Biochemical Properties of Dipeptidyl Peptidase III Purified from Porcine Skeletal Muscle

Miguel Angel Sentandreu and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjassot, Valencia, Spain

Dipeptidyl peptidase III (EC 3.4.14.4) was purified from porcine skeletal muscle by ammonium sulfate fractionation and by chromatography and rechromatography on a Biosept-DEAE HPLC column. The enzyme was purified 2329-fold with a 19% recovery. The relative molecular mass was estimated to be 82 000 Da by SDS−PAGE and the maximum activity was reached at pH 8.0 and 40 °C. The enzyme had a wider range of hydrolysis on peptide substrates than over dipeptidyl-AMC derivatives, but without any endopeptidase activity. On the basis of the kinetic parameters and the substrates studied, the tetrapeptide Gly-Gly-Phe-Leu (des-Tyr1 Leu-enkephalin) showed the best affinity for DPP III. The serine peptidase inhibitor 3,4-DCI, along with the cysteine protease inhibitors p-CMB and DTNB were potent inhibitors of DPP III. Chelating and reducing agents also affected considerably the enzyme activity. Co^{2+} was proved to markedly increase the enzyme activity. Zn^{2+}, Fe^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+} inhibited the activity, while Ca^{2+} and Mg^{2+} had no effect. The dipeptide Tyr-Tyr exerted an important inhibitory effect on DPP III activity.

Keywords: *Dipeptidyl peptidase III; muscle; purification; peptide hydrolysis; capillary electrophoresis; HPLC*

INTRODUCTION

Dipeptidyl peptidases (DPP) are a group of proteases which hydrolyze dipeptides from the NH− termini of peptides and proteins. There are four different well-described DPP activities, depending on their substrate specificity, optimum pH, subcellular location, molecular weight, and catalytic properties, named DPP I (EC 3.4.14.1), DPP II (EC 3.4.14.2), DPP III (EC 3.4.14.4), and DPP IV (EC 3.4.14.5) (Mc Donald and Barrett, 1986). New DPP activities have been found in other sources such as rat brain membranes (Hui, 1988), *Dicyostelium discoideum* (Atkinson et al., 1995) and *Pseudomonas* sp. (Ogasawara et al., 1996), in which the characteristics for the described enzymes do not fit into these four previously established groups. The role of DPP is related to peptide processing for different purposes such as regulation of bioactive peptide hormones like insulin, glucagon, angiotensins, and enkephalins, etc. (Lee and Snyder, 1982; Shimamori et al., 1986; Hazato et al., 1984) or protein turnover (Mc Donald and Schwabe, 1977) in the normal cell cycle. However, the role of these enzymes during meat aging and processing of meat products has not yet been clearly established. It is assumed that they contribute to muscle protein degradation, generating dipeptides from the NH− terminus of polypeptides resulting from the action of muscle proteinases, i.e., cathepsins B, D, and L, calpains I and II and the multicatalytic proteinase complex (Toldrá et al., 1996,1997). Other group of exopeptidases, the aminopeptidases, have been directly related with the increase of free amino acids occurring in post-mortem muscle during meat aging (Toldrá et al., 1995), but especially during the dry-curing of meat products, such as dry-cured ham (Toldrá and Flores, 1998). In fact, the complete purification and characterization of the main aminopeptidases of pork muscle, have been recently reported (Nishimura et al., 1991, 1992; Flores et al., 1993, 1996). Of the four dipeptidyl peptidases, DPP III is the only one which is located in the cytosol. It was discovered by Ellis and Nuenke in 1967, and they called it "dipeptidyl arilamidase III". Other names for this enzyme have been "red cell angiotensinase", "neutral angiotensinase", or "enkephalinase B" (Mc Donald and Barrett, 1986). There are no previous works about purification of DPP III in muscle, except a hydrolyzing activity from rat muscle extracts, but with no further characterization (Parsons and Pennington, 1976). This is the first time that DPP III is purified to homogeneity and fully characterized in muscle. Thus, all the information obtained will be referred and compared to DPP III purified and characterized from other sources either in mammalian or nonmammalian cells, like brain (Lee and Snyder, 1982; Hazato et al., 1982; Smyth and O'Cuinn, 1994), human placenta (Shimamori et al., 1986), porcine spleen (Lynn, 1991), *Sacharomyces cerevisiae* (Watanabe et al., 1990) or *Dicyostelium discoideum* (Chan, 1985). Some work has been done concerning DPP III in pork muscle, but in all of them the DPP III activity was measured directly in the muscle soluble extract, with neither specific assay conditions nor characterization (Blanchard and Mantle, 1996; Toldrá et al., 1996; Blanchard et al., 1993). Thus, we found it necessary to achieve a complete study of pork muscle DPP III to get a better knowledge of the role of this enzyme in subsequent stages of proteolysis during meat aging and ripening and its possible contribution to flavor development in processed meat products.

* Author for correspondence (fax, 34 6 3636301; e-mail, ftoldra@iata.csic.es).

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EXPERIMENTAL PROCEDURES

Materials. The substrates such as peptides, di-peptidyl methyl 7-amido-4-methylcoumarin (AMC) derivatives, Ala-Ala *p*-nitroanilide, chemical agents, inhibitors, and cations were obtained from Sigma (St. Louis, MO), except Arg-Arg-AMC, Z-Arg-Arg-AMC, Gly-Arg-AMC, Ala-Arg-AMC, Arg-Arg and Gly-Arg which were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Protein standards for SDS−PAGE were from Bio-Rad (Richmond, VA). The Biosept-DEAE HPLC anion exchange column (75 × 7.8 mm) was purchased from Phenomenex (Torrance, CA).

Enzyme Assays. The standard assay for DPP III activity was performed by using 0.5 mM of L-arginyl-arginyl-7-amido-4-methylcoumarin (Arg-Arg-AMC) as substrate, in 50 mM sodium tetraborate/potassium phosphate buffer, pH 8.0, containing 0.05 mM Co^{2+}. The reaction mixture (300 μL) was incubated in a multiwell plate at 37 °C for 20 min, and the generated fluorescence was determined in a multiscan fluorometer (Fluoroskan II, Labsystems, Finland), using excitation and emission wavelengths of 355 and 460 nm, respectively. Three replicates were measured for each experimental point. One unit of DPP III activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of substrate per hour at 37 °C.

Aminopeptidase activity was determined in the same way as DPP III but using 0.1 mM arginyl-AMC as substrate, and 100 mM phosphate buffer, pH 6.5, as reaction buffer. After incubation for 15 min at 37 °C, the fluorescence was determined as above. One unit of aminopeptidase activity was defined as the amount of enzyme which hydrolyses 1 μmol of substrate per hour at 37 °C.

DPP III Extraction. Unless indicated, all steps were performed at 4 °C. Samples (16 g) of muscle *Biceps femoris*, with no visible fat or connective tissue, were homogenized in 160 mL of 100 mM citric acid buffer, pH 5.0, by using a polytron (three strokes, 10 s each at 27 000 rpm with cooling in ice) homogenizer (Kinematica, Switzerland). The homogenate was then centrifuged at 17000*g* for 20 min and the supernatant filtered through glass wool. This soluble extract was then fractionated with ammonium sulfate, collecting the precipitate formed between 20 and 50% saturation by centrifugation at 12000*g* for 20 min. The pellet was then redissolved in 15 mL of 50 mM Tris-HCl buffer, pH 6.0, containing 50 mM NaCl, and centrifuged at 1000*g* for 2 min. The supernatant was dialyzed overnight against the same buffer, the sample was then recentrifuged at 1,000 g for 2 min, and then the supernatant filtered through a 0.22 μm membrane filter.

Anion Exchange Chromatography. The chromatographic separation was carried out in a biocompatible (titanium) 1050 Hewlett-Packard liquid chromatograph (Palo Alto, CA), equipped with a variable-wavelength UV detector fixed at 280 nm. Two milliliters of dialyzed sample were injected in the column, which was previously equilibrated with 20 mL of 10 mM Tris-HCl buffer, pH 6.0, containing 50 mM NaCl. Elution was first with 10 min of isocratic gradient with the equilibration buffer, and then with a linear salt gradient from 50 to 250 mM NaCl in the same buffer, at a flow rate of 1 mL/min. The eluted fractions (1 mL) were collected and assayed for DPP III and aminopeptidase activity. The two fractions with maximum DPP III activity were mixed and stored at 4 °C. The DPP III active fractions were concentrated to half volume with a 4 mL centrifugal filter, 10 kDa cutoff (Ultrafree, Millipore), by centrifugation at 3000*g* for 5 min. The concentrate was applied to the same column in the same conditions, but equilibrated now with 20 mL of 10 mM Tris-HCl buffer, pH 6.0, containing 100 mM NaCl and then with a linear gradient from 100 to 200 mM NaCl. The eluted fractions (1 mL) were assayed for DPP III activity, and those showing activity were pooled and stored at 4 °C.

Determination of Protein Concentration. Protein concentration in extracts and eluted fractions was determined by the method of Smith et al. (1985), using bicinchoninic acid as reagent and bovine serum albumin as standard. The eluted fractions from the chromatographic separation were also monitored at $\lambda = 280$ nm.

Electrophoresis. The purity and molecular weight of DPP III was estimated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), using 10% polyacrylamide gels and silver staining. SDS−PAGE standard proteins (myosin, β-galactosidase, phosphorilase B, bovine serum albumin, ovalbumin, carbonic anhidrase, trypsin inhibitor, lysozyme, and aprotinin) were simultaneously run for molecular mass estimation.

Substrate Specificity. The activity of the purified DPP III was assayed through the standard enzyme assay against Arg-Arg-AMC, Gly-Arg-AMC, Ala-Arg-AMC, Ala-Ala-*p*-nitroanilide (Ala-Ala-pNA), Lys-Ala-AMC, Gly-Pro-AMC, and Z-Arg-Arg-AMC with a final concentration of 0.5 mM in the reaction mixture.

The activity of DPP III against a variety of peptides (shown in Table 3) was also determined. Enzyme solution (100 μL) was added to 500 μL of standard assay buffer. The reaction mixture, containing 0.5 mM of each peptide, was incubated at 37 °C in a water bath, and aliquots (40 μL) were taken at different times (up to 6 h). Ten microliters of 0.6 M acetic acid solution were added to each aliquot to stop the reaction. Each sample was then further analyzed by capillary electrophoresis in a 270A Capillary Electrophoresis system (Applied Biosystems, Foster City, CA), equipped with a 72 cm fused silica capillary (50 cm to detector) and an UV single wavelength detector (200 nm). Samples were vacuum-injected (2 s), and the electrophoretic run was +20 kV at 35 °C in 50 mM phosphate buffer, pH 7.5, containing 50 mM hexanosulfonic acid. The peptide cleavage was expressed as the amount of peptide hydrolyzed per hour and was referred as a percentage of the hydrolysis of the peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro, which was given a value of 100%.

Kinetic Studies. The kinetic parameters for the hydrolysis of the synthetic substrates Arg-Arg-AMC and Ala-Arg-AMC were estimated by Lineweaver−Burk plots, using a range of concentrations of 20−100 μM and 20−200 μM, respectively. The enzyme activity was measured through the standard assay. Kinetic studies were also performed on the hydrolysis of the following peptides: Phe-Gly-Gly-Phe; Gly-Gly-Phe-Leu; Arg-Phe-Arg-Ser (30−100 μM) and Arg-Arg-Lys-Ala-Ser-Gly-Pro (100−250 μM). Fifty microliters of enzyme solution were added to 250 μL of each peptide in the standard assay buffer. After incubation for 2 h at 37 °C, the reaction was stopped by adding 75 μL of 0.6 M acetic acid to the reaction mixture. Samples were analyzed by capillary electrophoresis, in the same way as the substrate specificity study, but this time samples were injected for 5 s. Standard curves of the reaction products Gly-Phe, Gly-Gly, Arg-Phe, and Arg-Arg were made in the range 20−60 μM.

Optimal pH and Temperature. The effect of temperature on DPP III activity was determined in the range from 5 to 60 °C. The reaction mixture was previously equilibrated in Eppendorf tubes, and the reaction was initiated by the addition of the enzyme. After incubation for different times, according to each temperature, the reaction was stopped by adding 300 μL of 0.25 M acetic acid solution to each tube and then samples were transferred to the multiwell plates. Fluorescence was measured through the standard method, and activity was expressed as a percentage of the activity at optimum temperature.

DPP III activity was tested on both the synthetic substrate Arg-Arg-AMC and the peptide Phe-Gly-Gly-Phe, in a pH range from 5.5 to 9.5, using 100 mM citric acid/200 mM disodium phosphate buffer (pH 5.5−7) and 50 mM sodium tetraborate/100 mM monopotassium phosphate buffer (pH 7.5−9.5). The hydrolysis of Arg-Arg-AMC was measured through the standard enzyme assay and expressed in the same way as the temperature assay. The hydrolysis of Phe-Gly-Gly-Phe at each pH value was determined by capillary electrophoresis, as described in the kinetic studies for peptides. Activity was expressed as a percentage of the activity at optimum pH.

Effect of Different Compounds on DPP III Activity. The effect of several potential inhibitors on the DPP III activity was tested following the standard enzyme assay in the

Table 1. Purification of DPP III from Porcine Skeletal Muscle

purification step	protein (mg)	total activity (U)	specific activity (U/mg)	yield (%)	purification (fold)
soluble fraction from crude extract	561.25	23.15	0.04	100	1
20−50% ammonium sulfate	72.05	55.21	0.77	238	18.6
Anion exchange:					
50−250 mM NaCl	0.944	15.77	16.7	68	404.8
100−200 mM NaCl	0.0454	4.36	96.07	19	2329.3

presence of the respective compound. The following compounds, in the stated concentrations, were studied: Phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (3,4-DCI), 4-(2-aminoethyl)benzosulfonyl fluoride hydrochloride (Pefabloc SC), E-64, *p*-chloromercuribenzoic acid (p-CMB), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.04−0.4 mM); pepstatin A (0.008−0.08 mM); EDTA and EGTA (0.008−4 mM), *o*-phenantroline (0.008−0.8 mM); dithiotreitol and cysteine (0.04−4 mM), 2-mercaptoethanol (0.4−8 mM); puromycin, bestatin, amastatin, leupeptin, diprotin A, and aprotinin (0.04, 0.4 mM); $ZnSO_4$, $FeSO_4$, $CuCl_2$, $CdCl_2$, HgO, $CaCl_2$, $MgCl_2$, and $CoCl_2$ (0.04−0.4 mM); ammonium sulfate (0.4−4%); and 7-AMC (0.04−0.08 mM). The inhibitory effect on DPP III activity of dipeptides listed in Table 6 were studied in the 0.04−0.4 mM concentration range, in the same way as the others. Activity at each assayed concentration was referred to controls, which were run simultaneously, with the absence of any chemical agent.

Enzyme Stability. Thermal stability of purified DPP III was evaluated by incubating the enzyme in 10 mM phosphate/citric acid buffer, pH 6.0 at 5, 15, 25, 37, 45 and 55 °C. Aliquots (170 μL) were taken at different times, according with the assayed temperature. Activity was measured through the standard enzyme assay and expressed as a percentage of initial activity before incubation.

RESULTS AND DISCUSSION

Enzyme Purification. The results of the DPP III purification from porcine skeletal muscle are summarized in Table 1. In the last purification step, we achieve a 2329-fold purification in relation to the soluble extract, the highest reached in any DPP III purification scheme reported previously from other sources (Shimamori et al., 1986; Smyth and O'Cuinn, 1994). The final recovery was 19%. During the purification of DPP III, some authors have proposed the use of bestatin in the reaction buffer in order to selectively inhibit aminopeptidase activities in the soluble extract, and avoid its possible interferences when using Arg-Arg-AMC as substrate (Hazato et al., 1984; Shimamori et al., 1986; Watanabe et al., 1990; Alba et al., 1995), whereas other authors have measured the DPP III activity in the soluble extract without any aminopeptidase inhibitor (Blanchard et al., 1993; Smyth and O'Cuinn, 1994; Blanchard and Mantle, 1996). In our case, the Arg-Arg-AMC hydrolyzing activity in the soluble extract was almost completely due to DPP III activity, being less than 4% of the differences found in Arg-Arg-AMC hydrolyzing activity in the presence or absence of bestatin in the reaction buffer (data not shown). This is in accord with the low activity of the purified alanyl aminopeptidase from pork muscle toward Arg-Arg-AMC (Flores et al., 1996). We, therefore, concluded that presence of bestatin was not necessary in the standard DPP III assay in any of the purification steps, especially taking into account that DPP III was efficiently separated from aminopeptidase activity in the first chromatographic step.

In the first chromatographic step (Figure 1a), DPP III eluted as a single peak at 150 mM Na Cl, being separated entirely from the aminopeptidase activity (fraction 42). In the second anion exchange chromatography (Figure 1b), DPP III eluted as a single activity peak, free from other contaminating proteins, as demonstrated by the SDS−PAGE (see Figure 2), indicating enzyme homogeneity, with a mobility corresponding to

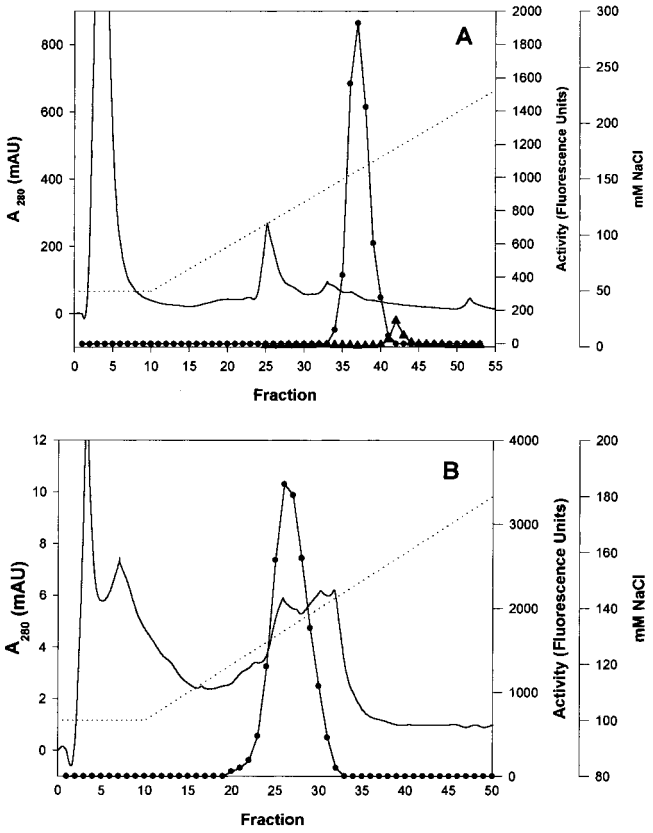

Figure 1. HPLC anion exchange purification of porcine muscle DPP III from the ammonium sulfate fractionation for (A) 50−250 mM NaCl linear gradient and (B) 100−200 mM NaCl linear gradient: (···) mM NaCl; (—) $A_{280\ nm}$; (●) DPP III activity; (▲) aminopeptidase activity.

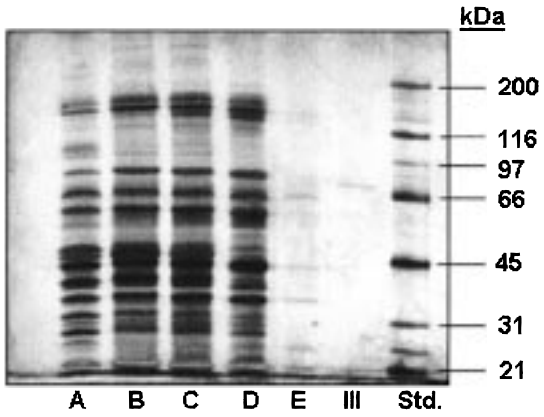

Figure 2. The 10% SDS−PAGE patterns at each purification step: (a) soluble fraction; (b) ammonium sulfate precipitate; (c) dialyzed and centrifuged ammonium sulfate precipitate; (d) former sample, filtered through 0.22 μm cutoff; (e) 50−250 mM NaCl anion exchange; (III) 100−200 mM NaCl anion exchange; and (Std.) standards.

Table 2. Activity of the Purified Porcine Muscle DPP III on Several Dipeptidyl-AMC Derivatives

substrate	relative activity[a] (%)
Arg-Arg-AMC	100.00
Ala-Arg-AMC	68.81
Ala-Ala-pNA	49.87
Gly-Pro-AMC	9.19
Lys-Ala-AMC	3.36
Gly-Arg-AMC	2.25
Z-Arg-Arg-AMC	0.00

[a] Expressed as a percentage of the activity against Arg-Arg-AMC, which was given a value of 100%.

a molecular mass of 82 000 Da, which is in accord with the Mr obtained for DPP III isolated from other sources, such as pig brain, rat brain, human placenta, human seminal plasma, or beef pituitary (Ellis and Nuenke, 1967; Lee and Snyder, 1982; Shimamori el al., 1986; Vanha-Perttula, 1988; Smith and O'Cuinn, 1994). In all cases, M_r was always ~80 000 Da. Some exceptions were the DPP III from the soluble fraction of monkey brain, with a M_r around 100 000 Da (Hazato et al., 1984) and that from porcine spleen, with M_r 66 000 Da (Lynn, 1991). In all cases, DPP III is considered to be a monomer, consisting of a single polypeptide chain.

Optimal pH and Temperature. The effect of pH on DPP III activity was examined on the hydrolysis of Arg-Arg-AMC and Phe-Gly-Gly-Phe. In both cases the enzyme was most active at basic pH, around 8.0. When Arg-Arg-AMC was used, the activity sharply decreased in acidic conditions, and with Phe-Gly-Gly-Phe the pH range of activity was narrower, with no activity detected in neutral or acidic conditions (data not shown). This is in accord with the optimal pH for DPP III obtained from other sources, which normally ranged between pH 8.0 and 9.0 (Smyth and O'Cuinn, 1994; Watanabe et al., 1990; Vanha-Perttula, 1988; Shimamori et al., 1986; Lee and Snyder, 1982). In contrast, previous works reported an optimal pH around the neutral region for the hydrolysis of peptide substrates (Hazato et al., 1984; Shimamori et al., 1988; Ellis and Nuenke, 1967).

The optimal temperature was found to be 40 °C. Higher temperatures resulted in a rapid decrease of the activity, while at 15 °C the enzyme showed only 6% of maximum activity (data not shown).

Substrate Specificity. Several synthetic AMC substrates and Ala-Ala-p-NA were studied (see Table 2). Arg-Arg-AMC was the most rapidly hydrolyzed by porcine muscle DPP III. This is the substrate commonly used to determine DPP III activity (Smyth and O'Cuinn, 1994); Ala-Arg-AMC and Ala-Ala-pNa are also hydrolyzed by the enzyme in a considerably high proportion (69 and 50%, respectively) in relation to Arg-Arg-AMC, as shown in Table 2. This is in agreement with the substrate specificity reported for DPP III from other sources (Hazato et al., 1984; Shimamori et al., 1986; Vanha-Perttula, 1988; Watanabe et al., 1990). However, the action of DPP III on fluorescence substrates has been reported to be mainly restricted to the hydrolysis of the Arg-Arg derivatives (Ellis and Nuenke, 1967; Lee and Snyder, 1982). Gly-Arg-AMC, Lys-Ala-AMC, and Gly-Pro-AMC, which are the usual substrates used to determine DPP I, DPP II, and DPP IV activity, respectively (Mantle, 1991; Lalu et al., 1987), are hydrolyzed in a very low proportion. No hydrolysis was observed against the blocked NH_2- termini Z-Arg-Arg-AMC, confirming that DPP III does not act as an endopeptidase, as previously reported (Mc Donald and Barrett, 1986; Watanabe et al., 1990).

Table 3. Hydrolysis of Peptides with Different N-Terminal Peptide Sequences by the Purified Porcine Muscle DPP III

peptide	relative activity[a] (%)
Gly-Gly-Phe-Leu (des-Tyr1 Leu-enkephalin)	273.15
Phe-Gly-Gly-Phe	157.99
Arg-Phe-Arg-Ser	147.01
Arg-Arg-Lys-Ala-Ser-Gly-Pro	100.00
Val-Gly-Ser-Glu	84.07
Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin)	83.55
Arg-Ser-Arg-His-Phe	78.23
Val-Tyr-Ile-His-Pro-Phe (des-Asp-Arg angiotensin II)	38.34
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II)	36.99
Gly-Arg-Gly-Asp	32.34
Arg-Gly-Asp-Ser	30.61
Ala-Gly-Ser-Glu	16.93
Arg-Arg-Pro-Tyr-Ile-Leu	0
Arg-Lys-Glu-Val-Tyr	0
Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin III)	0
Tyr-d-Ala-Gly-Phe-Leu-Arg	0
Gly-Gly-Phe	0
Tyr-Gly-Gly	0

[a] Expressed as a percentage of the activity against Arg-Arg-Lys-Ala-Ser-Gly-Pro, which was given a value of 100%.

Table 4. Kinetic Parameters of Porcine Muscle DPP III

substrate	K_m (μM)	V_{max} [μmol/(h mg)]	V_{max}/K_m [U/(mg μM)]
Arg-Arg-AMC	231.53	49.26	0.213
Ala-Arg-AMC	85.4	14.7	0.172
Gly-Gly-Phe-Leu	174.6	158.73	0.909
Phe-Gly-Gly-Phe	39.05	4.82	0.123
Arg-Phe-Arg-Ser	299.06	46.73	0.156
Arg-Arg-Lys-Ala-Ser-Gly-Pro	124.05	12.66	0.102

DPP III usually shows a wider range of activity on real peptide substrates than on AMC derivatives (Mc-Donald and Barrett, 1986), as also observed in our study (see Table 3). This broad specificity has also been observed by other authors (Ellis and Nuenke, 1967; Lee and Snyder, 1982; Smyth and O'Cuinn, 1994). Angiotensins and enkephalins (see Table 3) are considered some of the best substrates for DPP III (Smyth and O'Cuinn, 1994; Lee and Snyder, 1982). Our results show that DPP III from porcine skeletal muscle hydrolyzed angiotensin II and desAsp-Arg-angiotensin II (see Table 3), although at a very low rate and not at all in the case of angiotensin III. It seems clear that porcine muscle DPP III has less affinity toward those peptides than DPP III from other sources, generally from brain of different mammalian species (Lee and Snyder, 1982; Hazato et al., 1984; Smyth and O'Cuinn, 1994) or from the red cells (Kokubu, 1969).

Leu-enkephalin (Table 3), however, is a better substrate than angiotensins, having a 84% of hydrolysis of the peptide reference. The peptide that most rapidly hydrolyzed, as also reported in DPP III from human placenta (Shimamori et al., 1988), was des-Tyr1-leu-enkephalin (see Table 3), with a 273% hydrolysis rate with respect to the peptide reference, and with best affinity, as can be seen from the kinetic parameters (Table 4). Other good substrates for DPP III were the sequences Phe-Gly-Gly-Phe and Arg-Phe-Arg-Ser (158% and 147%, respectively). DPP III also hydrolyzed the tetrapeptides Val-Gly-Ser-Glu and Arg-Ser-Arg-His-Phe. Little hydrolysis was observed against Arg-Gly-Asp-Ser and Gly-Arg-Gly-Asp and was almost negligible

Table 5. Effect of Different Chemical Agents on the Activity of Porcine Muscle DPP III[a]

compound	0.04 mM	0.4 mM
PMSF	99	101
Pefabloc-SC	109	98
3,4-DCI	18	11
p-CMB	16	10
DTNB	35	4
E-64	111	113
puromycin	92	80
bestatin	100	89
amastatin	86	67
leupeptin	104	98
diprotin A	100	97
aprotinin	100	83

[a] The activity with no chemical agent added was taken as 100%.

in the case of Ala-Gly-Ser-Glu. Although DPP III cleaves both Gly-Gly and Tyr-Gly from desTyr1-Leu-enkephalin and Leu-enkephalin, respectively, it is unable to split the same dipeptides from Gly-Gly-Phe and Tyr-Gly-Gly, confirming that DPP III is not able to hydrolyze tripeptides (Ellis and Nuenke, 1967; Smyth and O'Cuinn, 1994). Besides, it is confirmed that DPP III cannot split peptide bonds containing a proline residue (Smyth and O'Cuinn, 1994).

Kinetic Studies. Kinetic parameters of DPP III for different substrates are presented in Table 4. The K_m obtained for the hydrolysis of Arg-Arg-AMC was considerably higher than previously reported (Smyth and O'Cuinn, 1994; Shimamori et al., 1986; Hazato et al., 1984), whereas the K_m toward Ala-Arg-AMC was somewhat similar than what Hazato et al. (1984) reported in monkey brain. In any case, it seems clear that hydrolytic activity of aminopeptidases toward aminoacyl-AMC derivatives is quite superior than hydrolytic activity of DPP III toward dipeptidyl-AMC derivatives (Flores et al., 1996). Between the peptide substrates, the affinity toward des-Tyr1 Leu-enkephalin was much greater than toward the rest of peptides assayed (Table 4).

Effect of Chemical Agents on DPP III Activity. The sulfonyl fluorides PMSF and Pefabloc SC exerted little effect on DPP III activity (see Table 5), in agreement with Watanabe et al. (1990), and less than data reported in previous works (Smyth and O'Cuinn, 1994; Shimamori et al., 1986; Vanha-Perttula, 1988). In contrast, the serine protease inhibitor 3,4-DCI strongly inhibited the enzyme activity, remaining only 11% of the initial activity in the presence of 0.4 mM of this compound (Table 5). This result seems to clarify the importance of the serine residue at the active site of the enzyme, in opposition to DPP III from other sources, where lower degrees of inhibition with serine protease inhibitors were obtained (Smyth and O'Cuinn, 1994; Vanha-Perttula, 1988; Lalu et al., 1987; Shimamori et al., 1986; Mc Donald and Schwabe, 1977). Hazato el al. (1984), described the almost total inhibition of DPP III when employing 1 mM of diisopropylfluorophosphate (DFP). However, this compound is highly toxic, whereas 3,4-DCI is relatively nontoxic (Salvesen and Nagase, 1989) and could thus be used in muscle DPP III characterization. DPP III was strongly inhibited by the cysteine protease inhibitors p-CMB and DTNB, but was unaffected by E-64, a cysteine protease inhibitor of microbial origin (Table 5). This seems to indicate that an −SH group of the enzyme could be involved in the activity. Chelating agents were also severe inhibitors of DPP III, especially *o*-phenantroline which suppressed

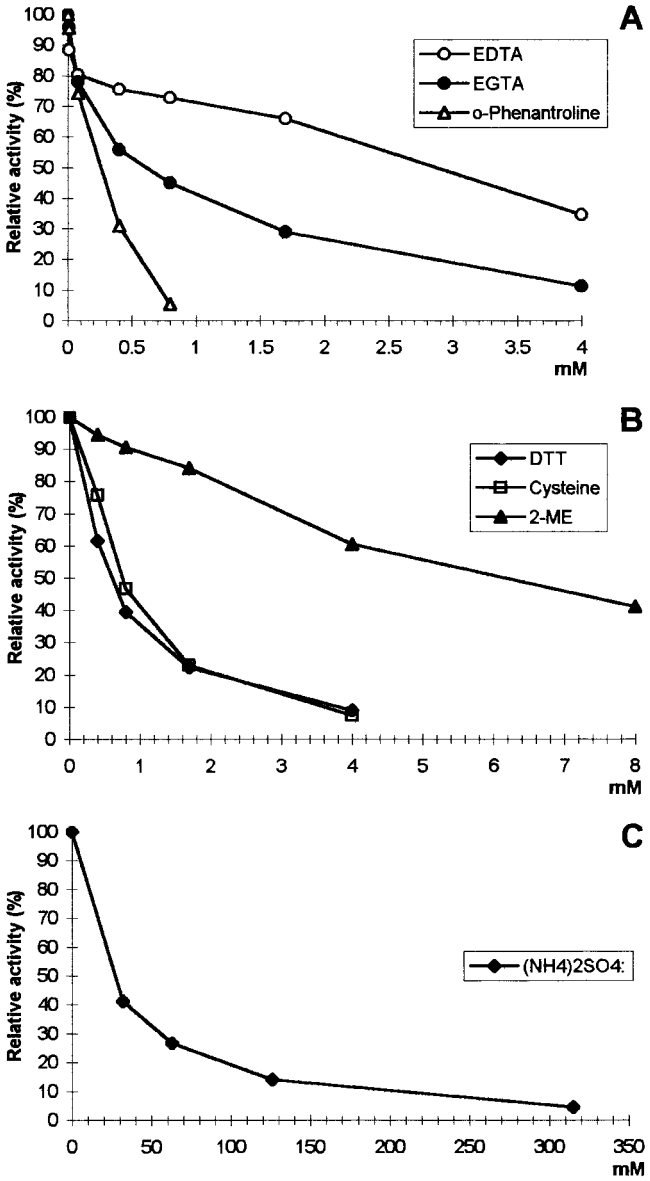

Figure 3. Effect of various inhibitors on the activity of porcine muscle DPP III. The activity with no inhibitor was taken as 100%.

95% of activity at 0.8 mM (see Figure 3A). The strong inhibition of DPP III by some sulfhydryl reagents and metallochelators was already observed by Ellis and Nuenke (1967), and seems to be a general pattern of DPP III (Smyth and O'Cuinn, 1994; Shimamori et al., 1986; Watanabe et al., 1990). However, DPP III from rat skin (Hopsu-Havu and Jansén, 1970) and from bull reproductive tissues (Agrawal and Vanha-Perttula, 1986) proved to be resistant to EDTA, while Ellis and Nuenke (1967) observed inhibition with EDTA only when previously incubated with 2-ME. As reported earlier (Shimamori et al., 1986; Ellis and Nuenke, 1967), and despite the sensitivity toward sulfydryl reagents, DPP III is thought to be a serine peptidase. In addition, reducing agents were not only useless as activators of the enzyme activity, but they caused a remarkable inhibition, especially in the case of cysteine and DTT, which approached 91−93% inhibition at 4 mM concentration (Figure 3B). This is of great importance, since the use of reducing agents, at 1−2 mM concentration, especially DTT, is quite usual in the enzyme assay of DPP III activity even during the purification steps.

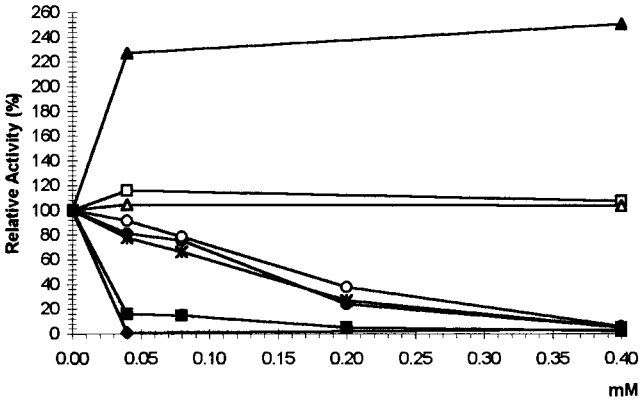

Figure 4. Effect on various divalent metal cations on the activity of porcine muscle DPP III; the activity with no cation was taken as 100%: (●) Zn^{2+}, (○) Fe^{2+}, (*) Cu^{2+}, (■) Cd^{2+}, (□) Ca^{2+}, (△) Mg^{2+}, (▲) Co^{2+}, and (◆) Hg^{2+}.

Some authors (Mantle, 1991; Mantle and Perry, 1990) were not able to obtain a chromatographic peak of DPP III activity, while others detected quite low activity levels of DPP III in the soluble extract of pork muscle (Blanchard et al., 1993; Blanchard and Mantle, 1996; Toldrá et al., 1996). From our results, we think that activity of DPP III in porcine skeletal muscle may be quite more relevant than previous reports to date and this must be taken into account when designing assay procedures for an accurate determination of DPP III activity in the soluble extracts of tissues.

Pepstatin A, a typical inhibitor of aspartic proteinases, has no effect on DPP III activity (data not shown), and among the inhibitors of microbial origin, only slight inhibition is observed at 0.4 mM except for leupeptin, which already exerts a moderate effect at 0.04 mM. Diprotin A, an inhibitor of DPP IV (Beynon and Salvesen, 1989) has practically no effect on DPP III activity (see Table 5). Ammonium sulfate, which was used in the purification scheme of DPP III, is a strong inhibitor of the activity (see Figure 3C). So, fractionation with this salt during the purification procedure requires further and complete dialysis in order to recover the maximal enzyme activity. The two principal aminopeptidases, alanyl aminopeptidade and aminopeptidase B, from pork muscle have been reported to be significantly inhibited by this salt (Flores et al., 1993, 1996).

Effect of Divalent Cations. Among the assayed divalent cations, Hg^{2+} is found to exert the most potent effect, since it completely inhibited the activity at 0.04 mM. Cd^{2+} also acted as a strong inhibitor, although in a lower degree. Zn^{2+}, Fe^{2+}, and Cu^{2+} had a more gradual inhibitory effect, as can be seen in Figure 4; Ca^{2+} and Mg^{2+} had almost no effect on DPP III activity. Co^{2+} was the only one which efficiently increased the enzyme activity, with a 2.5-fold stimulation at 0.4 mM. This stimulatory effect of Co^{2+} on DPP III activity was already observed by Shimamori et al. (1988) in human placenta, by Watanabe et al. (1990) in *S. cerevisiae*, and by other authors working with other exopeptidases (Flores et al., 1996; Pritchard and Coolbear, 1993; Fricker and Snyder, 1982). Thus, we decided to add 0.05 mM of Co^{2+} in the standard assay buffer for DPP III.

Effect of Dipeptides and 7-AMC. Dipeptides constitute the final end product of DPP III action. As most of the exopeptidases are usually feed-back inhibited, some dipeptides were assayed. The aromatic dipeptide Tyr-Tyr showed an important inhibitory effect (see Table 6), although less than what Lee and Snyder (1982) and Smyth and O'Cuinn (1994) reported for DPP III from rat and pig brain, respectively. Arg-Arg and Tyr-Ala also inhibited in a considerable percentage. Ala-Tyr and Arg-Phe had a moderate inhibitory effect, and

Table 6. Effect of Various Dipeptides on the Activity of Porcine Muscle DPP III[a]

dipeptide	0.04 mM	0.2 mM	0.4 mM
Gly-Phe	98	90	87
Gly-Arg	103	101	102
Lys-Ala	109	107	97
Gly-Pro	97	94	93
Tyr-Tyr	66	21	10
Tyr-Ala	86	67	50
Ala-Tyr	88	78	74
Arg-Ala	95	94	88
Lys-Lys	93	95	96
Arg-Phe	100	84	72
Arg-Arg	85	63	52

[a] Results are expressed as a percentage of control with no dipeptide added.

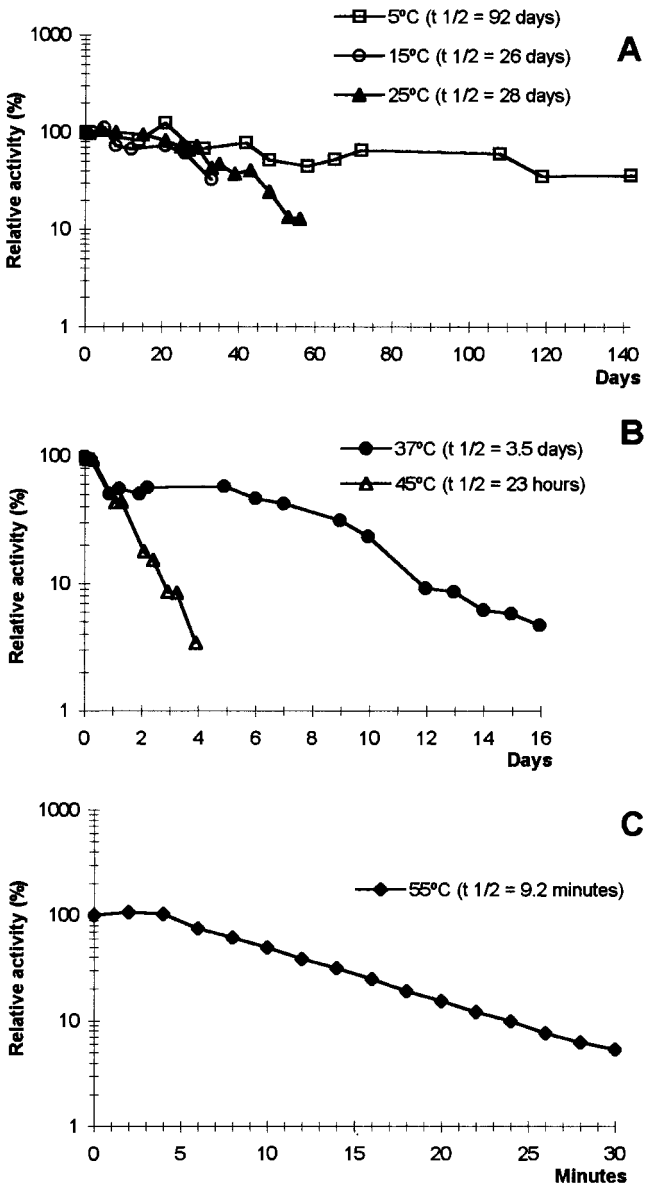

Figure 5. Stability of porcine muscle DPP III at different temperatures: 5, 15 and 25 °C (A), 37 and 45 °C (B), and 55 °C (C). Enzyme activity with time course was referred to initial activity before incubation, which was taken as 100%.

the rest of the assayed dipeptides (Gly-Phe, Gly-Arg, Lys-Ala, Gly-Pro, Arg-Ala and Lys-Lys) had little or no effect on the enzyme activity. Due to the important effect of the coumarin-based inhibitor 3,4-DCI on the activity of DPP III, the action of the 7-aminomethylcoumarin on the enzyme activity was studied, because it constitutes the fluorescent group of many synthetic substrates used in determining and characterizing enzymes. This group showed a considerable inhibitory effect on the activity of DPP III, 34% inhibition at 0.04 mM and 48% at 0.08 mM. So, short incubation times are desirable when employing this kind of substrates, to minimize the feed-back inhibition.

Enzyme Stability. Thermal stability of DPP III was determined at different temperatures from 5 to 55 °C. From what we see in Figure 5, DPP III is quite stable in storage up to 25 °C, the same temperature that Flores et al. reported for alanyl aminopeptidase and aminopeptidase B from porcine muscle. In fact, this latter enzyme was highly stable up to 35 °C (Flores et al., 1993; 1996). With higher temperatures, the loss of DPP III activity increases progressively until 55 °C, where the enzyme is fully inactivated within 30 min (Figure 5).

As we have seen, DPP III is able to split dipeptides from the NH− termini of several peptides sequences, which would not be well-hydrolyzed by aminopeptidases, according to previous work (Flores et al., 1996). Thus, those dipeptides could be present in the final product, contributing to its sensory characteristics.

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