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Characterization of Novel Cysteine Proteases from Germinating Cotyledons of Soybean [*Glycine max* (L.) Merrill]

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The enzymatic properties of novel cysteine proteases D3-α and β which were purified from germinating soybean cotyledons were investigated. The enzyme activities were exhibited in the presence of a thiol reagent, such as 2-mercaptoethanol, and apparently inhibited by E-64, a cysteine protease inhibitor. Hydrolytic activities toward carbobenzoxy-Phe-Arg-MCA were detected at a pH above 4.0. The optimum temperature for activities was about 40°C. The isoelectric point of D3-α and β was 4.4 and 4.7, respectively. The molecular mass of D3-α and β, measured by MALDI/TOF mass spectrometry, was 26,178 and 26,429 Da, respectively. The substrate specificities of the enzymes were examined using peptide-MCAs and peptides, and cathepsin L-like broad specificity was observed at pH 4.0. These results demonstrated that these enzymes are cysteine endopeptidases [EC 3.4.22.-] like papain [EC 3.4.22.2].

Key words: cysteine protease, *Glycine max*, proteolysis, seed germination, storage protein.

We have been searching for proteases which degrade storage proteins and their fragments to oligopeptides or amino acids in germinating seeds of the soybean, *Glycine max*. Investigation of purified proteases, their substrate specificities, structures, and localization in tissues with their antibodies, could be clarify the protein degradation system in protein bodies of soybean. We have already reported the isolation and analysis of the structure of a group of key intermediary fragments of about 30 kDa, termed c30, derived from degradation of β-conglycinin, a major storage protein of soybean (*1*). We purified c30 from extracts of cotyledons of *G. max* (L.) Merrill cultivar Kegon, 7 days after imbibition (d7). To search for a protease which digests c30, we constructed a protease assay with c30 as substrate. Using this assay, we discovered cysteine proteases in extracts from d7 to d10 cotyledons of *G. max* (L.) Merrill cultivar Kegon. The protease activity in extracts appeared after day 7 in the presence of a thiol reagent, and the total activity in cotyledons increased with seedling growth. We could not detect these proteases without a thiol reagent.

In this work, we purified proteases from the acidic extract of 10-day cotyledons by ammonium sulfate precipitation and successive chromatographies on Phenyl Sepharose, Mono Q, and Sephacryl S-200 HR. Using a thiol reagent, we purified two proteases, D3-α and β, with N-terminal sequence homology to the cysteine protease of the papain superfamily. These enzymes degraded not only c30 but also intact β-conglycinin and glycinin *in vitro* (Suzuki, S., Asano, M., Kawai, M., Miwa, T., and Shibai, H., unpublished findings). Here, we report the enzymatic properties of the purified D3-α and β.

MATERIALS AND METHODS

Plant Material—Soybean seeds, *G. max* (L.) Merrill cv. Kegon, were purchased from Sakata-no-Tane. The seeds were sown on wet vermiculite (day 0), and allowed to germinate in a phytotron maintained at 25°C under a 12-h light/12-h dark cycle. Water was supplied once a day. On day 10, cotyledons were dissected away from other tissues, rinsed with distilled water, and frozen at −80°C until use.

Reagents—Polyacrylamide gel, Multigel (15/25) (T=15-25%), was purchased from Dai-Ichi Pure Chemical. Peptide-MCA substrates for proteases, neurotensin, LH-RH, E-64, CA-074, and leupeptin, were purchased from Peptide Institute. Glucagon, pepstatin A, AEBSF, and 3,4-DCI were purchased from Sigma. pNA substrates were purchased from Bachem. Cystatin (egg white) was purchased from Takara. All other chemicals were of analytical grade.

Enzyme Assays—Proteolytic activity toward c30 (*1*) was measured as follows. The enzyme was incubated with c30 (0.5 mg/ml final) in 50 mM sodium acetate buffer pH 4.0 (or other buffers at different pHs when necessary), containing 200 mM NaCl, 2 mM 2-ME, and 0.02% (w/v) NaN_3 for 15 h at 30°C. The decrease in the amount of c30 was monitored by SDS-PAGE with an Image Master II (Phar-

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; AMC, 7-amino-4-methylcoumarin; 3,4-DCI, 3,4-dichloroisocoumarin; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanigino)butane; FAB-MS, fast atom bombardment mass spectrometry; LH-RH, luteinizing hormone releasing hormone; MALDI, matrix assisted laser desorption-ionization; MCA, 4-methylcoumaryl-7-amide; 2-ME, 2-mercaptoethanol; p-NA, *para*-nitroanilide; PVDF, poly(vinylidene difluoride); TOFMS, time of flight mass spectrometry; Z, carbobenzoxyl.

macia Biotech). One unit of c30 activity is defined as the amount of enzyme needed to digest 1 μg of c30 in 1 min at 30°C.

The enzyme activity toward peptide-MCA substrates was assayed as follows. D3-α or β (375 μl) in 50 mM sodium acetate buffer pH 4.0 (or other buffers at different pHs when necessary), containing 200 mM NaCl and 10 mM 2-ME was pre-incubated for 3 min at 37°C in a water bath. The reaction was started by adding 125 μl of substrate solution, 40 μM Z-Phe-Arg-MCA in 0.2% DMSO (Z-Arg-Arg-MCA, Arg-MCA, or Bz-Arg-MCA was used when necessary) to enzyme solution. The mixture was incubated for at 37°C for 3 min, and the reaction was stopped by adding 750 μl of E-64 solution in sodium acetate buffer, pH 4.0. The amount of AMC released was measured with a Hitachi spectrofluorometer F-4000 at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. One unit of activity was defined as that releasing 1 μmol of AMC/min (*2*).

The effects of pH, temperature, and inhibitors were examined by a modification of the methods described above.

Purification of D3-α and β—All purification procedures were performed at 4°C. Enzyme activities were detected by the c30 assay at each fractionation and purification step. Cotyledons (600 g) were homogenized and extracted with 3 liters of 10 mM sodium acetate buffer, 140 mM NaCl, pH 4.0. The extract was filtered through gauze and paper and centrifuged (20,000 $\times$ *g*, 30 min). The supernatant was collected, filtered through two layers of filter paper (Whatman no. 2), and adjusted to pH 6.5 with NaOH. After incubation for 18 h, the extract was centrifuged (28,000 $\times$ *g*, 30 min). The supernatant (968 mg proteins) was concentrated about 20 times in a concentrator with a polyethersulfone membrane (Millipore). Proteins were precipitated by treatment 80% saturation of ammonium sulfate for 18 h. The precipitate was then collected by centrifugation at 20,000 $\times$ *g* for 30 min, and was dissolved in 50 mM potassium phosphate buffer (pH 6.2, buffer A) containing 20% saturated ammonium sulfate, and dialyzed against same buffer. The solution was then centrifuged at 20,000 $\times$ *g* for 30 min. The enzymes were purified from this supernatant by successive chromatographies on Phenyl Sepharose HP 26/10 (Pharmacia Biotech), Mono Q (Pharmacia Biotech), and Sephacryl S-200 HR (Pharmacia Biotech). The column of Phenyl Sepharose HP 26/10 (2.6 $\times$ 10 cm) was equilibrated with buffer A containing 20% saturated ammonium sulfate (0.86 M). Proteins were eluted with decreasing ammonium sulfate concentration from 0.86 to 0 M. The fractions containing c30-degrading activity were collected and dialyzed against buffer A. The active fractions were applied to a Mono-Q 10/10 column (1 $\times$ 10 cm) equilibrated with buffer A and eluted with increasing NaCl concentration from 0 to 0.32 M. The fractions containing c30-degrading activity were coffected, concentrated, and dialyzed against buffer A containing 0.1 M NaCl. The solution was then applied to a Sephacryl S-200 HR 26/60 column (2.6 $\times$ 60 cm) equilibrated with buffer A containing 0.1 M NaCl. The active fractions were collected, concentrated, and pooled. Purity of each enzyme was analyzed by SDS-PAGE, determination of N-terminal sequence, and isoelectric point.

Protein Assays—Protein concentration was measured by use of a Bio-rad Protein Assay Kit with bovine γ globulin as a standard.

Analysis of N-Terminal Sequences of Purified Enzymes—The N-terminal sequences of purified D3-α and β were determined. The purified enzymes were each blotted on PVDF membrane and applied to a protein sequencer (ABI, Model 476A).

Measurement of Isoelectric Point—The isoelectric points of D3-α and β were measured using the Multiphor II system (Pharmacia Biotech). About 20 μg of purified D3-α or β was loaded on an Immobiline Dry Gel strip (pH 4.0-7.0, 18 cm, Pharmacia Biotech) and subjected to isoelectric focusing. The Coomassie Brilliant Blue-stained gel strips were analyzed to determine the isoelectric point.

Determination of Molecular Mass by MALDI/TOFMS—Molecular masses of D3-α, and β were measured by MALDI/TOF mass spectrometry (KOMPACTM-ALDI III, SHIMADZU-KRATOS). BSA was used for calibration.

Action on Some Oligopeptides—Substrate specificity was studied by using neurotensin, LH-RH, and glucagon (*3*). Two sets of experimental conditions were used to determine the major and minor cleavage sites of each peptide.

a) To find the major cleavage sites of peptides, each peptide (50 nmol of neurotensin or LH-RH, or 28.7 nmol of glucagon) was incubated with 0.02 U (defined by c30 assay) of D3-α or D3-β in 50 mM ammonium acetate buffer, 2 mM 2-ME, pH 4.0, at 30°C for 0-120 min. The molar ratio of the enzyme to peptide was 1:5,000 (neurotensin or LH-RH), or 1:2,800 (glucagon). The reaction was terminated by freezing in an ethanol bath. The enzyme was lyophilized and stocked at −20°C.

b) Further digestion of peptides was investigated with other conditions, as follows. Each peptide (50 nmol of neurotensin or LH-RH, or 28.7 nmol of glucagon) was incubated with 1.0 U (defined by c30 assay) of D3-α or D3-β in 50 mM ammonium acetate buffer, 2 mM 2-ME, pH 4.0, at 30°C for 20 h. Under these conditions, the molar ratio of the enzyme to peptide was about 1:100 (neurotensin or LH-RH), or 1:57 (glucagon).

Papain was also used to digest neurotensin and LH-RH for comparison (*3*). Digestion was performed in 50 mM ammonium carbonate buffer, pH 4.0, containing 10 mM 2-ME. Under conditions a, the molar ratio of papain to peptide was about 1:500, and under conditions b, 1:20.

The cleavage sites of peptides by the enzymes were determined by two methods, sequencing of each peptide fractionated by HPLC, and direct analysis of the reaction mixture by FABMS (*4*).

For HPLC and sequence analysis, an aliquot of the digestion mixture was purified by reverse phase HPLC using an Inertsil ODS-2 column (4.6 $\times$ 250 mm, GL Science). The peptides were eluted with a linear gradient of 5-60% acetonitrile delivered at 1 ml/min for 30 min, using Milli-Q water containing 0.085% TFA as solution A, and acetonitrile containing 0.08% TFA as solution B. The absorbance was monitored at 210 nm on Waters System 600E. The peak fractions were lyophilized, then sequenced with an Applied Biosystem 476A protein sequencer system.

For FABMS analysis, an aliquot of the digestion mixture was lyophilized, then sequenced by FABMS. The sequence of each fragment peptide was determined by comparison of

its obtained molecular weight (mass unit) with the computer-calculated mass unit.

RESULTS

Purification and Molecular Properties of D3-α and β—D3-α and β were purified about 134- and 128-fold in 7.3 and 8.6% yield, respectively (0.53 mg of D3-α and 0.65 mg of D3-β from 600 g wet weight of cotyledons, Table I). Figure 1 shows the chromatogram of first column, Phenyl Sepharose. D3-α and β were separated by this first chromatography. Figure 2 shows the SDS-PAGE profiles of purified D3-α and β under reducing conditions. A single protein band was detected on each lane. The molecular size of the purified D3-α and β was estimated to be approximately 29,000 Da each by SDS-PAGE under both reducing and non-reducing conditions. This indicated that each purified D3 was composed of single polypeptide chain. The N-terminal sequence of D3-α was DKLPESVDWRKEGAVPPVKDQGGXGSXWAF, and that of D3-β was DKLPDSVDWRKEGAVPPVKDQGG. The isoelectric points were 4.4 (D3-α) and 4.7 (D3-β). The molecular mass of D3-α and β measured by MALDI/TOFMS was 26,178 and 26,429 Da, respectively.

Effects of Protease Inhibitors on the Activity—The effects of various protease inhibitors on the proteolytic activity of D3 are summarized in Table II. The activity of D3-α and β was not detected in the absence of a thiol reagent, such as 2-ME. The activity with 2-ME was apparently inhibited by the cysteine protease inhibitors, E-64, leupeptin, and egg white cystatin. The specific inhibitor of cathepsin B, CA-074 (*5*), did not inhibit D3 activity. Metal chelator and other protease inhibitors showed little or no inhibitory effect. The profiles of inhibitory activity against D3-α and β were almost the same.

Effects of pH and Temperature on Activity of D3-α and β—D3-α and β were active around pH 4.0 using c30 as substrate (Fig. 3a). When a synthetic substrate, Z-Phe-Arg-MCA, was used, D3-α and β exhibited activities above pH 4.0. D3-α showed weaker activity under basic conditions (above pH 7.5) than D3-β (Fig. 3b).

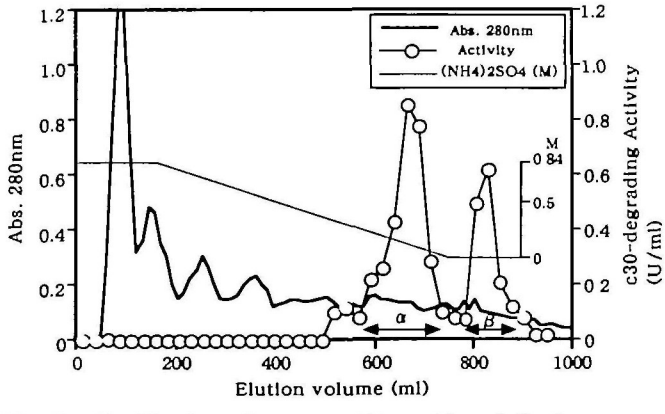

Fig. 1. **Purification of protease D3 on Phenyl Sepharose HP column.** The partially purified sample obtained by precipitation with ammonium sulfate was applied to a Phenyl Sepharose HP 26/10 column. After washing the column with 50 mM potassium phosphate containing 0.02% sodium azide and 0.86 M ammonium sulfate (pH 6.2), proteins were eluted with decreasing ammonium sulfate concentration from 0.86 to 0 M. C30-degrading acitivity was measured by densitometric analysis. Arrows in the figure indicate the collected fractions containing protease D3-α and β.

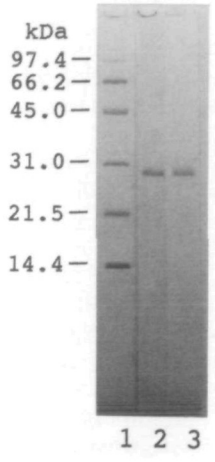

Fig. 2. **SDS-PAGE of the purified D3-α and β.** SDS-PAGE was performed under reducing condition using PAG, gradient gel (*T*=15-25%) by the method of Laemmli. For detection of total proteins, the gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratries). Lane 1, molecular size markers: rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa); lane 2, purified D3-β; lane 3, purified D3-α.

TABLE II. **Effects of protease inhibitors on D3-α and β.** The enzyme solution was incubated with inhibitors on ice for 30 min, and then the residual activity was measured.

Inhibitor	Final concentration	% control activity D3-α	D3-β
Control		100	100
E-64	0.2 μM	5.7	4.5
CA-074	0.2 μM	100	100
Leupeptin	50 μM	0.3	0.2
Cystatin (egg white)	1.5 μM	0.3	0.3
3,4-DCI	500 μM	52.9	66.2
AEBSF	500 μM	100	100
Pepstatin A	50 μM	100	100
NaCl	2.5 M	37.0	33.0

TABLE I. **Purification of protease D3-α and β.**

Step	Total protein (mg)		Total activity (U)		Specific activity (U/mg)		Yield (%)		Purification	
Extract	968		590		0.61		100		1	
	α	β	α	β	α	β	α	β	α	β
Phenyl Sepharose	35	13	241	93	7.0	7.4	41	16	11	12
Mono-Q	8.7	1.0	66	53	7.6	51	11	9.0	12	84
Sephacryl S-200	0.53	0.65	43	51	82	78	7.3	8.6	134	128

U: One enzyme unit was defined as the amount of enzyme required to digest 1 μg of c30 in 1 min at 30°C.

The effect of temperature on the activities of D3-α and β was examined by incubating the enzymes at various temperatures at pH 4.0 with c30 or Z-Phe-Arg-MCA as substrate (Fig. 4, a and b). D3-α and β were completely inactivated at 60°C (data not shown).

Substrate Specificity of D3-α and β—Four compounds, Z-Phe-Arg-MCA (substrate for cathepsin L and cathepsin B), Z-Arg-Arg-MCA (substrate for cathepsin B), Arg-MCA (substrate for cathepsin H), and Bz-Arg-MCA (substrate for soybean protease (*6*), trypsin, papain *etc.*), were tested as substrates for D3. The specific activities toward Z-Phe-Arg-MCA at pH 6 were 10.5 U/mg (D3-α) and 12.5 U/mg (D3-β). Z-Arg-Arg-MCA was also hydrolyzed by D3-α (4.3 U/mg) and β (6.9 U/mg) at pH 6. D3-α and β did not hydrolyze Arg-MCA (at pH 4, 6, or 8). These results indicated that the enzymes had no cathepsin H-like exopep-

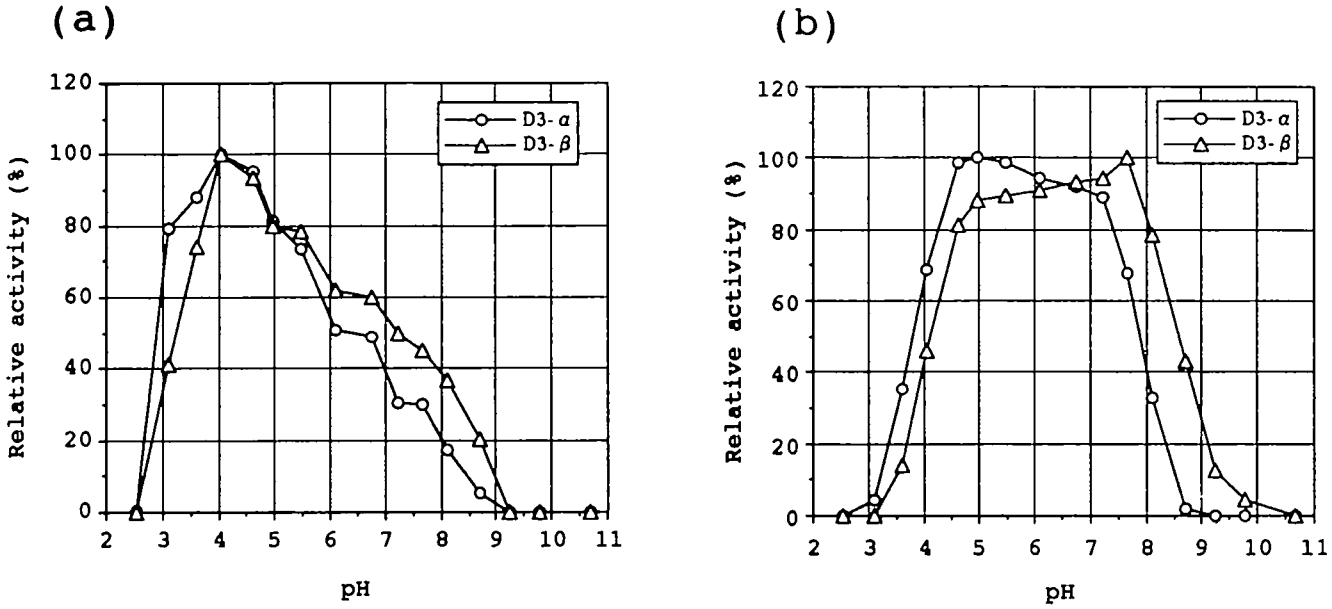

Fig. 3. **Effects of pH on activity measured with protein substrate, c30 (a) and Z-Phe-Arg-MCA substrate (b).** Activity was measured as described under "MATERIALS AND METHODS." The 50 mM buffers of sodium formate (pH 2.5-3.5), sodium acetate (pH 4.0-5.5), Tris-HCl (pH 6.0-9.0), and sodium carbonate (pH 9.0-12.0) were used. (a) D3-α or β (25 mU, c30 activity unit, about 0.35 μg of protein) and substrate were incubated for 18 h at 30°C in 200 μl of buffer solution. (b) D3-α or β (0.5 mU, c30 activity unit, about 7 ng of protein) and substrate were incubated for 5 min at 37°C in 500 μl of buffer solution.

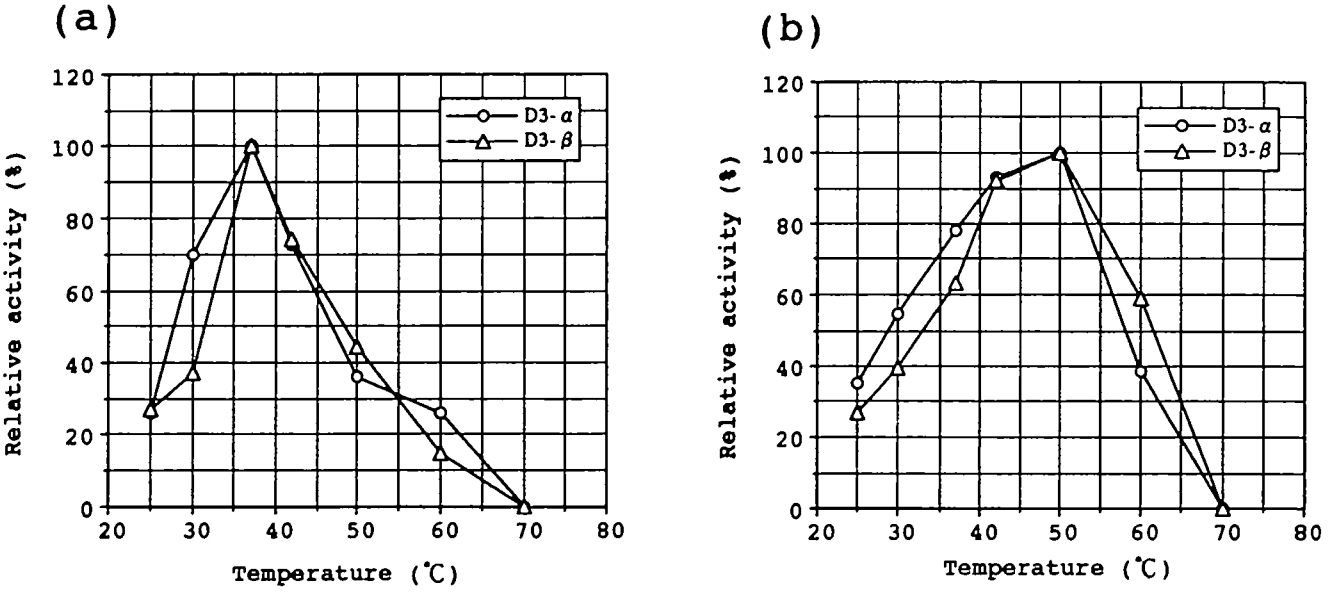

Fig. 4. **Effects of temperature on activity measured with protein substrate, c30 (a) and Z-Phe-Arg-MCA substrate (b).** Activity was measured as described under "MATERIALS AND METHODS." (a) D3-α or β (25 mU, c30 activity unit, about 0.35 μg of protein) and substrate were incubated for 6 h at pH 4.0, in 200 μl of 50 mM sodium acetate buffer. (b) D3-α or β (0.5 mU, c30 activity unit, about 7 ng of protein) and substrate were incubated for 5 min at pH 4.0 in 200 μl of 50 mM sodium acetate buffer.

tidase activity. We also confirmed that the enzymes had no exopeptidase activity toward Leu-pNA, Ala-pNA, Gly-pNA, Glu-pNA, and Phe-pNA (data not shown).

The crude enzyme extracted from cotyledons, also contained an endopeptidase that hydrolyzed Bz-Arg-MCA at pH 8, which was reported by Nishikata (*6*). D3 did not hydrolyze Bz-Arg-MCA at pH 4, 6, or 8. The separation of this serine protease and D3 was confirmed by measurement of activity toward Bz-Arg-MCA in D3 fractions.

The substrate specificities of D3 were examined by use of neurotensin, LH-RH, and glucagon, which were previously used to examine the substrate specificities of cathepsins L and B. A summary of the actions of D3-α and β on oligopeptides is shown in Fig. 5. Since D3-α and β gave identical results, we concluded that D3-α and β have the same specificity. The following results were obtained for D3-α.

With neurotensin, two peptides, Glu^4-Asn^5-Lys^6-Pro^7-Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} m/z 1,285.7 and Pyr^1-Leu^2-Tyr^3 m/z 406.2 were found initially, but Asn^5-Lys^6-Pro^7-Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} m/z 1,156.7 and Pyr^1-Leu^2-Tyr^3-Glu^4 m/z 535.2 were also detected as minor components of the reaction mixture. By HPLC purification, Glu^4-Asn^5-Lys^6-Pro^7-Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} and Asn^5-Lys^6-Pro^7-Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} were eluted in the same peak, and their molecular ratio was about 5:1 (estimated by sequence analysis with ABI 476A). Thus Tyr^3-Glu^4 was hydrolyzed preferentially by D3-α. Glu^4-Asn^5-Lys^6-Pro^7-Arg^8 m/z 643.3 and Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} m/z 661.4 were also detected after further incubation, suggesting that the Arg^8-Arg^9 bond of neurotensin is cleaved by D3-α, but the cleavage rate is much lower than that of Tyr^3-Glu^4. With papain, the major cleavage sites were Glu^4-Asn^5 and Arg^8-Arg^9. With cathepsin L, no cleavage occurred at Arg^8-Arg^9.

With LH-RH, two major fragments, Pyr^1-His^2-Trp^3-Ser^4 m/z 540.3 and Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$ m/z 661.5, were obtained. Pyr^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6 m/z 760.4 and Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$ m/z 441.3 were also detected in the reaction mixture initially as minor components. After overnight incubation, Tyr^5-Gly^6 (m/z 239.1) was also detected in the hydrolysate with other peptides. D3-α and β cleaved LH-RH at two sites, the Ser^4-Tyr^5 and Gly^6-Leu^7 linkages, of which the Ser^4-Tyr^5 linkage seemed to be the more effectively hydrolyzed. In our comparative study, papain hydrolyzed LH-RH at only the Gly^6-Leu^7 linkage. On the other hand, cathepsin L cleaved the Gly^6-Leu^7 linkage mainly, and also cleaved the Ser^4-Tyr^5 linkage and Leu^7-Arg^8 linkages. D3-α and β, papain, and cathepsin L showed similar tendency to prefer a bulky or hydrophobic residue at the P2 site of the substrate but slight difference in the case of LH-RH.

Peptides found by hydrolysis of glucagon were analyzed by FABMS. Mass spectrometry especially facilitated the analysis of this complex peptide mixture. Thr^7-Ser^8, Asp^{15}-Ser^{16} and Gln^{24}-Trp^{25} were cleaved effectively (Fig. 5). The susceptible bond at the initial site of cleavage of glucagon by D3 was similar to that of papain and cathepsin L (*3*).

No dipeptidylcarboxypeptidase activity like that of cathepsin B was detected (*3*).

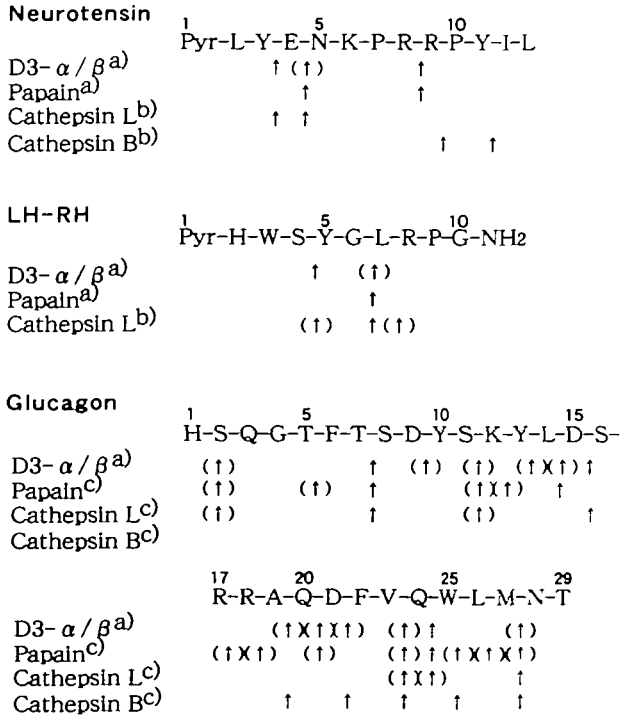

Fig. 5. **Cleavage sites of peptides by D3 and other cysteine proteases.** The digest was analyzed as described under "MATERIALS AND METHODS." Arrows represent major cleavage sites, ↑, and minor cleavage sites (↑). The references are (a) this study, (b) Katsunuma, N., *et al.* (*3*), and (c) Barrett, A.J. and Kirschke, H. (*2*).

DISCUSSION

The N-terminal residues of D3-α (30 residues) and β (23 residues) were found to be homologous to those of other cysteine proteases (*7, 8*). The activity profiles of D3-α and β with respect to inhibitors, activators and synthetic peptide-MCA substrates exhibited the characteristics of a cysteine endopeptidase like papain [EC 3.4.22.2].

Z-Phe-Arg-MCA, a substrate of cathepsin L, was a better substrate for D3 than Z-Arg-Arg-pNA, a substrate of cathepsin B. The specific inhibitor of cathepsin B, CA-074 (0.2 μM), did not inhibit D3. These synthetic substrate and specific inhibitor profiles suggested that D3-α and β resembled cathepsin L rather than cathepsin B. The specificities for peptides supported this speculation.

The cleavage specificities of D3-α and β for oligopeptides resembled those of papain and cathepsin L, which latter participates in cellar protein degradation (*3*). We studied the difference between D3-α and β to examine their roles in the degradation protein. Their enzymatic properties, molecular weight, substrate specificity, pH profile, and temperature profile were all similar, except for pI and specific activity under basic conditions (Table II and Fig. 3). Thus we deduced that D3-α and β are isozymes. The differences in pI, 4.4 and 4.7 for D3-α and β, respectively, and specific activity under a basic conditions might be caused by differences in the surface electric charge of the enzymes.

The physiological role of isozymes D3-α and β in cotyledon is not clear. However, only D3-α and β degraded *in vitor* the intermediary fragments, c30, derived from β-

conglycinin degradation. Thus we speculate that D3-α and β contribute to the degradation to oligopeptides of fragments of storage proteins that have been cleaved by other proteases, previously reported (*9-11*), in the germinating stage of soybean seeds. The homology with oryzain α, thought to be a major cysteine protease in the degradation of storage protein in rice seeds, supported our speculation (*8*). Many cysteine proteases and genes encoding cysteine endopeptidases have been isolated from plants, monocotyledons (*12-15*), and dicotyledons (*16-19*). In 1987, Shutov and Vaintraub suggested the participation of proteases in the mobilization of storage proteins during seed germination (*20*), and concluded that cysteine endopeptidases play an important role at this stage. Characterization of D3-α and β in the present study revealed that they optimally digest protein substrates under mild acidic conditions. This property also suggested that these cysteine endopeptidases are involved in the protein degradation process in protein bodies and the vacuolar system.

Detailed studies on the primary structures of D3-α and β and attempts to isolate cDNA clones are underway.

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