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Purification and characterization of a novel glutamyl aminopeptidase from chicken meat

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Abstract

A novel glutamyl aminopeptidase (aminopeptidase A, EC 3.4.11.7) was purified from chicken meat by ammonium sulfate fractionation, ethanol fractionation, heat treatment, and successive column chromatographies of DEAE-Sepharose CL-6B and Sephadex G-200. The purified enzyme migrated as a single band on SDS-PAGE. The molecular weight of this enzyme was found to be 55,000 and 550,000 by SDS-PAGE and Sephadex G-200 column chromatographies, respectively. This enzyme hydrolyzed Glu- and Asp-, but not Leu-, Arg-, and Ala-2-naphthylamide (-2NA) at all. The optimum pH and temperature for hydrolysis of Glu-2NA was 7.5. and 70°C, respectively. Reducing agents such as cysteine and dithiothreitol inhibited the activity of this enzyme at concentrations of 1 mM. However, the activation by Ca^{2+} and the inhibition by amastatin were not observed.

Keywords: Glutamyl aminopeptidase; Chicken; Meat; Glutamic acid; Umami

1. Introduction

In a previous paper (Maehashi, Matsuzaki, Yamamoto, & Udaka, 1999), we described that through a hydrolysis of chicken protein by bromelain, the hydrolysate rich in free glutamic acid having an obvious umami taste was obtained. It was presumed that some peptidases present in the raw material acted on the peptides during the hydrolysis of chicken protein, and that glutamic acid had been released from these various peptides. Since the formation of free glutamic acid was not observed from the chicken protein which was denatured by boiling, it seemed to be due to the action of peptidase which was contained in chicken protein. It has been reported that chicken meat contains aminopeptidase C (Nishimura, Kato, Okitani, & Kato, 1991) and H (Rhyu, Nishimura, Kato, Okitani, & Kato, 1992) which contribute to an increment of free amino acids during aging (Nishimura, Okitani, Rhyu, & Kato, 1990). However, those aminopeptidases have low specificity toward glutamic acid residue. Aminopeptidase which liberates glutamic acid or aspartic acid from N-terminus of peptide is called glutamyl aminopeptidase or aminopeptidase A (EC 3.4.11.7). This enzyme, which has been found in microorganisms (Niven, 1991; Oh, Park, Lee, Kim, & Nam, 1997; Strater, Sherratt, & Colloms, 1999), and organs and serum of various animals; that is, rat kidney (Glenner & Folk, 1961), rabbit small intestine (Andria, Marzi, & Auricchio, 1976), chicken egg-white (Petrovic & Vitale, 1990), porcine intestinal brush-border membrane (Benajiba & Maroux, 1980),and so on, is a membrane-bound metallopeptidase that contains the consensus zinc-binding motif (HEXXH) (Hooper, 1994).Angiotensin II, a key element in the brain or kidney renin-angiotensin system, has been reported to be a good substrate for glutamyl aminopeptidase in vivo (Cheung & Cushman, 1971; Nagatsu, Nagatsu, Yamamoto, Glenner, & Mehl, 1970).There is, however, no report on glutamyl aminopeptidase in chicken meat, and it's application for the taste improvement of foods. The present paper deals with purification and characterization of a novel glutamyl aminopeptidase in chicken meat.

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2. Materials and methods

2.1. Materials

Chicken breast meat was obtained from the local food store. DEAE-Sepharose CL-6B and Sephadex G-200 were purchased from Amerciam Pharmacia Biotech, Co. (Uppsala, Sweden). Amino acid-2-naphthylamides were purchased from Bachem (Bubendorf, Switzerland). Pepstatin, amastatin, leupeptin, and all other inorganic reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chemically synthesized Glu-Glu-Asn, Ala-Glu-Asp and Glu-Pro-Ala-Asp were kindly donated by Ito Ham Foods Inc.

2.2. Assay of aminopeptidase activity and protein content

The standard assay for glutamyl aminopeptidase was performed using glutamyl 2-naphthylamide (Glu-2NA) as substrate. After the 50 μl of enzyme solution had been incubated with 500 μl of 0.5 mM substrate in 10 mM Tris-HCl (pH 7.5) at 37 °C for 30 min, 500 μl of 0.7%HCl in ethanol and 500 μl of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol were added to the reaction mixture, in that order, to stop the enzyme reaction. The red color that developed was measured at 540 nm and the 2-naphthylamine released from Glu-2NA was determined.

One unit of the enzyme activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of substrate per min at 37 °C. Specific activity was expressed as U/mg protein.

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard. The fractions eluted from the chromatographic system were monitored at 280 nm.

2.3. Enzyme extraction

Chicken breast meat was trimmed to remove fat and connective tissues and then minced. Minced meat (944 g) was homogenized with 3 volumes of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl in a Waring blender for 1 min. The homogenate was then centrifuged at 7000 rpm for 30 min, and the resulting supernatant was filtered through four layers of gauze to remove the floating fat.

2.4. Enzyme purification

The crude extract from chicken meat was fractionated with ammonium sulfate at 4 °C. The precipitate formed 30% saturation was collected by centrifugation at 10,000 *g* for 20 min, dissolved in the 500 ml of 10 mM Tris-HCl buffer, pH 8.0, and added 2 volume of cold ethanol. After stirring a few minutes, the supernatant was collected by centrifugation at 10,000 *g* for 20 min. The precipitate obtained from 67% ethanol fractionation was collected and suspended in the buffer to the dialysis for 3 days. The dialyzate was centrifuged to remove insoluble materials and the supernatant was pooled. The resultant supernatant was heated at 60 °C for 20 min, and after being filtered with filter paper it was subjected to separation on a column of DEAE-Sepharose CL-6B, which was eluted with a gradient of 10 mM Tris-HCl buffer and the same buffer containing 0.1 M NaCl at pH 8.0. Pooled fractions were dialyzed and applied to a Sephadex G-200 column ($\phi 1.5 \times 85$ cm). Fractions containing glutamyl aminopeptidase activity were pooled and used as the purified enzyme.

2.5. Enzyme characterization

The activity of the purified glutamyl aminopeptidase was measured against several aminoacyl-2-naphthylamide derivatives (Glu-, Asp-, Ala-, Arg-, Leu-2NA) as substrates (0.5 mM) in 10 mM Tris-HCl buffer (pH 7.5) as standard assay medium. The hydrolytic activity toward synthetic oligopeptides Glu-Glu-Asn, Ala-Glu-Asp, and Glu-Pro-Ala-Asp (0.5 mM) were examined by determination of amino acids liberated using HPLC after incubation with the enzyme in 10 mM Tris-HCl pH 7.5 at 37 °C for 1 h. The effect of potential inhibitors or activators was tested by incubating under the standard assay condition with the following chemicals: puromycin, leupeptin, iodoacetic acid, EDTA, amastatin, cysteine, dithiothreitol (DTT), and glutathione.

The effect of metal salts was determined under the standard assay condition with respective salts: $CuSO_4$, $NiCl_2$, $ZnSO_4$, $CaCl_2$, $MnSO_4$, and $MgSO_4$.

The optimum temperature was measured at various temperatures (40–100 °C) in 10 mM Tris-HCl buffer, pH 7.5 for 10 min. Thermal stability of the purified glutamyl aminopeptidase was determined by incubation of the enzyme in the same buffer, at 40–80 °C for 20 min. Aliquots were taken to measure the remaining activity using the standard assay. Activity is expressed as a percentage of initial activity before incubation.

The optimum pH was measured at 37 °C for 30 min in reaction mixtures with different buffer systems, 10 mM sodium phosphate (pH 6.0–8.0), and 10 mM Tris-HCl (pH 7.0–8.5). For the pH stability determinations the purified enzyme samples were incubated at 37 °C in McIlvaine buffer (pH 4.0–6.0) and 10 mM Tris-HCl buffer (pH 7.0–9.0) for 60 min. Aliquots were taken to measure the remaining activity using the standard assay. Activity is expressed as a percentage of initial activity before incubation.

2.6. Estimation of molecular weight

The approximate molecular weight of the purified enzyme under native conditions was determined by gel filtration on Sephadex G-200 column (ϕ1.5×95 cm). The column was calibrated with Blue Dextran 2000, ovalbumin (MW 43,000), aldolase (MW 128,000), catalase (MW 232,000) and ferritin (MW 440,000). And the molecular weights under reducing conditions were estimated by SDS-PAGE according to the procedure of Leammli (1970) using 10% polyacrylamide gel slab. Molecular weight markers were phosphorylase b (94,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000). The proteins were stained with Coomassie Brilliant Blue R-250.

2.7. pI determination

The isoelectric point was determined by the isoelectirc focusing of native protein, performed on electrophoresis apparatus using Ampholine PAGplate pH 3.5–9.5 (Amersham Pharmacia Biotech).

3. Results

3.1. Purification of the glutamylaminopeptidase

A glutamyl aminopeptidase was purified from chicken breast meat by measuring it's activity with glutamyl 2-naphthylamide (Glu-2NA) as substrate. The meat homogenate was centrifuged and the resultant supernatant (the crude extract) was made 30% ammonium sulfate fractionation and 67% ethanol precipitate fractionations. After dialyisis, the resultant supernatant was heated at 60 °C for 20 min, and it was subjected to separation on a column of DEAE-Sepharose CL-6B. A linear (0–0.1 M) NaCl gradient in 10 mM Tris-HCl buffer (pH 8.0) revealed that the active fraction was eluted at 0.03 M NaCl (Fig. 1). This step was very effective for purification since the bulk of proteins was not adsorbed on the column. The active fractions eluted from the DEAE-Sepharose CL-6B column were pooled and concentrated to further separation. The enzyme solution was applied to a Sephadex G-200 column and its active fractions were combined and used as the purified enzyme. The results of purification are summarized in the Table 1. Chicken glutamyl aminopeptidase was purified from 1 kg of meat to a single protein band on SDS-PAGE (Fig. 3). Approximately 900-fold purification was achieved with an overall yield of 1.5%.

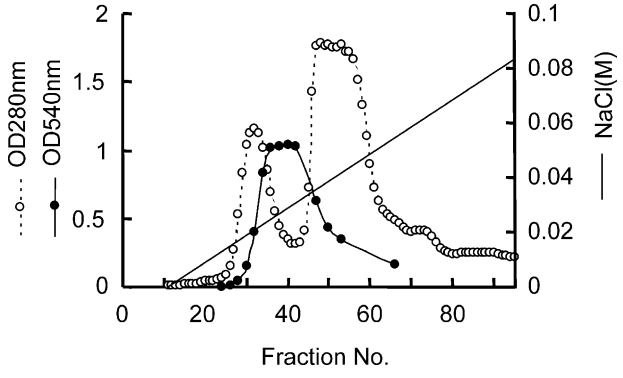

Fig. 1. Separation of chicken glutamyl aminopeptidase on DEAE-Sepharose CL-6B column. The experimental details are described in the text.

3.2. Enzyme characterization

The apparent molecular weight of the purified enzyme was estimated to be 550 and 55 kDa by a Sephadex G-200 column and SDS-PAGE, respectively (Figs. 2 and 3). This result indicates that this enzyme comprises ten subunits with the same molecular mass.

Table 1
Purification of chicken meat glutamyl aminopeptidase

Purification step	Total protein (mg)	Specific activity[a] (U/mg)	Purity (fold)	Recovered activity (%)
Crude extract	47,970	12.4	1	100.0
30% (NH4)2SO4 pptn	15,350	24.6	2	63.4
67% ethanol pptn	892	382	30	57.1
Heat treatment (60 °C, 20 min)	597	757	60	75.8
DEAE-Sepharose CL-6B	18.5	4480	360	13.9
Sephadex G-200	0.8	10,930	880	1.5

[a] The activity was measured using Glu-2NA as a substrate.

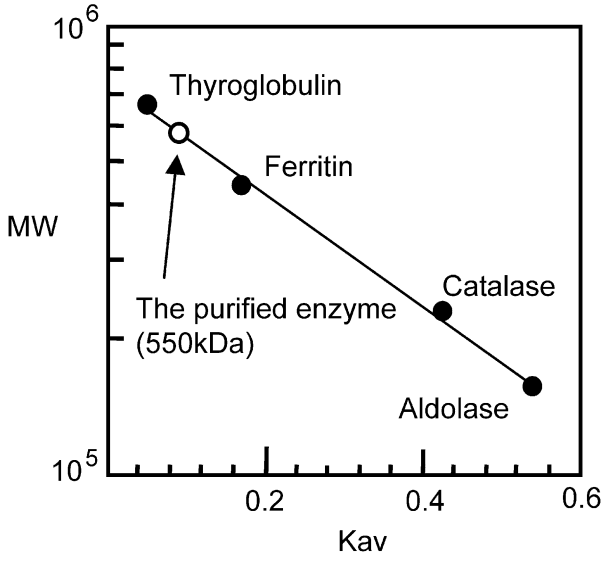

Fig. 2. Molecular weight estimation of the purified enzyme on Sephadex G-200.

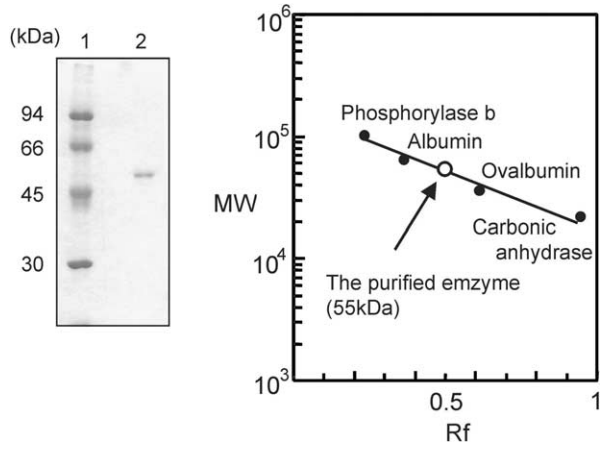

Fig. 3. Molecular weight estimation of the purified enzyme by SDS-PAGE. Lane 1, molecular weught marker; lane 2, the purified emzyme on Sephadex G-200 column.

The isoelectric focusing electrophoresis established pI 5.1 of the enzyme.

The effect of pH on the enzyme activity was examined on the hydrolysis of Glu-2NA. The optimal pH for the enzyme activity was around 7.5. The enzyme was stable around pH 6.0 while it was inactivated at pH 4.0, after incubation at 37 °C for 60 min in various buffer system.

Enzyme activities at various temperatures were measured by incubating the reaction mixtures in 10 mM Tris-HCl buffer (pH 8.0) at designated temperatures. The optimal temperature was found to be 70 °C. The enzyme was shown to be still stable at temperatures up to 65 °C for 20 min. But no activity was observed above 80 °C, indicating thermal inactivation (Fig. 4). The activity was still active after 1 h at 60 °C.

The hydrolytic activity of the purified enzyme on various synthetic peptides was examined. The enzyme was assayed with 0.5 mM 2-naphthylamide(2-NA) derivatives

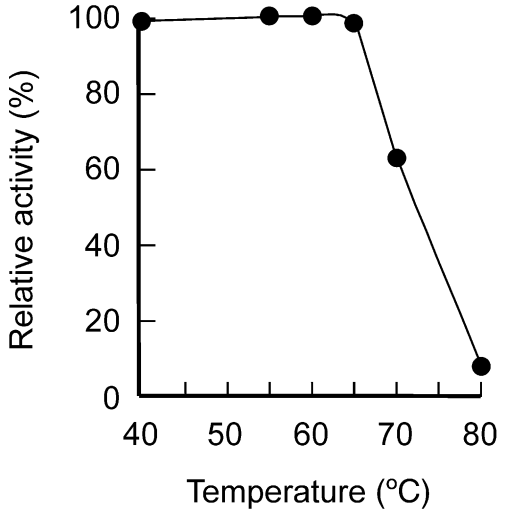

Fig. 4. Thermal stability of the purified enzyme. The experimental details are described in the text.

of amino acids or synthetic oligopeptides. The enzyme had high activity against Glu-2NA and 25% of it against Asp-2NA, whereas Leu-2NA, Arg-2NA and Ala-2NA were not hydrolyzed (Table 2). Synthetic oligopeptides, Glu-Glu-Asn was hydrolyzed, but Ala-Glu-Asp and Glu-Pro-Ala-Asp were not hydrolyzed (data not shown). It was considered that this enzyme cannot cleave X-Pro bond, as many other aminopeptidases. Casein hydrolysate by bromelain, as a natural substrate, was hydrolyzed by the enzyme to release a lot of glutamic acid, aspartic acid and leucine, and small amounts of glycine, alanine, phenylalanine, lysine, and histidine. This might indicate the existence of the dipeptides having those amino acid residues at the C-terminus in that hydrolysate.

The effects on enzyme activity of metal ions, reducing reagents and protease inhibitors were examined by adding designated substances at a final concentration of 0.05–10 mM to reaction mixtures and incubating them at 37 °C for 30 min. The results were shown in Tables 3–5. Among the metal ions tested, Cu^{2+} and Zn^{2+} inhibited the enzyme activity, whereas Ca^{2+}, Mn^{2+} and Mg^{2+} had no effect. Ca^{2+} is known to activate glutamyl aminopeptidases from mammals (Danielsen, Noren, Sjostrom, Ingram, & Kenny, 1980; Glenner, McMillan, & Folk, 1962) and chicken egg-white (Petrovic & Vitale, 1990). The enzyme was sensitive to the reducing reagents, such as cysteine, dithiothreitol, glutathione, and 2-mercaptoethanol. Since the enzyme activity was recovered when the reducing reagents were removed, the inhibitions by them were considered to be reversible. Probably the inhibition is caused by the dissociation into subunits

Table 2
Hydrolytic activity of the purified emzyme towards 2-naphthylamide derivatives of amino acids

Xaa in Xaa-2NA	Relative activity (%)
Glu	100
Asp	25
Leu	0
Arg	0
Ala	0

Table 3
Effects of metal salts on the activity of purified enzyme

	Conc (mM)	Relative activity (%)
None		100
$CuSO_4$	0.1	90
	1	31
$NiCl_2$	1	81
$ZnSO_4$	1	26
$CaCl_2$	1	94
	10	84
$MnSO_4$	1	90
$MgSO_4$	1	100

Table 4
Effects of reducing agents on the activity of purified enzyme

	Conc (mM)	Relative activity (%)
None		100
Cysteine	0.1	73
	1	19
	10	8
Glutathione	10	7
Dithiothreitol	0.1	49
	1	10
	10	2

Table 5
Effects of protease inhibitors on the activity of purified enzyme

Inhibitor	Conc (mM)	Relative activity (%)
None		100
Iodoacetic acid	1	98
	10	0
EDTA	1	100
	10	2
Amastatin	0.05	97
Leupeptin	0.5	94
Puromycin	0.5	66

Enzyme and inhibitor were preincubated for 30 min at room temperature in 10 mM Tris–HCl buffer, pH 7.5.

because of the structure of this enzyme comprising ten subunits. The enzyme was inhibited by iodoacetic acid at 10 mM but not by EDTA at 10 mM, and similar properties have been reported for aminopeptidase H from chicken skeletal muscle. Amastatin, a typical inhibitor of glutamyl aminopeptidases, did not affect this enzyme activity.

4. Discussion

Some aminopeptidases originated from chicken has been reported, that is, aminopeptidases C (Nishimura et al., 1991) and H (Rhyu et al., 1992) from skeletal muscle, aminopeptidase Ey (Midorikawa, Abe, Yamagata, Nakajima, & Ichishima, 1998) from egg yolk, glutamyl aminopeptidase from egg white (Petrovic & Vitale, 1990), and intestinal aminopeptidase (Gal-Garber & Uni, 2000). All aminopeptidases above have high activity toward leucyl-2NA except for egg white glutamyl aminopeptidase. The enzyme in this study showed some similarity to egg white glutamyl aminopeptidase in substrate specificity and optimum pH. However, it was reported that glutamyl aminopeptidase in egg white had a molecular weight of 320,000, and was sensitive to amastatin and activated by $CaCl_2$ and $MnCl_2$, whereas the enzyme in this study had a molecular weight of 550,000 with 10 subunits, and was insensitive to amastatin, $CaCl_2$, and $MnCl_2$. A great similarity between egg white, porcine kidney and human serum glutamyl aminopeptidase exists in pH optimum and thermal stability which is enhanced by the presence of Ca^{2+} ions (Lalu, Lampelo, Kortelainen & Perttula, 1984; Petrovic & Vitale, 1990; Tobe, Kojima, Aoyagi, & Umezawa, 1980). An interesting feature of the enzyme in this study was that its activity was inhibited by reducing agents. This property seemed to distinguish this enzyme from other aminopeptidases found in chicken or glutamyl aminopeptidases in various sources. There is only one report that complete inactivation was obtained with 2 mM DTT in aminopeptidase A from *Streptococcus cremoris* (Exterkate & De-Veer, 1987). From the present work, this enzyme was considered to be a novel glutamyl aminopeptidase on the basis of molecular mass, effect of Ca^{2+}, amastatin, and reducing agents.

The determination of the complete amino acid sequence of glutamyl aminopeptidase in the mouse (Wu, Lahti, Air, Burrows & Cooper, 1990), human (Li, Wang & Cooper, 1993; Nanus et al., 1993), porcine (Hesp & Hooper, 1997), rat (Lee, Tomioka, Takaki, Masumoto, & Saido, 2000), and nematode (Smith et al., 1997) shows the presence of consensus sequence HEXXH (385–389) found in the zinc-metalloprotease family (Hooper, 1994; Jongeneel, Bouvier, & Bairoch, 1989). The nucleotide sequence of chicken intestine aminopeptidase was 99% identical to that of chicken egg yolk (Midorikawa et al., 1998). The deduced amino acid sequence of chicken intestinal aminopeptidase includes the functional conserved sequence, HEXXH domain, which is rich in potential Zn^{2+} ligands.

It has been believed that in chicken muscle, aminopeptidases C and H are the major aminopeptidases in the production of free amino acids during storage. The neutral aminopeptidases have been surveyed from bovine, porcine, and chicken skeletal muscles and the existence of some aminopeptidases in each muscle were shown (Nishimura et al., 1990). In their study, however, glutamyl aminopeptidase was not detected in chicken muscle. The discrepancy between their finding and ours was considered that they surveyed aminopeptidases using the assay system containing 2 mM DTT, which was shown to be inhibitory on glutamyl aminopeptidase in this study. Since the glutamyl aminopeptidase described here has high specificity for glutamyl residue and thermostability, it may be useful to improve the taste of meat during processing or cooking by increasing glutamic acid content. The more detailed properties of the emzyme are currently under investigation. And the molecular cloning and characterizations of the gene are also in progress.

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