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Purification and characterization of a soluble methionyl aminopeptidase from porcine skeletal muscle

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Abstract

A soluble aminopeptidase was purified from porcine skeletal muscle by ammonium sulfate fractionation and two successive anion exchange chromatographic procedures. The enzyme eluted at 0.17 M NaCl, had a relative molecular mass of 53 KDa (by SDS-polyacrylamide gel electrophoresis) and was activated by sulfydryl compounds. Activity was optimal at pH 7.5 and 40°C and showed broad aminopeptidase and low endopeptidase activities. The aminopeptidase exhibited maximal activity against Met-, Lys-, Ala-, and Leu-7-amido-4-methyl-coumarin (-AMC), while Pro-AMC was not hydrolyzed. Inhibition of enzyme activity was observed in the presence of sulfydryl reagents, iodoacetic acid, puromycin, leupeptin and amastatin, but it was not affected by serin and aspartic protease inhibitors, EDTA and bestatin. The enzyme activity was not inhibited by sodium chloride and, therefore, the enzyme has potential for contributing to the generation of free amino acids in cured pork meat products.

1. Introduction

Aminopeptidases catalyze the cleavage of amino acids from the amino terminus of protein or peptide substrates. They are classified by the number of amino acids cleaved, with respect to the efficiency with which residues are removed and location, susceptibility to inhibition by bestatin, metal ion content and the pH at which maximal activity is observed (Taylor, 1993a). These enzymes generally have a broad substrate specificity, acting at neutral pH, although the rates of cleavage of physiological substrates do not parallel rates of cleavage of peptide analogues (Taylor, 1993b).

Aminopeptidases are involved in the generation of free amino acids in meat and meat products (Flores, Aristoy & Toldrá, 1998; Nishimura et al., 1988, 1990) and consequently, are important in the improvement of taste during storage of the meats (Kato, Rhue & Nishimura, 1989; Toldrá & Flores, 1998). Four aminopeptidases, alanyl, arginyl, leucyl and pyroglutamyl aminopeptidases, have been described in porcine skeletal muscle (Flores, Aristoy & Toldrá 1993, 1996) and their role in the processing of meat products reported (Toldrá, Flores & Aristoy, 1995, Toldrá, 1998). In fact, their possible contribution to the development of pork meat flavor has been elucidated by studying the effect of curing agents on their activities (Flores, Aristoy & Toldrá, 1997).

Alanyl aminopeptidase (AAP) is the major aminopeptidase activity peak found in porcine and human skeletal muscles (Flores et al., 1996; Lauffart & Mantle, 1988) and has broad substrate specificity. Arginyl aminopeptidase (RAP) or aminopeptidase B is a chloride activated enzyme hydrolyzing basic amino acids (Flores et al., 1993; Toldrá, Falkous, Flores & Mantle, 1996). Leucyl aminopeptidase (LAP) is a zinc metalloenzyme with an alkaline optimal pH that hydrolyzes leucine and other hydrophobic amino acids (Flores et al., 1997; Ledeme, Vincent-Fiquet, Hennon & Plaquet, 1983) and pyroglutamyl aminopeptidase (PGAP) that specifically hydrolyzes the pyroglutamyl amino acid at basic pH (Flores et al., 1997; Mantle, Lauffart & Gibson, 1991).

Alanyl, arginyl, leucyl, pyroglutamyl and glutamyl aminopeptidases have been also described in human skeletal muscle (Lauffart & Mantle, 1988), being found in the soluble fraction of human kidney except for glutamyl aminopeptidase (Mantle, Lauffart, McDermott & Gibson, 1990). These enzymes have also been detected in porcine skeletal muscle (Flores et al., 1997). But none of these authors reported aminoendopeptidase activity in the soluble fraction.

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Several aminoendopeptidase activities have been found in muscle. Cathepsin H (EC 3.4.22.16) has been described as an aminoendopeptidase because it acts equally on blocked and unblocked low-molecular mass substrates (Kirschke & Barrett, 1987). The molecular mass is about 26 KDa by SDS-PAGE and its optimum pH for hydrolysis 6.5. Cathepsin H hydrolyzes Bz-Arg-NHNap as an endopeptidase as well as Arg-NHNap and it does not hydrolyze Pro-X bonds in peptides. Okitani, Nishimura, Otsuka, Matsukura and Kato (1980) described a hydrolase H from rabbit skeletal muscle possessing endo and aminopeptidase activities although with a molecular mass of 340 KDa. This enzyme was also characterized (Okitani, Nishimura & Kato, 1981) as a thiol protease with maximal activity at pH 7.5–8.0, inhibited by monoiodoacetic acid, leupeptin and not affected by EDTA, PMSF and pepstatin. Moreover, this enzyme has been isolated and characterized from porcine, bovine and chicken muscles (Nishimura, Rhyu & Kato, 1991; Nishimuro, Rhyu, Kato & Arai, 1994; Rhyu, Nishimura, Kato, Okitani & Kato, 1992) with similar characteristics.

A number of aminopeptidases have been identified in many tissues but it is difficult to establish which aminopeptidases are present in porcine skeletal muscle bearing in mind the problem of the multiple substrate specificity of many of these enzymes. The object of this work was to isolate and characterize an aminopeptidase with endopeptidase activity to determine its relative activity in the processing of meat and meat products.

2. Materials and methods

2.1. Materials

The fluorimetric, aminoacyl-7-amido-4-methylcoumarin (-AMC), the colourimetric aminoacyl-*p*-nitroanilide (-*p*Na) substrates and the inhibitors were all obtained from Sigma (St. Louis, MO) except Lys-AMC and Met-AMC which were from Bachem (Bubendorf, Switzerland), phenylmethanesulfonyl fluoride (PMSF) was from Boehringer (Mannheim, Germany) and iodoacetic acid and Glu-pNa from Fluka (Buchs, Switzerland). Protein standards for electrophoresis were obtained from BioRad (Richmond, VA). The anion exchange column Resource Q (1 ml) was from Pharmacia Biotech (Uppsala, Sweden). Muscle *Biceps femoris* removed from six-month-old pigs just after death was used for enzyme extraction.

2.2. Assay of aminopeptidase activity.

The standard assay for methionyl aminopeptidase was performed using 0.15 mM Ala-AMC as substrate, in 100 mM phosphate buffer, pH 7.5 containing 10 mM dithiothreitol. In order to measure the activity of methionyl aminopeptidase in meat extracts 0.05 mM bestatin was added to the standard assay. The reaction mixture (300 μL) was incubated in a multiwell plate at 37°C for 15 min. The fluorescence liberated was measured in a fluorophotometer Fluoroskan II (Labsystems, Helsinki, Finland) using 360 and 440 nm as excitation and emission wavelengths, respectively. Three replicates were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 μmol of substrate by the enzyme per hour at 37°C.

Optimal pH and temperature for methionyl aminopeptidase were determined in the ranges 5.0–9.0 and 5–55°C, respectively. The optimal concentrations of reducing agents such as 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) were determined by assaying the enzyme activity at 37°C in 100 mM phosphate buffer, pH 7.5 with the addition of different concentrations, 0–10 mM for DTT and 0–30 mM for 2-ME, in order to obtain a standard assay for methionyl aminopeptidase.

Arginyl and alanyl aminopeptidases were also measured to avoid interference with methionyl aminopeptidase activity. Arginyl aminopeptidase (RAP) activity was measured using 0.1 mM Arg-AMC as substrate, in 50 mM phosphate buffer, pH 6.5 containing 0.2 M NaCl (Flores et al., 1993). Alanyl aminopeptidase (AAP) was measured using 0.1 mM Ala-AMC as substrate, in 100 mM phosphate buffer, pH 6.5 containing 2 mM 2-mercaptoethanol (Flores et al., 1996).

2.3. Enzyme extraction

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

2.4. Enzyme purification.

The soluble fraction was fractionated with ammonium sulphate at 4°C. The precipitate formed between 40 and 60% saturation was collected by centrifugation at 10,000 *g* for 20 min, dissolved in the minimum volume of 100 mM Tris–HCl buffer, pH 6.0 and dialysed overnight against the same buffer. The dialysed sample was centrifuged at 1000 *g* for 5 min, and the supernatant filtered through a 0.22 μm membrane filter.

Two milliliters of dialysed sample were injected in an FPLC system with a Resource Q (1ml) anion exchange column (Pharmacia Biotech, Uppsala Sweden) previously equilibrated with 10 mM Tris–HCl pH 6.0,

containing 0.1 M NaCl, 0.1% (v/v) 2-ME, 0.02% (w/v) sodium azide. The column was eluted at 1 ml/min, applying an initial isocratic period with the equilibration buffer (10 min) followed by a linear salt gradient, 0.1–0.25 M NaCl for 30 min and, finally, 5 min with 0.25 M NaCl. Forty-five fractions were collected (1 ml each) and assayed for aminopeptidase activity. The detection of AAP and RAP was assayed using the specific reaction buffers described before. The activity of methionyl aminopeptidase was detected using its specific reaction buffer as described before and also measuring the endopeptidase activity with 0.1 mM N-Bz-Arg-AMC in 100 mM phosphate buffer pH 7.5 with 10 mM DTT.

The three fractions with maximum methionyl aminopeptidase activity were mixed and stored at 4°C. These fractions were concentrated to half volume by centrifugation in a 10 KDa filter (Ultrafree, Millipore) at 3000 *g* during 10 min. The concentrate was applied to the same column previously equilibrated with 10 mM Tris–HCl, pH 6.0, containing 0 M NaCl, 0.1% (v/v) 2-ME, 0.02% sodium azide. The column was eluted at 1 ml/min with an isocratic period with the equilibration buffer (10 min) followed by a linear salt gradient from 0.0 to 0.2 M NaCl for 30 min and then 5 min with 0.2 M NaCl. The fractions (1 ml) were collected and assayed for aminopeptidase activity.

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.

The molecular mass and purity of the purified methionyl aminopeptidase was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% 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.

2.5. Enzyme characterization

The activity of the purified methionyl aminopeptidase was measured against several aminoacyl-AMC (Met-, Lys-, Ala-, Leu-, Phe-, Arg-, Gly-, Tyr-, Ser-, pGlu-, Val-, Pro-, N-Bz-Arg-, N-CBZ-Phe-Arg-, and Z-Arg-Arg-AMC) and aminoacyl-pNA (Leu-, Arg-, Glu-, Ala-pNA) derivatives as substrates (0.15 mM) in 100 mM phosphate buffer pH 7.5, 10 mM DTT as standard assay medium.

The kinetic parameters of the purified methionyl aminopeptidase were estimated for the aminoacyl-AMC and aminoacyl-pNA substrates by Lineweaver–Burk plots using 0.01–0.20 mM and 0.05–0.3 mM substrate concentrations, respectively. The enzyme activity on aminoacyl derivatives was measured by the standard assay and the rate of hydrolysis was followed continuously at 37°C. The absorbance when using aminoacyl-pNa derivatives was detected continuously at 410 nm in a microplate reader EL×800 from Biotek Inc (Winooski, VT, USA).

The effect of potential inhibitors or activators was tested by incubating the purified enzyme in the standard assay medium in the presence of the following chemicals: bestatin (0.025–0.5 mM), puromycin (0.025–0.5 mM), N-trans-epoxysuccinyl-L-leucyl-amido-4-guanidinobutane (E-64) (0.1–0.5 mM), phenylmethanesulfonylfluoride (PMSF) (0.01–1.5 mM), arphamenine B (0.1–1.0 mM), leupeptin (0.1–1.0 mM), pepstatin A (0.01–0.1 mM), amastatin (0.01–0.05 mM), EDTA (1–15 mM), iodoacetic acid (2–10 mM), NaCl (100–500 mM) and ammonium sulfate (100–500 mM). The effect of metal cations was determined in the presence of the respective salts: $CaCl_2$ and $CoCl_2$ at (0.25–1.0 mM), $CuCl_2$ (0.05–0.15 mM), $HgCl_2$ and ZnSO4 (0.05–0.5 mM). In all cases, activity was assayed in the standard assay medium. Controls in the absence of the chemical agents were simultaneously run.

Thermal stability of the purified methionyl aminopeptidase was determined by incubation of the enzyme in 100 mM phosphate pH 7.5, at the following temperatures: 5, 15, 25, 37, 45 and 55°C. Aliquots were taken at different times to measure the remaining activity using the standard assay. Activity is expressed as a percentage of initial activity before incubation.

3. Results

3.1. Purification of the aminopeptidase

The results of a typical purification are summarized in Table 1. The first chromatographic step on the anion exchange column gave three peaks showing aminopeptidase activity (Fig. 1A). Peak I, showing Arg-AMC hydrolyzing activity, eluted at 0.1 M NaCl and based on its properties was identified as arginyl aminopeptidase (RAP) previously purified from porcine skeletal muscle (Flores et al., 1993). Peaks II and III eluted at 0.17 and 0.20 M NaCl, respectively. Peak III hydrolyzed Arg-AMC and Ala-AMC at pH 6.5, but did not show activity against Ala-AMC at pH 7.5 in the presence of 0.05 mM bestatin or against N-Bz-Arg-AMC. So, peak III corresponds to the previously characterized alanyl aminopeptidase (AAP) (Flores et al., 1996). Peak II hydrolyzed Ala-AMC at pH 7.5 in the presence of 0.05 mM bestatin, and also showed activity against Arg-AMC at pH 6.5, lower activity against Ala-AMC at pH 6.5 and low activity against N-Bz-Arg-AMC at pH 7.5. In order to better separate peak II from peak III (Fig. 1A), a second chromatographic step was done and gave a purified peak at 0.17 M NaCl (Fig. 1B). The purified peak hydrolyzed Ala-AMC at pH 7.5 in the presence

of 0.05 mM bestatin and had low activity against Ala-AMC at pH 6.5. The whole procedure gave a 1410-fold purification of the enzyme and a yield of 2.3% (Table 1).

The purified aminopeptidase gave a single band, free from other contaminating proteins, on SDS-PAGE (Fig. 2, line F) with a mobility corresponding to a molecular mass of approximately 53 KDa.

3.2. Enzyme characterization.

The influence of pH and temperature on the activity of the purified enzyme was studied (data not shown). The activity on Ala-AMC derivative was maximal at pH 7.5. The enzyme activity decreased sharply to less than 20% of the maximal activity when assayed at pH 6.0 while almost 40% of the activity was recovered at pH

Table 1
Purification of porcine muscle methionyl aminopeptidase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1233.40	17.36	0.014	100.0	1.0
Soluble fraction	589.68	12.68	0.022	73.1	1.5
40–60% $(NH_4)_2SO_4$	248.10	9.75	0.039	56.2	2.8
FPLC (0.1–0.25 mM)	0.11	1.05	9.518	6.0	676.3
FPLC (0.0–0.20mM)	0.02	0.40	19.851	2.3	1410.4

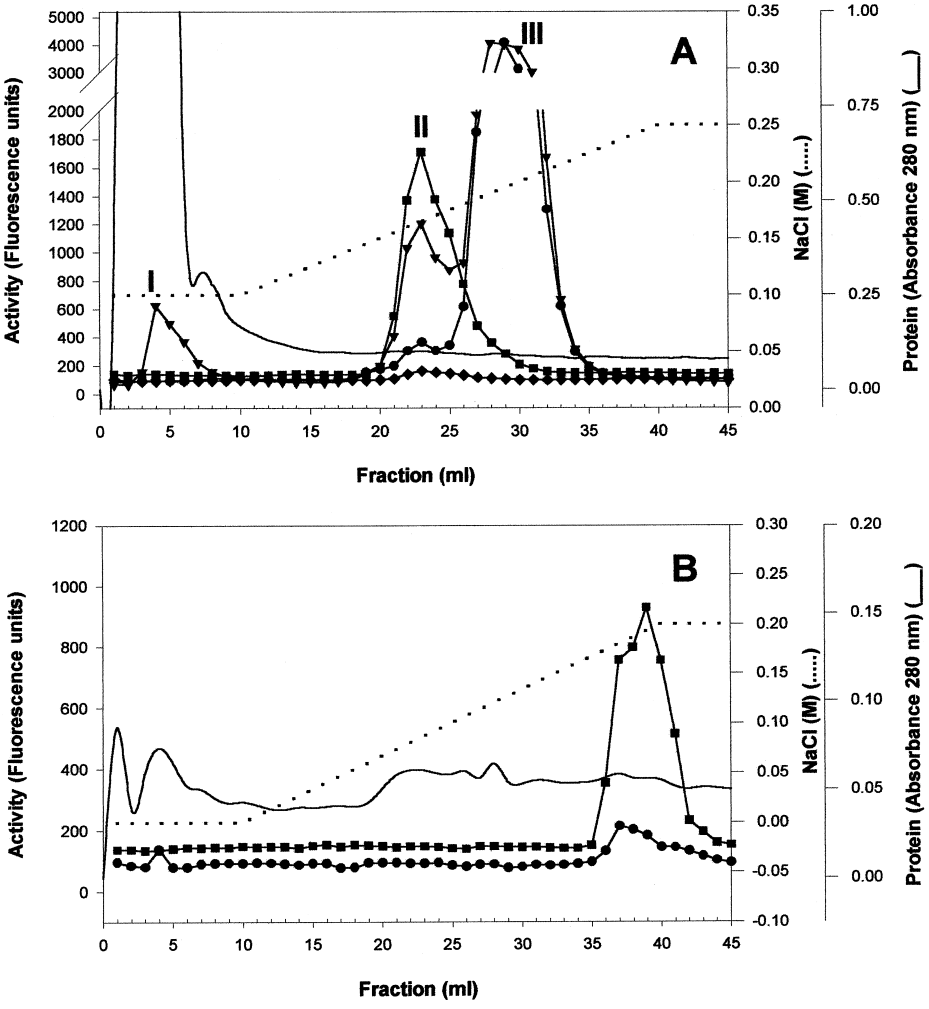

Fig. 1. FPLC anion exchange purification of methionyl aminopeptidase from porcine muscle after ammonium sulfate fractionation: (A) 0.1–0.25 M NaCl linear gradient and (B) 0.0–0.2 M NaCl linear gradient. (....) NaCl; (—) $A_{280\ nm}$; (●) AAP activity (Ala-AMC at pH 6.5); (■) Methionyl aminopeptidase activity (Ala-AMC at pH 7.5 in presence of 0.05 mM bestatin); (▲) RAP activity (Arg-AMC with NaCl); (◆) Endopeptidase activity (N-Bz-Arg-AMC at pH 7.5).

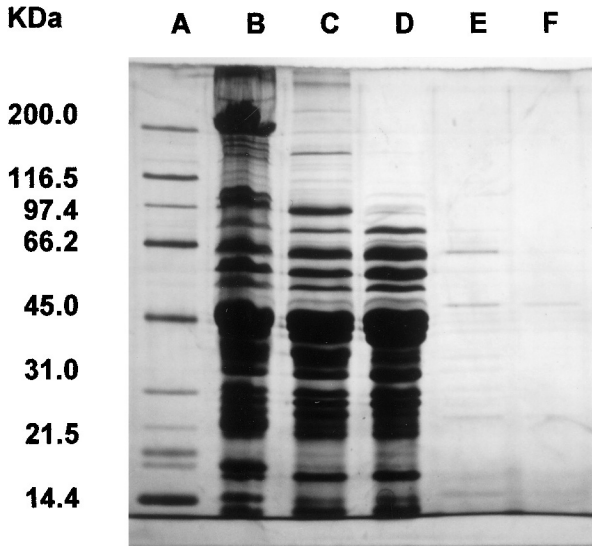

Fig. 2. Twelve per cent SDS-PAGE patterns at each step of the purification process: (A) protein standards, (B) crude extract, (C) soluble fraction, (D) dialyzed after ammonium sulfate precipitation, (E) peak II from anion exchange FPLC 0.1–0.25 M NaCl, (F) anion exchange FPLC 0.0–0.2 M NaCl.

9.0. The optimum temperature was 40°C. The enzyme was rapidly inactivated at 5 and 55°C, while at 15°C about 30% of the optimal activity remained.

The purified enzyme hydrolyzed a broad range of aminoacyl derivatives (Table 2). Methionyl-AMC derivative was the substrate most rapidly hydrolyzed, therefore the proposed name of methionyl aminopeptidase (MAP). The enzyme did not show activity against Pro-AMC derivative and exhibited low activity towards endopeptidase substrates.

Kinetic parameters for purified methionyl aminopeptidase are shown in Table 3. The enzyme had high affinity for Leu-, Tyr- and Met-AMC derivatives although the highest V_{max} were for Ala-, Leu- and Met-AMC. It was inhibited by high concentrations of the substrates (data not shown) as also reported for AAP (Flores et al., 1996). In the case of the aminoacyl-pNa derivatives, the enzyme showed the highest affinity for Leu-pNa although the highest V_{max} was for Glu-pNa.

The effect of the chemical agents on purified methionyl aminopeptidase is shown in Fig. 3. The purified enzyme was activated by reducing agents such as DTT and 2-ME (Fig. 3F) being 4 times more active in the presence of 10 mM DTT. Bestatin (Fig. 3A) and arphamenine B (Fig. 3B), typical inhibitors of exopeptidases, caused no inhibition while puromycin and amastatin (Fig. 3AC) inhibited it. The serin protease inhibitor PMSF (Fig. 3B) and aspartic protease inhibitor pesptatin A (Fig. 3C) did not give any inhibition. The cystein protease inhibitors E-64 (Fig. 3A) and leupeptin (Fig. 3B) inhibited the activity. However, metal chelators such as EDTA (Fig. 3D) did not affect the activity. Iodoacetic acid gave a strong inhibition of the activity (Fig. 3D). NaCl did not affect the activity although the enzyme was partially inhibited by ammonium sulphate (Fig. 3E). The effect of cations on the activity was also determined (Fig. 4). Ca^{2+} increased the activity by about 80% while Co^{2+} reduced the activity by about 50% (Fig. 4A). Hg^{2+}, Cu^{2+} and Zn^{2+} caused complete inhibition (Fig. 4B)

Thermal stability of the purified enzyme was studied by measuring the remaining activity after incubation at different temperatures (Fig. 5). The enzyme rapidly lost its activity above 55°C, being fully inactivated in 10 min. The enzyme was more stable at low temperatures, 5 and 15°C (half-life of about 5 days) than 25 and 37°C (half-life of about 1 day).

4. Discussion

The most remarkable characteristics of the purified methionyl aminopeptidase were its aminopeptidase activity with broad substrate specificity and low endopeptidase activity, it was not inhibited by bestatin, did not contain metal ions and had maximum activity at pH 7.5. All these properties distinguish this enzyme from

Table 2
Activity of purified methionyl aminopeptidase on various fluorimetric and colorimetric aminoacyl-derivatives

Substrate	Activity (%)[a]	Substrate	Activity (%)
Met-AMC	100	Val-AMC	3
Lys-AMC	81	Pro-AMC	0
Ala-AMC	57	N-Bz-Arg-AMC	4
Leu-AMC	48	N-CBZ-Phe-Arg-AMC	5
Phe-AMC	36	Z-Arg-Arg-AMC	4
Arg-AMC	31	Leu-pNA	47
Gly-AMC	26	Arg-pNA	43
Tyr-AMC	19	Glu-pNA	26
Ser-AMC	19	Ala-pNA	19
pGlu-AMC	6		

[a] Activity is expressed relative to Met-AMC substrate.

Table 3
Kinetic parameters of purified methionyl aminopeptidase

Substrate	K_m (mM)	V_{max} (μmol/h.mg)	V_{max}/K_m (U/mg.mM)
Leu-AMC	0.037	2.60	70.3
Met-AMC	0.078	2.32	29.8
Gly-AMC	0.078	1.36	17.4
Tyr-AMC	0.027	0.44	16.3
Phe-AMC	0.118	1.52	12.9
Ala-AMC	0.198	2.95	14.9
Arg-AMC	0.259	1.58	6.1
Ser-AMC	0.126	0.48	3.8
Leu-pNA	0.120	3.56	29.7
Ala-pNA	0.252	3.56	14.1
Glu-pNA	8.98	12.16	1.4

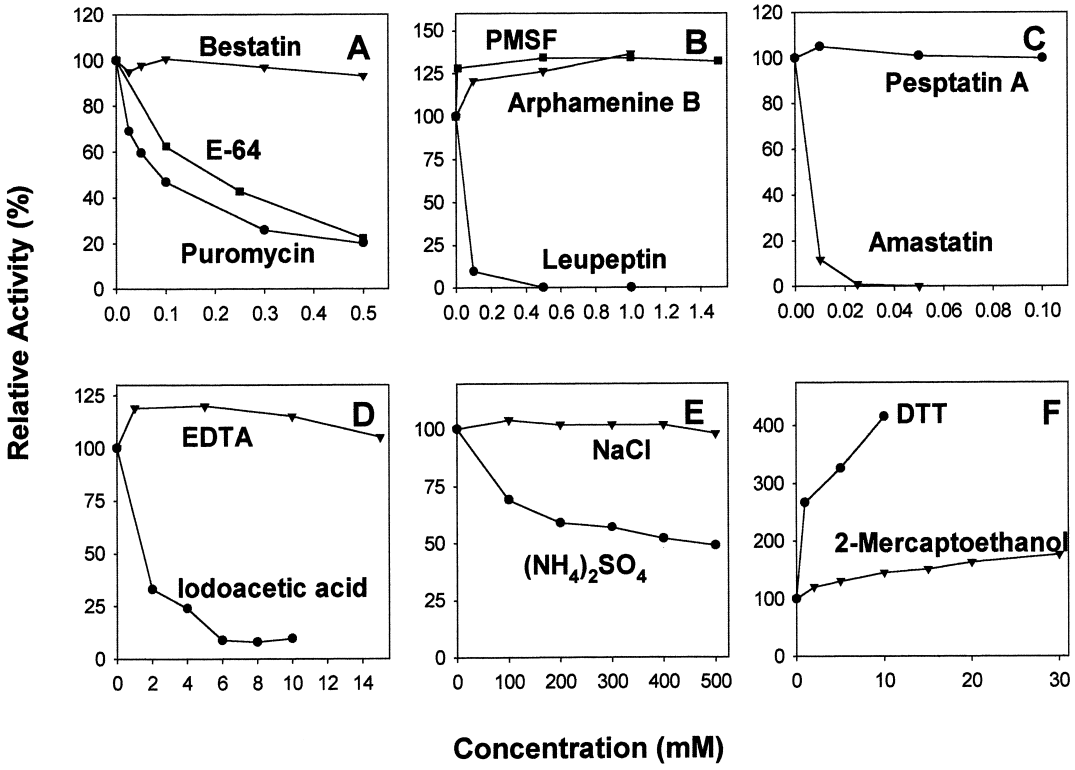

Fig. 3. Effect of chemical agents on the activity of purified methionyl aminopeptidase. The activity in the absence of the chemical agent was 100%.

other aminopeptidases found in porcine skeletal muscle. RAP (Flores et al., 1993) and AAP (Flores et al., 1996) were separated from methionyl aminopeptidase (Fig. 1A peaks I and III) and their characteristics were very different from the purified methionyl aminopeptidase. Also, leucyl (LAP) and pyroglutamyl (PGAP) aminopeptidases from porcine skeletal muscle (Flores et al., 1997) are different from the purified enzyme because LAP and PGAP have activity at basic pH and are specific against Leu- and pyroglu- in the amino terminus. Therefore, the purified enzyme constitutes a new aminopeptidase from porcine skeletal muscle.

The activity of the purified methionyl aminopeptidase was optimal at 40°C and pH 7.5 as found with the aminopeptidase H from porcine, bovine, chicken and rabbit skeletal muscles (Nishimura et al., 1991, 1994; Okitani et al., 1981; Rhyu et al., 1992). The activity profile of the purified aminopeptidase was similar to that of methionine aminopeptidase (McDonald & Barrett, 1986) and for aminopeptidase H (Nishimura et al., 1991). But the purified aminopeptidase showed low endopeptidase activity (Table 2), only 4 or 5% of the endo-substrates were hydrolysed in comparison to the Met-AMC derivative. From these results, it seems that the enzyme acts more as an aminopeptidase. As can be deduced for the Km values, the affinity of the enzyme for Leu-, Tyr, and Met-AMC was higher than with other aminoacyl derivatives, although, the chemical nature of the group situated at the C-terminus of the derivative is also significant in defining substrate specificity. Leu- and Ala-AMC derivatives gave lower K_m values than their respective Leu- and Ala-pNa derivatives (Table 3). Moreover, the aminopeptidase H purified from porcine muscle (Nishimura et al., 1991) had a molecular mass of 340 KDa by gel filtration and 51 KDa by SDS-PAGE, this is close to the 53 KDa band obtained on SDS-PAGE for our purified methionyl aminopeptidase.

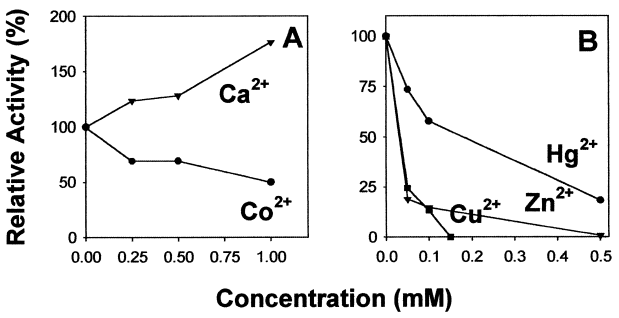

Fig. 4. Effect of various divalent cations on the activity of purified methionyl aminopeptidase. The activity in the absence of cation was 100%.

The inhibition of the purified enzyme by puromycin and amastatin is characteristic of muscle aminopeptidases (McDonald & Barret, 1986) although bestatin, a typical inhibitor of aminopeptidases, did not affect its activity. This is, in part, similar to aminopeptidase H (Rhyu et al., 1992) and methionyl aminopeptidase (Freitas, Termignoni & Guimaraes, 1985) since neither

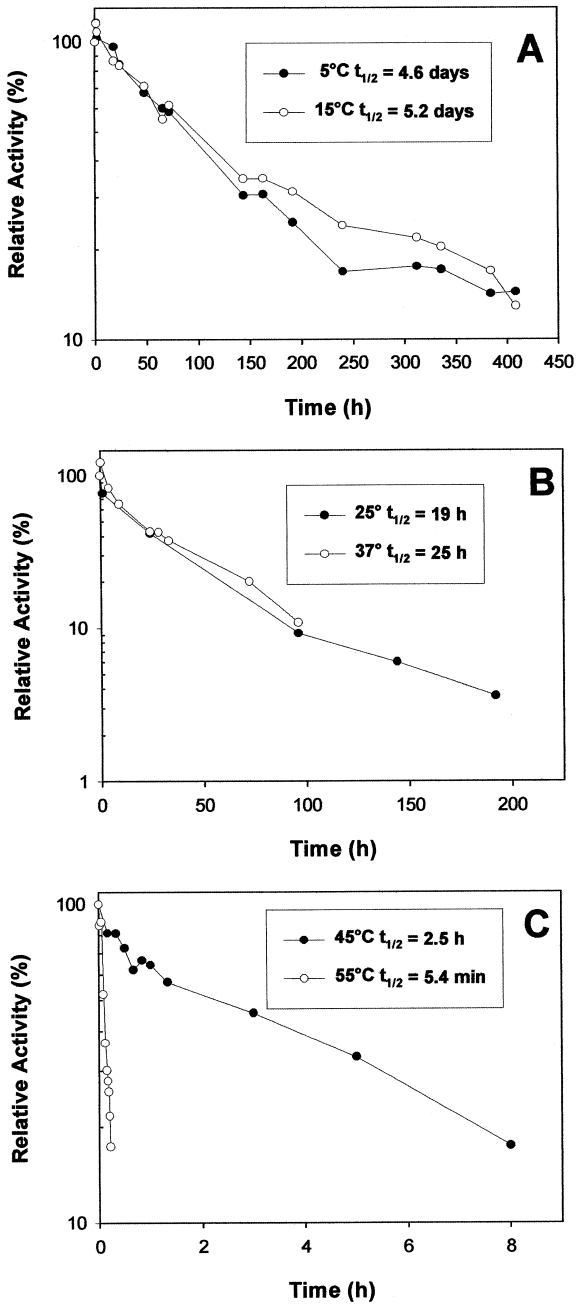

Fig. 5. Thermal stability of purified methionyl aminopeptidase. (A) Incubation at 5° and 15°C, (B) incubation at 25 and 37°C, (C) incubation at 45 and 55°C. Enzyme activity was referred to the activity before incubation, which was taken to be 100%.

were inhibited by bestatin or puromycin. On the other hand, Nishimura et al. (1991, 1994) reported 30% inhibition on aminopeptidase H activity in the presence of 0.1 mM puromycin similar to the inhibition obtained in our purified enzyme. Inhibition by thiol groups reagents and activation by reducing agents were also seen as reported for aminopeptidase H (Rhyu et al., 1992) and methionyl aminopeptidase (Freitas et al., 1985), indicating that sulphydryl groups may be essential for the aminopeptidase activity. However, the purified enzyme was not affected by PMSF and pestatin A but was inhibited by iodoacetic acid. The inhibition caused by ammonium sulphate was less than that caused on other aminopeptidases (Flores et al., 1996). The lack of EDTA inhibition indicated that the methionyl aminopeptidase is different to other metallo-aminopeptidases (McDonald & Barrett, 1986) although it is in accord with aminopeptidase H (Nishimura et al., 1991, 1994) and methionyl aminopeptidase (Freitas et al., 1985). The activation produced by calcium and the inhibition produced by Co^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+} has not been reported before.

The existence of three defined aminoendo-hydrolases has been recognised. The first, was cathepsin H (EC 3.4.22.16) that hydrolyzed endo substrates such as Bz-Arg-NHNap as well as amino derivatives Arg-NHNap (Rothe & Dodt, 1992), at an optimum pH of 6.5 and having a molecular mass of 26 KDa (Kirschke & Barrett, 1987). The second hydrolase H was isolated and characterised by Singh and Kalnitsky (1978, 1980) and showed aminoendopeptidase activity and inhibition by bestatin but not by iodoacetic acid. These properties are clearly different from those described for our purified methionyl aminopeptidase. The third, aminopeptidase H possessed amino and endopeptidase activities (Okitani et al., 1980, 1981). This enzyme was characterised as a thiol protease with maximum activity at pH 7.5–8-0 and was not affected by bestatin, EDTA, PMSF and pepstatin (Nishimura et al., 1991, 1994; Rhyu et al., 1992). It is clear that the purified methionyl aminopeptidase did not fit with the properties of cathepsin H or hydrolase H and although it was similar to the properties of aminopeptidase H in that it had low endopeptidase activity. On the other hand, the purified aminopeptidase was similar to the methionine aminopeptidase (EC 3.4.11.18), peptidase M (MAP) found in many tissues (Freitas et al., 1985; McDonald and Barret, 1986). This enzyme was characterized by its arylamidase activity on Met-NNap substrate at neutral pH, being also capable of hydrolysing Leu-, Arg-, Ala- and Lys-NNap substrates and was not inhibited by bestatin (Freitas et al., 1985) although this was a membrane bound enzyme. The enzyme was of special interest due to its possible contribution to the post-initiation cleavage of the terminal methionine from nascent peptides (McDonald & Barret, 1986).

One of the remarkable characteristics of the purified methionyl aminopeptidase was its lack of inhibition by sodium chloride. In the production of cured pork products, the use of high concentrations of sodium chloride is common (Flores & Toldrá, 1993). Salt is the main curing agent that produces an effect on aminopeptidase activity (Flores et al., 1997). AAP was inhibited by NaCl although RAP was activated by it (Flores et al., 1997). AAP is an aminopeptidase with broad substrate specificity (Flores et al., 1996) while RAP is specific

against basic amino acids (Flores et al., 1993). Therefore, the generation of free amino acids during the ageing of meat and in the processing of cured pork products may be due to the action of the methionyl aminopeptidase because of its broad substrate specificity, optimal activity at neutral pH and activity in the presence of sodium chloride.

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