HPLC Purification and Characterization of Soluble Alanyl Aminopeptidase from Porcine Skeletal Muscle

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A soluble alanyl aminopeptidase (EC 3.4.11.14) from porcine skeletal muscle was purified by ammonium sulfate fractionation and anion-exchange HPLC. The enzyme eluted at 0.31 M NaCl, had a relative molecular mass of 106 000 (by SDS−polyacrylamide gel electrophoresis), and was markedly stimulated by sulfhydryl compounds, Co^{2+} and Ca^{2+}. The enzyme exhibited maximum activity at pH 6.5 and 50 °C and showed a broad substrate specificity hydrolyzing aromatic, aliphatic, and basic aminoacyl bonds, but did not show endopeptidase activity. The affinity of the enzyme toward dipeptides was increased in the presence of an aromatic amino acid and the C-terminal side. Inhibition of enzyme activity was obtained in the presence of metal-chelating agents, sulfhydryl reagents, bestatin, amastatin, and puromycin. The enzyme was stable at temperatures below 15 °C and had a higher stability, 60% of initial activity after 3.5 months of incubation at −25 °C, in the presence of 50% ethylene glycol.

Keywords: *Alanyl aminopeptidase; cytosolic aminopeptidase; HPLC purification; peptide degradation; thiol-activated aminopeptidase*

INTRODUCTION

Aminopeptidases are enzymes widely distributed in skeletal muscle and usually named according to their preference in hydrolyzing an amino acid from the N-terminal side (McDonald and Barret, 1986). Aminopeptidases are believed to act in concert to completely degrade the peptides resulting from proteolysis into single amino acids sequentially from the N-terminal side (Lauffart and Mantle, 1988). In fact, an increase in the concentration of free amino acids has been reported in postmortem muscle during meat ageing (Okitani et al., 1981; Nishimura et al., 1988, 1990) and even in long-processed meats like dry-cured ham (Aristoy and Toldrá, 1991; Toldrá et al., 1992). In that case, aminopeptidase activity was detected even after more than 8 months of processing (Toldrá et al., 1992), suggesting that these enzymes were involved in the latter stages of protein degradation (Toldrá, 1992). The effect of curing agents on this enzyme activity were also tested (Toldrá et al., 1993). In a previous work (Flores et al., 1993), aminopeptidase B was successfully purified in a fast way from pork skeletal muscle through anion-exchange HPLC.

On the other hand pork muscle also contains another important muscle aminopeptidase known as soluble alanyl aminopeptidase (EC 3.4.11.14), which has not yet been studied in pork muscle. This enzyme has appeared under several names in the scientific literature including alanyl aminopeptidase, thiol-activated aminopeptidase, puromycin-sensitive aminopeptidase, cytosol aminopeptidase III (McDonald and Barret, 1986), major aminopeptidase (Mantle et al., 1983), aminopeptidase M-like enzyme (Ishiura et al., 1987), and aminopeptidase C (Nishimura et al., 1991). It is the aminopeptidase found in the highest amount in skeletal muscle (Mantle et al., 1983; Ishiura et al., 1987; Lauffart and Mantle, 1988; Nishimura et al., 1991) and has been also found in other tissues such as brain (Schnebli et al., 1979; Wagner et al., 1981; Hersh and Mcketvy, 1981; McDermott et al., 1985), liver (Kawata et al., 1982; Hiroi et al., 1992), kidney (Mantle et al., 1990), and lens (Sharma and Ortwerth, 1986). The object of this work was to gain a working knowledge of soluble alanyl aminopeptidase from porcine skeletal muscle in order to understand its possible role in processed pork meat and meat products.

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EXPERIMENTAL PROCEDURES

Materials. (Aminoacyl)-7-amido-4-methylcoumarin (AMC) substrates and the inhibitors were obtained from Sigma (St. Louis, MO) except bestatin, soybean trypsin inhibitor and phenylmethanesulfonyl fluoride (PMSF) which were purchased from Boehringer (Mannheim, Germany). Protein standards for electrophoresis were obtained from BioRad (Richmond, VA). The anion-exchange PL-1000 SAX column (50 × 5 mm, 8 μm particle size) was purchased from Hewlett-Packard (Palo Alto, CA). Muscle *Biceps femoris* was removed from six-month-old pigs just after death and used for the enzyme extraction.

Assay of Aminopeptidase Activity. The standard assay for soluble alanyl aminopeptidase was performed at 37 °C during 15 min by using 0.1 mM of L-alaninyl-7-amido-4-methylcoumarin (Ala-AMC) as substrate in 100 mM phosphate buffer, pH 6.5, 2 mM 2-mercaptoethanol (2-ME). The reaction was stopped by the addition of 100 mM sodium acetate/chloroacetate buffer, pH 4.3 (Barret, 1980). The fluorescence was measured at 360 and 440 nm as excitation and emission wavelengths, respectively, in a Shimadzu RF-5000 spectrofluorophotometer. One unit of enzyme activity (U) was defined as the release of 1 μmol of substrate hydrolyzed by the enzyme per hour at 37 °C. Four replicates (samples + controls) were measured for each experimental point. Optimal pH and temperature for soluble alanyl aminopeptidase were determined in the ranges of 5.0 to 8.5 and 5 to 75 °C, respectively.

The optimal concentration of reducing agents such as 2-ME and dithiothreitol (DTT) was determined by assaying the enzyme activity at 37 °C in 100 mM phosphate buffer, pH 6.5, with the addition of different concentrations (0−5 mM) of the respective agents in order to obtain a standard assay for soluble alanyl aminopeptidase. Activation by cobalt was tested in 100 mM phosphate buffer pH 6.5 in the presence or absence of 2 mM 2-ME, and the activation by Ca^{2+} (0−25 mM) in 100 mM Tris-HCl, pH 6.5, was also tested.

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Enzyme Extraction. Ten grams of muscle *Biceps femoris*, with no visible fat and conective tissue, was homogenized in 50 mL of 50 mM phosphate buffer containing 5 mM EGTA, pH 7.5 by using a polytron (three strokes, 10 s each at 27 000 rpm with cooling in ice) homogenizer (Kinematica, Switzerland). The extract was centrifuged at 10 000*g* for 20 min at 4 °C and the supernatant, filtered through glass wool (soluble fraction), was used for futher purification.

Enzyme Purification. *Ammonium Sulfate Fractionation.* Solid ammonium sulfate was added to the soluble fraction to give 40% saturation, and the mixture was allowed to stir for 30 min at 4 °C. Once centrifuged at 10 000*g* for 20 min, solid ammonium sulfate was added to the clear supernatant to a final saturation of 60% and left to stand for 2 h. The precipitate was collected by centrifugation, dissolved in a minimum volume of 100 mM Tris-HCl buffer pH 7.0, containing 0.02% (w/v) sodium azide, and dialyzed overnight against the same buffer.

Anion Exchange Chromatography. The chromatographic separation was carried out in a biocompatible (titanium) 1050 Hewlett-Packard liquid chromatograph equipped with a variable-wavelength UV detector (280 nm). The column was previously equilibrated by elution of 5 mL of 10 mM Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl, 0.1% (v/v) 2-ME, and 0.02% (w/v) sodium azide (Nishimura et al., 1990). The dialyzed sample was filtered through a 0.45 μm nylon membrane filter and injected (250 μL) into the system. The column was eluted at 0.5 mL/min for 9 min with the equilibration buffer, then, with a linear salt gradient (0.1−0.4 M NaCl) for 20 min and, finally, with 0.4 M NaCl for 9 min. Thirty-four fractions (0.5 mL each) were collected and assayed for aminopeptidase activity using different fluorescent aminoacyl-AMC substrates (L-alanyl-, L-arginyl- and L-leucyl-AMC).

Electrophoresis. The molecular mass and purity of the purified soluble alanyl aminopeptidase was determined by SDS polyacrylamide gel electrophoresis (SDS−PAGE) using 10% polyacrylamide gels and staining with Coomassie blue R-250 (Laemmli, 1970) or silver (Merril et al., 1981). Standard proteins were simultaneously run for molecular mass identification.

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

Substrate Specificity. The activity of the purified enzyme was measured against several aminoacyl-AMC derivatives (L-phenylalanine, L-alanine, L-arginine, L-leucine, L-tyrosine, L-serine, L-proline, glycine, L-glutamic acid, L-pyroglutamic acid, L-lysine, L-methionine, L-valine, Gly-Arg, Arg-Arg, Gly-Pro, Ala-Ala-Phe, Z-Arg-Arg, and *N*-CBZ-Phe-Arg) as substrates (0.1 mM) in 100 mM phosphate buffer pH 6.5, 2 mM 2-ME as standard assay medium.

The determination of kinetic parameters for hydrolysis of aminoacyl-AMC substrates were estimated by Lineweaver−Burk plots using 0.025−0.200 mM of substrate. The rate of hydrolysis was followed by measuring the continuous fluorescence liberated in the standard assay medium using a Fluoroskan II fluorophotometer (Labsystems, Helsinki, Finland) equipped with a thermostatted compartment (37 °C).

The hydrolysis of peptide and oligopeptide substrates by the soluble alanyl aminopeptidase was determined in a reaction mixture containing the peptide (0.2−3.0 mM) in the standard assay medium. At different times of incubation at 45 °C, to accelerate the reaction, an aliquot (40 μL) of the reaction mixture was taken and the reaction was stopped by adding 10 μL of 500 mM sodium acetate/chloroacetate buffer, pH 4.3, constituting the sample for the capillary electrophoretic analysis. A 270A Capillary Electrophoresis system (Applied Biosystems, Foster City, CA) with a fused silica capillary, 72 cm (50 cm to detector) in length and 50 μm i.d., was used for the analysis. Injection of sample was by vacuum for 4 s. Detection was performed by UV absorption at 200 nm. Electrophoretic runs were made by using 20 mM citric acid pH 2.5 as electrolyte and applied voltage of +30 kV, and the temperature was controlled at 35 °C.

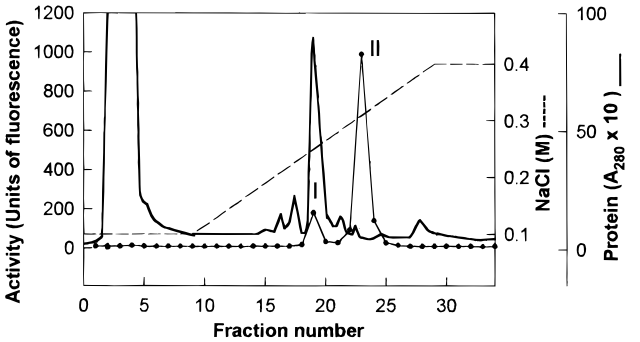

Figure 1. Anion exchange HPLC of dialyzed sample after ammonium sulfate fractionation, following the experimental procedure described under materials and methods. Alanyl (●) aminopeptidase activity is reported as units of fluorescence/0.05 mL fraction.

Table 1. Purification of Porcine Muscle Soluble Alanyl Aminopeptidase

	protein (mg)	total activity (U)	specific activity (U/mg)	recovered activity (%)	purification (fold)
crude extract	1860	120.2	0.065	100	1.0
soluble fraction	540	90.0	0.017	75	2.6
40−60% $(NH_4)_2SO_4$	150	84.0	0.560	70	8.8
anion exchange	1	52.8	52.800	44	830.0

Inhibition. The effect of potential inhibitors was tested by incubating the purified enzyme in the standard assay medium and in the presence of the following inhibitors: amastatin (0−0.2 mM), bestatin (0−0.5 mM), (*N-trans*-epoxysuccinyl-L-leucyl-amido)(4-guanidinobutane (E-64) (0−0.5 mM), leupeptin (0−0.3 mM), 1,10-phenanthroline (0−0.25 mM), puromycin (0−0.5 mM), pepstatin A (0−0.145 mM), PMSF (0−2 mM), *p*-hydroxymercuribenzoic acid (*p*-HMB) (0−1 mM), *p*-chloromercuribenzoic acid (*p*-CMB) (0−1 mM), EDTA (0−15 mM), soybean trypsin inhibitor (0−4.9 μM), cupric acetate (1 mM), mercuric chloride (1 mM), and ammonium sulfate (0−0.5 M). Controls with the absence of inhibitor were simultaneously run.

Determination of Enzyme Stability. Thermal stability of the purified enzyme was determined by incubation of the enzyme in a 100 mM phosphate buffer, pH 7.0, at the following temperatures: 5, 15, 25, 37, 50, and 65 °C. Frozen stability of the purified enzyme at −25 °C in the presence and absence of ethyleneglycol was also determined. The effect of pH on enzyme stability was determined by incubating the purified enzyme at 25 °C in 100 mM phosphate buffer, pH 5.0, 6.0, 7.0, and 8.0. In all cases, the activity was expressed as a percentage of the activity remaining at each time interval using as control the value obtained at time 0 with the standard activity assay.

RESULTS

Purification of the Enzyme. The results of a typical purification are summarized in Table 1. The procedure gave >830-fold purification of the enzyme, a 44% in yield. Alanyl aminopeptidase elutes from the anion exchange column as a single peak of activity at 0.31 M NaCl (peak II in Figure 1) and is completely inhibited by 0.1 mM puromycin, an effective specific inhibitor (Wagner et al., 1981; Kawata et al., 1982; Mantle et al., 1983; McDonald and Barret, 1986). SDS−polyacrylamide gel electrophoresis in 10% gel of the purified enzyme revealed by staining with both Coomassie blue or silver a single protein band, indicating homogeneity of the enzyme with a mobility corresponding to a molecular mass of 106 000 (data not shown).

Table 2. Activity of the Purified Soluble Alanyl Aminopeptidase on Various Aminoacyl-AMC Derivatives

substrate-AMC	activity (%)[a]	substrate-AMC	activity (%)[a]
Phe-	210.0	Val-	3.3
Lys-	130.0	γ-Glu-	0.0
Met-	124.0	Pglu-	0.0
Ala-	100.0	Gly-Arg-	2.5
Leu-	98.0	Lys-Ala-	0.7
Arg-	64.0	Arg-Arg-	4.8
Tyr-	10.7	Gly-Pro-	1.8
Ser-	7.3	Ala-Ala-Phe-	5.1
Pro-	5.8	*N*-CBZ-Phe-Arg	0.0
Gly-	5.0	Z-Arg-Arg-	0.0

[a] Activity is expressed relative to the alanine derivative.

Table 3. Kinetic Parameters of Porcine Muscle Soluble Alanyl Aminopeptidase

	K_m (μM)	V_{max} [μmol/(h mg)]	V_{max}/K_m [U/(mg μM)]
Leu-AMC	9	112	12.44
Arg-AMC	9	59	6.56
Lys-AMC	26	129	4.96
Met-AMC	29	175	6.03
Phe-AMC	43	268	6.23
Ala-AMC	52	133.1	2.56

This value is very similar to those reported for the enzyme purified from other sources e.g. 102 000 in human skeletal muscle (Mantle et al., 1983; Lauffart and Mantle, 1988), 100 000 in bovine brain (Hersh and Mckelvy, 1981), and 96 000 in bovine lens (Sharma and Ortwerth, 1986) and porcine liver (Kawata et al., 1982). Peak I corresponds to a mixture of peptidases (Flores et al., 1993).

Effect of pH and Temperature. The influence of pH on the activity of the purified enzyme was studied (data not shown). The activity on alanyl-AMC derivative was maximal at pH 6.5 and is similar to that by Wagner et al. (1981). Other authors have found maximal activity at pH 7.0 for other species (Schnebli et al., 1979; Kawata et al., 1982; Aratani et al., 1984; Ishiura et al., 1987; Hiroi et al., 1992). The enzyme activity sharply decreased to less than 10% of the maximal activity when assayed at acid (pH = 5.0) or basic (pH = 8.0) conditions. A complete inactivation is achieved in the pH regions below 5.0 and higher than 8.5. Enzyme reaction rate increases up to 50 °C (data not shown) which is very similar to 55 °C reported by other authors (Sharma and Ortwerth, 1986). The specific activity of the purified enzyme assayed at 50 °C was two times that obtained at 37 °C.

Substrate Specificity. The purified enzyme hydrolyzes a broad range of aminoacyl-AMC derivatives as shown in Table 2. Phenylalanyl-AMC derivative is the substrate most rapidly hydrolyzed as also reported by Ishiura et al. (Ishiura et al., 1987). The enzyme does not show endopeptidase activity when using N-terminal side-blocked substrates as expected (Schnebli et al., 1979; Wagner et al., 1981; Ishiura et al., 1987) but only a slight activity toward di- and tripeptidyl-AMC substrates. In fact this enzyme is reported to interfere in the assay of di- and tripeptidyl peptidases (Mantle, 1991). The enzyme shows the highest affinity toward leucyl- and arginyl-AMC although the highest V_{max} is to phenylalanyl- and methionyl AMC substrates (Table 3). High concentrations of the substrates produce a feedback inhibition (data not shown) (Schnebli et al., 1979; Kawata et al., 1982).

Table 4 shows the activity of the aminopeptidase toward dipeptides and oligopeptides when they were incubated at 45 °C. The enzyme does not show any activity toward Ala-Ala, Ala-Gly, Ala-Leu, and two natural dipeptides of the meat, anserine and carnosine (data not shown). The affinity of the enzyme is highest when an aromatic amino acid is located in C-terminal position of the dipeptide but longer peptides (between 3 to 5 residues) are better enzyme substrates than shorter ones.

Table 4. Hydrolysis of Peptides and Oligopeptides by Porcine Muscle Soluble Alanyl Aminopeptidase

	K_m (mM)	V_{max} [μmol/(h mg)]	V_{max}/K_m [U/(mg mM)]
Ala-Tyr	0.36	0.64	1.78
Ala-Phe	0.39	0.92	2.36
Ala-Trp	0.72	1.64	2.28
Ala-Met	1.22	2.81	2.30
Ala-Leu	n.h.[a]	n.h.	n.h.
Ala-Gly	n.h.	n.h.	n.h.
Leu-Phe	0.13	0.28	2.15
Leu-Met	0.61	3.06	5.02
Ala-Ala	n.h.	n.h.	n.h.
TriAla	0.07	10.10	2.75
TetraAla	0.87	8.10	9.31
PentaAla	1.30	9.30	7.15

[a] n.h. = not hydrolyzed.

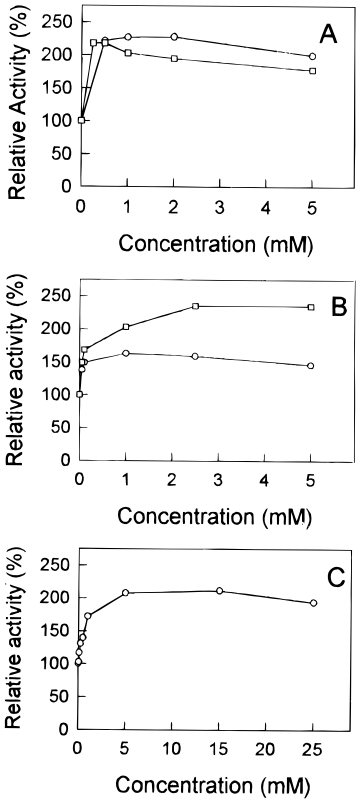

Figure 2. Effects of various activators on the activity of the soluble alanyl aminopeptidase. The final concentration of the enzyme was 3.8 μM and the activity was determined by the standard assay. The activity without activator was taken as 100%; (A) 2-ME (○) and DTT (□), (B) Co^{2+} in presence of 2-ME (○) and absence of 2-ME (□); (C) Ca^{2+}(○).

Activation. The purified enzyme is activated by reducing agents such as DTT and 2-ME as shown in Figure 2A, with concentrations of 0.2 and 0.1 mM, respectively, for half-maximal activation . The effect of 2-ME is reflected in a higher activation than DTT with a maximum at 1.0−2.0 mM as also reported by Mantle et al. (1983) and Hiroi et al. (1992). The presence of cobalt in the standard assay medium activates the enzyme but especially in the absence of 2-ME (Figure

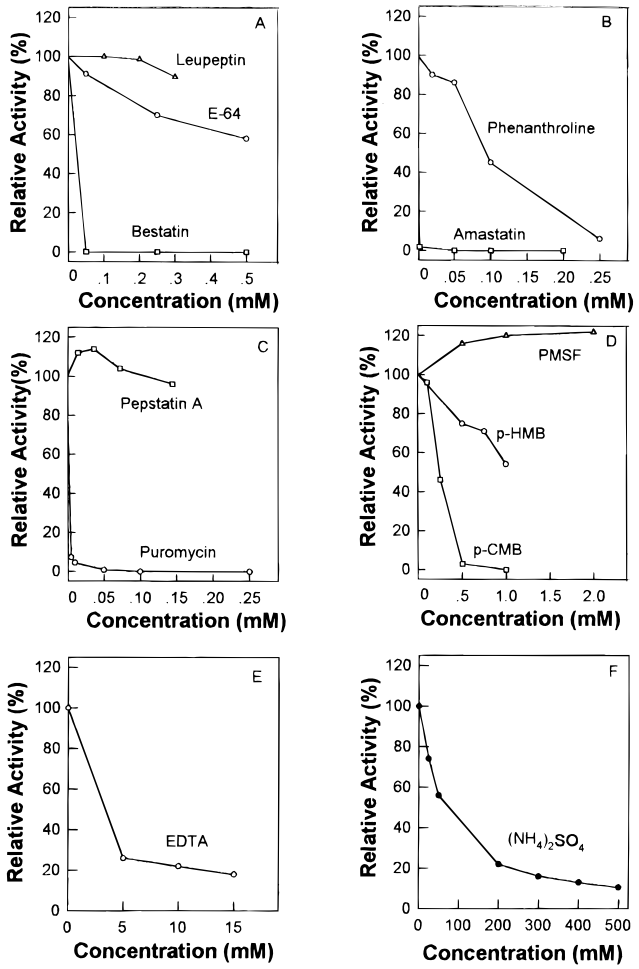

Figure 3. Effects of various inhibitors on the activity of the soluble alanyl aminopeptidase. The final concentration of the enzyme was 3.8 μM, and the activity was determined by the standard assay. The activity with no inhibitor was taken as 100%.

2B) with 0.05 mM concentration for half-maximal activation. While the addition of Ca^{2+} (see Figure 2C) enhances the enzyme activity with a maximum at 5−15 mM (Mantle et al., 1983; McDermott et al., 1985) and 0.1 mM concentration for half-maximal activation.

Effects of Inhibitors. Classical peptidase inhibitors such as bestatin (Suda et al., 1976; Umezawa et al., 1976) and amastatin (Aoyagui et al., 1978) inhibit 100% of the enzyme activity at 0.05 mM (see Figure 3A) and 98% at 0.002 mM (see Figure 3B), respectively. The enzyme is slightly affected by E-64 and leupeptin (cysteine protease inhibitors of microbial origin) as shown in Figure 3A. Pepstatin A (see Figure 3C), a typical inhibitor of aspartyl proteases (Mantle et al., 1983; Ishiura et al., 1987), as well as the serine protease inhibitors (Mantle et al., 1983; Nishimura et al., 1991; Hiroi et al., 1992) PMSF (see Figure 3D), and soybean trypsin inhibitor (data not shown) do not inhibit the purified enzyme. However metal chelators such as 1,10-phenanthroline and EDTA strongly affect the enzyme activity (Figure 3, parts B and E, respectively). Puromycin is an an effective inhibitor of the purified enzyme at a concentration of 0.1 mM (Figure 3C), very close to that reported by other authors (Wagner et al., 1981; Sharma and Ortwerth, 1986; Hiroi et al., 1992). *p*-CMB and *p*-HMB are sulfhydryl reagents reported as effective inhibitors of soluble alanylaminopeptidase (Hersh and Mckelvy, 1981; Sharma and Ortwerth, 1986; Ishiura et al., 1987; Hiroi et al., 1992). In our case, 1 mM of

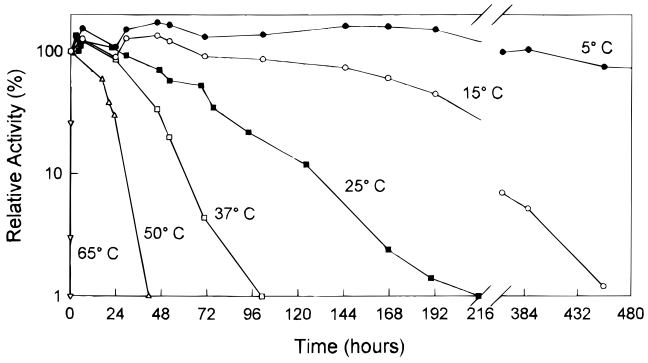

Figure 4. Thermal stability of soluble alanyl aminopeptidase. The purified enzyme was incubated in 100 mM phosphate buffer (pH 7.0) at the temperatures shown. The activity measured immediately after elution from the chromatographic system was taken as 100%.

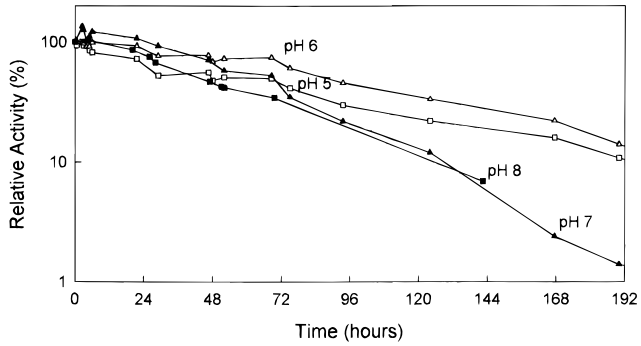

Figure 5. pH stability of soluble alanyl aminopeptidase. The purified enzyme was incubated at 25 °C in 100 mM phosphate buffer at the pH shown. The activity measured immediately after elution from the chromatographic system was taken as 100%.

p-CMB was enough for a complete inhibition while *p*-HMB gives 50% of inhibition (Figure 3D). On the other hand, 1 mM of Cu^{2+} and Hg^{2+} completely inhibited the enzyme activity (data not shown). The purified enzyme is strongly inhibited by ammonium sulfate (Figure 3F) so that dialysis is essential for an effective elimination of this agent and recovery of the enzyme activity.

Enzyme Storage and Stability. Thermal stability of the purified enzyme is studied by measuring the remaining activity after incubation at different temperatures (see Figure 4). The enzyme loses its activity very fast at temperatures higher than 50 °C, and even at 37 °C the enzyme remains active for less than 4 days. However the enzyme is quite stable at temperatures below 25 °C as also reported by Sharma and Ortwerth (1986). On the other hand, when storing the enzyme at −25 °C in 10 mM Tris-HCl buffer, pH 7.0, containing 0.01% (w/v) sodium azide, 0.05% (v/v) 2-ME, 0.15 M NaCl, and 50% ethylene glycol, the activity decreases very slowly, remaining 96% of its initial activity after 1 month and 60% after 3.5 months. However the storage of the enzyme in the absence of ethylene glycol results in a rapid loss of activity. In fact, more than 40% is lost in just 3 days (data not shown). The effect of pH on the stability of the enzyme is also shown (see Figure 5). The enzyme is more stable between pH 5.0 and 6.0 but its stability markedly decreases at pH higher than 7.0.

DISCUSSION

McDonald and Barret (1986) classified intracellular aminopeptidases into several groups according to their

subcellular localization and substrate specificity. Our purified aminopeptidase has a molecular mass of 106 000, is activated by sulfhydryl compounds, and is inhibited by metal-chelating agents, sulfhydryl-reactive reagents and puromycin. All these properties are very similar to the soluble alanyl aminopeptidase as reported in the classification above (McDonald and Barret, 1986). Similar properties have been found for enzymes from other origins but having different names, such as in the case of rat liver (Hiroi et al., 1992) or bovine lens cytosol aminopeptidase III (Sharma and Ortwerth, 1986), chicken muscle aminopeptidase C (Ishimura et al., 1991), human aminopeptidase M-like (Ishiura et al., 1987), and human kidney alanyl aminopeptidase (Mantle et al., 1990).

The purified porcine muscle alanyl aminopeptidase has a broad substrate specificity toward various amino acid-AMC (Table 2) which is slightly different from that of the major aminopeptidase from human skeletal muscle (Mantle et al., 1983). The slight activity toward di- and tripeptidyl-AMC substrates (Table 2) could interfere in its assay (Mantle et al., 1983; Mantle, 1991), although other authors have not found it (Ishiura et al., 1987). The apparent activity toward di- and tripeptidyl-AMC substrates would be the result of amino acid hydrolysis from the N-terminal side and the fluorescence generated when the last amino acid is reached (Sharma and Ortwerth, 1986). The kinetic parameters obtained for the alanyl aminopeptidase with aminoacyl-AMC substrates are different than others using *p*-nitroanilide and 2-naphthylamide substrates (Schnebli et al., 1979; Sharma and Ortwerth, 1986; Hiroi et al., 1992). This suggests that not only the amino acid in the N-terminal side is important in the catalytic process but also the adjacent amino acid or, in this case, the synthetic group is affecting the interaction between the enzyme and the substrate. The study of the hydrolysis of peptides shows that the aromatic properties of the second amino acid produces the highest affinity (Kawata et al., 1982; Aratani et al., 1984). The length of the oligopeptide also affects the hydrolysis rate (Table 4) (Ishimura et al., 1991; Hiroi et al., 1992).

On the other hand, the purified enzyme appears to have an essential cysteinyl residue since the activity is enhanced by sulfhydryl compounds (DTT, 2-ME) and inhibited by sulfhydryl reagents (*p*-CMB, *p*-HMB) as also reported for other sources such as human muscle (Ishiura et al., 1987), bovine brain (Hersh and Mckelvy, 1981), rat liver (Hiroi et al., 1992), and bovine lens (Sharma and Ortwerth, 1986). Furthermore, the addition of Cu^{2+} or Hg^{2+} completely inhibits the enzyme activity. Thus, our purified enzyme can be considered as a thiol-activated aminopeptidase and the addition of 0.1 mM puromycin results in a complete loss of enzyme activity as expected for this kind of enzyme (Wagner et al., 1981; Hersh and Mckelvy, 1981; Sharma and Ortwerth, 1986; Hiroi et al., 1992).

Metal chelators (1,10-phenantroline and EDTA) strongly affect the enzyme activity. Sharma and Ortwerth (1986) reported that the native enzyme contains bound metal ions and, in addition, a weak binding site for a metal ion which may serve as a regulator of the enzyme (Garner and Behal, 1974). The activation produced by calcium on the soluble alanyl aminopeptidase has been reported for the enzyme from human brain (0.5−1 mM Ca^{2+}) (McDermott et al., 1985) which needs less concentrations of calcium for the activation than the enzyme from porcine muscle (0−15 mM Ca^{2+}). Arylamidases with similar characteristics to our purified enzyme have been studied in skeletal muscle from different species such as rat (Parsons and Pennington, 1976; Parsons et al., 1979), chicken (Ishimura et al., 1991), rabbit (Otsuka et al., 1980; Okitani et al., 1981) and human (Mantle et al., 1983; Ishiura et al., 1987; Lauffart and Mantle, 1988). Recently, an aminopeptidase from human osteoclastomas has been described to have similar properties to the soluble alanyl aminopeptidase from porcine muscle (Page and Warburton, 1994) but with special affinity toward tyrosyl derivatives. This enzyme also prefers the presence of hydrophobic amino acids at the C-terminal position for the hydrolysis of dipeptides.

Many aminopeptidases have been isolated and characterized to date for medical purposes, for the purposes of trying to understand their biological significance in the in vivo activity of the cell. However, the action of muscle exopeptidases is very important for meat quality since these enzymes are involved in the postmortem breakdown of protein and peptide substrates and the release of free amino acids detected during meat ageing (Nishimura et al., 1988, 1990) and longer processes such as dry-curing of hams and sausages (Toldrá et al., 1992, 1993; Toldrá, 1992). The action of muscle aminopeptidases during these processes and their significance for flavor development is presently the object of further research in our laboratory.

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