Purification and Properties of Aminopeptidase H from Bovine Skeletal Muscle

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Bovine aminopeptidase H was detected and purified from fresh bovine skeletal muscle by ammonium sulfate fractionation and successive column chromatographies of DEAE-cellulose, Ultrogel AcA34, phenyl-Sepharose CL-4B, hydroxylapatite, and DEAE-cellulose again. The purified enzyme migrated as a single band on SDS–PAGE. The molecular weight of this enzyme was found to be 52 000 and 400 000 by SDS–PAGE and Ultrogel AcA34 column chromatographies, respectively. The optimum pH for hydrolysis of L-leucine-2-naphthylamide (Leu-NA) was 7.5. This activity was strongly inhibited by monoiodoacetic acid and leupeptin but was not affected by EDTA, pepstatin, and phenylmethanesulfonyl fluoride. Bovine aminopeptidase H was shown to hydrolyze α-*N*-benzoyl-L-arginine-2-naphthylamide (BANA) as well as L-leucine-2-naphthylamide (Leu-NA). The K_m values for the hydrolysis of Leu-NA and BANA were 3.13 and 2.38 mM, respectively. This enzyme strongly acted on Met-, Leu-, Lys-, Ala-, Glu-, and Ser-NA and hardly acted on Pro-NA.

Keywords: *Aminopeptidase H; conditioning; meat taste*

It is well-known that beef flavor (aroma and taste) is improved during the storage of beef for 2–3 weeks. However, in our previous paper (Nishimura *et al.*, 1988b), beef taste was not improved when the beef, 4 days after slaughter, was stored for another 8 days at 4 °C. Furthermore, the rate of increase in free amino acids during this additional storage of beef for 8 days has shown to be smaller than that during the storage of chicken and pork. This fact seemed to be one of the reasons why beef taste was not improved, because free amino acids such as glutamic acid largely contribute to meat taste (Kato *et al.*, 1989). Free amino acids have been shown to be released from the peptides by the action of neutral aminopeptidases during storage (Okitani *et al.*, 1974). Neutral aminopeptidases in skeletal muscle which have been reported are leucine aminopeptidase (EC 3.4.11.1) (Joseph and Sanders, 1966), aminopeptidases B (EC 3.4.11.6) (Mantle *et al.*, 1985; Ishiura *et al.*, 1987), C (Ishiura *et al.*, 1987; Otsuka *et al.*, 1976, 1980; Mantle *et al.*, 1983; Nishimura *et al.*, 1991a, 1992), and H (Okitani *et al.*, 1980, 1981a; Nishimura *et al.*, 1983, 1991b; Rhyu *et al.*, 1992), pyroglutamyl aminopeptidase (EC 3.4.11.8) (Lauffart and Mantle, 1988), DAP III (Parsons and Pennington, 1976), DAP IV (EC 3.4.21.26) (Kar and Pearson, 1981), and several dipeptidases (Smith, 1948a–c; Okitani *et al.*, 1981b).

Recently, we have shown that aminopeptidases C and H are major ones contributing to the release of free amino acids during the storage of rabbit, chicken, and porcine muscles by a comparison of the substrate specificity of aminopeptidases with the pattern of free amino acids released during storage (Nishimura *et al.*, 1988a, 1990). Furthermore, aminopeptidase H has not been shown to be detected in the beef 4 days after slaughter (Nishimura *et al.*, 1990). Spanier *et al.* (1990) have reported that activities of enzymes in beef gradually decrease during storage at 4 °C. Therefore, bovine aminopeptidase H seemed to be unstable during the post-mortem aging of beef and not to be detected in the beef 4 days after slaughter.

In this work, we tried to clarify whether or not aminopeptidase H was present in bovine muscle by using a fresh bovine muscle just after slaughter. As a result, bovine aminopeptidase H was first detected in and then purified from fresh skeletal muscle, and some of its properties were clarified.

MATERIALS AND METHODS

Materials. Bovine skeletal muscle (M. longissimus dorsi) was removed from the carcass within 1 h after exanguination and cooled on ice. After being cooled, it was trimmed to remove fat and connective tissues and then minced with a meat chopper at 4 °C. All of the following procedures were carried out at 4 °C.

DEAE-cellulose (DE-52 preswollen) was purchased from Whatman (Maidstone, U.K.) and Ultrogel AcA34 from LKB. Phenyl-Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxylapatite and cytochrome *c* were bought from Bio-Rad and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. Ferritin, catalase, and bovine serum albumin were obtained from Boehringer Mannheim GmbH (Germany). Thyroglobulin, ovalbumin, chymotrypsinogen, and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Leupeptin and pepstatin were purchased from Peptide Institute (Osaka, Japan).

Preparation of Crude Extract from Bovine Muscle. After bovine skeletal muscle obtained from one carcass was cut into three blocks, the blocks were stored for 2, 5, or 12 days, respectively. After each storage, the block was immediately minced, homogenized with 3 volumes of 40 mM Tris-HCl buffer (pH 7.2), and centrifuged at 10000*g* for 20 min. The supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 7.2) for 4 h and assayed against substrates for aminopeptidases.

Purification of Bovine Aminopeptidase H. Bovine aminopeptidase H was purified according to the method used to purify porcine aminopeptidase H in a previous paper (Nishimura *et al.*, 1991b).

Minced muscle (600 g) was homogenized with 3 volumes of 40 mM Tris-HCl buffer (pH 7.2) containing 0.1% mercaptoethanol (MCE) and centrifuged at 9000*g* for 15 min. The

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supernatant was subjected to ammonium sulfate fractionation. The precipitate obtained between 45 and 65% ammonium sulfate saturation was collected and dialyzed against 10 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl, 0.1% MCE, and 1 mM NaN_3 (buffer A). The dialysate was put on a DEAE-cellulose column (3.5 × 20 cm) previously equilibrated with buffer A. The enzyme was eluted with a linear concentration gradient of NaCl in buffer A. The active fractions eluting at 0.16 M NaCl were concentrated and applied to an Ultrogel AcA34 column (2.4 × 81 cm). The fractions showing both BANA and Leu-NA hydrolyzing activity were dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1.5 M $(NH_4)_2SO_4$ and 0.1% MCE and then were subjected to a phenyl-Sepharose CL-4B column. The enzyme was eluted by lowering the $(NH_4)_2SO_4$ concentration. The active fractions were collected, dialyzed against 10 mM phosphate buffer (pH 7.2) containing 0.1% MCE, applied to a hydroxylapatite column, and then eluted by raising the concentration of phosphate buffer to 300 mM. The active fraction was applied to a DEAE-cellulose column again after dialysis.

Enzyme Assay. The enzyme activities against amino acid-NA and BANA were measured according to the method in our previous paper (Nishimura et al., 1991b). After the enzyme had been incubated with 0.5 mM substrate in 0.1 M phosphate buffer (pH 8.0) containing 1 mM dithiothreitol at 37 °C for 1–60 min, 0.4 mL of 0.23 M HCl in ethanol and 0.4 mL of 0.06% *p*-(dimethylamino)cinnamaldehyde in ethanol were added to the reaction mixture to halt the enzyme reaction. The red color that developed was measured at 540 nm, and the 2-naphthylamine released from amino acid-NA and BANA was determined.

Protein Determination. The concentration of proteins was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor the protein peaks on the column chromatographies.

SDS–PAGE. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (1970) using 7.5% gels. The proteins were stained with Coomassie brilliant blue R-250.

Molecular Weight Determination. Gel filtration was accomplished on an Ultrogel AcA34 column (1.8 × 98 cm) equilibrated with 10 mM Tris-HCl (pH 7.2) containing 0.1% mercaptoethanol. Thyroglobulin (M_r = 669 000), ferritin (450 000), and catalase (240 000) were used as standard proteins. The molecular weight of the subunit of aminopeptidase H was determined by SDS–PAGE. Bovine serum albumin (M_r = 67 000), ovalbumin (45 000), and chymotrypsinogen (25 000) were used as standard proteins.

RESULTS

Changes in Activities of Crude Extract against Leu- and Glu-2-naphthylamides (-NA) during Storage of Bovine Muscle. After a crude extract was immediately prepared from each meat stored for 2, 5, or 12 days, respectively, it was assayed. Activities against Leu- and Glu-NA were lower in the extract from beef stored for a long time than for short time (Figure 1). Especially, the activity against Glu-NA rapidly decreased in the beginning of the storage, indicating that aminopeptidase H, which preferably hydrolyzes Glu-NA, is very unstable.

Purification of Aminopeptidase H from Bovine Muscle. The bovine aminopeptidase H was first detected by using a fresh muscle in this research.

The results of the purification process are summarized in Table 1. Aminopeptidase H was purified 120-fold over crude extract, and 1.1 mg of enzyme was isolated from 600 g muscle with a yield of 0.6%. The aminopeptidase H thus obtained gave a single band on SDS–PAGE (Figure 2). The large loss of activity during DEAE-cellulose column chromatography was caused by the separation of aminopeptidase H from the fractions containing aminopeptidase C, which has a higher arylamidase activity than aminopeptidase H.

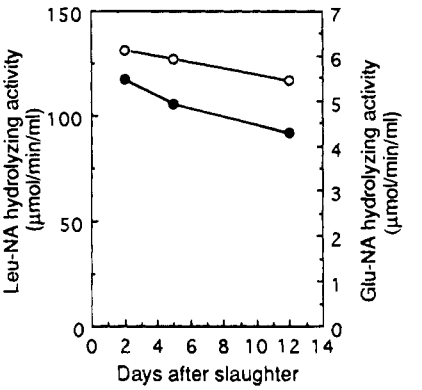

Figure 1. Changes in activities of crude extract against Leu- and Glu-2-naphthylamides (-NA) during the storage of bovine muscle. Crude extract was prepared from bovine muscles stored at 4 °C for 2, 5, and 12 days after slaughter. Activities of crude extracts against Leu-NA (○) and Glu-NA (●) were measured.

Table 1. Purification of Bovine Aminopeptidase H[a]

purification step	total protein (mg)	sp act. (nmol min^{-1} mg^{-1})	purity (fold)	recov of act. (%)
crude extract	21914	0.057	1.0	100
45–65% $(NH_4)_2SO_4$ pptn	7403	0.114	2.0	67.6
first DEAE-cellulose	94.1	1.57	27.5	11.8
Ultrogel AcA34	16.8	1.74	30.5	2.3
phenyl-Sepharose CL-4B	4.2	2.45	43.0	0.8
hydroxylapatite	1.6	6.19	109	0.8
second DEAE-cellulose	1.1	6.84	120	0.6

[a] Aminopeptidase H was purified from 600 g of bovine muscle. The activity against Leu-NA was measured. The details of the assay are given under Materials and Methods.

Some Properties of the Purified Aminopeptidase H. *Molecular Weight.* The molecular weight of aminopeptidase H was estimated to be 400 000 on Ultrogel AcA34 column chromatography (Figure 3) and 52 000 on SDS–PAGE, indicating that this enzyme comprises eight subunits with the same molecular weight.

pH Optimum. The activity against Leu-NA was measured in 50 mM potassium phosphate and ammonium buffers of various pH. The optimum pH was around 7.5 (Figure 4).

K_m Values. The K_m values of aminopeptidase H were 3.1 and 2.4 mM for Leu-NA and BANA, respectively. The former was 10 times that of porcine aminopeptidase H.

Effects of Inhibitors. As shown in Table 2, the enzyme was inhibited strongly by monoiodoacetic acid and leupeptin and weakly by puromycin. However, it was not affected by EDTA, PMSF, and pepstatin. These results suggest that it is a cysteine protease.

Substrate Specificity. This enzyme showed a broad substrate specificity for amino acid-NAs (Table 3). Met-, Leu-, Lys-, Ala-, Glu-, and Ser-NA have been shown to be susceptible to aminopeptidase H. Val- and Gly-NA were slightly hydrolyzed. Pro-NA was not hydrolyzed by aminopeptidase H at all.

DISCUSSION

We have found and purified aminopeptidases H in rabbit, chicken, and porcine skeletal muscles in previous studies. However, no aminopeptidase H had been previously detected in bovine skeletal muscle. In this study, Glu-NA hydrolyzing activity in bovine skeletal

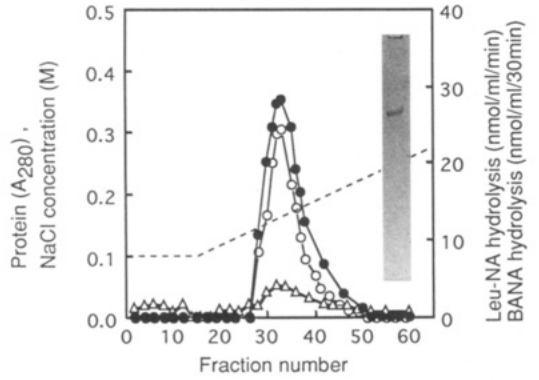

Figure 2. Rechromatography of bovine aminopeptidase H on DEAE-cellulose column. The active fractions (1.6 mg) from hydroxylapatite column were pooled and dialyzed against 10 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl and 0.1% MCE. The dialysate was applied to the DEAE-cellulose equilibrated with the same buffer. Fractions of 3 mL were collected. The purified enzyme was applied to a 7.5% polyacrylamide gel containing SDS. (△) Protein; (●) Leu-NA hydrolyzing activity; (○) BANA hydrolyzing activity; (- - -) NaCl concentration.

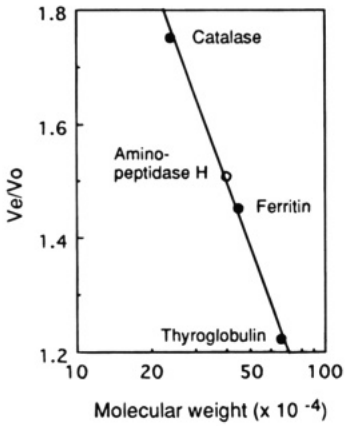

Figure 3. Determination of molecular weight of bovine aminopeptidase H on Ultrogel AcA34. Bovine aminopeptidase H and 5 mg of catalase, ferritin, and thyroglobulin were put on Ultrogel AcA34 column and eluted with 10 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl and 0.1% MCE. The void volume (V_0) was determined with blue dextran, and then the elution volume (V_e) for each protein was measured.

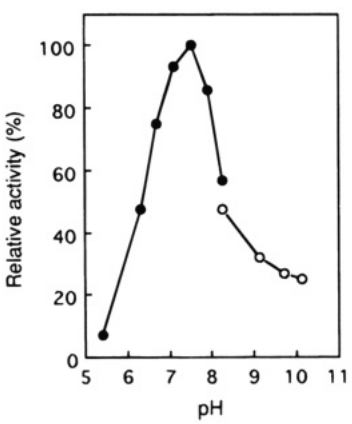

Figure 4. Effect of pH on the activity of bovine aminopeptidase H. The activity was measured using 0.5 mM Leu-NA in potassium phosphate (●) or ammonium (○) buffer.

muscle has been shown to be unstable during storage, indicating that the aminopeptidase H, which mostly contributes to Glu-NA hydrolysis in muscle aminopeptidases, is very unstable during the storage of bovine muscle. Spanier *et al.* (1990) have shown that the activities of many enzymes in bovine muscle homogenate reached a maximum by 3.5 h postslaughter and

Table 2. Effects of Protease Inhibitors on Activity of Bovine Aminopeptidase H[a]

inhibitor	concn (mM)	rel act. (%) against Leu-NA	rel act. (%) against BANA
none		100	100
EDTA	1	100	100
iodoacetic acid	10	0	0
PMSF	1	100	100
pepstatin	0.1	100	83.8
leupeptin	0.1	62.2	25.0
puromycin	0.1	87.8	77.9

[a] The activities against Leu-NA and BANA were measured in the presence of each inhibitor. Details of the assay are given under Materials and Methods.

Table 3. Hydrolytic Activity of Bovine Aminopeptidase H against 2-Naphthylamide Derivatives of Amino Acids (Xaa-NA)[a]

Xaa in Xaa-NA	rel act. (%)	Xaa in Xaa-NA	rel act. (%)
Leu	100	Ser	55.9
Met	101	Val	7.5
Lys	72.8	Gly	1.2
Ala	70.0	Pro	0
Glu	61.2		

[a] The enzyme was incubated with a Xaa-NA in 0.1 M potassium phosphate (pH 8.0) containing 1 mM dithiothreitol at 37 °C. Details of the assay are given under Materials and Methods.

then there was a gradual decline in enzyme activity. Using fresh bovine muscle has led us to the first detection of bovine aminopeptidase H. It seems to be necessary that this bovine enzyme be extracted from fresh muscle taken immediately after slaughter.

The properties of bovine aminopeptidase H were very similar to those of rabbit (Okitani *et al.*, 1980, 1981a; Nishimura *et al.*, 1983), chicken (Rhyu *et al.*, 1992), and porcine (Nishimura *et al.*, 1991b) aminopeptidases H. That is, the activities of these aminopeptidases H were greatest around pH 7.5−8.0. They were inhibited by monoiodoacetic acid and were not sensitive to EDTA, PMSF, or pepstatin. Furthermore, these enzymes preferably acted on Glu- and Ser-NA as well as Leu-, Met-, Ala-, and Lys-NA and did not hydrolyze Pro-NA. However, there are some different properties among these aminopeptidases H. The molecular weights of bovine and rabbit aminopeptidases H were 400 000 and 340 000, respectively. The former consisted of 8 subunits with the same molecular weight of 52 000, while the latter had three kinds of subunits whose molecular weights were 52 000, 72 000, and 91 000. It is still not clear whether the subunits of 72 000 and 91 000 of rabbit aminopeptidase H are essential or not. More detailed research is needed to elucidate this problem. The K_ms of bovine aminopeptidase H for Leu-NA and BANA were 3.1 and 2.4 mM, respectively, while those of porcine enzyme for Leu-NA and BANA were 0.37 and 1.25, respectively. It should be noted that the K_m of the former for Leu-NA was 10 times that of the latter. These active and/or binding sites of both enzymes to Leu-NA seem to be a little different, because other properties were very similar between them. The comparison of amino acid sequences of active and binding sites between the two enzymes will make this problem clear.

The rate of increase in free amino acids during the storage of beef has been shown to be smaller than in chicken and pork (Nishimura *et al.*, 1988b), and there was no improvement of the taste of beef during the additional storage for 8 days from 4 to 12 days after slaughter. This seemed to be caused by two factors. The first was that bovine aminopeptidase H was more

unstable than the chicken and porcine ones. The increase of free amino acids seems to occur in the beginning of the storage after slaughter. The second reason was that the K_m of bovine aminopeptidase H was smaller than that of the chicken and porcine ones. Those indicated that the period for the storage of beef should be longer to improve the flavor of beef.

In other tissues, cathepsin H in liver (Kirschke *et al.*, 1976) and some peptidases in rabbit lung (Singh and Kalnitsky, 1978, 1980) and rat skin (Jarvinen and Hopsu-Havu, 1975) have been found and characterized as a group of aminoendopeptidases such as an aminopeptidase H. Though these aminoendopeptidases have activities of both exopeptidase and endopeptidase, their properties were quite different from those of aminopeptidase H. Optimum pH (pH 7.5) for endopeptidase activity of aminopeptidase H was higher than those of rat skin (pH 5.8), rat liver (pH 6.0), and rabbit lung (pH 6.5). On the other hand, optimum pH (pH 7.5) for exopeptidase activity of aminopeptidase H was also higher than that of rat liver (pH 6.0), rat skin (pH 7.0), and rabbit lung (pH 7.2). Furthermore, these aminoendopeptidases, except for aminopeptidase H, possess much lower molecular weights than that of aminopeptidase H (400 000). The molecular weights of aminoendopeptidases of rat liver, rat skin, and rabbit lung are 28 000, 27 000, and 26 000–29 000, respectively. It is absolutely clear that aminopeptidase H is different from the low molecular weight aminoendopeptidases. It should be resolved that aminopeptidase H exists in other tissues except for muscle.

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