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Partial Purification and Characterization of a Novel Soybean Protease Which Is Inhibited by Kunitz and Bowman-Birk Trypsin Inhibitors

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A novel serine protease has been partially purified from dry seeds of the soybean (*Glycine max*) cultivar Keburi by cryoprecipitation at pH 6.4, fractional precipitation with ammonium sulfate, and a series of column chromatographic procedures on DEAE-Sepharose, SP-Sepharose, and Arginine-Sepharose 4B. Some properties of the purified enzyme were studied. The protease hydrolyzed the native storage globulins of soybean seeds, such as the α subunit of β-conglycinin, at a pair of arginine residues, Arg126-Arg127. The proteolysis of the α subunit in the purified $\alpha_2\beta$ molecule of β-conglycinin apparently followed first order kinetics. The enzyme was inhibited by both soybean Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor in a competitive manner. Moreover, the enzyme could catalyze the specific proteolysis of the A3 polypeptide of the purified G5 glycinin at the Arg99-Gly100 linkage, or the carboxyl side of the Arg98-Arg99 paired basic residues.

Key words: β-conglycinin, glycinin, serine protease, soybean, trypsin inhibitor.

The proteolytic activities of soybean [*Glycine max* (L.) Merrill] seeds have been investigated principally at the maturing and germinating stages to determine the mechanisms of the formation, processing and degradation of the storage proteins in seeds. Several proteolytic enzymes have been isolated, partially purified and characterized. Most of the proteases in the earlier studies were studied using casein or synthetic oligopeptides as substrates, while several enzymes in later studies were found to act on the naturally occurring storage proteins in soybean seeds: a protease acting on 7S globulin (*1, 2*), an acidic carboxyl endopeptidase and a metalloendopeptidase acting on the α and α' subunits of β-conglycinin (*3*), a sulfhydryl protease acting on Kunitz trypsin inhibitor and the acidic subunit of glycinin (*4*), a protease acting on Bowman-Birk protease inhibitor (*5*), a processing enzyme acting on proglycinin (*6, 7*), a protease acting on the α and α' subunits of β-conglycinin (*8, 9*), and a serine protease acting on the acidic and basic subunits of glycinin (*10, 11*). These proteolytic enzymes were found in maturing or germinating cotyledons, but their activities were very weak or undetectable in resting dry seeds.

Recently, we detected a protease in resting dry seeds of the cultivar "Keburi" and genetically related strains of soybeans, which lack the α' subunit of β-conglycinin (*12*). The protease acted very specifically on the native α subunit of β-conglycinin, cleaving only one linkage, Arg126-Arg127, but it was inactive against the native β subunit under these conditions. Preliminary characterization showed that the proteolytic activity exhibited a maximum in the pH range between 8 to 9, and it was inhibited by antipain, (4-amidinophenyl)methanesulfonylfluoride, aprotinin, and leupeptin, but not by bestatin, chymostatin, E-64, EDTA, pepstatin, or phosphamidon. These facts suggested that the enzyme was a neutral/alkaline serine protease of soybean. Nishikata (*13*) isolated a similar trypsin-like protease from soybean seeds, but he stated that the enzyme acted on synthetic oligopeptide substrates, although it was inert to protein substrates, including soybean reserve proteins. Moreover, his enzyme was not inhibited by soybean trypsin inhibitors. On the other hand, Qi *et al.* (*8, 9*) reported a protease which cleaved the α and α' subunits of β-conglycinin to give a product with a molecular weight similar to that of the β subunit. However, their enzyme was different from ours in terms of the optimal pH (4.0) and the hydrolysis of the α and α' subunits in successive steps. Our enzyme thus appeared to be a novel protease of soybean seeds. However, our previous experimental results were obtained with a crude extract from dry seeds of soybean, and a question remains as to whether multiple proteases might exhibit such characteristics. Therefore, characterization of the protease (substrate specificity, type of inhibition, *etc.*) should be performed with the purified enzyme.

In the present study, we have attempted to characterize the purified enzyme, and we report here the purification and some properties of the neutral/alkaline serine protease from the seeds of the soybean cultivar Keburi. This is the first report that the soybean protease is inhibited by Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)butane; 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); APMSF, (4-amidinophenyl)methanesulfonylfluoride; MCA, 4-methylcoumaryl-7-amide; Boc, *t*-butyloxycarbonyl.

MATERIALS AND METHODS

Soybean Seeds—Three different kinds of cultivars or strain of soybeans were used in the present experiments. The seeds of the soybean cultivar Keburi were harvested in a field belonging to Fuji Oil in Ibaraki Prefecture in 1991 and 1992. The seeds harvested in 1991 were used for the preparation of the single molecular species $\alpha_2\beta$ of β-conglycinin after storage at room temperature for about one year ("old crop"), while those harvested in 1992 were used for the purification of the protease after brief storage at −20°C ("new crop"). For the preparation of a single molecular species of glycinin, $(A3B4)_6$ (*14, 15*), we used the seeds of a new strain of soybean, which lacks the glycinin genes Gy_1, Gy_2, Gy_3, and Gy_4 and contains only gene Gy_5 expressing the G5 subunit, A3B4 (*16*). The strain was kindly supplied by K. Kitamura, the National Agriculture Research Center, Tsukuba, and was cultivated in Fuji Oil's field in Tsukuba in 1992. Seeds of the standard cultivar Enrei, harvested in Toyama and Fukui Prefectures in 1992, were also used in this experiment for comparison, as well as for the preparation of β-conglycinin containing the α, α', and β subunits.

Chemical Reagents—Soybean Kunitz trypsin inhibitor was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. The molar concentration of the Kunitz inhibitor was calculated using the molecular weight of 21,500. Bowman-Birk proteinase inhibitor from soybean was purchased from Sigma Chemical (lot no. 50H8095). The molar concentration was calculated by assuming a molecular weight of 8,000. Arginine-Sepharose 4B was purchased from Pharmacia Biotech. All other chemicals were of analytical grade.

Preparation of β-Conglycinin—A single molecular species of β-conglycinin, $\alpha_2\beta$, was prepared for use as the substrate in the measurement of specific proteolysis of the α subunit from the seeds of the old crop Keburi, which lacked the α' subunit of β-conglycinin. The seeds were powdered in an electric mill, and the flour was defatted twice with 10 times its weight of *n*-hexane at room temperature and then dried overnight. The defatted flour was mixed with 12 times its weight of 30 mM Tris-HCl buffer solution, pH 8.0, containing 10 mM 2-mercaptoethanol. The slurry was stirred for 1 h and then centrifuged to remove the insoluble material. The supernatant was used as the soybean extract. β-Conglycinin was prepared from the extract by the method of Thanh and Shibasaki (*17*) with some modifications including the removal of γ-conglycinin by isoelectric precipitation at pH 5.7 (*18*) and fractional precipitation with ammonium sulfate according to the method of Iwabuchi and Yamauchi (*19, 20*). The isolation and purification of the four components, α_3, $\alpha_2\beta$, $\alpha\beta_2$, and β_3, of β-conglycinin were performed by anion-exchange chromatography according to the method of Thanh and Shibasaki (*17*) with some modifications, in the presence of 0.02% NaN_3 to prevent growth of microorganisms. All procedures were done at 4°C and in the presence of 10 mM 2-mercaptoethanol and 100 μM antipain to prevent the proteolysis of β-conglycinin. A 3 g aliquot of the β-conglycinin fraction was dissolved in a minimum amount of 35 mM potassium phosphate buffer, pH 7.6, containing 10 mM 2-mercaptoethanol and 0.4 M NaCl (Standard Buffer) and passed through a Sephadex G-25 column, 2.5×40 cm, equilibrated with 19 mM potassium phosphate buffer, pH 7.8, containing 0.2 M NaCl and 10 mM 2-mercaptoethanol. The passed protein solution was applied to a DEAE-Sephadex A-50 column, 2.5×100 cm, equilibrated with the same buffer, and elution was performed with a linear increase in NaCl concentration from 0.2 to 0.4 M (2 liters each) at a flow rate of 30 ml/h. The appropriate fractions, containing the α and β subunits at a ratio of 2 : 1, were collected and pooled after 90% saturation with ammonium sulfate. The homogeneity of the purified $\alpha_2\beta$ molecular species was determined by HPLC.

Standard β-conglycinin containing all three kinds of subunits, α, α', and β, was prepared from cv. Enrei seeds, and partially purified chromatographically as described above.

Preparation of G5 Glycinin $(A3B4)_6$—Glycinin G5 (*15*) was prepared from the soybean seeds of a strain containing only glycinin gene Gy_5, with other glycinin genes deleted. The protein was purified by cryoprecipitation at pH 6.4, followed by fractional precipitation with ammonium sulfate according to Iwabuchi and Yamauchi (*19, 20*).

Electrophoresis and Electroblotting onto PVDF Membranes—In order to identify and determine the subunits of β-conglycinin, SDS-PAGE was performed according to the method of Laemmli (*21*) 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 (*22*). The protease preparations during the course of purification were also analyzed by native PAGE in the absence of SDS. The proteins were located by silver staining. The proteolytic activity was detected fluorometrically under ultraviolet light after the gels had been sprayed with a 100 μM Boc-Gly-Arg-Arg-MCA solution.

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.* (*8*). In the present experiments, the purified β-conglycinin $\alpha_2\beta$ was used as the substrate. One hundred microliter of 1% β-conglycinin $\alpha_2\beta$ solution dissolved in the Standard Buffer was mixed with 50 μl of 0.4 M potassium phosphate buffer, pH 8.0, containing 0.02% NaN_3. The proteolysis was initiated by the addition of 50 μl of protease solution. The final concentration of the α subunit of β-conglycinin $\alpha_2\beta$ was calculated to be 57.5 μM. The reaction mixture, 200 μl, was incubated at 30°C for 1 to 4 h, unless otherwise stated. At appropriate time intervals, 40 μl of the reaction mixture was withdrawn, diluted with 160 μl of water and added to 800 μl of SDS solution. Then 22 μl of the treated solution was charged on a gel and analyzed by SDS-PAGE, as described in the previous section. After staining, the difference in absorbance of the bands of the α-subunit of β-conglycinin $\alpha_2\beta$ before and after the enzymatic reaction was determined with a densitometer. As the reaction was first order, as will be shown later, the rate of decrease in the concentration of the α subunit during the first 1 min was calculated using the slope of the semi-logarithmic plot. One unit of the 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 at an initial α subunit concentration of 57.5

μM, in 200 μl of reaction mixture. The native β subunit in the substrate was used as the internal standard, because it was not degraded by this protease under the conditions used. The enzyme assay for the $\alpha_2\beta$ molecule of β-conglycinin was used throughout the purification of the protease.

Determination of Protein—Protein was determined by the dye-binding method of Bradford (*23*) 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 (*24-26*).

High-Performance Liquid Chromatography (HPLC)—The analysis of the molecular species of β-conglycinin and its hydrolyzed products was performed with a Shimadzu HPLC LC-10A system. For ion-exchange chromatography, a Shim-pack PA-DEAE column, 8×100 mm, was used with NaCl-gradient elution.

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.* (*27*) 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).

Purification of Protease—Soybean seeds of the cultivar Keburi new crop, 200 g, were powdered in an electric mill, and the flour was extracted with 10 times its weight of 30 mM Tris-HCl buffer solution, pH 8.0, containing 0.02% NaN_3 at 4°C for 2 h. The insoluble materials were removed by centrifugation at 10,000×g, followed by filtration to prepare a clear extract. The pH of the solution was adjusted to 6.4, and the solution was chilled to 4°C and left standing overnight. The cryoprecipitates, containing the bulk of the protease, were collected by centrifugation (crude 11S fraction) and dissolved in a small amount of the Standard Buffer without 2-mercaptoethanol. When the solution was brought to 50% saturation with ammonium sulfate, the protease activity was concentrated in the precipitates. The precipitates were collected by centrifugation and dissolved in 40 ml of the Standard Buffer without 2-mercaptoethanol at 4°C, and the insoluble material was spun off. The supernatant was passed through a Sephadex G-25 column equilibrated with 35 mM potassium phosphate buffer, pH 8.0, to remove ammonium sulfate. The solution was divided into 20 equal parts, and a 16 ml aliquot of the solution containing 240 mg protein was applied to a DEAE-Sepharose Fast Flow column, 2.5×25 cm, equilibrated with the same buffer; the protein was eluted with increasing NaCl concentration from 0 to 0.4 M in the buffer solution. The active fractions were collected and precipitated once by 80% saturation with ammonium sulfate. The precipitates obtained from 20 repeated runs were collected by centrifugation and dissolved in a small amount of the Standard Buffer without 2-mercaptoethanol. The solution was passed through a Sephadex G-25 column equilibrated with 35 mM potassium phosphate buffer, pH 8.0, to remove the ammonium sulfate. The solution was divided into four equal parts and each aliquot was separately applied to an SP-Sepharose Fast Flow column, 2.5×25 cm, equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl concentration from 0 to 0.2 M in the buffer solution. The active fractions from four runs were collected, diluted with three times the volume of water, and concentrated by adsorption on an SP-Sepharose column followed by elution with a small volume of the buffer solution, pH 8.0, containing 0.2 M NaCl. The solution was dialyzed against 5 mM potassium phosphate buffer, pH 7.0. The dialyzed solution was divided into eight equal parts (10 ml each), and each aliquot was passed through an Arginine-Sepharose 4B column, 1.6×12 cm (*28*). The protease was eluted from the column at 0.4 M NaCl concentration in the same buffer solution, and all of the active fractions from eight runs were pooled and stored in a refrigerator.

RESULTS

Purification of Soybean Protease—The proteolytic activity was easily extracted in dilute buffer solution at pH 8 from seeds of the soybean cultivar Keburi; it was concentrated in the crude 11S fraction precipitating at pH 6.4 and 4°C. Typical patterns after SDS-PAGE of the proteolytic digestion of the α subunit in the molecular species $\alpha_2\beta$ of β-conglycinin are shown in Fig. 1. As will be discussed later, the activity often could not be detected directly in the crude extract from the seeds because of the inhibitory effect of the excess amounts of Kunitz and Bowman-Birk trypsin inhibitors present in the crude extract, but strong proteolytic activity appeared after removal of the inhibitors from the cryoprecipitates of the protease at pH 6.4. The protease was further fractionated with ammonium sulfate, the activity being concentrated in the fraction precipitated at 50% saturation of the salt. Next, it was

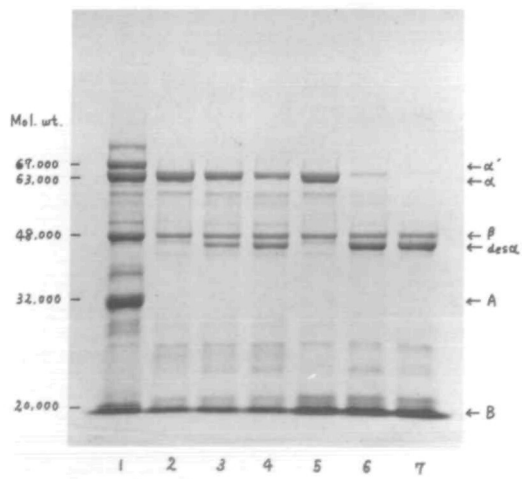

Fig. 1. **SDS-PAGE of proteolytic digest of the α subunit in the molecular species $\alpha_2\beta$ of β-conglycinin by soybean protease.** β-Conglycinin $\alpha_2\beta$, 57.5 μM, in 35 mM Tris-HCl buffer, pH 8.0, (200 μl) was digested with a seed extract or a solution of the cryoprecipitation fraction (protein concentration 0.5%) at 30°C. Lane 1, Extract from soybean seed cv. Enrei; lane 2, β-conglycinin $\alpha_2\beta$; lane 3, $\alpha_2\beta$ + the extract from cv. Keburi, 2 h; lane 4, $\alpha_2\beta$ + the extract from cv. Keburi, 4 h; lane 5, β-conglycinin $\alpha_2\beta$; lane 6, $\alpha_2\beta$ + cryoprecipitate fraction, 2 h; lane 7, $\alpha_2\beta$ + cryoprecipitate fraction, 4 h.

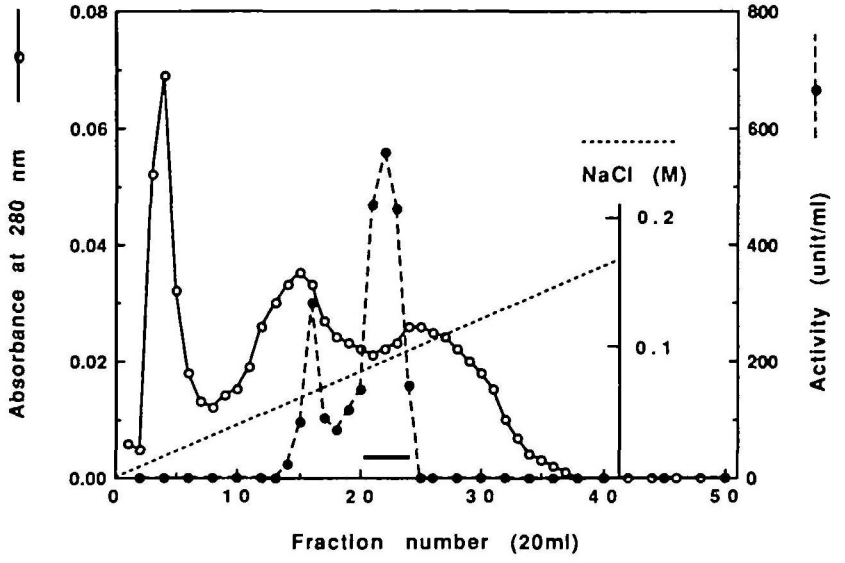

Fig 2. **Ion-exchange chromatography of soybean protease on an SP-Sepharose Fast Flow column, 2.5×25 cm.** Elution was performed with a linear gradient of NaCl concentration from 0 to 0 2 M in 35 mM phosphate buffer, pH 8, containing 0 02% sodium azide. The flow rate was 0 5 ml/min. The horizontal bar represents the collected fractions of purified components.

TABLE I. **Purification of soybean protease from 200 g of seeds (cv. Keburi).**

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification
Crude extract	80,400	(1,278,000)	(15 9)		
Cryoppt.	47,100	1,498,000	31.8	100	1
$(NH_4)_2SO_4$ ppt	6,020	820,000	136	55	4 3
DEAE-Sepharose	218	509,000	2,334	34	73
SP-Sepharose	70	448,000	6,400	30	201
Arg-Sepharose	3.2	37,470	11,710	2.5	368

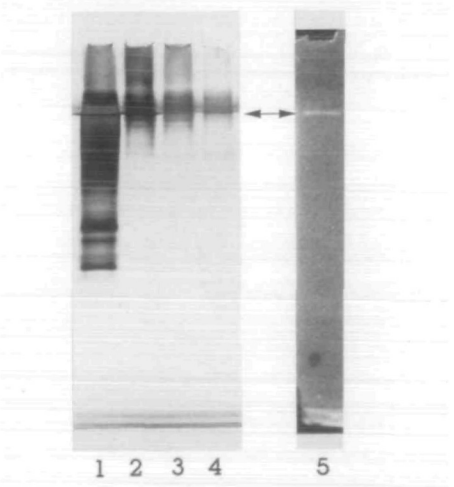

Fig. 3 **Silver-stained gel following undenatured electrophoresis without SDS.** Lane 1, the seed extract; lane 2, DEAE-Sephadex-passed fraction; lane 3, SP-Sephadex fraction; lane 4, Arginine-Sepharose fraction; lane 5 fluorescent band of protease from Arginine-Sepharose fraction.

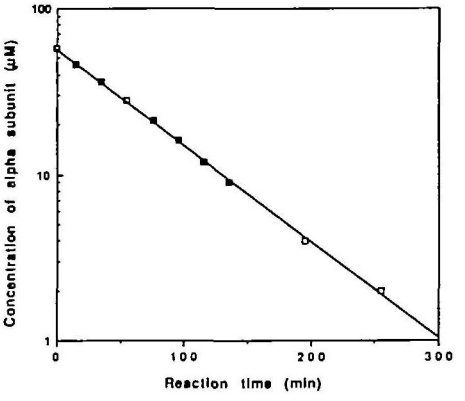

Fig 4 **First-order reaction in the proteolysis of the α subunit of β-conglycinin.** β-Conglycinin $\alpha_2\beta$, 57 5 μM, in 200 μl of K-phosphate buffer, pH 8 0, was digested with 120 units of protease at 30°C.

purified by successive DEAE-Sepharose, SP-Sepharose, and Arginine Sepharose 4B chromatographies. The protease activity was not adsorbed on DEAE-Sephadex. The chromatographic pattern on SP-Sephadex is shown in Fig. 2. The results of a typical purification are summarized in Table I. The enzyme was relatively stable under the purification conditions, and it was purified 368-fold from the cryoprecipitates (crude 11S fraction). The purification from the crude seed extract reach 600-fold. As shown in Fig. 3, the protease was still not homogeneous in the final step on silver-stained gels of native PAGE, but the activity staining showed a single band of the enzyme.

Properties of Soybean Protease—As described in the previous report (*12*), the protease found in the crude extract of soybean seeds (cultivar Keburi) was identified as a neutral/alkaline serine protease. We further characterized the enzyme using a purified preparation. The time course of the hydrolysis of the $\alpha_2\beta$ molecular species of soybean β-conglycinin by the protease was first-order, as shown in Fig. 4. In this action on the $\alpha_2\beta$ molecule, the

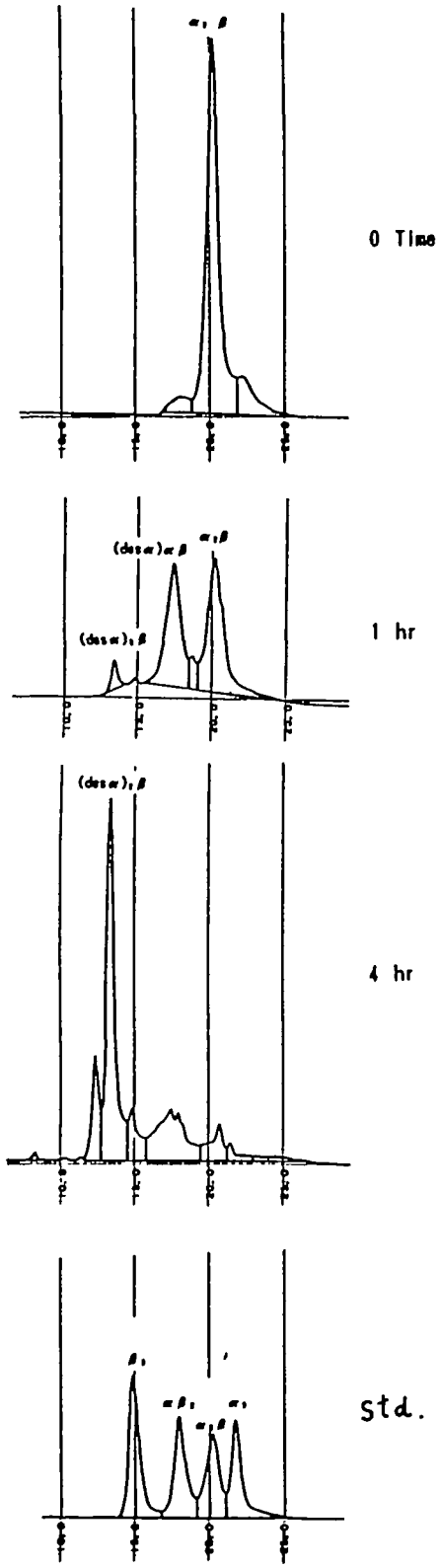

Fig. 5. **HPLC of the reaction mixture from the proteolysis of $\alpha_2\beta$ by soybean protease.** β-Conglycinin $\alpha_2\beta$, 57.5 μM, in 35 mM Tris-HCl buffer, pH 8.0, (200 μl) was digested with 50 units of the protease at 30°C. At appropriate time intervals, an aliquot (20 μl) of the reaction mixture was withdrawn and subjected to HPLC analysis.

proteolysis of the two α subunits in the molecule proceeded equally, with retention of the quaternary structure of the β-conglycinin trimer. The weakly acidic α subunit lost the highly acidic Val1-Arg126 fragment to form the basic

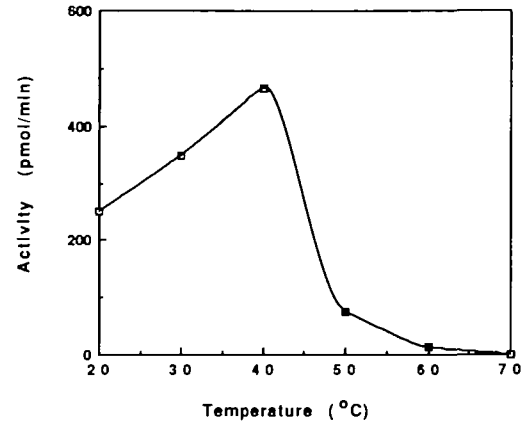

Fig. 6. **Temperature dependence of proteolytic activity of soybean protease.** The measurement of the activity was performed under the conditions described in "MATERIALS AND METHODS," except that the activity was measured at various temperatures for 60 min, with 350 units of the enzyme in 200 μl of 150 mM Tris-HCl buffer, pH 8.0. The activity was expressed in pmol of the α subunit disappearing during the first 1 min from the initial concentration of 57.5 μM α.

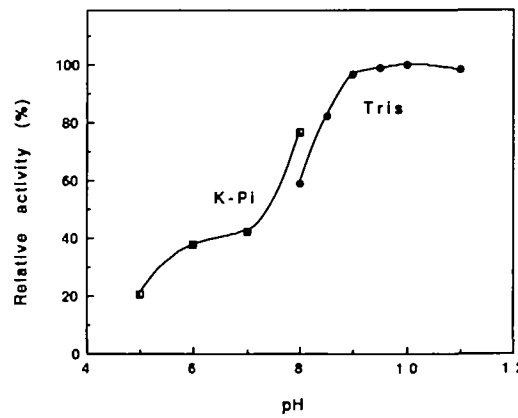

Fig. 7. **Effect of pH on the proteolytic activity of soybean protease.** The reaction mixture, 200 μl, containing 120 units of the protease and 57.5 μM $\alpha_2\beta$ in 0.1 M K-phosphate (pH 5-8) or 0.1 M Tris-HCl (pH 8-11), was incubated at 30°C for 1 and 4 h. The activities are represented as relative values.

TABLE II. **Effects of inhibitors and effectors on the protease activity.**

Inhibitor or effector	Conc.	% control activity
Leupeptin	100 μM	0
Aprotinin	100 μM	0
Antipain	100 μM	0
Iodoacetic acid	1 mM	100
p-Chloromercuribenzoate	1 mM	100
EDTA	1 mM	100
$CaCl_2$	1 mM	100
$MnCl_2$	1 mM	100
$FeCl_2$	1 mM	100
$CoCl_2$	1 mM	100

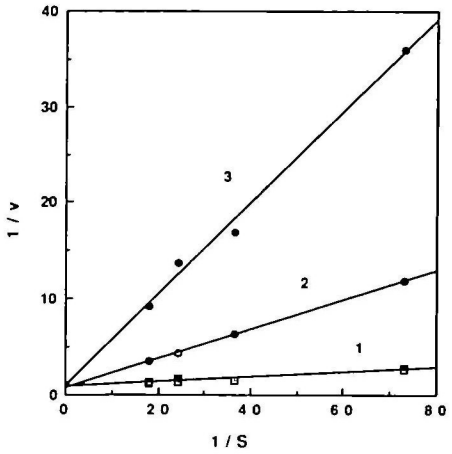

Fig 8 **Lineweaver-Burk plot of the proteolytic activity in the presence and absence of soybean Kunitz and Bowman-Birk inhibitors.** β-Conglycinin $\alpha_2\beta$, 13.7 to 57.5 μM, in 200 μl of 150 mM Tris-HCl buffer, pH 8.0, was digested with 120 units of protease at 30°C for 60 and 120 min in the absence and presence of inhibitors 1, no inhibitor, 2, 4.76 μM Bowman-Birk inhibitor, 3, 4.43 μM Kunitz inhibitor.

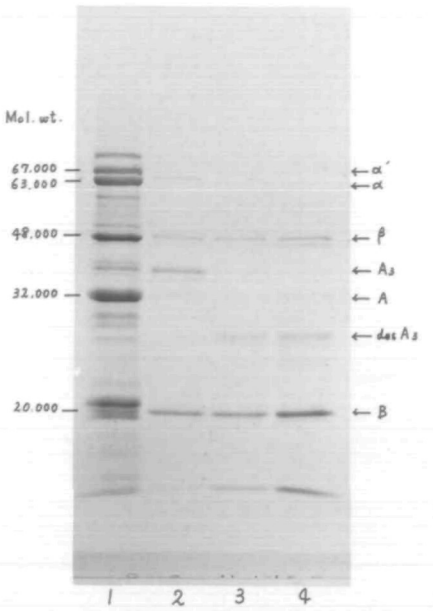

Fig 9. **SDS-PAGE of the products of limited proteolysis of the A3 polypeptide in the G5 glycinin molecule $(A3B4)_6$.** G5 glycinin (0.5%) was digested with 50 units of the protease in 200 μl of 35 mM K-phosphate buffer, pH 8.0, at 30°C. Lane 1: extract from soybean seed cv. Enrei, lane 2, G5 glycinin, lane 3, G5 glycinin + the soybean protease.

des(1-126)α subunit, so that ion-exchange chromatography, as shown in Fig. 5, revealed the transformation of the $\alpha_2\beta$ molecule to the βdes(1-126)α_2 molecule through $\alpha\beta$des(1-126)α in two steps. The K_m value for the α subunit was estimated to be 34.25 μM from the Lineweaver-Burk plot, as will be shown later (Fig. 8).

The temperature dependence of the proteolysis is shown in Fig. 6. The activity decreased markedly at temperatures over 40°C, while the enzyme in solution was very stable at temperatures as low as 5°C for several months, with almost no loss of activity in the presence of 0.02% NaN_3. Next, the effect of pH on the proteolytic activity is shown in Fig. 7. The crude extract of the seeds showed the maximum activity at pH between 8 to 9, while the partially purified preparation shows the highest activity over pH 9 to 11. Such high stability in the alkaline region has also been reported for a serine protease from soybean seeds (*10, 11*).

Inhibitory Experiments—The effects of proteinase inhibitors and effectors such as binary cations are shown in Table II. The enzyme was inhibited by leupeptin, aprotinin, and antipain, but the activity was not affected by iodoacetic acid, *p*-chloromercuribenzoate, EDTA, or binary cations such as calcium. Moreover, as we have stated in the previous report (*12*), the proteolytic activity was completely inhibited by antipain and APMSF, and the enzyme cleaved the post-arginyl peptide linkage in the α subunit of β-conglycinin. These data suggested that the enzyme may belong to the trypsin family. Therefore, we examined the inhibitory effect of Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor from soybean seeds on this protease. The Kunitz inhibitor was strongly inhibitory (Fig. 8), and the inhibition was competitive. The inhibition constant, K_i was calculated to be 0.265 μM. The Bowman-Birk inhibitor displayed weaker competitive inhibition, the K_i being 0.885 μM.

Action on Glycinin Subunits—Glycinin, another major storage soybean globulin, also contains several R-R linkages, some of which should be accessible to the protease. In fact, we found in the previous study (in Fig. 1 in Ref. *12*) that the amount of A3 polypeptide of the glycinin G5 subunit in the crude extract from cv. Keburi was markedly diminished during incubation of the extract. We then tried to confirm the digestion using purified glycinin G5 and the partially purified protease. Figure 9 shows that the A3 polypeptide is indeed accessible for limited proteolysis. SDS-PAGE showed that the molecular weight of one of the major products was about 18,000. The relative rate of proteolysis of the A3 polypeptide was as rapid as that of the α subunit of β-conglycinin. The product was subjected to electroblotting onto a PVDF membrane, and the amino-terminal amino acid sequence was analyzed. The result showed a sequence of Gly-Ser-Arg-Ser-Gln-Gln-Gln-Leu-, corresponding to the sequence from Gly100 to Leu107 of the A3 polypeptide, in which the sequence around the scissile site (from 95 to 105) is -Gln-Ser-Ser-Arg-Arg-Gly-Ser-Arg-Ser-Gln-Gln-. Therefore, proteolysis occurred at the Arg99-Gly100 peptide linkage in this case.

DISCUSSION

In the previous report (*12*), we preliminarily characterized a neutral/alkaline serine protease from dry seeds of the soybean cultivar Keburi. However, possible contamination by other kinds of proteases, such as the protease reported by Qi *et al.* (*9*), in the crude extract of soybean seeds could not be excluded in the previous experiments using a crude preparation. The purified enzyme after the Arginine-Sepharose 4B chromatography in the present investigation had no such contaminating protease activity. We concluded that the protease was a trypsin-type protease, because it was inhibited by soybean Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor. In both cases, the inhibitions were found to be competitive, and the inhibition

constants were determined to be 0.265 and 0.885 μM, respectively. Since the scissible points in the α and α' subunits of β-conglycinin were the Arg-Arg linkages, the inhibitors presumably combine with the enzyme at the substrate-binding site. The present experimental results are the first finding of inhibitory effects of both Kunitz and Bowman-Birk inhibitors on a protease of soybean origin. Two kinds of neutral/alkaline serine proteases have been found in soybean seeds. Nishikata (*13*) purified a protease from seeds of the soybean cultivar Toyosuzu. The protease acted on benzoyl-L-arginine-MCA and several naturally occurring peptides, such as angiotensin II, serum thymic factor, and bradykinin potentiator B, at the carboxyl ends of arginyl or lysyl residues, but it could not hydrolyze protein substrates, including soybean 7S and 11S globulins. Moreover, various protein inhibitors of trypsin, such as soybean trypsin inhibitor, were ineffective in the proteolysis of synthetic or peptide substrates. Akhtaruzzaman *et al.* (*10, 11*) reported a partially purified serine protease which was active on glycinin A4A5B3 at pH 8 or higher. However, the scissile site of the substrate did not involve basic amino acid residues; the Tyr378-Asn379 linkage in the B3 polypeptide was identified as the bond cleaved. Moreover, the activity of their enzyme was not affected by soybean trypsin inhibitor. Therefore, the protease in the present investigation is considered to be a novel and highly arginine-specific serine protease in soybean seeds. One of the reasons for the failure to detect in previous investigations with crude extract of soybean seeds would be the strong inhibitory effect of Kunitz and Bowman-Birk inhibitors present in the extracts. Kunitz and Bowman-Birk inhibitors are known to be distributed widely in the cotyledon tissues of soybean seeds (*29, 30*) and can be easily extracted, but the concentrations of these inhibitors in the extract and the relative inhibitory activities of the multiple components of Bowman-Birk inhibitors (*31, 32*) on the present protease are not known. However, Anderson *et al.* (*33*) reported the contents of trypsin inhibitors to be 50 mg per g of soybean flour, as estimated by determination of the inhibitory activity on trypsin. This amount would be sufficient to inhibit the protease strongly in the crude extract, competitively with the natural substrates, glycinin and β-conglycinin. We happened to find the proteolytic activity in the crude extract of the 1992 soybean seeds, probably because the crops harvested in 1992 exhibited relatively strong proteolytic activity and a low content of natural inhibitors. When using the seeds harvested in 1991, 1993, and 1994, almost no proteolytic activity could be detected in the crude extract; it was apparent only after the removal of the inhibitors by cryoprecipitation of the protease fraction at pH 6.4.

As for the substrate specificity of the protease under investigation, the α-subunit of β-conglycinin was cleaved at the Arg-Arg linkage, while the A3 polypeptide of G5 glycinin was cleaved at the carboxyl side of the Arg-Arg linkage quite specifically. This fact suggests that the protease seems to recognize the paired basic amino acid residues in the protein substrates.

Recent investigations have shown that many physiological peptides or proteins are produced from their precursors by cleavage at paired basic amino acid residues. The Kex 2 protease from yeast cells and furin from mammalian cells are typical proteinases exhibiting such a processing function (*34, 35*), but our protease was not affected by EDTA and calcium ions. On the other hand, the storage proteins of plant seeds are often produced by processing of the proproteins in the accumulation step to form the mature proteins. Usually, a specific processing enzyme cleaves the site after Asn residues (*36*). In the case of soybean globulins, glycinin seems to be formed by the processing of proglycinin to afford acidic and basic polypeptides by such limited proteolysis, and the protease has been highly purified (*6, 7, 37*). However, Nielsen (*38*) has pointed out that the carboxy-terminus of the acidic polypeptide was Lys, not Asn, in the A2 polypeptide component, which is the only one for which the amino acid sequence of the expressed protein component has been completely determined, rather than deduced from the cDNA sequences. This result suggested another possible cleavage site at the linker region between the acidic and basic polypeptides; Nielsen suggested that paired basic amino acids, which were found in such linker regions of glycinin precursors, would be important recognition signals. At any rate, the present soybean protease is the first enzyme recognizing paired basic amino acid residues to have been found in plant tissues. At the present stage of investigation, however, it is not clear whether the soybean protease has a processing function, acting on precursors of unknown active proteins, or whether it acts as a processing enzyme at the maturation stage of soybean storage proteins. The distribution or localization of the enzyme in the tissues may provide a clue to the significance of the enzyme. Further experiments are needed on the precise substrate specificity using synthetic peptide-MCA and naturally occurring neuropeptides containing paired basic amino acid sequences.

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