Purification and Properties of an Aminopeptidase from Rabbit Skeletal Muscle

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An aminopeptidase active on L-Val-L-Val-L-Val-L-Ala was purified from rabbit skeletal muscle by the method including ammonium sulfate precipitation, DEAE-cellulose chromatography, gel-filtration on Sephadex G-200, rechromatography on DEAE-cellulose, hydroxylapatite chromatography and rechromatography on Sephadex G-200. Polyacrylamide gel disc electrophoresis showed that the enzyme thus obtained was homogeneous. The specific activity of the purified enzyme was 1500 times that of the original muscle extract. The enzyme had an optimal pH in a range of 6.0~7.6 and was stable in pH 6.1~8.1. Molecular weight of the enzyme was estimated to be 160,000 from the result of gel-filtration on Sephadex G-200. The enzyme showed specificity for tri-, tetra-, penta-, and hexapeptides. The analytical data of liberated amino acids showed that the enzyme was an aminopeptidase active on these oligopeptides. The enzyme was strongly inhibited by *N*-ethyl-maleimide and EDTA.

We have been studying on the proteolysis in rabbit skeletal muscle.^{1~3)} In our recent paper,³⁾ we found that, besides an acidic proteolytic system consisting of cathepsins, there was another proteolytic system active in a neutral pH region, and it was constituted of various proteinases and peptidases.

It is well known that various kinds of peptidases are present in animal tissues to hydrolyze oligopeptides.⁴⁾ It is, however, not yet certain that these peptidases contribute to the hydrolysis of tetrapeptides or larger oligopeptides in animal tissues. Ellis and Perry,⁵⁾ Auricchio *et al.*,⁶⁾ and Donlon and Fottrell⁷⁾ have reported the presence of a tetrapeptide hydrolysis activity in tissues. However, they have not purified and not examined the properties of the enzyme in detail. Thus, there are only a few reports made so far on the proteases which hydrolyze such oligopeptides. The mechanism of the oligopeptide hydrolysis in tissues seems

to be also unknown.

In the present paper,** we describe the methods for the purification and the properties of a muscle aminopeptidase that hydrolyzes tri-, tetra-, penta-, and hexapeptides. For the purification we employ a synthetic oligopeptide, VVVA, as substrate for convenience, although this aminopeptidase can hydrolyze also other various oligopeptides.

MATERIALS AND METHODS

Muscles. The muscle (*longissimus dorsi*) of rabbit was obtained from the carcass immediately after slaughter and minced with a meat chopper.

Chemicals. VVVA, Gly-Gly-Ile-Ala, Lys-Gly-Ile-Ala, Thr-Gly-Ile-Ala, Ile-Gly-Ile-Ala, Glu-Gly-Ile-Ala, D-Ala-Gly-Ile-Ala, Ala-Gly-Phe-Ala, Met-Gly-Phe-Ala, Val-Gly-Phe-Ala and Ala-Leu-Gly-Ala-Leu-Gly were synthesized by the methods reported by Katakai.⁸⁾ Gly₂, Gly₃, Z-Gly-Phe, Val-Tyr-Val, and Gly-Leu-Tyr were obtained from Protein Research Foundation, Osaka, Val₂, Val₃, Val₄ and Val₅ were from Bachem Inc., California, Ile-Gly, Leu-Gly, Val-Gly, His-Gly, Ser-Gly, Phe-Gly, Thr-Gly, Tyr-Gly and Met-Gly were from Cyclo Chemical Co., California. The peptides other than D-Ala-Gly-Ile-Ala, were all

** The following abbreviations are used: VVVA, Val-Val-Val-Ala; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; IAA, monoiodoacetic acid; DTT, dithiothreitol; DIPP, diisopropylphosphorofluoridate.

constituted of L-amino acids. DEAE-cellulose was obtained from Brown Co. and Whatmann Co. (DE-52), hydroxylapatite from Seikagaku-Kogyo, Japan, calibration proteins from Boehringer-Mannheim-Japan, Japan. All other chemicals were of guaranteed reagent grade.

Assay. One-fifth milliliter of enzyme in 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol was preincubated for 3 min and incubated with 0.2 ml of 1 mM substrate in 0.01 M Tris-HCl buffer (pH 7.2) at 37°C for 20 min. The incubation was stopped by adding 2.0 ml of cold 2.0 M citrate buffer (pH 5.0). After 1.6 ml of the ninhydrin reagent⁹⁾ were added to the reaction mixture, the whole mixture was boiled at 100°C for 15 min and then cooled in running water. The optical density was measured at 570 nm. The enzyme activity was expressed as follows: one unit = an amount of the enzyme which produces one μ mole amino acid per min at 37°C. The production of amino acid during the incubation was measured by the ninhydrin method described above.

Protein concentration. Optical densities were measured at 260 nm and 280 nm with a Hitachi Spectrophotometer (EPS-3T), and a protein concentration was calculated by the method of Layne.¹⁰⁾

RESULTS

Purification of the enzyme

Step 1) Extraction and ammonium sulfate precipitation. All the procedures were performed at 4°C. Minced muscle (1100 g) was added to 3300 ml of 0.04 M Tris-HCl buffer (pH 7.2) and homogenized with a Waring blender for 1 min. The homogenate was centrifuged at $4500 \times g$ for 15 min. To the supernatant (crude extract) were added 2-mercaptoethanol up to 4 mM and ammonium sulfate up to 55% saturation. The mixture was centrifuged at $4500 \times g$ for 15 min. Ammonium sulfate was added again to the supernatant up to 75% saturation, and then centrifuged. The precipitate was collected and dissolved in 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol, and it was dialyzed against the same buffer to obtain an ammonium sulfate precipitate fraction.

Step 2) DEAE-cellulose column chromatography. The ammonium sulfate precipitate fraction (480 ml) was applied to a DEAE-cellulose (Brown Co.) column (4.5 $\times$ 30 cm)

previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol. After the column was washed up to tube No. 20 with the same buffer, the enzyme was eluted with a linear gradient of increasing concentration of sodium chloride from 0 to 0.4 M in the same buffer (Fig. 1). The eluate was collected in 20-ml fractions. The fractions from No. 98 to No. 103 were pooled.

FIG. 1. Chromatography of the Ammonium Sulfate Precipitate Fraction on DEAE-cellulose.

The peak fractions from No. 98 to No. 103 were pooled. ●—●, VVVA hydrolysis activity; ○—○, protein (O.D.₂₉₀); —, NaCl.

Step 3) Gel-filtration on Sephadex G-200. The enzyme (120 ml) eluted from the DEAE-cellulose column was concentrated with a membrane filtration apparatus (Diafilter 10-T), and subjected to the gel-filtration on a Sephadex G-200 column (1.5 $\times$ 120 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.2)

FIG. 2. Gel Filtration on Sephadex G-200.

The peak fractions from No. 33 to No. 38 were pooled. ●—●, VVVA hydrolysis activity; ○—○, protein (O.D.₂₈₀).

containing 4 mM 2-mercaptoethanol. Filtration was performed with the same buffer, and flow rate was 5 ml/hr. The eluate was collected in 3-ml fractions. The result is shown in Fig. 2. The fractions from No. 33 to No. 38 were pooled.

Step 4) Rechromatography on DEAE-cellulose. The enzyme (18 ml) eluted from the gel-filtration was applied to a DEAE-cellulose (Whatman DE-52) column (1.5×30 cm). The elution was carried out with a linear gradient of increasing concentration of sodium chloride from 0 to 0.4 M in the same buffer, and the eluate was collected in 3-ml fractions. The result is shown in Fig. 3. The fractions from No. 55 to No. 60 were pooled.

FIG. 3. Rechromatography on DEAE-cellulose (Whatman DE-52).

The fractions from No. 55 to No. 60 were pooled. ●—●, VVVA hydrolysis activity; ○—○, protein (O.D.₂₉₀); —, NaCl.

Step 5) Hydroxylapatite column chromatography. The enzyme (23.7 ml) eluted from the DEAE-cellulose column was dialyzed against 1 mM potassium phosphate buffer (pH 7.2) containing 4 mM 2-mercaptoethanol, and subjected to a hydroxylapatite column (0.9×5.5 cm) previously equilibrated with the same buffer. After the column had been washed with the same buffer, the elution was made in a step-wise manner with 0.03 M, 0.04 M, 0.08 M, 0.10 M and 0.15 M potassium phosphate buffers (pH 7.2) all containing 4 mM 2-mercaptoethanol. The eluate was collected in 3-ml fractions. The result is shown in Fig. 4. The fractions from No. 11 to No. 13 were pooled.

FIG. 4. Hydroxylapatite Column Chromatography. A, B, C, D, E and F; respectively 0.001 M, 0.03 M, 0.04 M, 0.08 M, 0.10 M and 0.15 M potassium phosphate buffer (pH 7.2) containing 4 mM 2-mercaptoethanol. ●—●, VVVA hydrolysis activity; ○—○, protein (O.D.₂₉₀).

Step 6) Rechromatography on Sephadex G-200. The enzyme (15 ml) eluted from the hydroxylapatite column was dialyzed against 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol. The dialyzed enzyme was concentrated with a membrane filtration apparatus, and subjected to the gel-filtration on a Sephadex G-200 column (1.5×120 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol. The filtration was performed with the same buffer at a flow rate of 5 ml/hr, and 3-ml fractions were collected. The result is shown in Fig. 5. The fraction No. 31 was used as a main enzyme fraction. The results obtained through the purification process are summarized in Table I. The enzyme was purified about 1500-folds, with a recovery of

FIG. 5. Rechromatography on Sephadex G-200. ●—●, VVVA hydrolysis activity; ○—○, protein (O.D.₂₉₀).

TABLE I. PURIFICATION OF RABBIT MUSCLE AMINOPEPTIDASE

Fraction	Specific activity (units/mg protein)	Recovery ^{a)} (%)
Crude extract	0.005	100
55%~75% (NH ₄) ₂ SO ₄ precipitate	0.022	172
DEAE-cellulose	0.649	19
Sephadex G-200	1.184	14
2nd DEAE-cellulose	2.450	6.7
Hydroxylapatite	4.111	5.0
2nd Sephadex G-200	7.338	1.3

^{a)} Calculated on the pooled fractions.

1.3%, over the crude extract.

Purity

The purity of the purified enzyme was detected by the polyacrylamide gel disc electrophoresis as described by Davis¹¹⁾ with 7.5% acrylamide gel at pH 8.4. Riboflavin was used instead of ammonium persulfate to initiate polymerization. After the electrophoresis had been made, protein was stained with amidoblack 10B and was traced in a densitometer (Gilford Spectrophotometer 240). As shown

FIG. 6. Acrylamide Gel Disc Electrophoresis of the Purified Enzyme.

Electrophoresis was performed at 20°C and 0°C at pH 8.4 by the method of Davis.¹¹⁾ Riboflavin was used for gel formation, and 2-mercaptoethanol was added into the gel (a final concentration of 4 mM). The protein (20 µg) was added, and an electric current of 5 mA/gel was applied. After the electrophoresis, protein was stained with amidoblack 10B, and the protein band was traced in a densitometer (Gilford spectrophotometer 240).

in Fig. 6, a single peak was detected and the enzyme activity was detected at the same position.

Molecular weight

Molecular weight of the enzyme was determined in the gel-filtration on Sephadex G-200 (Fig. 5). Ferritin, catalase, aldolase, bovine serum albumin, hen egg albumin and cytochrome-c were used as marker proteins. As shown in Fig. 7, the molecular weight was estimated to be 160,000.

FIG. 7. Molecular Weight Determination of the Enzyme.

Molecular weight of the enzyme was determined on a Sephadex G-200 column (1.5 × 120 cm). Molecular weight was estimated to be 160,000. ⊙, ferritin; ▲, catalase; □, aldolase; △, bovine serum albumin; ■, hen egg albumin; ○, cytochrome-c; ●, the enzyme.

Effect of pH on the enzyme activity and stability

The optimal pH for the enzyme activity was determined using the buffers in a pH range from 5.3 to 8.4. As shown in Fig. 8, the enzyme was most active between pH 6.0 and 7.6. The enzyme was fairly stable between pH 6.1 and 8.1 at 37°C in the buffers of a pH range from 3.2 to 8.1 (Fig. 9).

Effect of temperature on the enzyme activity and stability

As shown in Figs. 10 and 11, the optimal temperature was approximately 37°C and the enzyme was more stable at lower temperatures.

Effect of metal ions on the enzyme activity

Effect of various metal ions on the enzyme activity was checked. The results are shown

Fig. 8. Effect of pH on the Enzyme Activity.

Assay: One-fifth ml of 1 mM VVVA in Tris-HCl buffer (pH 7.2) was incubated with 1.26 μ g of the purified enzyme in 0.025 ml of 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol and 0.175 ml of 0.1 M Tris-maleate buffer (at various pH values) containing 4 mM 2-mercaptoethanol, at 37°C for 20 min. Liberation of amino acid was measured with ninhydrin.

Fig. 9. Effect of pH on the Stability of the Enzyme.

The enzyme (1.26 μ g) in 0.025 ml of 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol was preincubated for 30 min with 0.025 ml of 0.1 M MacIlvain buffer (at various pH values) containing 4 mM 2-mercaptoethanol. 0.15 ml of 0.3 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol and 0.2 ml of 1 mM VVVA in 0.01 M Tris-HCl buffer (pH 7.2) were added to the mixture. Incubation was performed at 37°C for 20 min.

in Table II. The divalent ions used have an inhibitory effect.

Effect of reagents

Effect of some reagents was checked. As shown in Table III. EDTA and NEM have an inhibitory effect.

Determination of the released amino acids

Amino acids released from VVVA and Ala-Leu-Gly-Ala-Leu-Gly during the incubation

Fig. 10. Effect of Temperature on the Enzyme Activity.

The activities were measured using 1.26 μ g of the enzyme under the standard assay conditions at various temperatures.

Fig. 11. Effect of Temperature on the Stability of the Enzyme.

After the enzyme (1.26 μ g in 25 μ l of 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol) had been preincubated for 30 min at various temperatures, the activity was assayed at 37°C under the standard assay conditions.

TABLE II. EFFECT OF METAL IONS ON THE ENZYME ACTIVITY

The enzyme (1.26 μ g) in 0.1 ml of 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol was incubated with 0.2 ml of 1 mM VVVA in 0.01 M Tris-HCl buffer (pH 7.2) and 0.1 ml of various metal ions at 37°C for 20 min.

Metal ion (final conc.)	Relative activity (%)
CaCl ₂ (1 mM)	31
MgCl ₂ (1 mM)	73
MnCl ₂ (1 mM)	38
ZnCl ₂ (1 mM)	2
CoCl ₂ (1 mM)	55
Pb(CH ₃ COO) ₂ (0.75 mM)	0
HgCl ₂ (1 mM)	0
CuSO ₄ (1 mM)	0
None	100

TABLE III. EFFECT OF CHEMICAL REAGENTS ON THE ENZYME ACTIVITY

After the enzyme (1.26 μ g) in 0.1 ml of 0.01 M Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol had been preincubated with 0.1 ml of various reagents at 37°C for 10 min, 0.2 ml of 1 mM VVVA in 0.01 M Tris-HCl buffer (pH 7.2) was added. Incubation was performed at 37°C for 20 min.

Reagent (final conc.)	Relative activity (%)
EDTA (1 mM)	10
<i>O</i> -Phenanthroline ^{a)} (0.75 mM)	85
IAA (4 mM)	84
NEM (0.75 mM)	60
NEM ^{a)} (2.5 mM)	4
DTT (1mM)	120
NaN ₃ (1 mM)	124
None (Water)	100

^{a)} In these studies the enzyme was preincubated with the reagents for 3 min.

with the enzyme at pH 7.2 and 37°C were analyzed with a Hitachi Amino Acid Autoanalyzer (KLA-5) (Fig. 12). Only valine was liberated from VVVA. Alanine was first liberated and successively leucine from Ala-Leu-Gly-Ala-Leu-Gly. Glycine was not de-

FIG. 12. Liberation of Amino Acids from VVVA (left) and Ala-Leu-Gly-Ala-Leu-Gly (right).

The enzyme was incubated with 0.5 mM peptide in 0.01 M Tris-HCl buffer (pH 7.2) containing 2 mM 2-mercaptoethanol. Liberated amino acids were determined with a Hitachi Amino Acid Autoanalyzer (KLA-5). ○—○, Ala; ●—●, Leu; □—□, Gly; ▲—▲, Val.

tected in this case.

Specificity of the enzyme

Table IV shows the relative rates of hydrolysis of various synthetic peptides by the enzyme, indicating that this is active on tri-, tetra-, penta- and hexapeptides, but not (or less active) on various dipeptides.

TABLE IV. RELATIVE ACTIVITY OF THE ENZYME FOR VARIOUS PEPTIDES

The rate of hydrolysis of VVVA is arbitrarily taken to be 1.00. The final concentration of each substrate was 0.5 mM.

Peptide	Relative activity	Peptide	Relative activity
<i>Dipeptides</i>		<i>Tetrapeptides</i>	
Gly ₂	0.00	Val ₄	0.98
Val ₂	0.00	VVVA	1.00
Ile-Gly	0.02	Gly-Gly-Ile-Ala	0.00
Leu-Gly	0.00	Lys-Gly-Ile-Ala	1.15
Val-Gly	0.01	Thr-Gly-Ile-Ala	0.10
His-Gly	0.00	Ile-Gly-Ile-Ala	0.19
Ser-Gly	0.04	Glu-Gly-Ile-Ala	0.92
Phe-Gly	0.05	D-Ala-Gly-Ile-Ala	0.00
Thr-Gly	0.00	Ala-Gly-Phe-Ala	1.94
Tyr-Gly	0.00	Met-Gly-Phe-Ala	1.76
Met-Gly	0.00	Val-Gly-Phe-Ala	0.31
Z-Gly-Phe	0.00	<i>Pentapeptide</i>	
<i>Tripeptides</i>		Val ₅	0.24
Val-Tyr-Val	0.66	<i>Hexapeptide</i>	
Val ₃	0.57	Ala-Leu-Gly-Ala-Leu-Gly	1.17
Gly ₃	0.00		
Gly-Leu-Tyr	0.00		

DISCUSSION

We have been studying on the proteolysis in the skeletal muscle,^{1~3)} and found that there was a proteolytic system active in a neutral pH region besides the one active in an acidic region.³⁾ We also found that there were various enzymes as members of this proteolytic system in a neutral pH region (unpublished). There are many kinds of work on the acidic proteolytic system composed of cathepsins,¹²⁾ but only a few reports have been presented as to the neutral proteolytic system in tissues.¹³⁾ On the other hand, the mechanism of protein degradation *in vivo* has not been clarified yet. Furthermore, it is considered that the major product formed by the proteinase action are large oligopeptides.¹⁴⁾ There are, however, also a few reports on the oligopeptidases^{5~7)} that hydrolyze larger oligopeptides than tripeptide and on the mechanism of their hydrolysis in tissues. Therefore, it may be important to study the oligopeptidase active in a neutral pH region.

It is considered that the ammonium sulfate precipitate fraction contains various kinds of peptidases. The high total VVVA hydrolytic activity of the ammonium sulfate precipitate fraction might be explained from the co-operative action of these peptidases. Separation of each enzyme by DEAE-cellulose column chromatography might result in the low recovery of the VVVA hydrolytic activity.

Figure 4 shows three peaks of the activity. In a preliminary investigation, however, only one peak was detected where the elution was made with the gradient in a range of 0.001 M to 0.2 M. The enzyme activity was lost in the process of the gradient elution, because the time required in this method was too long to keep its activity. Therefore, the step-wise elution was made in a shorter period of time. It was thus successful to obtain fractions with high activity (Fig. 4).

In the case of the polyacrylamide gel disc electrophoresis, 2-mercaptoethanol was added at a final concentration of 4 mM to the gel in order to recover the full enzyme activity.

Riboflavin was used as the catalyst for polymerization of acrylamide, because it was disclosed that there was a problem of the unexpected formation of some artifacts from ammonium persulfate in the case of SH-protease.¹⁵⁾

Inhibition of the enzyme by EDTA suggests that metal ion is essential to the activity. The divalent ions tested here affected the enzyme activity (Table II). The enzyme was also inhibited by NEM, and lost the activity when buffer containing no 2-mercaptoethanol was used. These results show that the SH-group is essential to the activity.

The results in Fig. 12 show that the enzyme splits off the amino-terminal amino acid from each peptide. The enzyme was, therefore, found to be an aminopeptidase. This muscle aminopeptidase shows specificity for tripeptides and larger oligopeptides (Table IV). It is considered that the muscle aminopeptidase is inactive (or less active) on the oligopeptides having glycine at the amino-terminal (Fig. 12 and Table IV). The results in Table IV also suggest that the muscle aminopeptidase prefers a straight-chain amino-terminal amino acid rather than a branched one.

There are many reports¹⁶⁾ on aminopeptidases. The cytosol aminopeptidase¹⁷⁾ (EC 3.4.11.1) formerly called leucine aminopeptidase hydrolyzes Leu-Gly, and is activated by Mn^{2+} and Mg^{2+} . The microsomal aminopeptidase¹⁸⁾ (EC 3.4.11.2) hydrolyzes the peptide having glycine at its amino-terminal, and is not affected by EDTA. These observations suggest that the muscle aminopeptidase presently studied differs from these aminopeptidases.

Proteolysis proceeds in the post mortem aging of meat and amounts of amino acids increase as a result of this proteolysis,¹⁹⁾ although the mechanism is not yet known in detail. The enzyme reported here hydrolyzes oligopeptides to amino acids, and is stable at pH 6.1. These results may suggest that this enzyme brings forth the production of amino acids during the post mortem aging and contributes to the taste of meat.

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