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Purification and Characterization of an N-Terminal Acidic Amino Acid-Specific Aminopeptidase from Soybean Cotyledons (Glycine max)

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A novel enzyme that catalyzes the efficient hydrolysis of Glu-Glu was isolated from soybean cotyledons by ammonium sulfate fractionation and successive column chromatographies of Q-sepharose, Phenyl sepharose, and Superdex 200. The apparent molecular mass of this enzyme was found to be 56 kDa and 510 kDa by SDS-polyacrylamide gel electrophoresis and Superdex 200 HR 10/30 column chromatography respectively. The enzyme had high activity against Glu-*p*-nitroanilide (*p*NA) and Asp-*p*NA, whereas Leu-*p*NA, Phe-*p*NA, Ala-*p*NA, and Pro-*p*NA were not hydrolyzed. The synthetic dipeptides Glu-Xxx and Asp-Xxx were hydrolyzed, but Xxx-Glu was not. The digestion of a Glu-rich oligopeptide, chromogranin A (Glu-Glu-Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH$_2$) using this purified enzyme was also investigated. Glutamic acid residues were cleaved one by one from the N-terminus. These observations indicate that the enzyme removes glutamyl or aspartyl residues from N-terminal acidic amino acid-containing peptides. It is thought that it was an N-terminal acidic amino acid-specific aminopeptidase from a plant.

Key words: N-terminal acidic amino acid-specific aminopeptidase; soybean; cotyledon; aspartyl aminopeptidase

Shutov and Vaintraub suggested a role of proteases in the mobilization of storage proteins at the cotyledon during seed germination.[1] In addition, many peptidases in cotyledon tissue that play important roles in the degradation process of storage proteins, have been reported.[2–5] Aminopeptidases catalyze the sequential removal of amino acids from the unblocked N termini of peptides and proteins. Various aminopeptidases with different substrate specificities are widely distributed in eukaryotes and prokaryotes.[6] They are generally classified in terms of their substrate specificities, the preference for a neutral, acidic, or basic amino acid in the P1 position. They play an important role in protein and peptide metabolism.[7]

We have been searching for proteases and peptidases that can degrade storage proteins and their fragments to oligopeptides or amino acids in germinating seeds of the soybean Glycine max. First we investigated the protein degradation process during germination in the protein bodies of soybeans, and identified a group of key intermediary fragments of approximately 30 kDa, called c30. These fragments were identified as being derived from the degradation of β-conglycinin, a major storage protein in soybeans.[8] Using an assay system capable of detecting hydrolytic activity toward c30, protease D3 was purified from the germinating cotyledons of soybeans.[9] Characterization of this enzyme showed that it was cysteine protease. Its specificities were examined using peptide-4-methylcoumary-7-amide (MCAs) and peptides hormones, and cathepsin L-like broad specificity was observed at pH 4.0.[9] These results indicate that protease D3 contributes to protein turnover in germinating soybean cotyledons, as do other cysteine proteases in animals and plants.[10,11] We also confirmed that it was difficult to digest native soybean storage proteins using commercially available proteases for food processing. Therefore, protease D3 probably plays a critical role in protein metabolism in soybean seeds and has enzymatic activity hydrolyzing native soybean storage proteins. Peptidases such as carboxypeptidase,[12] prolidase,[13] and leucine-aminopeptidase[14] have also been reported in soybean cotyledons. Glycinin and β-conglycinin, major storage proteins of soybean, are abundant in acidic amino acid sequences such as -Glu-Glu- and -Glu-Asp-.[15] Aminopeptidase, which liberates glutamic acid or aspartic acid from the N-terminus of peptide, is called glutamyl aminopeptidase[16–18] or aspartyl aminopeptidase.[19,20] These enzymes have been reported to be present in mammals, yeast, and filamentous fungi,[20–22] but have not been purified from plants.

We speculated that glutamyl aminopeptidase or aspartyl aminopeptidase exists in the germinating soybean cotyledone and plays an important role in soybean germination. In this study, we looked for acidic amino acid specific aminopeptidase in soybean cotyledons with Glu-Glu as a substrate, and isolated a novel aminopeptidase from the soybean cotyledon that removes acidic amino acids sequentially from N-terminus acidic amino acid-containing peptides.

Materials and Methods

Plant material. Soybean seeds, Glycine max [L.] Merrill cv. Kegon, were purchased from Sakata-no-Tane (Yokohama, Japan). They were sown on wet vermiculite (0 DAI) 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 days 0–10, the cotyledons alone were dissected from the other tissues, rinsed with distilled water, and frozen at $-80\,^{\circ}$C until use.

Reagents. Polyacrylamide gel, Multigel (15/25) (T = 15–25%) was purchased from Dai-Ichi Pure Chemical (Tokyo). A glutamic acid assay kit was from Yamasa (Tokyo). Chromogranin A and E-64 were from the Peptide Institute (Osaka, Japan). AEBSF was from Sigma-Aldrich Japan (Tokyo), and *p*NA substrates for aminopeptidase were from Bachem (Bubendorf, Switzerland). Synthetic dipeptides containing Glu and/or Asp were from the Peptide Institute and Kokusan Chemicals (Tokyo). All other chemicals were of analytical grade.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed under reducing conditions using Multigel (15/25) (T = 15–25%) by the method of Laemmli.[23]

Protein assays. The protein concentration was measured with a Bio-Rad Protein Assay Kit (Bio Rad, Hercules, CA) with bovine γ-globulin as the standard.

Enzyme assays. Enzyme activity was determined by quantitative analysis of Glu released from Glu-Glu. L-α-Glu-Glu (0.02 ml of 50 mM), 0.02 ml of 100 mM HEPES buffer (pH 7.2), and 0.14 ml of H_2O were mixed and preincubated at $37\,^{\circ}$C for 5 min, and then 0.02 ml of enzyme solution was added and the mixture was incubated at $37\,^{\circ}$C for 20 min. The reaction was terminated by adding 0.05 ml of 50% aqueous acetic acid. The quantity of glutamic acid was determined with a glutamic acid assay kit and by conventional amino acid analysis by the ninhydrin method. One unit of enzyme activity corresponded to the release of 2 μmol of Glu from Glu-Glu per min at $37\,^{\circ}$C.

Other dipeptidase activity was measured by the method described above, and conventional amino acid analysis was conducted by the ninhydrin method, but replacing substrate Glu-Glu with another dipeptide, Glu-Lys, Glu-Gly, Glu-Ala, Glu-Phe, Glu-Thr, Glu-Ser, Glu-Asp, γ-Glu-Glu, Pro-Glu, Lys-Glu, Phe-Glu, Ala-Glu, Ser-Glu, Asp-ε-N-Lys, Asp-Lys, Asp-Ala, Asp-Glu, Asp-Phe, Asp-Asp, or γ-Glu-Leu.

Aminopeptidase activities were measured using amino acid-*p*NA (Glu, Asp, Leu, Phe, Gly, Ala, and Pro). The enzyme reaction was initiated by mixing 0.05 ml of enzyme solution, 0.02 ml of 50 mM amino acid-*p*NA substrate, 0.02 ml of 100 mM HEPES buffer (pH 7.2), and 0.11 ml of H_2O, and the mixture was incubated at $37\,^{\circ}$C for 20 min. The reaction was terminated by adding 0.05 ml of 50% aqueous acetic acid. The absorbance of the reaction mixture was measured at 410 nm to evaluate the releasing activity of *p*NA from amino acid-*p*NA. One unit of enzyme activity corresponded to the release of 1 μmol of amino acid from amino acid-*p*NA per min at $37\,^{\circ}$C.

Search for Glu-Glu hydrolyzing peptidase. We looked for Glu-Glu hydrolyzing peptidase from soybean cotyledons. Each soybean cotyledon obtained from 0–10 DAI was homogenized in ice-cold buffer (20 mM potassium phosphate buffer, pH 7.0, 200 mM NaCl, 10 mM 2-ME, 2 mM NaN$_3$) at a fresh-weight: buffer ratio of 1 g: 5 ml.

The filtrates, filtered through gauze, were centrifuged at 32,000 *g* for 30 min. The supernatants were filtered with filter paper (Whatman No. 2, Whatman Japan, Tokyo) to give the crude extract.

Purification of peptidase.

Extraction. Purification procedures were performed as described below. All were performed below $10\,^{\circ}$C.

Cotyledons (600 g) of 7 DAI were homogenized and extracted with 3 liters of 20 mM potassium phosphate buffer (pH 7.0), 200 mM NaCl, 0.1 mM AEBSF, 10 μM E-64, and 10 mM 2-ME. The extract was filtered with gauze, and then centrifuged (32,000 $\times$ *g*, 30 min). The supernatant was collected and filtered through two layers of filter paper (No. 514A, Advantec, Tokyo). The supernatant (19,780 mg of proteins) was concentrated about 20 times with a concentrator with a polyethersulfone membrane (Minitan, Millipore, Billerica, MA).

Ammonium sulfate precipitation. The pH of the extract was adjusted to pH 7.0 with NaOH, and a 40% saturation was made with ammonium sulfate (763 g/3,150 ml). The solution was stirred for 6 h and centrifuged at 32,000 $\times$ *g* for 30 min at $4\,^{\circ}$C. The supernatant was saturated with 65% saturation ammonium sulfate (564 g/3,400 ml). To recover the precipitated proteins, the supernatant was stirred for 15 h at $4\,^{\circ}$C and then centrifuged at 32,000 $\times$ *g* for 30 min. The precipitates were dissolved in 120 ml of 50 mM potassium phosphate buffer, 100 mM NaCl (pH 7.0, 2 mM NaN$_3$) and dialyzed against the same buffer (2 liters $\times$ 5, 16 h) at $7\,^{\circ}$C. After dialysis, the solution was centrifuged at 32,000 $\times$ *g* for 20 min at $4\,^{\circ}$C and then filtered through a 0.2 μm filter (Nalgene Labware, Rochester, NY).

Chromatography on Q sepharose HP. An anion exchange column, HiLoad Q Sepharose HP 26/10 (GE Healthcare Japan, Tokyo) was equilibrated with 50 mM potassium phosphate buffer and 100 mM NaCl (pH 7.0, 2 mM NaN$_3$). The enzyme solution (5,160 mg protein/190 ml) was divided into eight aliquots, and each aliquot was subjected to the column. The column was then washed with a 5-fold volume of the buffer. After washing, the absorbed active fraction was eluted with an increasing linear gradient of 100 mM to 250 mM NaCl in an 8-fold column volume of the buffer. The active fractions were collected and concentrated from 2,000 ml to 90 ml with an ultracentrifugation unit (Minitan). After 11.88 g of ammonium sulfate was added to this concentrate and dissolved, the solution was centrifuged at 32,000 $\times$ *g* for 10 min to give the supernatant.

Phenyl sepharose HP hydrophobic chromatography. The supernatant obtained at step 3 above was divided into two aliquots, and each aliquot was applied to Phenyl Sepharose HP 26/10 (GE Healthcare Japan, Tokyo). The column was equilibrated with 50 mM potassium phosphate buffer containing ammonium sulfate (1.0 M). After the addition of the sample, the column was washed with a 5-fold column volume buffer. The absorbed active component was then fractionated in a decreasing linear gradient of ammonium sulfate concentration from 1.0 M to 0 M. The active fractions were collected and concentrated from 180 ml to 9.8 ml with an ultracentrifugation unit (Minitan and Centriprep 10 (Millipore)). The concentrate was centrifuged at 32,000 $\times$ *g* for 10 min to give the supernatant.

*Superdex 200 gel filtration chromatography.* The supernatant obtained at step 4 above was fractionated by gel filtration through HiLoad 26/60 Superdex 200 pg. The supernatant was divided into two aliquots, and each aliquot was applied to the column which had been equilibrated with 50 mM potassium phosphate buffer and 100 mM NaCl (pH 7.0, 2 mM NaN$_3$). Elution was performed with the same buffer. The active fractions were concentrated to give a purified enzyme. The purity of the enzyme was analyzed by SDS–PAGE and an analytical grade gel filtration column, Superdex 200 HR 10/30 with an AKTA FPLC system.

Effects of pH and temperature. The pH dependency of the enzyme activity was determined in the following manner.

The enzyme reaction buffers used were sodium acetate buffer (pH 4.0, 4.5, 5.0, 5.5, 6.0), potassium phosphate (pH 6.0, 7.0), Tris–HCl buffer (pH 7.0, 7.8, 8.3, 8.8), and sodium carbonate buffer (pH 9.0, 9.5, 10.0). The purified enzyme (180 μl, 3.5 mU) was prepared in 50 mM buffer at each pH value and pre-incubated for 5 min at $30\,^{\circ}$C. The substrate, Glu-Glu (20 μl, 5 mM for reaction), was added to each sample. The reaction mixture was stirred and incubated for 20 min at $30\,^{\circ}$C. The reaction was terminated by adding 20 μl of 50% aqueous acetic acid. The quantity of glutamic acid was determined with a glutamic acid assay kit.

Measurement of the optimum temperature of the purified enzyme was carried out as follows: The substrate, Glu-Glu (20 μl, 5 mM for reaction) was added to 50 mM sodium acetate buffer (pH 6.0), and 180 μl of the reaction solution obtained (substrate solution) was pre-incubated for 5 min at each temperature (25 $^{\circ}$C, 30 $^{\circ}$C, 37 $^{\circ}$C, 42 $^{\circ}$C, 50 $^{\circ}$C, 60 $^{\circ}$C, and 70 $^{\circ}$C). The purified enzyme (7 mU) was then added. The reaction mixture was stirred and incubated for 20 min at each temperature. The reaction was terminated by adding 20 μl of 50% aqueous acetic acid. Enzyme activity was determined by the method described above.

Thermal stability. Determination of the thermal stability of the purified enzyme was carried out as follows: The purified enzyme in 50 mM sodium acetate buffer (pH 6.0) was incubated for 80 min at 25 $^{\circ}$C, 42 $^{\circ}$C, 50 $^{\circ}$C, 60 $^{\circ}$C, and 70 $^{\circ}$C. The treated enzyme solution (7 mU) was then added to 180 μl of Glu-Glu at a final concentration of 5 mM, followed by incubation for 20 min at $30\,^{\circ}$C. The reaction was
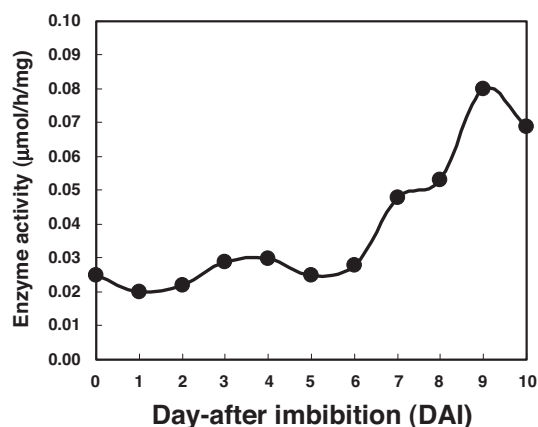
Fig. 1. Course of Changes in the Enzyme Activity of Glu-Glu Hydrolyzing Peptidase.

The cotyledons (1 g) were homogenized and extracted with 5 ml of 50 mM sodium phosphate buffer (pH 7.0), 200 mM NaCl, and 10 mM 2-ME. The extract was filtered with gauze and then centrifuged (16,000 × g, 30 min). The supernatant was collected and filtered through two layers of filter paper. The enzyme activities (units/h/mg of protein) of Glu-Glu hydrolyzing peptidase were measured in each extract of cotyledons from 0 DAI to 10 DAI. Results are the means for two independent experiments.

terminated by adding 20 μl of 50% aqueous acetic acid. Residual activity was determined by the method described above.

Detection of hydrolysis of chromogranin A by the purified enzyme. Hydrolysis of chromogranin A, an oligopeptide containing five glutamic acids at the N-terminal, was examined by the purified enzyme, as follows: 500 mM ammonium carbonate buffer (0.02 ml), 0.05 ml of 5 mM chromogranin A, 0.08 ml of H_2O, and 0.05 ml of the purified enzyme (6 mU) were incubated at 37 °C. A 0.02-ml portion of the reaction solution was sampled at 0, 1, and 15 h. The reaction was stopped with 0.02 ml of 50% aqueous acetic acid. This reaction solution was analyzed for molecular weight by MALDI-TOF mass spectra obtained on a Kompakt Maldi III (Shimazu-Kratos, Kyoto, Japan) mass spectrometer using 2,5-dihydroxybenzoic acid (DHBA) and 2-(4-hydroxyphenylazo)benzonic acid (HABA) as a matrix.

Results and Discussion

Enzyme activity of soybean cotyledons each day after imbibition

The new enzyme was detected by measuring its activity with Glu-Glu as substrate. L-α-glutamylglutamic acid (Glu-Glu) hydrolyzing activity was present in each cotyledon extract from 0 DAI to 10 DAI (Fig. 1). In a previous study, we found that the total amounts of proteins, major storage proteins, and their fragments decreased as time passed during soybean germination and seedling growth. Finally, the proteins in the cotyledon disappeared by 10 DAI.[8] In contrast, the enzyme activity (μmole/h/mg of protein) of this peptidase was higher after 6 DAI (Fig. 1). Hence we purified this enzyme from the cotyledons of 7 DAI in view of the results from the enzyme activity of cotyledons obtained each day after imbibition, as described above. In addition, the influence of various protease inhibitors was investigated. We found that extraction efficiency increased very much with the addition of AEBSF and E-64 (data not shown). Hence the soybean cotyledon homogenate for enzyme purification was prepared with the buffer solution containing them.
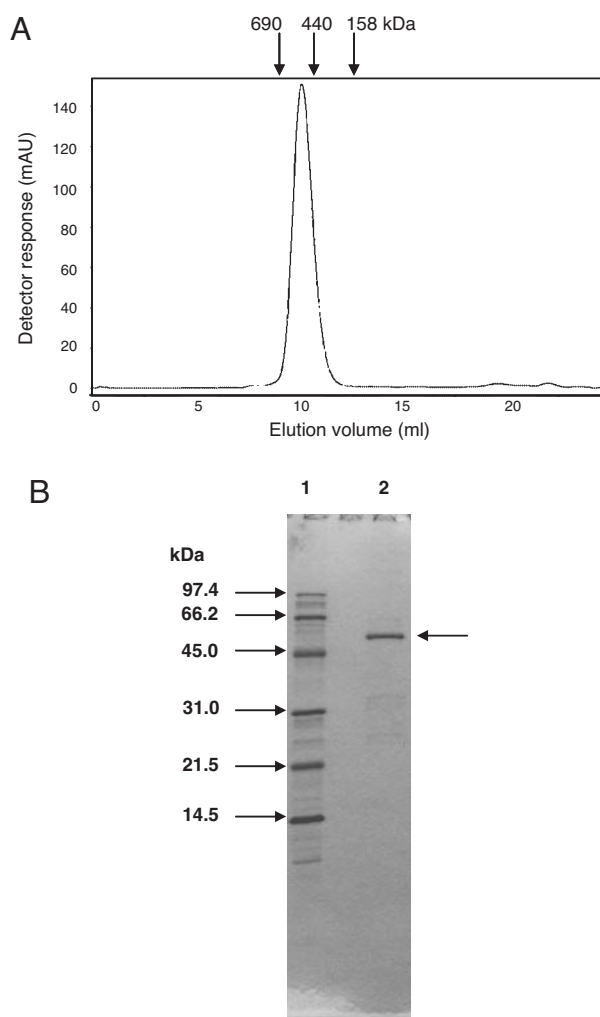
Fig. 2. Separation of Purified Glu-Glu Hydrolyzing Peptidase by Chromatography.

A, Analytical gel filtration of purified Glu-Glu hydrolyzing peptidase. The purity of the enzyme was analyzed with an analytical grade gel filtration column, Superdex 200 HR 10/30 with an AKTA FPLC system. Thyroglobulin (690 kDa), ferritin (440 kDa), and aldorase (158 kDa) were used as molecular size markers. B, Results of SDS–PAGE analysis of the purified Glu-Glu hydrolyzing peptidase. Lane 1, marker proteins; lane 2, the purified enzyme. 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) were used as molecular size markers. The arrow indicates the position of the purified Glu-Glu hydrolyzing peptidase.

Purification of the enzyme

A novel N-terminal acidic amino acid specific aminopeptidase was purified from soybean cotyledons of 7 DAI. The soybean cotyledon homogenate was filtrated and centrifuged, and it was precipitated with 65% ammonium sulfate. It was successively fractionated by chromatographies on columns of Q-sepharose and Phenyl sepharose. The active fraction collected from Phenyl sepharose was applied to a Superdex 200 column, and its active fractions were combined and used as the purified enzyme. The purification steps for the enzyme are summarized in Table 1. The specific activity of the purified enzyme was determined to be 66.3 units/mg with a yield of 35% when Glu-Glu was used as substrate.

Table 1. Purification of N-Terminal Amino Acid-Specific Aminopeptidase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Extract	20,000	150	7.6×10^{-3}	100	1.0
Ammonium sulfate precipitation	5,200	200	3.9×10^{-2}	130	5.1
Q-sepharose	710	150	2.2×10^{-1}	100	29
Phenyl sepharose	9.2	72	7.8	47	1000
Superdex 200	0.8	53	66	35	8700

U, One unit of enzyme activity corresponded to the release of 2 μmol of Glu from Glu-Glu per min at 37 °C.

Table 2. Effects of Potential Inhibitors on the Activity of the Purified Enzyme

Chemical	Concentration (mM)	Relative activity (%)
None		100
$ZnCl_2$	0.4	68.3
$MnCl_2$	0.4	107
$CoCl_2 \cdot 6H_2O$	0.4	97.1
$CaCl_2 \cdot 2H_2O$	0.4	106
EDTA	5.0	83.5
EDTA	20	68.1
DTT	0.1	32.0
o-Phenanthroline	2.0	47.1

Activity was expressed as the percentage of activity remaining after a 10-min incubation of the enzyme in the presence of the potential inhibitor. Activity was measured with substrate Glu-Glu. Results are the means for three independent experiments.

Table 3. Substrate Specificity toward Amino Acid-*p*NA

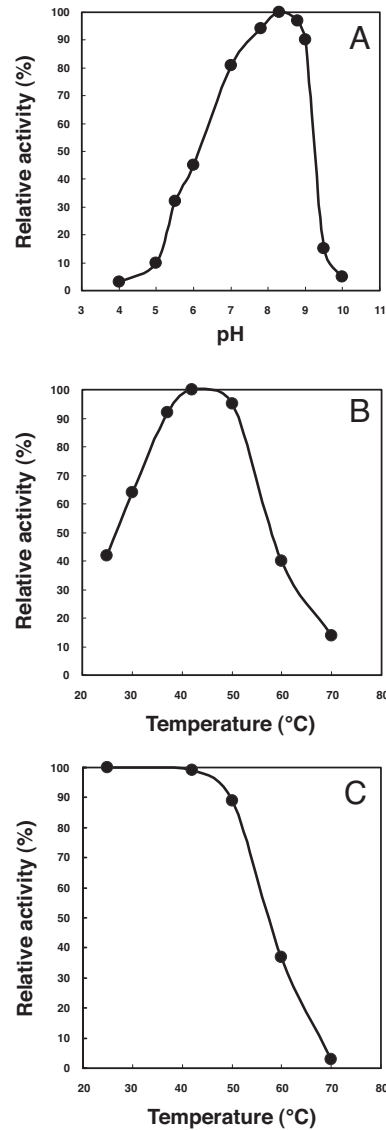| X-*p*NA	Specific activity (U/mg)
Glu-*p*NA	319
Asp-*p*NA	132
Leu-*p*NA	0
Phe-*p*NA	0
Gly-*p*NA	0
Ala-*p*NA	0
Pro-*p*NA	0

U, One unit of enzyme activity corresponded to the release of 1 μmol of amino acid from amino acid-*p*NA per min at 37 °C. Results are the means for two independent experiments.


Fig. 3. Characteristics of the Purified Glu-Glu Hydrolyzing Peptidase.

A, pH dependency of hydrolysis activity toward Glu-Glu at 30 °C. B, Optimal reaction temperature for the hydrolysis of Glu-Glu at pH 6.0. C, Thermal stability of the purified Glu-Glu hydrolyzing peptidase. The purified enzyme was incubated at temperatures for 80 min. Results are the means for two independent experiments.

Enzyme characterization

The apparent molecular mass of the purified enzyme was estimated to be 510 kDa by Superdex 200 HR 10/30 column chromatography (Fig. 2A), and 56 kDa by SDS–PAGE (Fig. 2B). This indicates that the enzyme was a multimeric homopolymer.

Figure 3 shows the enzymatic properties of the purified enzyme. It exhibited more than 80% activity from pH 7 to 9 at 30 °C (Fig. 3A). The optimum reaction temperature was approximately 45 °C at pH 6.0 (Fig. 3B). The enzyme was stable at temperatures below 50 °C over an 80-min incubation at pH 6.0 (Fig. 3C).

To test the influence of several reagents on the purified enzyme, enzyme activity towards Glu-Glu was measured following a 10-min pre-incubation with several reagents (Table 2). The enzyme was inhibited by Zn^{2+}, EDTA, *o*-phenanthroline, and DTT. Therefore, it is thought that metal influences its activity. Various amiopeptidases with various substrate specificities are distributed widely in prokaryotes and eukaryotes. Most aminopeptidases are metalloproteases, and are inhibited by chelating agents.[24] However, DTT also clearly inhibited the activity. The primary structure of the enzyme is currently under investigation. Molecular cloning and characterization of the gene are also in progress. We intend to come to a conclusion as to the enzyme type after confirming the homology of the enzyme and various aminopeptidaes.

Substrate specificity

The hydrolytic activity of the purified enzyme on various synthetic peptides was examined. First, the substrate specificity of the enzyme was determined using seven sets of *p*NA substrate. It had high activity against Glu-*p*NA and Asp-*p*NA, whereas Leu-*p*NA, Phe-*p*NA, Gly-*p*NA, Ala-*p*NA, and Pro-*p*NA were not hydrolyzed
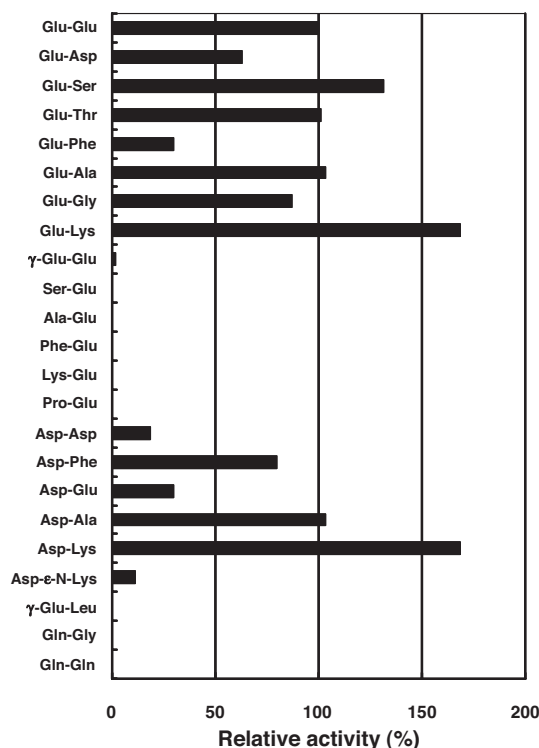
Fig. 4. Activities of the Purified Enzyme toward Various Peptide Substrates.

The specific activity toward Glu-Glu was defined as 100%. Results are the means for two independent experiments.

(Table 3). Secondly, 21 synthetic dipeptides containing Glu and/or Asp were evaluated for the reactivity of the enzyme. Glu-Xxx and Asp-Xxx were hydrolyzed, but Xxx-Glu was not (Fig. 4). This suggests that the enzyme exhibits substrate specificity for unblocked N-terminal acidic amino-acid residues. Next we investigated the reaction of a Glu-rich oligopeptide, chromogranin A (Glu-Glu-Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2) as substrate. The resulting peptides were detected by MALDI-TOF MS analysis (Fig. 5). We detected the production of Glu-Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2 and Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2 after 1 h. After 15 h, Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2, Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2 and Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2 were also generated. It is thought that the enzyme cleaved to the first N-terminal acidic amino-acid residue of the peptides. These results indicate that the enzyme is the N-terminal acidic amino acid-specific aminopeptidase from the germinating soybean cotyledon. Its substrate specificity was similar to aspartyl aminopeptidase (EC 3.4.11.21) and to glutamyl aminopeptidase (EC 3.4.11.7). In particular, the behavior of the enzyme showed similarity to those of the aspartyl aminopeptidases from yeast and *Aspergillus oryzae* in its enzymatic characterization.[20–22] Aspartyl aminopeptidase has been reported to be present in mammals, yeast, and filamentous fungi, but has not previously been purified from plants.[20–22] To confirm that the enzyme was aspartyl aminopeptidase, we intend to determine the amino acid sequence of the enzyme and to compare them of the known aspartyl aminopeptidases.
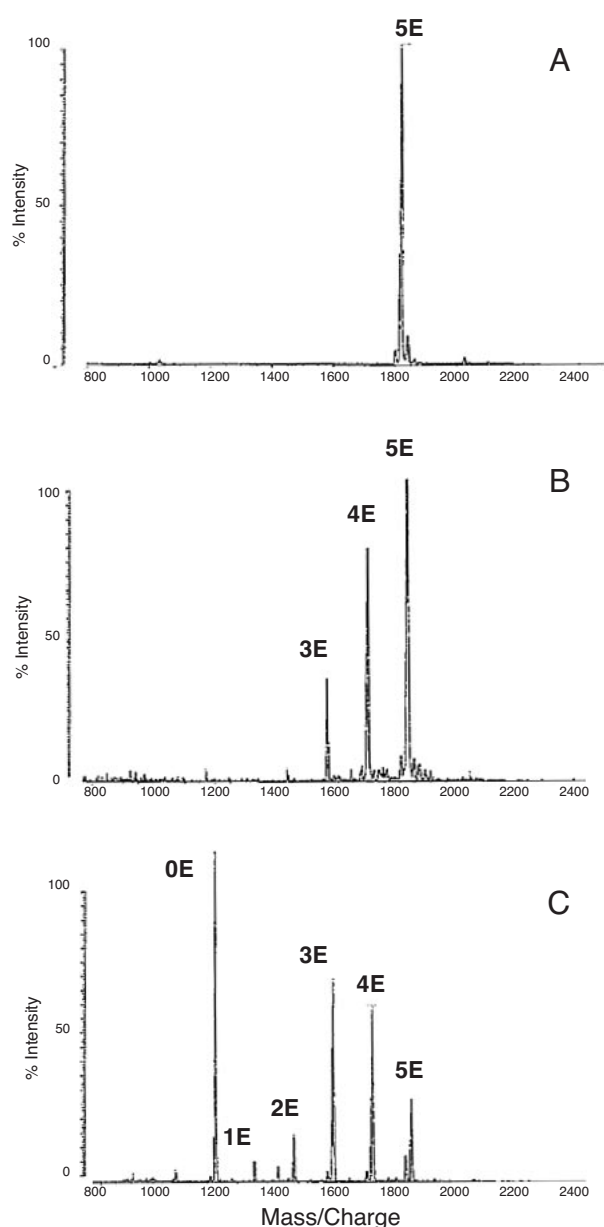
Fig. 5. Time Course of the Reaction of Chromogranin A.

The change over time of chromogranin A (Glu-Glu-Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2) in the reaction mixture was analyzed. The change in its molecular weight was analyzed by MALDI TOF-MS analysis. Reaction times were as follows: A, 0 h; B, 1 h; C, 15 h. The products of the digestion of chromogranin A (EEEEEMAVVPQGLFRG-NH_2, 5E) with the purified enzyme are labeled 4E, 3E, 2E, 1E, and 0E. The products and their molecular weights were as follows: 5E, EEEEEMAVV-PQGLFRG-NH_2, $[M + H]^+$ 1,821; 4E, EEEEMAVVPQGLFRG-NH_2, $[M + H]^+$ 1,692; 3E, EEEMAVVPQGLFRG-NH_2, $[M + H]^+$ 1,563; 2E, EEMAVVPQGLFRG-NH_2, $[M + H]^+$ 1,434; 1E, EMA-VVPQGLFRG-NH_2, $[M + H]^+$ 1,305; 0E, AVVPQGLFRG-NH_2, $[M + H]^+$ 1,175. These molecular weights are in agreement with the theoretical values.

The proteolysis of seed storage proteins is a critical step in the process of germination and early growth. In soybean seedling cotyledons, proteolysis of storage proteins is also initiated by proteolytic enzymes with narrow substrate specificity. One such initiating enzyme is protease C1 in soybean.[25] Protease C1 cleaves wherever an exposed portion of the polypeptide chain bears a string of acidic amino acid residues.[26] Cleavage occurred between two acidic amino acid residues in a

region of the polypeptide that bears many other acidic amino acids residues. There are acidic amino acid-rich regions in two main storage proteins of soybean, the β-conglycinin N-terminal and the C-terminal of the glycinin acidic chain.[15] It is thought that acidic amino acid-specific aminopeptidase played an important role in germinating soybean cotyledone. In this study, we searched for aminopeptidase in germinating soybean cotyledons with Glu-Glu as substrate, and isolated a novel N-terminal acidic amino acid-specific aminopeptidase. This aminopeptidase removed acidic amino acids sequentially from N-terminus acidic amino acid-containing peptides. Furthermore, 13 acidic dipeptides (Glu-Glu, Glu-Asp, Glu-Ser, Glu-Thr, Glu-Phe, Glu-Ala, Glu-Gly, Glu-Lys, Asp-Asp, Asp-Phe, Asp-Glu, Asp-Ala, and Asp-Lys) hydrolyzed by this enzyme were peptides isolated from a soy sauce.[27] Therefore, it is thought that the enzyme contributes to the improvement of the hydrolysis rate of soybean protein. We believe it might be possible to use the enzyme in a variety of food processing applications.

Here we report on the characterization of a novel N-terminal acidic amino acid-specific aminopeptidase from germinating soybean cotyledone. Studies of its genetic properties and structural analysis are necessary, and are underway.

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