Leucine Aminopeptidase in Extracts of Swine Muscle

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1. Leucine aminopeptidase (EC 3.4.1.1) has been demonstrated in swine muscle at a level of activity one-fifth that of the swine kidney. 2. The enzyme has been purified 110-fold by precipitation with ammonium sulphate, heat treatment and chromatography on Sephadex G-100. 3. The enzyme is heat-stable, but is rapidly inactivated below pH 7. It requires Mg^{2+} or Mn^{2+} for activity. The Michaelis constant for leucine amide with Mg^{2+} -activated enzyme is $5.0 \times 10^{-3} M$. 4. Muscle leucine aminopeptidase is very similar to the kidney enzyme.

This paper follows work in other Laboratories on proteolytic enzymes in muscle (Smith, 1948; Snoko & Neurath, 1950; Koszalka & Miller, 1959; Bodwell & Pearson, 1964). We have now shown that L-leucine amide, L-leucylglycine and L-leucylglycylglycine are hydrolysed rapidly by extracts of swine muscle, and that this activity is stimulated and stabilized by Mg^{2+} or Mn^{2+} . We have characterized the enzyme responsible by studying its properties in crude extracts and have partially purified it. It is heat-stable and sensitive to pH below 7.0. It resembles swine-kidney leucine aminopeptidase (EC 3.4.1.1) (Spackman, Smith & Brown, 1955; Smith & Spackman, 1955; Bryce & Rabin, 1964a,b).

MATERIALS AND METHODS

Chemicals

Substrates. L-Leucine amide, L-leucylglycine and L-leucylglycylglycine were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) or British Drug Houses Ltd. (Poole, Dorset). Homogeneity was checked by paper chromatography in butan-2-ol-3% (w/v) ammonia (3:1, v/v).

Inorganic salts, acids and alkalis. These were A.R. grade. The $(NH_4)_2SO_4$ was recrystallized twice from EDTA solution and once from water.

Ninhydrin reagent. This was made by dissolving 0.2 g. of A.R. ninhydrin (British Drug Houses Ltd.) in 95 ml. of ethanol with 3 ml. of water. Collidine (2 ml.) was added just before use. Copper reagent was made by dissolving 4 ml. of saturated $Cu(NO_3)_2$ solution in 96 ml. of acetone and acidifying with 0.2 ml. of conc. HNO_3 .

Buffers

All pH measurements were made at 2°. Tris (Koch-Light Laboratories Ltd.) was recrystallized twice from EDTA and

once from water; it moved as a single spot on paper chromatograms. It was made up as 5 mM-buffer, pH 8.4, with HCl. Tris- Mg^{2+} buffer was prepared from tris buffer by adding $MgCl_2$ to 5 mM.

Extraction procedure

Extraction medium. A 0.3 M-KCl-50 mM- NaH_2PO_4 solution was adjusted to pH 7.5 at 2° with saturated CO_2 -free NaOH solution.

Swine muscle. Pigs of 120 lb. live weight were killed with a captive bolt. The longissimus dorsi muscle and the hams were rapidly removed and put on ice. The muscle was frozen solid at -18° within 30 min. of death.

Preparation of extracts. All operations were at 2°. The sarcoplasmic proteins were obtained in solution by homogenizing muscle with twice its weight of extraction medium for 1 min. in an Ato-Mix blender (Measuring and Scientific Equipment Ltd.). The fibre and coarse debris were removed by centrifuging at 5000 g for 30 min. in the 6 × 250 ml. head of the Sorvall RC-2 centrifuge. The supernatant was retained and stored on ice until required.

Enzyme assay

We used the pH-stat method of Bryce & Rabin (1964a). Crude extracts were initially incubated for 1 hr. in 2.5 mM- $MgCl_2$ at 40°. Extracts that had been dialysed against tris- Mg^{2+} buffer did not need activation, and were brought to 40° and assayed at once. Samples (2 ml.) were pipetted into a reaction mixture containing leucine amide or leucylglycine (0.14 mM) and $MgCl_2$ (0.05 mM). Hydrolysis of the substrate was followed with the Radiometer pH-stat, with 0.1 N-HCl.

The specific activity is expressed as μ moles of leucine amide hydrolysed/min./mg. at pH 8.4, 40° and 10.1, with saturating substrate and Mg^{2+} . This is essentially the unit used by Bryce & Rabin (1964a).

We used Mg^{2+} -activated enzyme in all our later experiments. This is preferred because Mn^{2+} solutions throw down a heavy brown deposit of $Mn(OH)_2$ after standing at pH 8.4. Mg^{2+} is probably the natural cofactor for leucine aminopeptidase (Bryce & Rabin, 1964b).

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Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) or by the measurement of E_{280} (Warburg & Christian, 1941).

RESULTS

Demonstration of activity

The specific activities of various samples were in the range 0.12–0.15 μ mole of leucine amide hydrolysed/min./mg. of protein (Fig. 1). Individual pigs and particular muscles did not differ by more than 0.02 unit. A sample of muscle was stored for 12 days at 2° and sampled daily for activity. The activity was constant for the whole period.

Enzyme activity was completely removed by dialysis against mM-EDTA, but was restored to its former value by incubation for 3 hr. with Mg^{2+} ; incubation with Mn^{2+} gave two to three times the former value.

Leucylglycine was hydrolysed at twice the rate of leucine amide. This ratio was found in crude extracts and in heat-treated fractions. Paper chromatography was used to check the results of many experiments. It was the only technique used to demonstrate the hydrolysis of L-leucylglycylglycine (Fig. 2). Spots were detected with ninhydrin reagent and fixed with copper reagent.

Effect of subsequent treatment of crude extract

Ammonium sulphate fractionation. Activity was precipitated continuously between 20% and 70% saturation with ammonium sulphate, mostly at

Fig. 1. Initial velocity of hydrolysis of leucine amide. The enzyme sample was the 40–70%-saturated $(NH_4)_2SO_4$ fraction that had been dialysed against tris- Mg^{2+} buffer.

Fig. 2. Drawing of paper chromatogram of the hydrolysis of L-leucylglycylglycine by muscle extracts at pH 8.4 and 40°. Extracts were preincubated with either $MgCl_2$ or $MnCl_2$ (2.5 mM) for 3 hr. in 5 mM-tris buffer, pH 8.4; samples (5 mg. of protein) were transferred to reaction mixtures of substrate (50 mM), $MgCl_2$ or $MnCl_2$ (2.5 mM) and tris (5 mM). Samples (10 μ l., 25 μ g. of protein) were removed on to paper at intervals and developed for 16 hr. (descending) with either butan-1-ol-acetic acid-water (4:1:5, by vol.) or butan-2-ol-3% ammonia (3:1, v/v). A, $MgCl_2$ or $MnCl_2$ present; B, $MgCl_2$ and $MnCl_2$ omitted; C, substrate omitted; D, enzyme omitted. Shading indicates the intensity of the spots.

Table 1. *Precipitation of leucine aminopeptidase with ammonium sulphate*

Crude extract was fractionated at 2° and pH 8.0 by successive additions of solid $(\text{NH}_4)_2\text{SO}_4$. Precipitates were collected by centrifugation and then dialysed against tris- Mg^{2+} buffer at 2° until free of sulphate.

Fraction	Specific activity (μ moles of leucine amide hydrolysed/min./mg.)	Total activity (μ moles of leucine amide hydrolysed/min.)
Extract	0.13	720
0-20%-satd. $(\text{NH}_4)_2\text{SO}_4$	Negligible	Negligible
20-30%-satd. $(\text{NH}_4)_2\text{SO}_4$	0.06	31
30-40%-satd. $(\text{NH}_4)_2\text{SO}_4$	0.08	106
40-50%-satd. $(\text{NH}_4)_2\text{SO}_4$	1.01	62
50-60%-satd. $(\text{NH}_4)_2\text{SO}_4$	0.23	191
60-70%-satd. $(\text{NH}_4)_2\text{SO}_4$	0.20	330
70%+ -satd. $(\text{NH}_4)_2\text{SO}_4$	Negligible	Negligible

Table 2. *Effect of heat treatment on leucine aminopeptidase*

Samples of enzyme [as 40-70%-saturated $(\text{NH}_4)_2\text{SO}_4$ fractions after dialysis] were taken quickly to the required temperature, held for 4 min. and cooled rapidly to 2°. Precipitated protein was removed by centrifugation and the supernatant assayed for activity and protein content.

Temp.	Protein left in solution (%)	Activity remaining (%)
(a) Original extraction at pH 7.0		
Control	(100)	(100)
60°	48	100
62	42	100
64	28	100
68	12	87
70	12	30
(b) Original extraction at pH 7.5		
Control	(100)	(100)
60°	48	78
65	25	78
68	16	78
70	10	87
72	8	55
74	7	51

50-70% saturation but with a smaller peak at 30-40% saturation (Table 1). In routine purifications we pooled the three fractions giving 40-70% saturation.

Heat treatment. The effect of heat on samples of enzyme stabilized with Mg^{2+} (Spackman *et al.* 1955) at pH 8.0 is shown in Table 2. Enzyme extracted at pH 7.5 appears to be more resistant to high temperatures than that extracted at pH 7.0. Considerable purification is effected by precipitating

Fig. 3. Effect of pH on enzyme stability. The 40-70%-saturated $(\text{NH}_4)_2\text{SO}_4$ fractions that had been dialysed were adjusted to the desired pH with N-HCl or N-NaOH at 2°, held for 4 min., readjusted back to pH 8.4, centrifuged and the supernatants assayed for protein and enzyme activity. ○, Total activity; ●, specific activity.

other (glycolytic?) enzymes. Above 76° the activity falls away very sharply.

Effect of pH on enzyme stability. The effect of pH on the molecular stability of leucine aminopeptidase is shown in Fig. 3. The enzyme is rapidly destroyed below pH 7.

Starch-gel electrophoresis. Electrophoretic mobility on starch gels was demonstrated with heat-treated fractions. We used the discontinuous buffer system of Poulik (1957) but modified it by making the gel buffer 5 mM with respect to magnesium chloride. Protein was located on thin slices of the gel with Amido Black 10B. Activity was

located by eluting 5mm. segments of the bulk of the gel into water by soaking, and incubating the eluates with leucine amide (20mM) and magnesium chloride (10mM); leucine was detected by paper chromatography. The activity moved 1.5–2.5cm. towards the anode in a wide but discrete band; it was not possible to relate this band to any of the protein bands (Fig. 4).

Effect of substrate concentration. Initial velocities of hydrolysis of leucine amide at concentrations in the range 1.0–14mM were measured. We used heat-treated (at 70°) enzyme of specific activity 2.07 μ -moles/min./mg. The results are plotted in Fig. 5. A Michaelis constant of 5.0×10^{-3} M was obtained.

Sephadex chromatography. Heat-treated fractions of specific activity 0.2–0.7 were run on Sephadex G-100 columns (Fig. 6). The activity always

emerged at the break-through peak. The purification effected was generally four- to five-fold.

Sequence of purification. We have operated the various fractionation procedures in sequence and have obtained a 110-fold purified preparation consistently from several batches of muscle (Table 3).

DISCUSSION

Leucine aminopeptidase (EC 3.4.1.1.) is Mg^{2+} - or Mn^{2+} -activated and can hydrolyse L-amino acid amides as well as peptides. Its preference is for a hydrophobic side chain, though so far no amino acid has been found resistant to it. Our preparations satisfy these requirements and this justifies our identification of leucine aminopeptidase in muscle. Moreover, the molecular properties we have described support this identification. The different relative rates of hydrolysis indicate that the specificity may not be exactly the same as that of kidney leucine aminopeptidase.

The rate of hydrolysis of leucylglycine and leucylglycylglycine is twice that of leucine amide, and this ratio persists through two fractionation stages. Purified leucine aminopeptidase hydrolyses both peptides at 70% of the rate for leucine amide (Spackman *et al.* 1955).

The two peptides are also hydrolysed by aminopeptidase of the type EC 3.4.1.2. which are not activated by metal ions. Hence muscle contains either two peptidases with the specificities of EC 3.4.1.1 and EC 3.4.1.2, which are not separated by ionic strength or heat fractionation, or one metal ion-activated peptidase similar to EC 3.4.1.1, but with a greater affinity for peptides relative to amides than is found in standard preparations from

Fig. 4. Starch-gel electrophoretograms of heat-treated 40–70%-saturated $(NH_4)_2SO_4$ fraction. Heat treatment was at (a) 60°, (b) 74°. Electrophoresis was at pH 8.9 for 5 hr. at 45 mA (constant current) at room temperature. Shading indicates the intensity of the bands.

Fig. 5. Lineweaver-Burk plot for the hydrolysis of L-leucine amide at pH 8.4, 40° and $I 0.05$. ●, Experimental points; —, regressed line.

Fig. 6. Sephadex G-100 chromatography of leucine aminopeptidase. The column (140 cm. $\times$ 1.0 cm.) was equilibrated with tris- Mg^{2+} buffer, pH 8.4, at 2°. Protein (112 mg.) of specific activity 0.45 was put on to the column and eluted at 8 ml./hr.; 3 ml. fractions were collected. —, Protein (E_{280}); ---, total activity. The arrows indicate fractions of high activity that were reserved.

Table 3. *Partial purification of swine-muscle leucine aminopeptidase*

Frozen swine muscle (1 kg.) was thawed, extracted and treated in the sequence shown. These are the results of one batch. Other batches differed in final yield by $\pm 5\%$ and in specific activity by ± 0.3 .

Stage	Protein (mg.)	Total activity (μ moles of leucine amide hydrolysed/min.)	Specific activity (μ moles of leucine amide hydrolysed/min./mg.)	Recovery (%)
1 Crude extract	47500	3800	0.08	100
2 40–70% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	25800	4100	0.20	110
3 Heat treatment (70°)	820	1900	2.30	47
4 Sephadex G-100 column	140	1330	9.2	33

kidney. The complete loss of activity when our extracts are treated with EDTA rules out the first possibility. It follows that muscle contains leucine aminopeptidase differing only slightly in its specificity from kidney leucine aminopeptidase. The level of activity we found is one-fifth that of kidney and one half that of the gut (Smith & Bergmann, 1944).

The activity is precipitated by ammonium sulphate continuously between 20% and 70% saturation, mostly at 50–70% saturation. This is similar to the precipitation of kidney enzyme, which occurs continuously between 30% and 70% saturation, and maximally at 50–70% saturation.

Dixon & Webb (1961) state that homogeneous proteins should be precipitated completely in about a 7–8% range of saturation. The phenomenon of continuous precipitation may indicate the presence of isoenzymes differing in their solubility. This suggestion is strengthened by our having shown a smaller peak of activity at 30–40% saturation. This would be settled by purifying the individual fractions to a point at which they could be differentiated by starch-gel electrophoresis.

We pooled the 40–70%-saturated ammonium sulphate fractions in many preparations to obtain the greatest increase in specific activity for the smallest loss of total activity. We achieved approximately twofold purification of enzyme compared with the fivefold purification obtained by Spackman *et al.* (1955).

Muscle leucine aminopeptidase is thermostable. It resembles the kidney enzyme (Spackman *et al.* 1955) but not the mucosal enzyme, which loses activity even when stored cold in Mn^{2+} solutions (Smith & Bergmann, 1944). This difference may be associated with the difference in site of action: the mucosal enzyme is extracellular but the kidney and muscle enzymes are intracellular.

Muscle leucine aminopeptidase is stable at pH 8.0 and is rapidly inactivated below pH 7.0; in this it closely resembles the kidney and gut enzymes (Spackman *et al.* 1955; Smith & Bergmann, 1944). However, muscle operates at pH 6.9 (Dubuisson,

1939) and this raises the question of the stability and activity of the enzyme in the tissues. There is little information yet to settle this question, but our findings that activity persists for 12 days *post mortem* and that the yield of enzyme from different pigs is constant indicate that the enzyme is stable in the tissues. Further, if the activity resembles that of the kidney enzyme, which is one-fourth as active at pH 6.9 as it is at the optimum pH 9.0 (Smith & Spackman, 1955), it is reasonable to suggest that the enzyme could be active in the tissues, once it were established that the substrates were accessible. These conclusions are important for an understanding of the role of the enzyme in protein metabolism.

The present study shows that leucine aminopeptidase obtained from swine muscle is similar to the leucine aminopeptidase from swine kidney. The Michaelis constant for muscle enzyme, $5.0 \times 10^{-3} \text{M}$, is close to that found for kidney enzyme, $5.21 \pm 0.43 \times 10^{-3} \text{M}$ (Bryce & Rabin, 1964a). The position of elution from Sephadex G-100 suggests a high molecular weight, which is unusual for a proteolytic enzyme. Kidney leucine aminopeptidase has a molecular weight of 300 000 (Spackman *et al.* 1955).

Highly purified swine-kidney enzyme (Spackman *et al.* 1955; Bryce & Rabin, 1964a) has 50 times the specific activity of our preparation after Sephadex chromatography. Since the enzymes appear to be similar we must assume that our preparation is still contaminated with other proteins.

It is premature to speculate on the role of muscle leucine aminopeptidase, but we suggest that it is probably concerned with the degradation of protein molecules that are no longer required in the metabolic machinery of the muscle (Whipple, 1956).

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