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Substrate Specificity of a Novel Serine Protease from Soybean [*Glycine max* (L.) Merrill]

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The substrate specificity of a novel serine protease isolated from soybean seeds, cultivar Keburi, was investigated using various peptide-MCAs and several neuropeptides involving single and paired basic amino acid sequences. The protease was quite specific for arginine residue at the P1 site of the active center, and it recognized paired Arg-Arg and cleaved at the linkage between Arg-Arg or after Arg-Arg in peptide and protein molecules. This is the first protease in plant tissues which resembles in substrate specificity the arginine-specific serine proteinases from porcine gastric and intestinal mucosa, recognizing paired basic amino acid sequences.

Key words: arginine-specific protease, serine protease, soybean, substrate specificity.

In our previous paper (*1*), we found a proteolytic activity toward the α subunit of β-conglycinin in resting mature seeds of soybean [*Glycine max* (L.) Merrill] cultivar Keburi. The proteolysis of the α subunit of β-conglycinin occurred quite specifically at a single linkage Arg126-Arg127 in the α subunit (*2*). The proteolytic activity was intense at pH 8 to 9, and it was inhibited by antipain, APMSF, aprotinin, and leupeptin. These results suggest that the protease under investigation is a novel trypsin-type neutral/alkaline serine protease, which has not previously been recognized in soybean seeds.

Later, we partially purified the protease and elucidated its enzymatic properties (*3*). The protease was purified from the extract of soybean seeds cultivar Keburi by cryoprecipitation at pH 6.4, ammonium sulfate fractionation, and successive chromatographies on DEAE-Sepharose, SP-Sepharose, and Arginine-Sepharose, the final specific activity was about 700 times that of the crude extract. The proteolysis of the α subunit in the purified $\alpha_2\beta$ molecule of β-conglycinin apparently followed first-order kinetics, the K_m value for the α subunit being estimated at 34.25 μM. The most striking finding was that the protease was inhibited by both Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor in a competitive manner. The inhibitor constants, K_i, for both inhibitors of soybean origin were found to be 0.265 and 0.885 μM, respectively. This is the first time that these inhibitors have been found to act on a plant protease. Moreover, the protease hydrolyzed the A3 polypeptide of glycinin G5 subunit (*4*). However, the cleavage point of A3 polypeptide was on the carboxyl side of the Arg98-Arg99 residues, not the linkage between the two basic amino acid residues. The previous experiments suggested that the trypsin-type serine protease of soybean seeds cultivar Keburi recognized the arginine residues in the protein substrates, though the scissile point was different in the two soybean proteins: the α subunit of β-conglycinin and the A3 polypeptide of glycinin.

In the present investigation, we tried to elucidate the substrate specificity more precisely using a series of synthetic peptide substrates containing 4-methylcoumarin-7-amide, and naturally occurring peptides containing single and paired basic amino acid sequences, *i.e.*, dynorphin A, bovine adrenal medulla dodecapeptide, and neurotensin.

MATERIALS AND METHODS

Preparation of Soybean Protease—The purification of the protease from soybean seeds may be summarized as follows (*3*). Soybean seeds (cultivar Keburi) were powdered and extracted with Tris-HCl buffer, pH 8. The pH of the soluble extract was adjusted to 6.4, and the solution was chilled to 4°C. The cryoprecipitate was extracted with Tris-HCl buffer, pH 7.6, and the protease was precipitated by 50% saturation with ammonium sulfate. The enzyme was then purified by successive chromatographies on DEAE-Sepharose Fast Flow, SP-Sepharose Fast Flow, and Arginine-Sepharose 4B. The final preparation had the specific activity of 11,710 units/mg, representing a 700-fold purification from the crude extract.

Chemical Reagents—Synthetic oligopeptide-MCA substrates and naturally occurring peptides, such as dynorphin A, BAM-12P, and neurotensin, were obtained from the Peptide Institute, Osaka. Bovine pancreas trypsin was purchased from Sigma. All other chemicals were of analytical grade.

Preparation of β-Conglycinin and Its Components—β-Conglycinin was prepared according to the method of Thanh

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; MCA, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; Boc, *t*-butyloxycarbonyl; BAPA, benzoyl-DL-arginine nitroanilide; APMSF, (4-amidinophenyl)methanesulfonyl-fluoride; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)butane; BAM-12P, bovine adrenal medulla dodecapeptide; HPLC, high-performance liquid chromatography; des(1-126)α, the α subunit of β-conglycinin in which the N-terminal 126 amino acids (Val1 to Arg126) have been deleted by proteolysis with soybean protease; PVDF, poly(vinylidene difluoride).

and Shibasaki (*5*) combined with the ammonium sulfate fractionation procedure of Iwabuchi and Yamauchi (*6, 7*) from soybean seeds cultivar Enrei. Single molecular species of β-conglycinin, $\alpha\beta_2$, $\alpha_2\beta$, and α_3, were purified by ion-exchange chromatography of β-conglycinin purified from soybean seeds cultivar Keburi, lacking the α' subunit, according to the method of Thanh and Shibasaki (*5*). The β_3 component was isolated and purified by the methods of Yamauchi *et al.* (*8*) and Sykes and Gayler (*9*) from the crude 11S fraction of soybean, or alternatively from the γ-conglycinin fraction of soybean according to the method of Sato *et al.* (*10*).

Assay of Proteolytic Activity toward β-Conglycinin—Limited proteolytic activity against the α subunit of β-conglycinin was measured by densitometric analysis of the stained gels after SDS-PAGE basically according to the method of Qi *et al.* (*11*). The purified β-conglycinin $\alpha_2\beta$ was used as the substrate. The proteolysis was performed at 30°C for 1 to 4 h in 200 μl of reaction mixture, containing 57.5 μM $\alpha_2\beta$ solution in potassium phosphate buffer, pH 8.0, and 0.02% NaN_3. The decrease in the α subunit was monitored by SDS-PAGE. One unit of proteolytic activity was defined as the amount of protease that caused the disappearance of 1 pmol of the α subunit in the first 1 min at 30°C under the assay conditions.

Assay of Activity toward Peptide-MCA Substrates—The enzyme assays using peptide-MCA substrates were performed by fluorometric determination of liberated 7-amino-4-methylcoumarin (AMC) according to the method of Morita *et al.* (*12*). To 3,940 μl of 50 mM Tris-HCl buffer solution, pH 8.0, containing 100 mM NaCl and 10 mM $CaCl_2$, was added 40 μl of 10 mM peptide MCA substrate dissolved in dimethyl sulfoxide. The enzyme solution, 50 units in 20 μl, was mixed with the substrate solution in a cell at 25°C. The liberation of AMC by enzymatic hydrolysis was monitored with a Hitachi fluorescence spectrophotometer, model MPF-2A, at 25°C for 120 s. The fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The activity was expressed as the increase in fluorescence intensity during 1 min. In the case of trypsin, 0.2 μg/20 μl of the enzyme was added to the reaction mixture (total 4 ml).

In order to detect the hydrolysis of Arg-Arg linkages, the reaction mixture was analyzed by HPLC to detect Arg-MCA, which was formed by the hydrolysis of substrates at Arg-Arg linkages. The reaction mixture, 100 μl, contained 1 mM peptide-MCA substrates and 1 unit of the enzyme in 300 mM Tris-HCl buffer solution, pH 8.0. The proteolysis was performed at 37°C for 2 h. Ten microliter aliquots of the digestion mixture were withdrawn and analyzed by reverse-phase HPLC on Millipore Puresil C18, 4.6×250 mm, with a linear gradient of acetonitrile from 10 to 90%, and the elution was monitored at 330 nm. Two products, AMC and Arg-MCA, were determined in digestion mixtures.

Cleavage of Naturally Occurring Peptides—Dynorphin A, BAM-12P, and neurotensin were digested with the soybean protease. Each peptide was dissolved in 0.1 M phosphate buffer, pH 8.0, at a concentration of 100 μM. The peptide solution, 100 μl, was mixed with 50 μl of 0.1 M phosphate buffer, pH 8.0, and 50 μl of the protease solution, 50 units, and the mixture was incubated at 30°C for 1 to 8 h. An aliquot of the digestion mixture was analyzed using reverse phase HPLC on a Millipore Puresil C18 column, 4.6×250 mm, under a linear gradient (1%/min) of acetonitrile from 10 to 60%, and the elution was monitored at 220 nm. The amino acid sequences of appropriate fractions were determined by the automated Edman method.

Electrophoresis and Electroblotting onto PVDF Membranes—In order to identify and determine the subunits of β-conglycinin and their proteolytic fragments, SDS-PAGE was performed according to the method of Laemmli (*13*) at room temperature with the use of 10% gels. The proteins were located with Coomassie Brilliant Blue R-250. The separated protein was electroblotted onto PVDF membranes (Immobilon Transfer, 0.45 μm, Millipore) and subjected to amino acid sequence analysis (*14*).

Determination of Protein—Protein was determined by the dye-binding method of Bradford (*15*) with human immunoglobulin as the standard. The concentrations of the purified β-conglycinin components were determined spectrophotometrically, using the absorption coefficient values ($E^{1\%}_{1cm}$ at 280 nm) of 3.62 for α_3 and 3.50 for $\alpha_2\beta$; these values were calculated from the molar absorption coefficients and the contents of tyrosine, tryptophan and cysteine in each molecule based on the amino acid sequences of the α and β subunits of β-conglycinin (*2, 16, 17*).

Amino Acid and Amino-Terminal Sequence Analyses—Amino acid analysis of the protein and peptide products was performed with an amino acid analyzer (L-8500, Hitachi) by the method of Spackman *et al.* (*18*) after the protein or peptide was hydrolyzed in 6 N HCl at 110°C for 48 h under nitrogen gas. Amino-terminal sequences of the proteolytic peptides were analyzed automatically by the Edman method with an automated sequencer (model 477A, Applied Biosystems) equipped with a PTH analyzer (model 120A).

RESULTS

Substrate Specificity on Peptide-MCA Substrates—The substrate specificity of the soybean protease was examined with various peptide-MCA substrates, and the results are shown in Table I. The table also shows the relative activity of bovine pancreas trypsin toward the same substrates for comparison. The soybean enzyme could hydrolyze peptide-MCA bonds containing only the Arg-MCA linkage, but not MCA compounds with other amino acid residues. The enzyme was most active toward the paired Arg-MCA substrates, such as Boc-Gly-Arg-Arg-MCA and Boc-Gln-Arg-Arg-MCA. It also hydrolyzed, to a lesser extent, Xaa-Arg-MCA containing one or more amino acid residues in front of the Arg residue, but did not hydrolyze Bz-Arg-MCA or Arg-MCA. Trypsin showed similar results toward peptide-MCA substrates, but it was active toward both Lys-MCA and Arg-MCA linkages, while the soybean protease was strictly specific to the Arg-MCA linkage.

In several peptide-MCA substrates, such as Boc-Gly-Arg-Arg-MCA, the Arg-Arg linkage was also hydrolyzed simultaneously, and Arg-MCA was formed as a product, which was not further hydrolyzed, as shown in Table I. We determined two products, Arg-MCA and AMC, in the digestion mixture by reverse-phase HPLC analyses. Table II shows the results for several peptide-MCA substrates containing an Arg-Arg linkage. All of the Arg-Arg linkages

of the substrates were hydrolyzed simultaneously, in addition to Arg-MCA linkages, by the soybean enzyme. The ratios of hydrolysis of the Arg-Arg and Arg-MCA linkages were different from one substrate to another. It is interesting to find that Boc-Arg-Val-Arg-Arg-MCA, a typical substrate for the kexin family, was more easily hydrolyzed between the two Arg residues than on the carboxyl side of the paired Arg residues. Any linkage between a non-Arg amino acid and Arg of the other substrates was not hydrolyzed. The serine proteases from porcine gastric mucosa (*19*) and from porcine intestinal mucosa (*20*) also showed similar substrate specificity to that of the soybean enzyme, but they were also active towards Lys residues, though to a much lesser extent, and were less specific for Arg than the soybean enzyme

Enzyme Activity on Naturally Occurring Peptide Substrates—Dynorphin A, BAM-12P, and neurotensin were subjected to hydrolysis by the soybean protease in order to examine the substrate specificity, because all three peptides contain paired Arg residues in the molecule as well as single Arg and/or Lys residues. In the case of dynorphin A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln, reverse-phase HPLC of the digestion products gave three peaks, while the intact dynorphin A almost disappeared, as shown in Fig. 1A. The amino acid sequence analysis of peak 1 showed two almost equally mixed peptides, Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln and Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln, while peak 2 and peak 3 each showed a single peptide, Tyr-Gly-Gly-Phe-Leu-Arg-Arg and Tyr-Gly-Gly-Phe-Leu-Arg, respectively. The heights of peak 2 and peak 3 were almost equal. These results showed that the soybean protease cleaved dynorphin A at two sites, the Arg6-Arg7 linkage and the Arg7-Ile8 linkage, almost equally. The single Arg or Lys was inert to the proteolysis.

In the case of BAM-12P, Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu (Fig. 1B), three fragments were found. Peaks 1, 2, and 3 were identified as Val-Gly-Arg-Pro-Glu, Tyr-Gly-Gly-Phe-Met-Arg-Arg, and Tyr-Gly-Gly-Phe-Met-Arg, respectively. Therefore, the Arg-Arg and Arg-Val linkages seemed to be cleaved, though the Arg-Val-Gly-Arg-Pro-Glu fragment was not detected on HPLC. Peak 2 was much higher than peak 3, so that the Arg-Val linkage seemed to be more effectively hydrolyzed than the Arg-Arg linkage in this case.

In the case of neurotensin, Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (Fig. 1C), only one fragment was identified: Arg-Pro-Tyr-Ile-Leu. This fact suggested that only the Arg-Arg linkage would be cleaved, but the cleavage rate was much lower than those of the other two peptides, probably because of the steric effect of Pro residues on both sides of Arg-Arg in neurotensin. In the cases of BAM-12P and neurotensin, no cleavage occurred after the single Arg or Lys. These results are summarized in Table III.

Action on α, α′ and β Subunits of β-Conglycinin—In the

TABLE I. **Enzyme activities toward various peptide-MCA substrates.**[a]

Substrate	Relative activity (%)	
	Soybean protease	Trypsin
Boc-Gly-Arg-Arg-MCA	100	47.3
Boc-Gln-Arg-Arg-MCA	91.2	83.4
Boc-Leu-Arg-Arg-MCA	46.9	44.7
Boc-Gly-Lys-Arg-MCA	44.8	44.2
Boc-Gln-Ala-Arg-MCA	23.8	100
Boc-Leu-Lys-Arg-MCA	21.4	42.2
Boc-Val-Pro-Arg-MCA	16.7	48.5
Bz-Arg-MCA	0	0
Arg-MCA	0	0
Boc-Glu-Lys-Lys-MCA	0	26.6
Boc-Val-Leu-Lys-MCA	0	13.9
Lys-MCA	0	0
Suc-Ala-Pro-Ala-MCA	0	0
Suc-Ala-Ala-Pro-Phe-MCA	0	0
Gly-Pro-MCA	0	0

[a]The relative activities are expressed as percent of the activity toward Boc-Gly-Arg-Arg-MCA for the soybean protease and toward Boc-Gln-Ala-Arg-MCA for trypsin. MCA: 4-methylcoumaryl-7-amide, Boc: *t*-butyloxycarbonyl, Bz: benzoyl, Suc: succinyl.

TABLE II. **Cleavage sites of peptide-MCA substrates containing Arg-Arg-linkages by the soybean protease.**

	mol% product to substrate used	
	Arg-MCA	AMC
Boc-Gly-Arg-Arg-MCA	8.2	28.6
Boc-Gln-Arg-Arg-MCA	6.6	26.1
Boc-Leu-Arg-Arg-MCA	20.9	13.4
Boc-Gln-Ala-Arg-MCA	0	6.8
Boc-Arg-Val-Arg-Arg-MCA	35.0	5.4

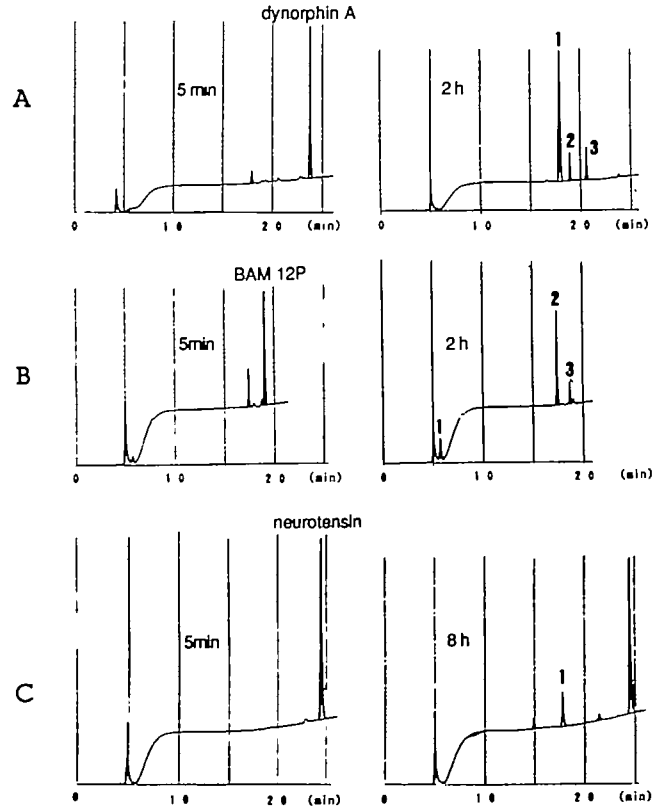

Fig. 1. **HPLC of the reaction mixture from the proteolysis of dynorphin A (A), BAM 12P (B), and neurotensin (C).** A 50 μM solution of each peptide in 0.1 M phosphate buffer, pH 8.0, (200 μl) was digested with 50 units of protease at 30°C for 2 to 8 h. At appropriate time intervals, a 10 μl aliquot, of the reaction mixture was withdrawn and subjected to HPLC analysis. The numerals indicate the fragments from peptides as explained in the text.

previous paper (*1*), we reported that the soybean protease under investigation hydrolyzed specifically the α subunit of β-conglycinin, when we used an α' subunit-lacking strain of soybean seeds. In that report, we suggested that the protease would hydrolyze the α' subunit of β-conglycinin as well, because both of the α and α' subunits contain Arg-Arg sequence in similar sites on their primary structures. Then we tried to digest a usual β-conglycinin purified from soybean seeds (cultivar Enrei) which contained α, α', and β subunits. The results of SDS-PAGE of the purified β-conglycinin and its digested product are shown in Fig. 2. The α and α' subunits of β-conglycinin disappeared during the proteolytic digestion for 4 h, and instead, a new band with a molecular weight of 47,000 increased. The densitometric determination of these bands during the course of digestion showed that the intensity of the new band formed always corresponded to the sum of those of the disappeared α and α' bands. This means that the both α and α' subunits give products of similar molecular weight. The amino-terminal sequence of this new band showed only one sequence, Arg-His-Lys-Asn-Lys-Asn-Pro-, which corresponded to the sequence of the α subunit from Arg127 to Pro133 and also to the sequence of the α' subunit from Arg143 to Pro149, as shown in Table IV (*21*). The results revealed that the cleavage occurred at the Arg142-Arg143 linkage of the α' subunit, similar to the Arg126-Arg127 linkage of the α subunit. In the previous report (*3*), we revealed that the digestion of the α subunit of β-conglycinin followed first-order kinetics, using the $\alpha_2\beta$ molecular species of β-conglycinin as the substrate. As the α' subunit should give similar kinetics, we plotted the decrease in the α, α', and β subunits of β-conglycinin purified from the soybean seeds (cultivar Enrei), as shown in Fig. 3. The decrease in the intensity of the α' subunit was linear, which means that the digestion of the α' subunit followed first-order kinetics, like that of the α subunit. The reactivity on the α' subunit was less than that on the α subunit. The activity on the α subunit, determined at the same concentrations of the α and α' subunits (the crossing point of the two curves in Fig. 3), was 3.7 times higher than that on the α' subunit.

Finally, we should point out the cleavage of the β subunit of β-conglycinin, probably by the same soybean protease. As seen in Figs. 2 and 3, the β subunit of β-conglycinin was almost inert to proteolysis during the present experimental period (4 h). However, we found that even the β subunit was specifically hydrolyzed over a long period. The purified β_3 component from the γ-conglycinin fraction prepared from cultivar Keburi was hydrolyzed by a contaminating protease in the fraction during the crystallization of the β_3 component in 0.15 M phosphate buffer, pH 7.6. After 1 and 3 months of crystallization, a part of the β subunit was hydrolyzed to form two fragments with molecular weights of about 28,000 and 20,000, as shown in the SDS-PAGE

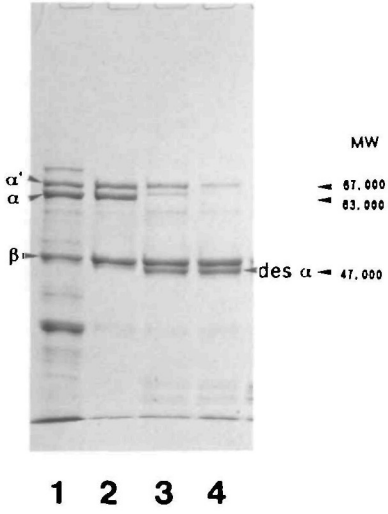

Fig. 2. **SDS-PAGE of the proteolytic digestion of the α and α' subunits of β-conglycinin purified from soybean seeds cv. Enrei.** A 0.5% β-conglycinin solution in 35 mM Tris-HCl buffer solution, pH 8.0, was incubated with 120 units of the soybean protease at 30°C for 4 h. Lane 1, seed extract from cv. Enrei; lane 2, purified β-conglycinin from cv. Enrei; lane 3, after digestion for 2 h; lane 4, after digestion for 4 h. The letters α, α', and β represent the α, α', and β subunits of β-conglycinin and des α represents the products of α and α' subunits digested with the soybean protease.

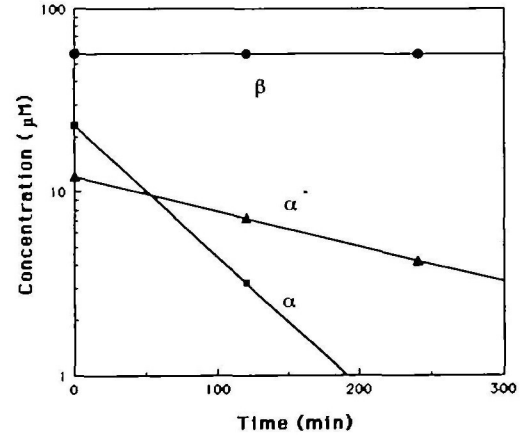

Fig. 3. **First-order reaction of the proteolysis of the α and α' subunits of β-conglycinin from soybean seeds cv. Enrei.** The conditions of the proteolytic reaction were the same as described in the legend to Fig. 2. The concentrations of the components were determined by densitometry of the stained bands on SDS-PAGE shown in Fig. 2.

TABLE III. **The cleavage specificity toward three peptides.** Each peptide (100 μM) was digested with 50 units of the soybean protease in 200 μl of 0.1 M K-phosphate buffer, pH 8.0, at 30°C for 1 to 8 h. Pyr: pyroglutamic acid. Arrows represent the cleavage sites by the soybean protease.

	Amino acid sequence and cleavage site
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-**Arg**-**Arg**-Ile- Arg-Pro- Lys-Leu-Lys-Trp-Asp-Asn-Gln
BAM-12P	Tyr-Gly-Gly-Phe-Met-**Arg**-**Arg**-Val- Gly- Arg-Pro- Glu
Neurotensin	Pyr-Leu-Tyr-Glu-Asn-Lys- Pro- **Arg**-**Arg**-Pro- Tyr- Ile- Leu

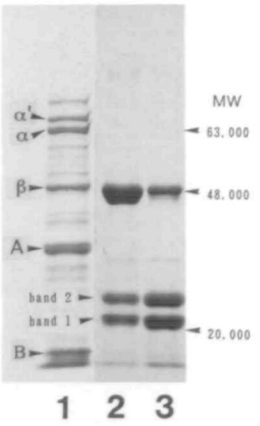

Fig. 4 **SDS-PAGE of the proteolytic digestion of the β_3 molecular species during the crystallization of the protein.** A roughly 1% solution of β_3 molecular species was dialyzed against 0.15 M potassium phosphate buffer, pH 7.6. Lane 1, extract from soybean seeds cv. Enrei; lane 2, β_3 after crystallization for 1 month; lane 3, β_3 after crystallization for 3 months. The letters α, α', β, A, and B represent the α, α', and β subunits of β-conglycinin, and acidic and basic polypeptides of glycinin, respectively. The numerals are the fragments of the β_3 component as explained in the text

TABLE IV. **Amino acid sequence and cleavage sites of three β-conglycinin subunits.** Arrows represent the cleave sites by the soybean protease. The residues underlined were confirmed by sequence analysis.

	Amino acid sequence and cleavage site	
	↓	
α	123-Ser-Glu-Leu-**Arg**-**Arg**-His-Lys-Asn-Lys-Asn-Pro-Phe-His	135
	↓	
α'	139-Arg-Glu-Pro-**Arg**-**Arg**-His-Lys-Asn-Lys-Asn-Pro-Phe-His	151
β	1 Leu-Lys-Val-Arg-Glu-Asp-Glu-Asn- - Asn-Pro-Phe-Tyr	12
α	322 Ile-Arg-Ala-Leu-Ser-Lys-Arg-Ala-Lys-Ser-Ser-Ser-Arg-	334
α'	357 Ile-Arg-Glu-Leu-Ser-Lys-His-Ala-Lys-Ser-Ser-Ser-Arg-	369
	↓	
β	195 Ile-Arg-Gln-Leu-Ser-**Arg**-**Arg**-Ala-Lys-Ser-Ser-Ser-Arg-	207

(Fig. 4), though the quaternary structure of the β_3 component was retained. The amino-terminal sequence analysis indicated that one fragment (band 2) showed the amino-terminal sequence of the β subunit, Leu-Lys-Val-Arg-Glu-, while the other (band 1) had the sequence Arg-Ala-Lys-Ser-Ser-. The result shows that the β subunit was cleaved at the Arg200-Arg201 linkage (Table IV). On the other hand, we could not detect any cleavage of β_3 of β-conglycinin purified from seeds of cultivar Enrei. Therefore, the cleavage of the β subunit of β-conglycinin from seeds of cultivar Keburi probably involves the action of contaminating protease in the fraction from the seeds of cultivar Keburi, and it seems likely to be due to the same enzyme discussed in this paper, because it occurred just between two Arg residues in the β subunit. It is very interesting that the β_3 preparation modified by limited proteolysis was more easily crystallized than the native β_3. The previous investigations on two vitellins, canavalin from jack bean and phaseolin from French bean, showed that these two storage proteins were cleaved specifically at a single site between two major domains with retention of the quaternary structures of the vicillin trimers, and the products could be crystallized more easily than the native proteins (*22, 23*). The cleaved site on the β subunit of β-conglycinin seemed to be similarly located between two possible major domains of the protein.

DISCUSSION

In the previous papers (*1, 3*), we reported the detection and purification of a characteristic protease from dry soybean seeds (cultivar Keburi and related strains). The enzyme exhibited various properties of a trypsin-type protease: an optimal pH higher than 8; inhibitions by various serine-protease inhibitors such as (4-amidinophenyl)methanesulfonyl fluoride, antipain, aprotinin, and leupeptin; proteolytic activity on the carboxyl side of Arg residues in naturally occurring soy proteins such as A3 polypeptide of glycinin and the α subunit of β-conglycinin; Inhibitions by Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor; selective adsorption on an Arginine-Sepharose 4B column.

In 1984, Nishikata (*24*) reported a trypsin-like protease purified from soybean seeds (cultivar Toyosuzu) using benzoyl-L-arginine *p*-nitroanilide as a substrate. The enzyme was most active between pH 8–10 and hydrolyzed synthetic substrates and oligopeptides exclusively at the carboxyl side of L-arginine and L-lysine. It was inhibited by diisopropyl fluorophosphate, tosyl-L-lysine chloromethyl ketone, leupeptin, and antipain. These properties showed the enzyme to be a trypsin-like serine protease. However, the protease reported by Nishikata is quite different from our protease purified from cultivar Keburi in the following properties: no inhibition by protein inhibitors of trypsin such as soybean trypsin inhibitor; no activity toward protein substrates such as bovine serum albumin, ovalbumin, myoglobin, lysozyme, soybean 7S and 11S globulins; Bz-Arg-MCA being the best substrate; and inhibitions by metal ions such as Ni^{2+}, Co^{2+}, Fe^{2+}, and Hg^{2+}. These properties suggested that our protease is a novel serine protease different from the enzyme reported by Nishikata. No other trypsin-like protease has been reported from soybeans.

In the present investigation, we studied the substrate specificity of the Keburi protease, which seemed to have a high specificity for Arg residues. This was supported by the results on the action toward various peptide-MCA substrates. The Keburi protease had a strict specificity for the Arg-MCA linkage, but not other amino acid-MCA linkages (including Lys-MCA), unlike the case of trypsin. Moreover, the Keburi protease did not act on Bz-Arg-MCA, which was the best substrate for the protease reported by Nishikata (*24*). The Keburi protease was most active towards Xaa-Arg-Arg-MCA, as shown in Table I. In the case of these paired Arg residues, the enzyme acted both on the Arg-Arg linkage and on the Arg-Xaa linkage on the carboxyl side of the Arg-Arg residues, as shown in Table II. The relative activities on these two sites were different, depending on the peptides. The hydrolysis of these two linkages was also observed in naturally occurring peptides, dynorphin A and BAM-12P, as shown in Table III. Table III further shows that the Keburi protease recognized the paired Arg residues but did not act on single Arg residues or Lys residues. Nishikata (*24*) reported that his protease from cultivar Toyosuzu acted on both single Arg and Lys residues in oligopeptides such as angiotensin II, serum thymic factor, bradykinin potentiator B and deamino-dicarba-arginine-

vasotocin. The substrate specificity of the Keburi protease to recognize paired Arg residues is a noteworthy characteristic of this enzyme, and this specificity for Arg-Arg residues was much stricter than that of other proteases, as described before (*19, 20*). Such a high arginine-specificity has not previously found in plant proteases.

However, we do not know why the enzyme hydrolyzed the linkage either between Arg-Arg residues or on the carboxyl side of Arg-Arg residues in the case of natural soybean globulins, though it acted on both sites in the cases of synthetic or naturally occurring oligopeptides. Probably, steric restrictions or the flexibility of the substrate determine the hydrolysis site. Besides the proteases under discussion in this paper, many proteases belonging to the kexin and furin families recognize paired basic residues. According to the proposed model for the substrate-binding site of human furin (*25*), the P1 site (*26*) of the Keburi protease should be strictly arginine-specific. As pointed out by Perona and Craik (*27*), knowledge of the three-dimensional structure of the enzyme will be necessary for elucidation of the mechanism of substrate determination of the enzymes of this class.

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