Purification and Properties of an Aminopeptidase from Buckwheat Seed

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An aminopeptidase was purified from buckwheat seed by using affinity chromatography, ion-exchange chromatography and chromatofocusing. The enzyme had a molecular weight of 37,000 as determined by gel filtration. The aminopeptidase activity, determined with L-leucine-*p*-nitroanilide (Leu-pNA) as the substrate, exhibited a pH optimum of 7.2. The *K_m* value for Leu-pNA was 140 μ M. The preferred substrates were L-leucine- β -naphthylamide and Leu-pNA, although there was also high activity against L-leucyl-L-alanine and L-leucinamide. Thiol antagonists were found to be potent inhibitors against the enzyme. The enzyme exhibited less or no sensitivity to the endogenous proteinase inhibitors, benzamidine and TPCK.

Buckwheat (*Fagopyrum esculentum* Mönch) seed constitutes an important source of dietary proteins in some parts of the world. Noodle made from buckwheat flour-water dough is a long-established, most acceptable product in Japan. The major storage proteins of buckwheat seed contribute greatly to the noodle-making quality of buckwheat flours. Any alterations of these proteins such as from proteolytic enzymes can have a beneficial or deleterious effect on the quality of resultant noodles from such flours. The storage proteins are synthesized during development of the buckwheat kernel together with other functional proteins including proteolytic enzymes. More information is needed on the properties of the proteolytic enzymes and their possible action on the storage proteins in buckwheat seed.

The present authors have presented evidence for the occurrence of trypsin inhibitors in buckwheat seed.^{1,2)} Although a proteolytic

enzyme, measured as the capacity to hydrolyze benzoyl-D,L-arginine-*p*-nitroanilide, has been shown to occur in buckwheat seed,^{3,4)} no attempt has been made to determine whether the trypsin inhibitors present in buckwheat seed could inhibit endogenous enzymes of the seed. In the course of studies devoted to elucidate this problem, the authors came to recognize that buckwheat seed should contain a proteolytic enzyme, which was presumably classified as a member of aminopeptidase. Sugiyama and Fukuba⁵⁾ have suggested its occurrence in the aqueous extract of commercial buckwheat flour. It is uncertain, however, whether this enzyme is associated with the noodle-making properties of flours made from buckwheat seed. Isolation and characterization of this enzyme will ultimately allow an evaluation of the influence of this enzyme towards the quality of resultant product from buckwheat seed.

The present paper describes the purification

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Abbreviations: Leu-pNA, L-leucine-*p*-nitroanilide; Leu- β NA, L-leucine- β -naphthylamide; Leu-NH₂, L-leucinamide; Leu-Gly, L-leucyl-glycine; Leu-Ala, L-leucyl-L-alanine; Gly-Leu, glycyl-L-leucine; Leu-Gly-Gly, L-leucyl-glycyl-glycine; Ac-Ala-pNA, *N*-acetyl-L-alanine-*p*-nitroanilide; Suc-[Ala]₃-pNA, succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide; Bzl-Cys-pNA, *S*-benzyl-L-cysteine-*p*-nitroanilide; γ -Glu-pNA, L- γ -glutamyl-*p*-nitroanilide; BApNA, α -*N*-benzoyl-D,L-arginine-*p*-nitroanilide; BTpNA, benzoyl-L-tyrosine-*p*-nitroanilide; SBTI, soybean trypsin inhibitor; pCMB, *p*-chloromercuribenzoic acid; TLCK, *p*-toluenesulfonyl-L-lysine chloromethyl ketone; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone.

and characterization of an aminopeptidase from buckwheat seed.

MATERIALS AND METHODS

Materials. Fresh and mature buckwheat seeds were purchased locally and stored at 4°C until use. Leu-pNA and BApNA were obtained from Nakarai Chemicals Co. and Boehringer Mannheim Co., respectively. All other substrates for enzyme assay were purchased from Nakarai Chemicals Co. Trypsin (from bovine pancreas), pepsin (from hog stomach mucosa), SBTI and ovalbumin were obtained from Sigma Chemicals Co. The buckwheat trypsin inhibitors used in this study were isolated in a partially-purified form on trypsin-bound Sepharose 4B as described previously.²⁾ AH-Sepharose 4B, DEAE-Sepharose CL-6B, polybuffer exchanger PBE 94, polybuffer PB 74, Sephacryl S-200 and Sephadex G-25 were products of Pharmacia Fine Chemicals. All other chemicals used were of analytical grade.

L-Leucine was used as a ligand for affinity chromatography. The ligand was coupled to AH-Sepharose 4B, with 1,6-diaminohexane as a spacer, using *N*-ethyl-*N'*-(3-dimethyl aminopropyl) carbodiimide hydrochloride. The column (6.0 × 4.0 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5).

Purification of the aminopeptidase. The purification procedure was conducted at 0–4°C. Finely-ground buckwheat seed meal was extracted with 0.05 M Tris-HCl buffer (pH 7.5) for 1 hr and a 5:1 buffer-to-meal ratio with stirring. After extraction, the suspension was clarified by centrifugation (10,000 × *g*, 15 min). An equal volume of the above buffer solution was then added to the supernatant. The solution obtained was applied on an affinity column of leucine-bound Sepharose 4B. Enzymes bound to the affinity resin were eluted with the same buffer containing 0.3 M NaCl. The active fraction obtained was further fractionated by ion-exchange chromatography on DEAE-Sepharose CL-6B in 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM 2-mercaptoethanol using a linear gradient of 0–0.35 M NaCl. The enzyme fraction obtained was then subjected to chromatofocusing, the enzyme solution being applied to a column (40 × 1.0 cm) of polybuffer exchanger PBE 94, which had been pre-equilibrated against 25 mM Tris-HCl buffer (pH 7.2). The active fraction by this procedure was pooled, desalted by chromatography on a column of Sephadex G-25, and lyophilized. Gel electrophoresis with 7.5% gel (pH 8.6) confirmed the homogeneity of the sample so obtained.

Enzymatic assays. Aminopeptidase activity was routinely assayed using Leu-pNA as the substrate according to the procedure of Apple.⁶⁾ Enzymes sufficient to give a convenient rate of hydrolysis were added to the reaction mixture which consisted of the following in the final

concentration: Leu-pNA, 2 mM; and Tris-HCl buffer (pH 7.2), 0.1 M. The reaction was carried out at 35°C for 20 min and stopped by the addition of 1.0 ml of 20% acetic acid. One unit of enzyme activity is defined as the conversion of 1 μ mol of substrate per minute. Specific activity is defined in terms of 1 unit/mg of protein. The hydrolytic activities of the enzyme against Ac-Ala-pNA, Bzl-Cys-pNA, γ -Glu-pNA, Suc-[Ala]₃-pNA, BApNA and BTpNA were determined by measuring the amounts of *p*-nitroaniline liberated from the substrates from their spectrophotometer readings at 410 nm; the activities against Leu- β NA, Leu-NH₂, Leu-Gly, Leu-Ala, Gly-Leu and Leu-Gly-Gly were determined by measuring the amounts of L-leucine liberated from the substrates with a Yanaco LC-8 amino acid analyzer.

Other analyses. The molecular weight of the enzyme was estimated by the molecular sieving method using Sephacryl S-200 (72 × 3.6 cm) in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl and 1 mM 2-mercaptoethanol.

The distribution of protein in the column effluents was determined by A_{280} measurements. Protein concentration was obtained using the method of Lowry *et al.*⁷⁾ Disc gel electrophoresis was carried out in 7.5% polyacrylamide gel at pH 8.6 by the procedure of Davis.⁸⁾

RESULTS AND DISCUSSION

Purification of the aminopeptidase

The buckwheat aminopeptidase was separated quantitatively from the bulk of the seed proteins by affinity chromatography on leucine-bound Sepharose 4B (Fig. 1). The protein peak which eluted with the starting buffer, 0.05 M Tris-HCl buffer (pH 7.5), showed no enzymatic activity. Enzymes bound to the affinity resin were eluted by 0.3 M NaCl in the same buffer. The active fraction obtained was fractionated by ion-exchange chromatography on DEAE-Sepharose CL-6B (Fig. 2). This chromatography resulted in the appearance of a single peak of enzyme activity. The enzymatically-active fraction was applied to a column of polybuffer exchanger PBE 94, which had been pre-equilibrated against 25 mM Tris-HCl buffer (pH 7.2). Polybuffer 74 at pH 4.0 (diluted 1:10 from the stock solution), 5 ml, was applied to the column, followed by 3–8 ml aminopeptidase solutions. Finally, polybuffer 74, pH 4.0, was added at a flow rate of 20 ml/hr. Aminopeptidase was separated with

FIG. 1. Affinity Column Separation of Buckwheat Aminopeptidase.

Fifty milliliters of the diluted aqueous extract were added to a column (6.0×4.0 cm) of leucine-bound Sepharose 4B. The arrow indicates a change of effluent from the extractant buffer to 0.3 M NaCl in the same buffer.

-----, A_{280} ; —, enzyme activity.

FIG. 2. Ion-exchange Chromatography of the Pooled Material Desorbed from Leucine-bound Sepharose 4B.

The ion exchanger (DEAE-Sepharose CL-6B) was equilibrated with an initial effluent buffer (0.05 M Tris-HCl buffer, pH 7.5). The linear gradient limit was 0~0.35 M sodium chloride in the equilibrating buffer and the total volume of the gradient was 800 ml.

-----, A_{280} ; —, enzyme activity; ·····, NaCl.

other inert proteins at pH 4.9 (Fig. 3). On polyacrylamide gel electrophoresis, a single band was detected for the purified enzyme, even if about 50 μ g of protein was applied.

The buckwheat aminopeptidase was purified

to a homogeneous state with a recovery of approximately 20% (Table I). The specific activity of the purified enzyme was 6.17 units/mg protein, representing approximately 110-fold purification over the aqueous extract.

FIG. 3. Chromatofocusing of the Active Fraction Obtained from DEAE-Sephacrose CL-6B Column Chromatography.

-----, A_{280} ; —, enzyme activity; - · - · -, pH.

TABLE I. PURIFICATION OF AN AMINOPEPTIDASE FROM BUCKWHEAT SEED

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Ratio
I. Aqueous extract	1706.4	96.6	0.06	100	1.0
II. Affinity resin column chromatography	33.3	72.0	2.16	74.5	38.1
III. DEAE-Sephacrose CL-6B column chromatography	9.7	39.1	4.03	40.5	71.0
IV. Chromatofocusing	2.9	17.9	6.17	18.5	108.9

Molecular weight estimation

The molecular weight of the buckwheat aminopeptidase, as estimated by molecular-sieve chromatography on Sephacryl S-200, was 37,000 (Fig. 4). Its molecular weight was a little lower in comparison with aminopeptidases found in other plant seeds.⁹⁻¹²⁾

pH Optimum and stability

Aminopeptidase activity, determined with Leu-pNA as the substrate, exhibited a pH optimum of 7.2 (Fig. 5A). The kinetic parameters for Leu-pNA as the substrate were determined to be: $K_m = 1.4 \times 10^{-4}$ M, and $V_{\max} = 4.5 \times 10^{-3}$ M/min/mg protein.

The effect of pH on the stability of the

enzyme was also studied. The enzyme was stable to changes of pH in the range of 4~8 (Fig. 5B). Below pH 4 and above pH 8 the enzyme significantly lost its activity. The thermal stability of the aminopeptidase at pH 7.2 has also been studied. The enzyme had a comparatively high degree of stability at 40°C; there was about 10% loss of its original activity after heating for 10 min at 40°C. The activity was much less stable at 50°C (data not shown).

Substrate specificity

The relative activities of the aminopeptidase against several substrates are presented in Table II. Of the substrates tested, Leu- β NA demonstrated the highest degree of suscepti-

FIG. 4. Determination of the Molecular Weight of the Buckwheat Aminopeptidase by Molecular-sieve Chromatography with Sephacryl S-200.

The arrow indicates the enzyme. Standard proteins and their molecular weights are: 1, ovalbumin (45,000); 2, pepsin (35,000); 3, trypsin (24,000).

TABLE II. SUBSTRATE SPECIFICITY OF BUCKWHEAT AMINOPEPTIDASE

Enzyme activity was determined using the indicated compound as substrate (2 mM). Assays were carried out in 100 mM Tris-HCl buffer (pH 7.2).

Activities are expressed relative to Leu-pNA.

Substrate	Relative activity (%)
Leu-pNA	100
Leu- β NA	208.7
Leu-NH ₂	19.8
Ac-Ala-pNA	nd
Suc-[Ala] ₃ -pNA	nd
Bzl-Cys-pNA	nd
γ -Glu-pNA	nd
BAPNA	nd
BTpNA	nd
Leu-Gly	11.8
Leu-Ala	55.9
Gly-Leu	17.1
Leu-Gly-Gly	8.6

nd, not detectable.

FIG. 5. The pH-dependence of the Activity (A) and the Stability (B) of Buckwheat Aminopeptidase.

(A), the enzyme activity was assayed in 0.05 M phosphate buffer at various pH levels, 35°C, for 20 min using Leu-pNA as the substrate.

(B), the enzyme solution (20 μ g/ml) was adjusted at the indicated pH with NaOH, NaCl or HCl (final ionic strength, 0.5).

The enzyme solution so obtained was incubated for 30 min at 37°C, and then the remaining enzymatic activity against Leu-pNA as the substrate was assayed.

bility to hydrolysis. In addition, the preferred substrates for the enzyme were Leu-pNA, Leu-Ala, Leu-NH₂, Gly-Leu and Leu-Gly. The enzyme was also found to liberate L-leucine from Leu-Gly-Gly, but the enzyme did not hydrolyze glycyl-glycine occurring from Leu-Gly-Gly. No activity towards Ac-Ala-pNA, Suc-[Ala]₃-pNA, Bzl-Cys-pNA and γ -Glu-pNA was observed. BAPNA and BTpNA, endopeptidase substrates, were also not hydrolyzed by the enzyme. These results suggest that this enzyme is a true aminopeptidase requiring a free L-amino group on the substrate. The buckwheat aminopeptidase showed a substrate specificity relatively similar to aminopeptidases found in pea (AP-1)¹³⁾ and *Agave americana*.¹⁴⁾ Although the buckwheat enzyme exhibited high hydrolytic activity against the peptides with NH₂-terminal leucine, its pattern of substrate specificity differs from that of a leucine aminopeptidase found in barley,¹⁵⁾ especially in terms of activities of each of the two enzymes against Leu- β NA as the substrate.

TABLE III. EFFECTS OF INHIBITORS AND ACTIVATORS ON BUCKWHEAT AMINOPEPTIDASE

Inhibitors or activators were pre-incubated with the enzyme for 10 min at 35°C before assaying. The substrate for the enzyme was Leu-pNA. All the indicated substances, except for buckwheat trypsin inhibitor (1 mg) and SBTI (1 mg), were added to 1 mM (concentration in the final reaction mixture). Conditions for these assays are in the text.

Substance added	Enzyme activity remaining (%)
None	100
EDTA	89
Citrate	99
Imidazole	103
Cysteine	86
Iodoacetic acid	57
Iodoacetamide	101
pCMB	30
2-Mercaptoethanol	105
Dithiothreitol	102
HgCl ₂	0
TPCK	87
TLCK	82
Benzamidine	101
Buckwheat trypsin inhibitor	96
SBTI	103

TABLE IV. EFFECTS OF VARIOUS IONS ON BUCKWHEAT AMINOPEPTIDASE

Ions were pre-incubated with the enzyme for 10 min at 35°C before assaying. The substrate for the enzyme was Leu-pNA. All the ions examined were added to 5 mM (concentration in the final reaction mixture).

Ion added	Enzyme activity remaining (%)
None	100
K ⁺	97
Na ⁺	95
Ca ²⁺	89
Mg ²⁺	93
Mn ²⁺	99
Hg ²⁺	0
Cu ²⁺	0.2

Enzyme inhibitors and activators

The results of the effects of the inhibitors and activators employed to classify peptide hydrolases are summarized in Table III. The sulhydryl reagents, pCMB and HgCl₂, were

shown to be potent inhibitors of the enzyme. Iodoacetic acid also inhibited the activity of the enzyme. On the other hand, the enzyme was not affected by several chelating reagents, *i.e.*, EDTA and citrate. The buckwheat trypsin inhibitors, SBTI, benzamidine and TPCK had little or no inhibitory capacity against the activity of the enzyme.

Various ions were also evaluated for their influence towards the enzyme (Table IV). Cupric ion, as well as Hg²⁺, almost completely diminished the enzyme activity. The enzyme was not affected by any of the other ions, including Mg²⁺ and Mn²⁺. These results suggest that the buckwheat aminopeptidase is a non-metallo, SH-peptidase (Tables III and IV).

Although the buckwheat aminopeptidase shows high affinity against L-leucine, this enzyme differs from the so-called leucine aminopeptidases, found in various mammalian sources,¹⁶⁾ with respect to these properties. The typical leucine aminopeptidases are activated by Mg²⁺ or Mn²⁺, and inactivated by chelating agents; and their molecular weight is usually high. On the other hand, in terms of antagonism by thiol inhibitors, non-sensitivity towards chelating agents and pH-activity profile, the buckwheat aminopeptidase resembles the aminopeptidases found in barley⁹⁾ and pea (AP-1).¹³⁾ These similarities probably reflect some analogies in the respective reaction mechanisms.

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