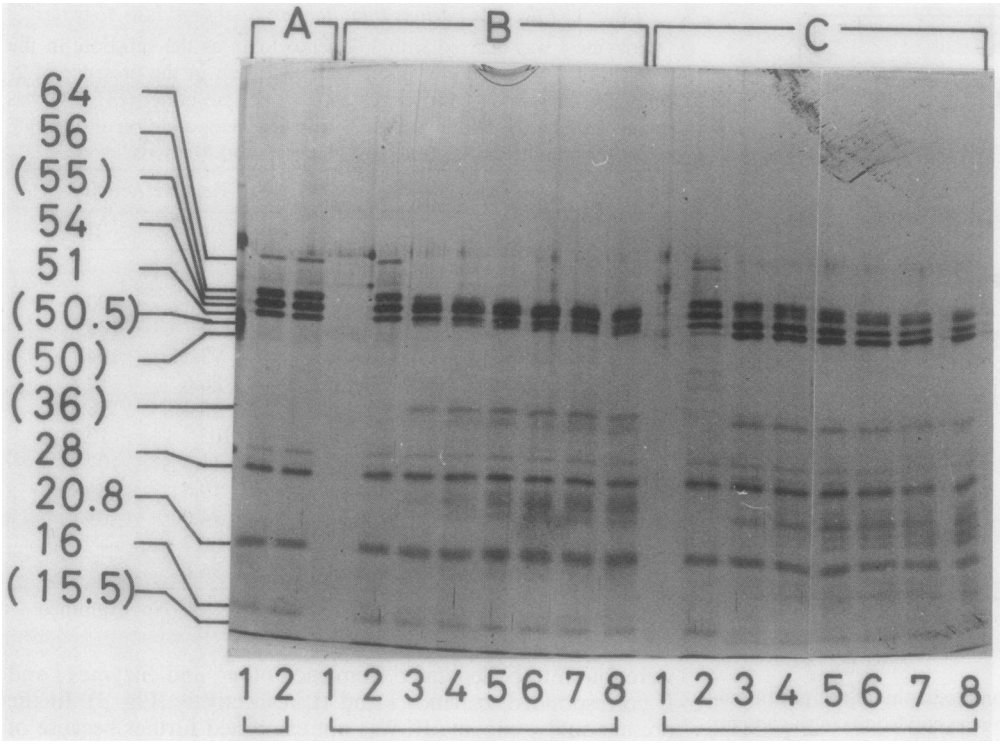

FIG. 6. Patterns of *in vitro* seed globulin digestion by enzymes I and II. Seed globulin solution was incubated with enzyme I or II at 30°C. Digestion products were analyzed by 10% polyacrylamide gel electrophoresis. The gel was stained by the silver staining method. Experimental details are described in "Materials and Methods". Mol wt ($\times 10^3$ D) of polypeptides were shown at the left side of electrophoretogram. Numbers in parentheses show mol wt of peptides which newly appeared after the incubation. Lanes A1 and A2 show seed globulin without the enzyme before and after the incubation for 24 h, respectively. B and C represent patterns of globulin degradation by enzyme I and II, respectively. Lane 1, the enzyme solution only; lanes 2 to 8, digestion patterns of the seed globulin at incubation times of 0, 3, 6, 9, 12, 15, and 18 h after the addition of enzyme.

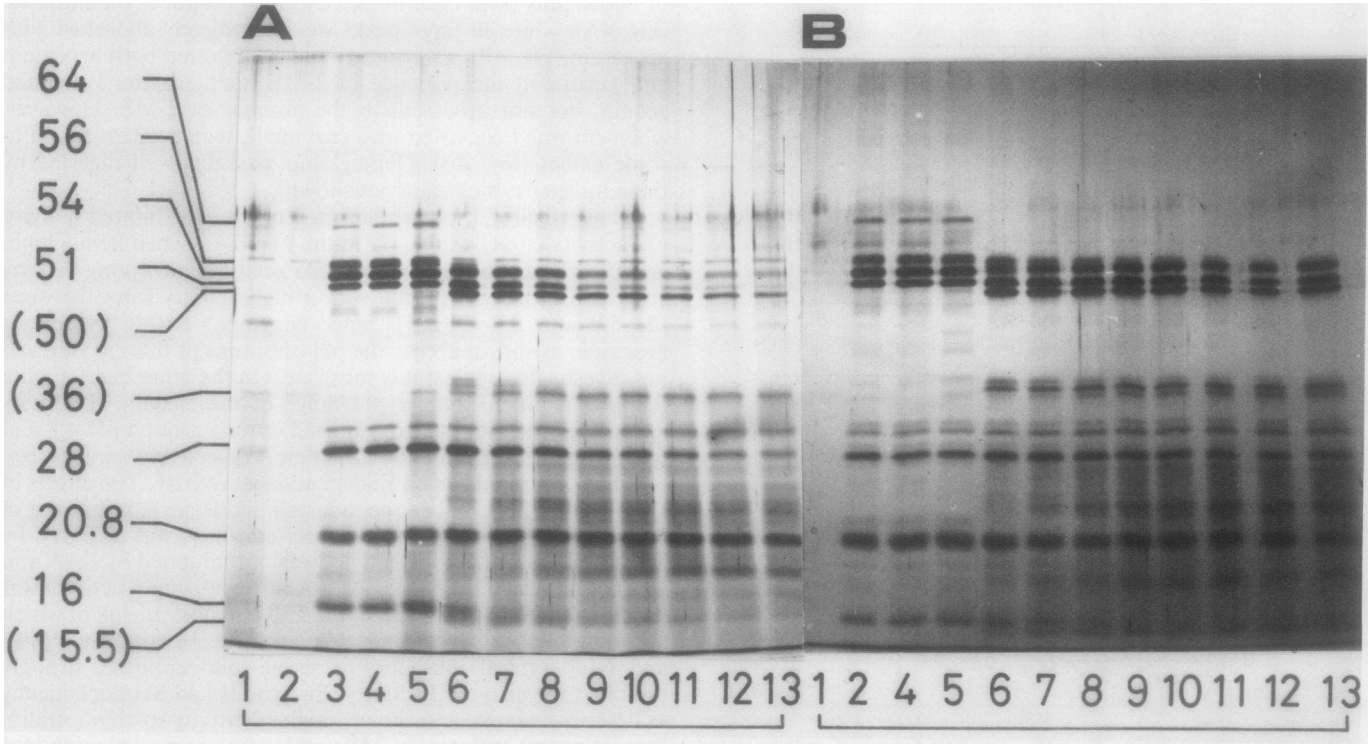

FIG. 7. Patterns of time course of *in vitro* seed globulin digestion by a crude enzyme (A) or enzymes I + II (B). Seed globulin solution was incubated with a crude enzyme solution or mixture of enzymes I and II at 30°C. Experimental details are as described for Figure 6. Lane 1, the enzyme solution only; lane 2, the enzyme solution after incubation for 24 h at 30°C; lane 3, seed globulin solution; lane 4, seed globulin solution after 24 h of incubation at 30°C; lanes 5 to 13, digestion patterns of the seed globulin at incubation times of 0, 3, 6, 9, 12, 15, 18, 21, and 24 h after the addition of enzyme.

by incubating *V. mungo* seed globulin in the presence of the crude enzyme solution or the two enzyme preparations. Enzyme I digested 64 and 56 kD subunits of the globulin within the initial 6 h, new polypeptides (55, 50.5, and 36 kD) accumulated, and the intensity of the 51 kD polypeptide decreased slowly thereafter (Fig. 6). Smaller products appeared gradually in a range of 25 to 30 kD.

Subunits 64, 54, and 16 kD were digested more rapidly by enzyme II than by enzyme I and new products at 50, 36, and 15.5 kD were detected within the initial 3 h. The intensity of the 56 kD subunit decreased gradually and a 50 kD subunit accumulated after 9 h. Smaller products (20–30 kD) appeared during further incubation. Time course studies of the digestion of the seed globulin by a crude enzyme clearly showed the disappearance of the three large polypeptides (56, 54, and 51 kD) and the appearance of 50 and 36 kD and numerous small polypeptides. The 64, 56, and 16 kD subunits were degraded rapidly within 3 h and a new product of 50 kD appeared. Limited degradation products in the range of 20 to 25 kD accumulated gradually.

Figure 7B shows globulin digestion patterns by the combination of the enzymes I and II. The 56 and 16 kD polypeptides were degraded rapidly, and an intense band of 50 kD peptide was produced. Smaller products (20–25 kD) were accumulated gradually. These patterns were virtually similar to the patterns obtained with a crude enzyme (Fig. 7A), although a subtle difference was found between them.

DISCUSSION

The present study showed that activities of at least two major endopeptidases increased in *Vigna mungo* cotyledons during germination. The increase of these two enzyme activities was delayed by the removal of embryonic axes (Figs. 1 and 2). We previously reported that in *V. mungo* seeds the degradation of storage proteins was depressed by the axis removal (16). It is thus likely that the two endopeptidases play a major role in the mobilization of storage proteins in *V. mungo* cotyledons during germination. The two enzymes have approximate molecular masses of 50 to 60 kD (enzyme I) and 20 to 30 kD (enzyme II) as estimated by Biogel A-O.5 m column chromatography (data not shown). Enzyme I is not a trypsin- or chymotrypsin-like enzyme but seems to be a serine-endopeptidase (Table I). Enzyme II was found to be a sulfhydryl-endopeptidase as judged by SH-dependency and inhibition by chymostatin, leupeptin, and antipain, all of which are known to affect sulfhydryl enzymes.

Seed endopeptidases which may participate in the degradation of storage proteins have been known to be sulfhydryl and acid enzymes in legume seeds (5, 24, 29). Serine-endopeptidases which participate in the degradation of legume seed storage proteins have not been reported. In the present study, besides a sulfhydryl type enzyme, a serine-endopeptidase was found in *V. mungo* cotyledons. The latter enzyme (enzyme I) hydrolyzes the seed globulin (Fig. 6). The mixture of enzymes I and II hydrolyzed the seed globulin more easily than either single enzyme (Fig. 7A). The globulin digestion pattern obtained with the combination of two enzymes was similar to that of the crude enzyme (Fig. 7B). These results suggest that storage proteins are mainly hydrolyzed cooperatively by enzymes I and II in *V. mungo* cotyledons during germination.

In vivo and *in vitro* studies of globulin digestion by using gel electrophoresis were reported for a number of legume seeds such as *Phaseolus vulgaris* (20, 21), *Vicia faba* (16), *Phaseolus aureus* (13), *Glycine max* (5, 7), *Pisum sativum* (2), and *Arachis hypogaea* (3). Chrispeels and collaborators (4) have reported that the 50 kD vicilin peptide was degraded to 20 to 30 kD peptides by a *Vigna radiata* sulfhydryl-endopeptidase, vicilin peptidehydrolase. They also found that carboxypeptidase participated concomitantly with the above endopeptidase in the degradation of vicilin peptides in *V. radiata* seeds (8). We previously reported that some kinds of exopeptidases including a carboxypeptidase increased in the cotyledons during germination of *V. mungo* seeds. In addition to the two endopeptidases, these exopeptidases will cooperatively hydrolyze the seed storage proteins. We are attempting the purification of the two endopeptidases to study more details of their physiological roles.

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