Survey of Neutral Aminopeptidases in Bovine, Porcine, and Chicken Skeletal Muscles

Toshihide NISHIMURA, Akihiro OKITANI,* Mee Ra RHYU and Hiromichi KATO

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
**Department of Animal Products Science and Technology, Nippon Veterinary and Zootechnical College, Musashino-shi 180, Japan*

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A survey of the total aminopeptidase activity of bovine, porcine, and chicken skeletal muscles at neutral pH was done, using the β-naphythylamide derivatives of nine amino acids. DEAE-cellulose column chromatography of the muscle extract found at least four types of aminopeptidases in bovine muscle and six types of aminopeptidases in porcine and chicken muscles. Aminopeptidase B and aminopeptidase C were commonly recognized in bovine, porcine, and chicken muscles. Hydrolase H was recognized in porcine and chicken muscles. Aminopeptidase C and hydrolase H had high activity against almost all substrates. The substrate specificities of both enzymes were fairly compatible with the pattern of free amino acids which increased during the storage of bovine, porcine, and chicken meats [*Agric. Biol. Chem.* 52, 2323 (1988)], indicating that aminopeptidase C and hydrolase H are responsible for the increment of free amino acids during aging of these muscles.

We have demonstrated that free amino acids and peptides contribute to the improvement of meat taste during the postmortem storage of meat.[1] Free amino acids and peptides are produced from the muscle proteins by the action of proteases during the storage of meat. The increase of free amino acids during the storage of meat is caused by aminopeptidases active at neutral pH.[2-6] Some aminopeptidases active at neutral pH have been found and/or purified from muscle tissue. Parsons and Pennington,[7] Parsons *et al.*,[8] and Jacobs *et al.*[9] found different forms of aminopeptidases in rat skeletal muscle. Joseph and Sanders[10] partially purified leucine aminopeptidase from porcine muscle. Mantle *et al.* purified a major aminopeptidase[11] and aminopeptidase B[12,13] from human skeletal muscle, and also reported leucyl, glutamyl, and pyroglutamyl aminopeptidases in human skeletal muscle.[14] Ishiura *et al.*[15] also purified an aminopeptidase M-like enzyme and aminopeptidase B from human skeletal muscle. In rabbit skeletal muscle, aminopeptidase C[16,17] and hydrolase H[18-20] were found and purified. Of these aminopeptidases, aminopeptidase C in rabbit skeletal muscle, a major aminopeptidase in human skeletal muscle, and aminopeptidase M-like enzyme in human skeletal muscle may be classified as the same enzyme, because they show broad substrate specificities and rapidly release the N terminal alanine in aminopeptidase substrates, and their activities are inhibited by chelating reagents. (In what follows, these enzymes are called aminopeptidase C.)

In rabbit skeletal muscle, the comparison of the substrate specificity of aminopeptidases with the pattern of free amino acids released during storage has shown that aminopeptidase C and hydrolase H were the major aminopeptidases contributing to the release of free amino acids during the storage of rabbit muscle.[21] Thus, the information on aminopeptidases has

increased recently. However, it has never been known how many kinds of aminopeptidases exist and which aminopeptidases contribute to the increase of free amino acids during the storage of bovine, porcine, and chicken meats which are supplied in large amounts for us.

In this work, we put extracts of bovine, porcine, and chicken skeletal muscles on a DEAE-cellulose column and identified the aminopeptidase activities of each fraction eluted from the column. Then, we clarified the substrate specificities of these aminopeptidases and compared them among bovine, porcine, and chicken muscles.

Materials and Methods

Materials. Bovine and porcine muscles (*longissimus dorsi*) were removed from the carcass four and one days after slaughter, respectively. Chicken muscle (*pectoralis superficialis*) was removed from the carcass immediately after slaughter. Each muscle was trimmed to remove fat and connective tissue and minced with a meat chopper. All procedures were done at 4°C.

DEAE-cellulose (DE-52) was purchased from Whatman Co., Leu-β-naphthylamide (NA) was obtained from the Protein Institute, and Ala-, Glu-, Gly-, Lys-, Met-, Pro-, Ser-, and Val-NA were purchased from Bachem Co. Benzoyl-Arg-β-naphthylamide (BANA) was obtained from Sigma Co.

Extraction and chromatography. Minced muscle (10 g) was homogenized with 30 ml of 40 mM Tris–HCl (pH 7.2) in a Waring blender for 1 min. The homogenate was centrifuged for 20 min at 10,000 *g*. The supernatant was dialyzed overnight against 10 mM Tris–HCl buffer (pH 7.2) containing 0.1% 2-mercaptoethanol (buffer A), and was pooled as a muscle extract. A portion (20 ml) of the muscle extract was put on a column (2.8 × 13 cm) of DE-52 that had been equilibrated with buffer A. After the unadsorbed proteins (breakthrough proteins) had been completely removed from the column by washing with buffer A, elution with a concentration gradient of NaCl in buffer A was started. Fractions (10 ml) were collected, and the aminopeptidase activity of each fraction was assayed by the hydrolysis of amino acid–β-naphthylamides (AA–NA).

Assay of aminopeptidases with amino acid–β-natphthylamides (AA–NA). AA–NA is widely used as the substrate of aminopeptidases. The enzyme activity against AA–NA was measured by the method of Matsutani *et al.*[22] After 0.2 ml of each fraction eluted from the column had been incubated at 37°C for 2–60 min with 0.2 ml of 1 mM AA–NA (except for 0.5 mM Val and 0.25 mM Gly) in 100 mM Tris–HCl, pH 7.2, containing 2 mM DTT, 0.4 ml of 0.23 N HCl in ethanol and 0.4 ml of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol were used to halt the enzyme reaction. The red color that developed was measured at 540 nm, and the β-naphythylamine released from AA–NA was measured.

Results

Aminopeptidase activities of the bovine, porcine, and chicken muscle extracts

As shown in Table I, Ala-, Lys-, Met-, and Leu-NA were preferentially hydrolyzed and Gly- and Val-NA were hydrolyzed a little by all these extracts. The hydrolyzing activity of bovine muscle extract against Glu-NA was lower than those of porcine and chicken muscle extracts. The sum of the values of hydrolyzing activities against all substrates was also lower in the extract of bovine muscle than in the extract of porcine or chicken muscle. These extracts were investigated by DEAE-cellulose column chromatography to examine the amounts of activities and substrate specificities of aminopeptidases in these extracts.

Chromatography of the porcine muscle extract

The chromatography of the porcine muscle extract on a DEAE-cellulose column gave the

Table I. HYDROLYSIS OF AMINO ACID–β-NAPHTHYLAMIDES (AA–NA) BY THE EXTRACTS OF PORCINE, CHICKEN, AND BOVINE MUSCLES

AA–NA	Activity (μmol β-naphthylamine/hr/0.2 ml[b])		
	Porcine	Chicken	Bovine
Ser	0.87	1.14	0.51
Glu	0.44	0.64	0.18
Pro	0.87	1.14	0.65
Gly	0.02	0.03	0.02
Ala	6.39	7.03	5.04
Val	0.14	0.17	0.12
Met	3.83	3.56	2.57
Leu	2.81	2.71	2.12
Lys	5.36	5.78	3.81
Total activity[a]	20.73	22.21	15.02

[a] Sum of the activity towards each amino acid–β-naphthylamide.

[b] 0.2 ml of extract corresponds to 0.05 g muscle.

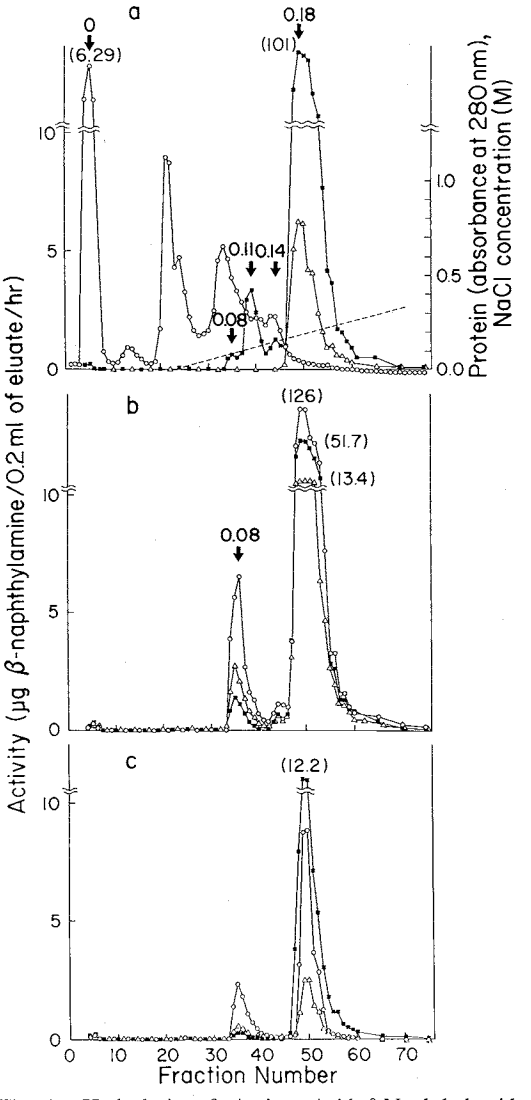

Fig. 1. Hydrolysis of Amino Acid–β-Naphthylamide (AA–NA) by Fractions from DEAE-cellulose Column Chromatography of Porcine Muscle Extract.

Experimental details are given in Materials and Methods. The numbers in parentheses show the activities in the most active fractions and the absorbance at 280 nm in the protein peak. The arrows indicate the position (NaCl concentration) where the hydrolyzing activities were eluted. Since the hydrolysis of Gly-NA by each fraction was very small, its value could not be plotted in this figure. (a): ○, protein; ---, NaCl; ■, Lys-NA; △, Glu-NA. (b): ○, Ala-NA; ■, Met-NA; △, Leu-NA. (c): ○, Pro-NA; ■, Ser-NA; △, Val-NA.

results shown in Fig. 1. Figure 1a indicates that a large portion of the protein put on was eluted as an unadsorbed breakthrough peak. The

Table II. Hydrolysis of Amino Acid–β-Naphthylamides AA–NA by Fractions from DEAE-Cellulose Chromatography of Porcine Muscle Extract

AA–NA	Hydrolysis of AA–NA				
	DEAE fraction				
	0[a]	0.08	0.11	0.14	0.18
Ser	+[b]	+	−	−	+++
Glu	+	−	−	−	+
Pro	+	+	−	−	++
Gly	+	−	−	−	+
Ala	+	+	−	+	++++
Val	+	+	−	−	+
Met	+	+	−	+	+++
Leu	+	+	−	+	+++
Lys	+	+	+	+	++++

[a] Numbers in this line indicate molar concentration of NaCl where the fractions eluted.

[b] Activity is classified by the amount of released β-naphthylamine as follows: −, 0 μg/hr/0.2 ml; +, 0–8; ++, 8–24; +++, 24–72; ++++, 72– .

hydrolyzing activity of each fraction was measured against the β-naphthylamide derivatives of nine amino acids (AA–NA). The activity against AA–NA appeared in five separated peaks eluted at 0, 0.08, 0.11, 0.14, and 0.18 M NaCl (Figs. 1a, b, and c). The hydrolytic activities of these peaks are summarized in Table II. The eluate at 0 M NaCl had a little activity against all substrates. The eluate at 0.08 M NaCl had activities against Ser-, Pro-, Ala-, Val-, Met-, Leu-, and Lys-NA, and hydrolyzed Ala-NA most rapidly. The eluate at 0.11 M NaCl was active against only Lys-NA. The eluate at 0.14 M NaCl had activities against Lys-, Ala-, Met-, and Leu-NA. The eluate at 0.18 M NaCl had the greatest activities against all the substrates except for Gly-NA, indicating that this eluate contained the major aminopeptidase(s) in muscle. This eluate hydrolyzed Ala-NA most rapidly. The hydrolyzing activity against Gly-NA was too small to give a peak.

Our previous work[21] showed that aminopeptidase C and hydrolase H in rabbit skeletal muscle were eluted around 0.18 M NaCl. In porcine muscle, the activity against BANA,

Table III. EFFECTS OF EDTA ON HYDROLYSIS OF AMINO ACID–β-NAPHTHYLAMIDES (AA–NA) BY 0.18 M FRACTION* FROM DEAE-CELLULOSE CHROMATOGRAPHY OF PORCINE SKELETAL MUSCLE EXTRACT

AA–NA	Activity (μg β-naphthylamine/hr/0.2 ml)	
	Without EDTA	With EDTA
Ala	70.0	18.5
Lys	54.7	12.7
Met	32.7	8.9
Leu	26.5	6.8
Ser	7.8	5.8
Glu	4.7	5.2
Pro	4.6	0.3
Val	1.5	0.4
Gly	0.1	0.05

* The 0.18 M fraction represents the pooled fractions (fraction numbers 48 to 53) shown in Fig. 1.

which is used as the substrate of hydrolase H, was also recognized at the fraction eluted around 0.18 M NaCl (data not shown). We tried to clarify to what extent of all the activities of this fraction was responsible for hydrolase H. After EDTA, the inhibitor for aminopeptidase C, had been added to the eluate around 0.18 M NaCl, the activity of eluate against each AA–NA was measured. The activities against Ala-, Lys-, Met-, Leu-, Pro-, and Val-NA was greatly inhibited by EDTA, but the activity against Glu-NA was not affected at all (Table III). These results indicate that most of the activity against AA–NA, except for Glu-NA and Gly-NA, could be caused by aminopeptidase C, and that the activity against Glu-NA would be mainly caused by hydrolase H.

Chromatography of the chicken muscle extract

The chicken muscle extract was chromatographed on a DEAE-cellulose column (Fig. 2). Most of the protein put on was eluted as an unadsorbed breakthrough peak. The hydrolyzing activity of each fraction was measured against the β-naphthylamide derivatives of nine amino acids (AA–NA). The activity against AA–NA appeared in six separated peaks eluted at 0, 0.05, 0.11, 0.14, 0.18, and 0.23 M NaCl

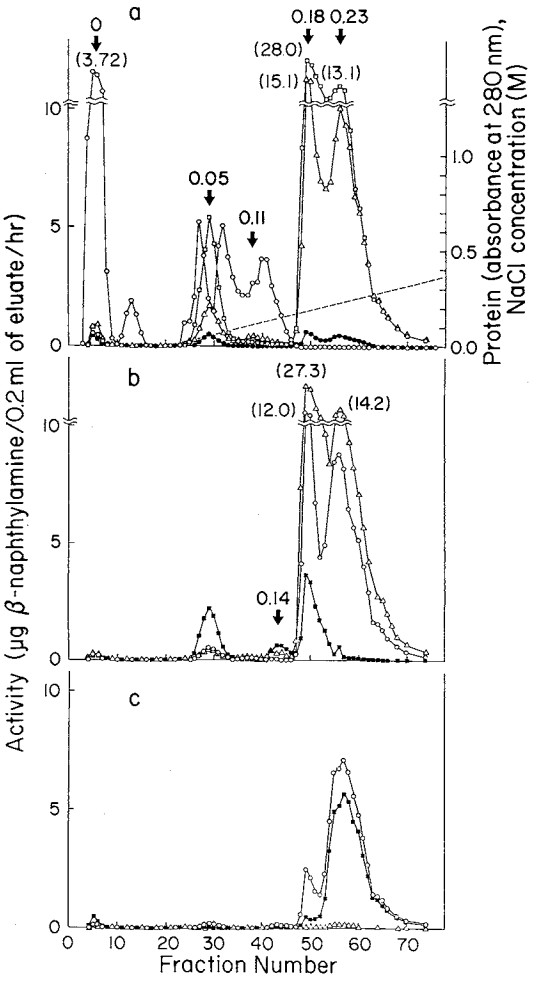

Fig. 2. Hydrolysis of Amino Acid–β-Naphthylamide (AA–NA) by Fractions from DEAE-cellulose Column Chromatography of Chicken Muscle Extract.

Experimental details are given in Materials and Methods. The numbers in parentheses show the activities in the most active fractions and the absorbance at 280 nm in the protein peak. The arrows indicate the position (NaCl concentration) where the hydrolyzing activities were eluted. (a): ○, protein; ---, NaCl; □, Ala-NA; △, Leu-NA; ●, Val-NA. (b): ○, Met-NA; ■, Pro-NA; △, Lys-NA. (c): ○, Ser-NA; ■, Glu-NA; △, Gly-NA.

(Figs. 2a, b, and c). The hydrolytic activities of these peaks are summarized in Table IV. The eluate at 0 M NaCl had a little activity against all substrates. The eluate at 0.05 M NaCl was active against all substrates except for Glu- and Gly-NA, and hydrolyzed Ala-NA most rapidly. The eluate at 0.11 M NaCl was active against

Table IV. Hydrolysis of Amino Acid–β-Naphthylamides (AA–NA) by Fractions from DEAE-Cellulose Chromatography of Chicken Muscle Extract

AA–NA	Hydrolysis of AA–NA					
	DEAE fraction					
	0[a]	0.05	0.11	0.14	0.18	0.23
Ser	+[b]	+	−	+	+	++
Glu	+	−	−	−	+	++
Pro	+	+	−	+	++	+
Gly	+	−	−	−	+	+
Ala	+	++	−	−	++++	+++
Val	+	+	−	−	+	+
Met	+	+	+	−	+++	++
Leu	+	+	+	−	++++	+++
Lys	+	+	−	+	++++	+++

[a] Numbers in this line indicate molar concentration of NaCl where the fractions eluted.

[b] Activity is classified by the amount of released β-naphthylamine as follows: −, 0 μg/hr/0.2 ml; +, 0–3; ++, 3–9; +++, 9–15; ++++, 15– .

Leu- and Met-NA. The eluate at 0.14 M NaCl was active against Ser-, Pro-, and Lys-NA. The eluates at 0.18 and 0.23 M NaCl had the greatest activities against all substrates except for Gly-NA. The former had higher activities against Ala-, Val-, Leu-, Met-, Pro-, and Lys-NA than the latter. The eluate at 0.18 M NaCl hydrolyzed Ala-NA most rapidly. The hydrolyzing activity against Gly-NA was small in all fractions.

Chromatography of the bovine muscle extract

The chromatography of the bovine muscle extract on a DEAE-cellulose column gave the results shown in Fig. 3. The majority of the protein put on was eluted as an unadsorbed breakthrough peak. The hydrolyzing activity of each fraction against the β-naphthylamide derivatives of nine amino acids was measured. The activity against AA–NA appeared in four separated peaks eluted at 0, 0.08, 0.11, and 0.18 M NaCl (Figs. 3a, b, and c). The hydrolyzing activities of these peaks are summarized in Table V. The eluate at 0 M NaCl had a little activity against all substrates except for Ser- and Glu-NA. The eluate at 0.08 M

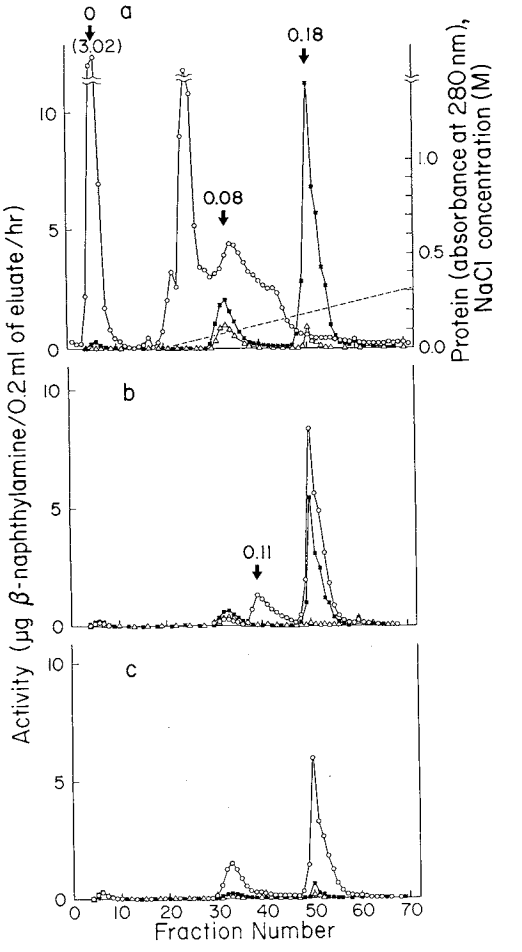

Fig. 3. Hydrolysis of Amino Acid–β-Naphthylamide (AA–NA) by Fractions from DEAE-cellulose Column Chromatography of Bovine Muscle Extract.

Experimental details are given in Materials and Methods. The numbers in parentheses show the activities in the most active fractions and the absorbance at 280 nm in the protein peak. The arrows indicate the position (NaCl concentration) where the hydrolyzing activities were eluted. Since the hydrolysis of Gly-NA by each fraction was very small, its value could not be plotted in this figure. (a): ○, protein; ---, NaCl; ■, Ala-NA; △, Pro-NA. (b): ○, Lys-NA; ■, Met-NA; △, Val-NA. (c): ○, Leu-NA; ■, Ser-NA; △, Glu-NA.

NaCl was active against all substrates except for Gly-NA, and hydrolyzed Ala-NA most rapidly. The eluate at 0.11 M NaCl was active only against Lys-NA. The eluate at 0.18 M NaCl had activities against all the substrates and hydrolyzed Ala-NA most rapidly. This eluate among all eluates showed the greatest activities

Table V. HYDROLYSIS OF AMINO ACID–β-NAPHTHYLAMIDES (AA–NA) BY FRACTIONS FROM DEAE-CELLULOSE CHROMATOGRAPHY OF BOVINE MUSCLE EXTRACT

AA–NA	Hydrolysis of AA–NA			
	DEAE fraction			
	0[a]	0.08	0.11	0.18
Ser	−[b]	+	−	+
Glu	−	+	−	+
Pro	+	+	−	+
Gly	+	−	−	+
Ala	+	++	−	++++
Val	+	+	−	+
Met	+	+	−	+++
Leu	+	+	−	+++
Lys	+	+	+	++++

[a] Numbers in this line indicate molar concentration of NaCl where the fractions eluted.

[b] Activity is classified by the amount of released β-naphthylamine as follows: −, 0 μg/hr/0.2 ml; +, 0–2; ++, 2–4; +++, 4–8; ++++, 8– .

against Ala-, Leu-, Met-, Lys-, and Ser-NA. The Glu- and Gly-NA hydrolyzing activities were small in all these fractions, and BANA hydrolyzing activity was not detected in all these fractions.

Discussion

In this paper, we have surveyed neutral aminopeptidases in porcine, chicken, and bovine muscles and shown that at least six aminopeptidases exist in porcine and chicken muscles and at least four aminopeptidases in bovine muscle

The activity of aminopeptidase eluted at 0 M NaCl was commonly recognized in porcine, chicken, and bovine muscles. Its substrate specificity was broad and the amount of its activity against the AA–NA tested was small. In rabbit muscle, it has been reported that activities against Val-NA, Val-Tyr-Val, and Val-Val-Val-Ala were eluted at 0 M NaCl and these were ascribed to an aminopeptidase.[6,21] The Val-NA hydrolyzing activity of our 0 M NaCl fraction in bovine, porcine, and chicken muscles also seems to be attributable to the same enzyme in rabbit muscle.[21]

The eluates at 0.05 M NaCl in chicken muscle and at 0.08 M NaCl in porcine and bovine muscles had broad substrate specificities and hydrolyzed Ala-NA most rapidly. So the hydrolyzing activities in these eluates seem to be the same aminopeptidase. This aminopeptidase is assumed to be similar to the aminopeptidase eluted at 0.09 M NaCl in rabbit skeletal muscle,[21] because these enzymes were eluted at similar NaCl concentrations on the DEAE-cellulose column chromatography and the rabbit aminopeptidase at 0.09 M NaCl also hydrolyzed Ala-NA most rapidly.

The eluates around 0.11 M NaCl in the chromatograms of the porcine and bovine extracts were active against only Lys-NA, and the eluate around 0.14 M NaCl in the chicken extract hydrolyzed Lys-NA most rapidly. These Lys-NA hydrolyzing activities seem to be those of aminopeptidase B. Mantle *et al.*[12,13] and Ishiura *et al.*[15] purified aminopeptidase B from rat and human skeletal muscles, respectively. Aminopeptidase B was thought to be a common aminopeptidase in skeletal muscle.

The activity eluted around 0.14 M NaCl in porcine muscle was specific for Ala-, Met-, Leu-, and Lys-NA and the activity eluted at 0.11 M NaCl in chicken muscle was also specific for Met- and Leu-NA. These enzymes may be the same.

Aminopeptidase C is known to have a broad substrate specificity, be most active in the neutral aminopeptidases against many amino acid-derivatives, and hydrolyze Ala-NA most rapidly.[11,14–17] In rabbit muscle, aminopeptidase C was eluted around 0.18 M NaCl on the DEAE-cellulose column chromatography.[21] The eluates at 0.18 M NaCl on the chromatograms of porcine, chicken, and bovine muscle extracts had the same properties as aminopeptidase C. Aminopeptidase C appears to be a major common aminopeptidase in skeletal muscle.

Hydrolase H[18–20] is an aminoendopeptidase with more hydrolyzing activity against Glu-NA than aminopeptidase C. This enzyme

also hydrolyzes BANA, which is an endopeptidase substrate. In rabbit muscle, hydrolase H was eluted around 0.18 M NaCl on the DEAE-cellulose column chromatography.[21] The eluates at 0.18 M NaCl on the chromatogram of porcine muscle and at 0.23 M NaCl on the chromatogram of chicken muscle had hydrolyzing activities against BANA and were more active against Glu- and Ser-NA than aminopeptidase C. So these eluates from porcine and chicken muscles are probably hydrolase H. It was supposed that hydrolase H in porcine and chicken muscles contributed to the release of Glu and Ser from peptides more rapidly than aminopeptidase C. On the other hand, no activity of hydrolase H was recognized in the fractions of the DEAE-cellulose column chromatography of bovine muscle extract. In this work, we had to use the bovine muscle stored at 4°C for 4 days after slaughter. It is unclear whether hydrolase H is inherently absent in bovine muscle or was inactivated during storage or DEAE-cellulose column chromatography. This problem must be resolved by using fresh bovine muscle.

It was shown that the hydrolyzing activities against most of AA–NA, especially Glu- and Ser-NA, were larger in porcine and chicken muscle extracts than in bovine muscle extract. This accounted for the result that the activity of hydrolase H, which has broad substrate specificity and hydrolyzes Glu- and Ser-NA more actively than aminopeptidase C, was not detected on DEAE-cellulose column chromatography of bovine muscle.

We demonstrated previously that the accumulation of free amino acids in bovine muscle during the storage was less than that in chicken and porcine muscles; the increase in Glu was especially small.[1] This may be because hydrolase H is not present in bovine muscle. Therefore, as in rabbit muscle,[21] aminopeptidase C and hydrolase H seemed to contribute to the increment of free amino acids, and especially the latter seemed to contribute to the increment of Glu during the storage of porcine and chicken muscles. The contribution of these aminopeptidases to the increment of free amino acids during storage may be identified by the examination of their activities against the peptides produced from the muscle proteins.

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