Purification and Biochemical Properties of Dipeptidyl Peptidase I from Porcine Skeletal Muscle

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Dipeptidyl peptidase I (DPP I; EC 3.4.14.1) was purified from porcine skeletal muscle after several steps such as heat treatment, ammonium sulfate fractionation, gel filtration chromatography, and HPLC anion exchange chromatography. The purified enzyme showed a native molecular mass of ~200 kDa on Sephacryl S-200 column chromatography. Two protein bands of 65 and 42 kDa were obtained by SDS−PAGE, indicating its oligomeric nature. Maximum activity was reached at pH 5.5 and 55 °C. DPP I shared some common substrate specificities, both on synthetic derivatives and on real peptides, with porcine muscle DPP III. The enzyme required reducing agents for full activation, although the halide requirement was not proved. DPP I was inhibited by the assayed cysteine peptidase inhibitors except *p*-CMB. The serine peptidase inhibitor 3,4-DCI also inhibited the enzyme as did the divalent cations Co^{2+}, Mn^{2+}, and Zn^{2+}. On the basis of its properties, DPP I may contribute to the generation of dipeptides during the processing of meat and/or meat products, including cooked ham.

Keywords: *Dipeptidyl peptidase I; exopeptidases; peptidases; pork muscle; peptide; meat*

INTRODUCTION

Dipeptidyl peptidase I belongs to a group of peptidases capable of hydrolyzing different dipeptide sequences from the amino termini of peptides. Although they have received different names in the past, they are currently known as dipeptidyl peptidases (DPP); DPP I (EC 3.4.14.1) and DPP II (EC 3.4.14.2) are located in the lysosomes, DPP III (EC 3.4.14.4) is located in the cytosol, and DPP IV (EC 3.4.14.5) is located in cell membranes (McDonald and Barrett, 1986). Their role in the regulation of bioactive peptides and protein turnover or their implication in some diseases has been widely studied (McDonald and Schwabe, 1977). DPPs, together with muscle aminopeptidases, are responsible for the complete breakdown of polypeptide fragments resulting from proteins, in an accumulation of free amino acids and dipeptides (Coffey and de Duve, 1968). Some evidence exists of a synergistic action of lysosomal exopeptidases in the intracellular protein degradation (Huang and Tappel, 1971); however, little is known concerning their contribution to the numerous postmortem changes occurring in skeletal muscle during storage and ripening and their relation to the increase in meat tenderness and flavor development of processed meat products. Although it is assumed they would contribute, together with aminopeptidases, to the extensive degradation of polypeptide fragments resulting from the activity of muscle endopeptidases (Toldrá et al., 1996, 1997), there is no clear evidence to confirm this activity. Thus, it is necessary to characterize the action of those peptidases in meat and meat products. In fact, DPP III has been recently purified and characterized from porcine skeletal muscle (Sentandreu and Toldrá, 1998a). Aminopeptidases are better studied enzymes in muscle (Nishimura et al., 1991, 1992; Flores et al., 1993, 1996), and they are related with the increase of free amino acids occurring during meat aging (Toldrá et al., 1995; Flores et al., 1998) and especially during the ripening of meat products such as dry-cured ham (Toldrá and Flores, 1998). DPP I was first discovered by Gutmann and Fruton in 1948 and has received different names such as cathepsin C, dipeptidyl aminopeptidase I, dipeptidyl arylamidase, glucagon-degrading enzyme, and dipeptidyl transferase. This latter name was due to the polymerase activity attributed to DPP I at basic pH (McDonald et al., 1969a). DPP I was recently reported as belonging to the papain family (Rawlings and Barrett, 1993), and typical sources have been lysosome-rich tissues such as spleen (McDonald et al., 1972a), liver (McDonald et al., 1969a), or kidney (Mantle, 1991). Although this enzyme may be identical to the lysosomal exopeptidase from nonmuscle sources that has been most extensively purified and characterized (McDonald and Schwabe, 1977), this is the first time a complete purification and study of the biochemical properties of DPP I from muscle has been carried out. A partial isolation of DPP I from rat (Parsons and Pennington, 1976) and human muscle (Bury and Pennington, 1975) has been reported, but this enzyme has not been characterized. Other studies of muscle DPP I have been restricted to the measurement of its activity directly in muscle soluble extracts (Kar and Pearson, 1978; Blanchard et al., 1993; Rosell and Toldrá, 1998), with no optimized conditions for the enzyme assay. The aim of this work is to advance the knowledge of muscle exopeptidases, more specifically DPP I, to better understand the intermediate stages of the proteolytic chain in post-mortem muscle, resulting in the final generation of small peptides and free amino acids.

EXPERIMENTAL PROCEDURES

Materials. Muscle Longissimus dorsi, from 6-month-old Landrace × Large White pigs, was used as enzyme source. Samples were cut and vacuum-packed between 12 and 24 h

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post-mortem and immediately frozen at −20 °C until their utilization. The substrates such as peptides, dipeptidyl-7-amido-4-methylcoumarin (dipeptidyl-AMC) derivatives, Ala-Ala-*p*-nitroanilide (Ala-Ala-pNA), chemical agents, protein standards for gel filtration, inhibitors, and cations were obtained from Sigma (St. Louis, MO), except Gly-Arg-AMC, Ala-Arg-AMC, Arg-Arg-AMC, and Z-Arg-Arg-AMC, which were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Protein standards for SDS−PAGE were from Bio-Rad (Richmond, CA). Both Sephacryl S-200 HR and the Resource-Q 1 mL anion exchange column (6.4 × 30 mm) were purchased from Pharmacia LKB (Uppsala, Sweden).

Enzyme Assays. The standard assay of DPP I activity was performed by using 0.5 mM Gly-Arg-AMC as substrate, in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 5 mM dithiotreitol. The reaction mixture (300 μL) was incubated in a multiwell plate at 37 °C for 20 min, and the fluorescence was determined in a multiscan fluorometer (Fluoroskan II, Labsystems), using excitation and emission wavelengths of 355 and 460 nm, respectively. Three replicates were measured for each experimental point. One unit of DPP I activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per hour at 37 °C.

Purification of DPP I from Porcine Skeletal Muscle. *Enzyme Extraction.* Unless indicated, all steps were performed at 4 °C. Sixteen grams of pork loin, with no visible fat or connective tissue, was obtained and homogenized in 160 mL of 100 mM citric acid buffer, pH 5.0, containing 5 mM EDTA by using a polytron (three strokes, 10 s each at 27000 rpm, with cooling in ice) homogenizer (Kinematica). The homogenate was then centrifuged at 17000*g* for 20 min and the supernatant collected and filtered through glass wool.

Heat Treatment and Ammonium Sulfate Fractionation. The volume of β-mercaptoethanol (β-ME) necessary to have a 5 mM concentration in the soluble extract was dropwise added. The extract was then heated to 55 °C for 30 min and chilled at 4 °C. The precipitated protein was removed by centrifugation at 12000*g* for 20 min, and the supernatant was then fractionated with ammonium sulfate, collecting the precipitate formed in the range 40−70%. After centrifugation at 12000*g* for 20 min, the pellet was gently redissolved in 5 mL of 50 mM Tris−maleate buffer, pH 6.0, containing 100 mM NaCl and 5 mM β-ME. Afterward, the redissolved protein was centrifuged at 1000*g* for 5 min and the supernatant filtered through a 0.45 μm membrane filter.

Gel Filtration Chromatography. The redissolved protein, once centrifuged and filtered, was applied onto a Sephacryl S-200 HR gel filtration column (1.5 × 100 cm) previously equilibrated with 50 mM Tris−maleate buffer, pH 6.0, containing 100 mM NaCl, 5 mM β-ME, and 0.02% sodium azide. Fractions (2 mL) were collected at a flow rate of 10 mL/h and assayed for DPP I activity. Fractions with maximal DPP I activity were pooled and dialyzed against 10 mM Tris−maleate buffer, pH 6.0, containing 5 mM β-ME. The dialyzed protein was then filtered through a 0.22 μm membrane filter and concentrated to 2 mL with a 15 mL centrifugal filter device having a 10 kDa cutoff (Ultrafree, Millipore, Bedford, MA).

HPLC 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. The concentrated protein was injected in a Resource-Q 1 mL column, previously equilibrated with 10 mL of 10 mM Tris−maleate buffer, pH 6.0, containing 5 mM β-ME. Elution was performed at a flow rate of 1 mL/min with a three-step NaCl gradient, consisting in an isocratic gradient with the equilibration buffer for 10 min, a linear salt gradient from 0 to 100 mM NaCl in the same buffer for 15 min, and finally a linear salt gradient from 100 to 500 mM in the same buffer for 25 min. Fractions of 1 mL were collected and assayed for DPP I activity. Those fractions with maximal DPP I activity were pooled and dialyzed overnight against 50 mM citric acid buffer, pH 5.0, containing 5 mM β-ME and then stored at 4 °C under nonoxidizing conditions.

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

Polyacrylamide Gel Electrophoresis. The purity and molecular weight of DPP I subunits were estimated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), using 12% polyacrylamide gels and silver staining. Standards (myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, and aprotinin) were simultaneously run for molecular mass estimation.

Substrate Specificity. DPP I activity was evaluated, through the standard enzyme assay, against the following synthetic substrates: Gly-Arg-AMC, Ala-Arg-AMC, Arg-Arg-AMC, Ala-Ala-pNA, Lys-Ala-AMC, Gly-Pro-AMC, Z-Arg-Arg-AMC, *N*-Cbz-Phe-Arg-AMC, glutaryl-Gly-Gly-Phe-AMC, *N*-succinyl-Leu-Tyr-AMC, and *N*-*t*-Boc-Val-Leu-Lys-AMC, in a final concentration of 0.5 mM in the reaction mixture.

DPP I was also evaluated for activity against the following peptides: Val-Tyr-Ile-His-Pro-Phe, Tyr-Gly-Gly-Phe-Leu, Met-Leu-Phe, Met-Ala-Ser, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Arg-Val-Tyr-Ile-His-Pro-Phe, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu, Ser-Asp-Gly-Arg-Gly, Gly-Arg-Gly-Asp, Gly-Gly-Phe-Leu, Phe-Gly-Gly-Phe, Ala-Gly-Ser-Glu, Val-Gly-Ser-Glu, Ala-Ala-Ala-Ala, Arg-Phe-Arg-Ser, Gly-Phe-Leu, Tyr-Gly-Gly, and Leu-Gly-Gly. One hundred microliters of enzyme solution 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 shaken-plate incubator, and aliquots (40 μL) were taken at different times (up to 20 h). Ten microliters of 1 M citric acid solution containing 1 mM iodoacetic acid was added to each aliquot to stop the reaction. Samples were vacuum-injected (up to 2 s) in a 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA), equipped with a 72 cm fused silica capillary (50 cm to detector) and a UV single-wavelength detector (200 nm). The electrophoretic run was +20 kV at 35 °C in 50 mM phosphate buffer, pH 7.5, containing 50 mM hexanesulfonic acid. The peptide cleavage was expressed as the amount of peptide hydrolyzed per hour and was referred as a percentage of the hydrolysis of Val-Tyr-Ile-His-Pro-Phe (des-Asp-Arg angiotensin II), which was given a value of 100%.

Kinetic Studies. The hydrolysis of the synthetic substrates Gly-Arg-AMC and Ala-Arg-AMC was studied in a concentration range from 25 to 500 μM through the standard enzyme assay. The kinetic parameters K_m and V_{max} were estimated by the double-reciprocal plot.

Optimal pH and Temperature. The pH optimum for the hydrolysis of Gly-Arg-AMC was investigated using 100 mM citric acid/200 mM disodium phosphate buffer at 37 °C in a pH range from 4.0 to 8.0. Activity at each pH value was expressed as a percentage of the activity at optimum pH.

The activity of DPP I against Gly-Arg-AMC in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 5 mM DTT, was studied in a temperature range from 5 to 90 °C. This substrate solution (250 μL) was previously equilibrated in Eppendorf tubes, and then the reaction was initiated by the addition of the enzyme solution (50 μL). After incubation for different times, according to each temperature, 300 μL of 100 mM glycine/NaOH buffer, pH 10.5, was added to stop the reaction. Samples were then transferred to a multiwell plate, and fluorescence was measured through the standard assay. Activity was expressed as a percentage of the activity at optimum temperature.

Effect of Chemical Agents. The effect of different chemical agents on the activity of DPP I was tested, through the standard assay procedure, in the presence of the respective compound at the following concentration ranges: dithiotreitol (DTT), β-mercaptoethanol (β-ME), and cysteine, 1−50 mM; NaCl, 1−100 mM; ammonium sulfate, 40−380 mM (0.5−5%); *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64),

Table 1. Purification of DPP I from Porcine Skeletal Muscle[a]

purifn step	protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purifn (-fold)
crude extract	1678.44	45	0.027	100	1
soluble extract	668.74	68.65	0.103	152.6	3.8
heat treatment	337.4	48.04	0.142	106.8	5.3
ammonium sulfate fractionation	110.13	26.2	0.238	58.2	8.9
gel filtration	34.97	36.01	1.03	80	38.4
anion exchange	4.28	13.14	3.07	29.2	114.6

[a] Enzyme activity was expressed as μmol of released AMC per hour at 37 °C. Enzyme assays and protein determination were performed as described under Experimental Procedures.

p-chloromercuribenzoic acid (*p*-CMB), iodoacetic acid, phenylmethanesulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (3,4-DCI), and 4-(2-aminoethyl)benzosulfonyl fluoride hydrochloride (Pefabloc-SC), 0.05−1 mM; EDTA and EGTA, 0.5−5 mM; $ZnSO_4$, $FeSO_4$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$, $HgCl_2$, puromycin, bestatin, leupeptin, diprotin A, and pepstatin A, 0.05−0.5 mM; 7-AMC, 0.05 and 0.1 mM. Activity at each assayed concentration was referred to controls, which were simultaneously measured in the absence of any chemical agent.

RESULTS AND DISCUSSION

Purification of Porcine Muscle DPP I. The enzyme DPP I was purified from pork loin as described under Experimental Procedures following homogenization of crude extract, heat treatment, ammonium sulfate fractionation, gel filtration chromatography, and HPLC anion exchange chromatography. The final enzyme preparation gave a specific activity of 3.07 units/mg, 114-fold higher than the activity of crude extract, with a final yield of 29% (Table 1). Other authors obtained higher purification folds, but by purifying DPP I from other sources such as goat brain (Pal et al., 1993), rabbit lungs (Paszkowski et al., 1983), or human spleen (McGuire et al., 1992), whereas DPP I from chicken kidney (Barceló et al., 1981) or rat liver (Nikawa et al., 1992) resulted in lower purification degrees. In all purification steps, the activity of DPP I was determined using Gly-Arg-AMC as substrate, without the addition of any specific aminopeptidase inhibitor because previous works concerning characterization of muscle aminopeptidases reported negligible or no activity of those enzymes toward Gly-AMC or Gly-Arg-AMC (Nishimura et al., 1991, 1992; Flores et al., 1993, 1996). Therefore, an interfering action of aminopeptidases through the sequential hydrolysis of Gly-Arg-AMC was not expected. To support this, the hydrolysis of Gly-Arg-AMC was measured in both crude and soluble extract in the presence and absence of bestatin, a strong inhibitor of the main aminopeptidases (Beynon and Salvesen, 1989), the differences in Gly-Arg-AMC hydrolyzing activity being lower than 2% (data not shown). Nishimura et al. (1991) reported that aminopeptidase H was unaffected by bestatin, but this enzyme was proved to be rather inactive against Gly-β-naphthylamide and, additionally, its optimum pH, ~8.0, allowed little activity in the acidic region. Therefore, it was not necessary to include any aminopeptidase inhibitor in the assay mixture when DPP I activity in any of the purification steps was determined.

In our purification scheme (Table 1), the soluble extract was submitted to 55 °C for 30 min, because DPP I has been reported to be stable upon exposure to heat

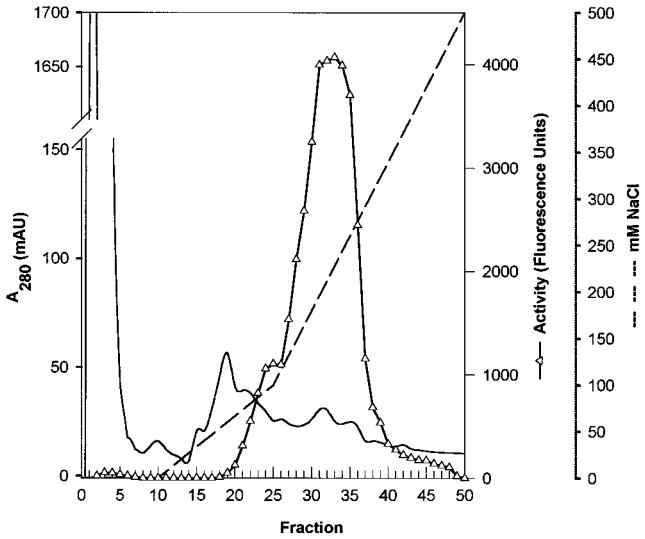

Figure 1. HPLC strong anion exchange chromatography of the pooled DPP I active fractions from gel filtration chromatography: (−) A_{280nm}; (△) DPP I activity; (- - -) mM NaCl.

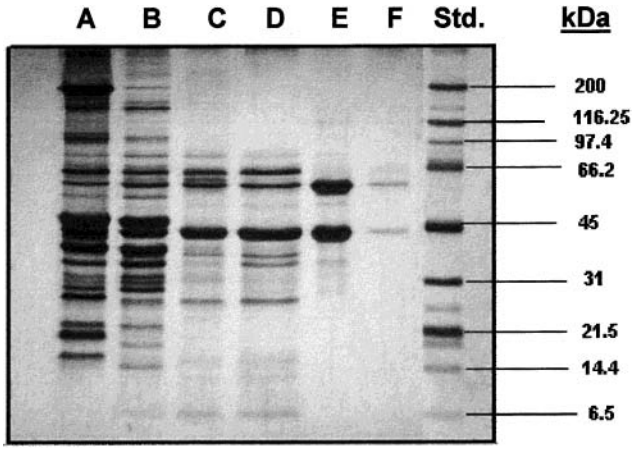

Figure 2. 12% SDS−PAGE from the pork muscle DPP I purification. Purification steps: (A) crude extract; (B) soluble extract; (C) heat-treated extract; (D) 40−70% ammonium sulfate fraction; (e) Sephacryl S-200 pool; (f) DPP I from the strong anion exchange purification. (Std) protein standards.

(Metrione et al., 1966; McDonald et al., 1972b). In our case, some DPP I activity was lost after heat treatment, but the specific activity increased as a result of the elimination of up to 45% of total protein (Table 1). Moreover, this step has the additional advantage of destroying the heat labile activities of enzymes such as carboxypeptidase A (EC 3.4.16.1), cathepsin D (EC 3.4.26.5), carboxypeptidase B (EC 3.4.18.1), and DPP II (McDonald et al., 1972a,b; McDonald and Schwabe, 1977; Pal et al., 1993). The 40−70% ammonium sulfate fraction was redissolved and applied directly to the Sephacryl S-200 HR column. During this chromatographic step, the low M_r proteins (<31 kDa) such as cathepsins B, H, and L were eliminated from the active DPP I fractions, as corroborated by SDS−PAGE (lane E in Figure 2). These active DPP I fractions were dialyzed, concentrated, and applied to the strong anion exchange column. The elution profile of DPP I was spread over several fractions, and some tailing was also observed (fractions 40−50 in Figure 1), but the most active fractions were concentrated in a well-defined peak, better than classical chromatographies previously reported by other authors (Planta and Gruber, 1964;

Lalu et al., 1987; Kuribayashi et al., 1993), who had already observed this phenomena. This may be explained by the complex oligomeric structure of DPP I, in which conformational changes may occur during eluting from the anion exchanger (Planta and Gruber, 1964). It has been quite common to store DPP I at 4 °C in the presence of reducing agents and NaCl (McDonald et al., 1972a; Paszkowski et al., 1983; Lalu et al., 1987; Pal et al., 1993; Dolenc et al., 1995). We found it convenient, in accordance with Barceló et al. (1981), to store DPP I under reducing conditions but in the absence of NaCl (data not shown). Halide salts seem to favor the gradual oxidation of the SH− groups, which are essential for DPP I activity. Therefore, it was necessary to dialyze pure DPP I fractions before storage to eliminate NaCl resulting from the anion exchange chromatography.

Molecular Mass. DPP I showed a molecular mass of 200 kDa on Sephacryl S-200, which is in agreement with the general features reported for this enzyme from different sources. The M_r of DPP I normally ranged between 180 and 210 kDa (McDonald and Barrett, 1986; Mantle, 1991; McGuire et al., 1992; Pal et al., 1993; Popovic et al., 1996). Under denaturing and reducing conditions, purified DPP I showed two protein bands with M_r of 65 and 42 kDa, respectively, in 1:1 ratio, as can be observed in Figure 2 (lane F). This evidence of oligomeric structure of DPP I was established and generally accepted many years ago (Metrione et al., 1970). However, the detailed structure of this enzyme has been under discussion for many years. As a common feature, when DPP I was run on SDS−PAGE under reducing conditions, one protein band of ∼21−24 kDa was observed and attributed to the M_r of one of the subunits that would constitute an octameric form for DPP I (Metrione et al., 1970; Paszkowski et al., 1983; McGuire et al., 1992; Pal et al., 1993). On the other hand, some authors have reported the dissociation of these subunits into heavy and light chains (Isidoh et al., 1991; Nikawa et al., 1992; Dolenc et al., 1995, 1996). In our case, under denaturing and reducing conditions, neither the 21−24 kDa band nor its dissociation into heavy and light chains was detected. McGuire et al. (1992) were not able to detect the heavy and light chains, even though they observed the 24 kDa band. According to our results, DPP I from porcine skeletal muscle would be a tetrameric enzyme with two pairs of subunits. A further and more accurate study about the structure of porcine muscle DPP I would be required to determine whether those protein bands of 65 and 42 kDa were due to an incomplete dissociation of the subunits or could be considered by themselves two different subunits of pork muscle DPP I because there are no previous reports about the DPP I structure from muscle.

Optimal pH and Temperature. DPP I exopeptidase activity was mainly expressed in the acidic region. The enzyme showed its optimum at pH 5.5, but it retained most of the activity in the pH range between 5 and 6.5 (data not shown), as reported for DPP I from rabbit lungs (Pazskowski et al., 1983), very similar to data from other sources (McDonald and Schwabe, 1977; Barceló et al., 1980; Mantle, 1991; McGuire et al., 1992; Pal et al., 1993; Dolenc et al., 1995). Incubation temperature influenced the enzyme activity of DPP I from porcine muscle against Gly-Arg-AMC, as shown in Figure 3. This enzyme reached its maximal activity in the range 45−55 °C, as also reported by Pazskowski et al. (1983) and Pal et al. (1993). DPP I also proved to be resistant to heat, as previously reported (Barceló et al., 1980; McGuire et al., 1992; Pal et al., 1993).

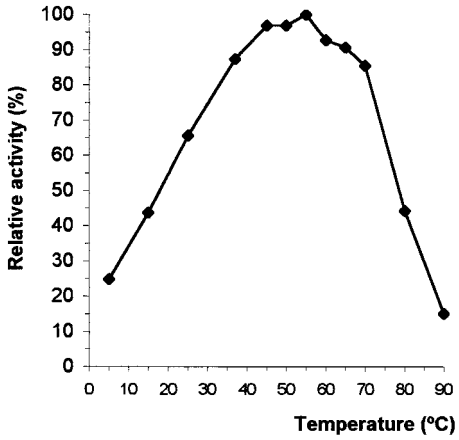

Figure 3. Effect of incubation temperature on the activity of DPP I from porcine skeletal muscle, expressed as a percentage of the activity at optimum temperature.

Table 2. Specificity of Porcine Muscle DPP I on Different Synthetic Dipeptidyl -AMC and -pNA Substrates

substrate	relative activity[a] (%)
Ala-Arg-AMC	123.7
Gly-Arg-AMC	100.0
Ala-Ala-pNA	13.3
Arg-Arg-AMC	0.0
Lys-Ala-AMC	0.0
Gly-Pro-AMC	0.0
Z-Arg-Arg-AMC	0.0
N-Cbz-Phe-Pro-AMC	0.0
glutaryl-Gly-Gly-Phe-AMC	0.0
N-succinyl-Leu-Tyr-AMC	0.0
N-*t*-Boc-Val-Leu-Lys-AMC	0.0

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

Substrate Specificity. Porcine muscle DPP I hydrolyzed the synthetic derivative Ala-Arg-AMC with a 124% hydrolysis rate in relation to Gly-Arg-AMC (see Table 2), which has been considered the best substrate for the determination of DPP I activity (Parsons and Pennington, 1976; Pazkowsky et al., 1983; McDonald and Barrett, 1986; Pal et al., 1993). McDonald et al. (1972a) reported the hydrolysis of Gly-Arg-βNA and Ala-Arg-βNA by DPP I from beef spleen but, in that case, the Ala-Arg-βNA hydrolyzing activity was only 72% of that of Gly-Arg-βNA, which would be indicative of variations due to different species and/or tissue sources of DPP I. Ala-Arg-AMC proved to be also a good substrate for porcine mucle DPP III (Sentandreu and Toldrá, 1998a). Thus, Ala-Arg-AMC could be used as a substrate of DPP I activity in muscle soluble extracts. Although this substrate is also hydrolyzed by porcine muscle DPP III (Sentandreu and Toldrá, 1998a), this enzyme would not interfere in the DPP I assay because it has no activity at pH 5.5 (Sentandreu and Toldrá, 1998b) and is almost totally inhibited in the presence of 4 mM DTT (Sentandreu and Toldrá, 1998a). Ala-Ala-pNA was also hydrolyzed by porcine muscle DPP I but to a lower degree (Table 2), as reported previously (McDonald et al., 1972a; Nikawa et al., 1992). Lys-Ala-AMC, Arg-Arg-AMC, and Gly-Pro-AMC, usual substrates for the determination of DPP II, DPP III, and DPP IV activity, respectively (McDonald and Barrett,

Table 3. Rates of Hydrolysis of Peptides with Different N-Terminal Peptide Sequences by Purified DPP I from Pork Muscle

peptide	relative activity[a] (%)
Val-Tyr-Ile-His-Pro-Phe (des-Asp-Arg-angiotensin II)	100.0
Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin)	68.2
Met-Leu-Phe	22.2
Met-Ala-Ser	9.3
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II)	3.8
Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin III)	0
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly	0
pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (neurotensin)	0
Ser-Asp-Gly-Arg-Gly	0
Gly-Arg-Gly-Asp	0
Gly-Gly-Phe-Leu (des-Tyr1-Leu-enkephalin)	0
Phe-Gly-Gly-Phe	0
Ala-Gly-Ser-Glu	0
Val-Gly-Ser-Glu	0
Ala-Ala-Ala-Ala	0
Arg-Phe-Arg-Ser	0
Gly-Phe-Leu	0
Tyr-Gly-Gly	0
Leu-Gly-Gly	0

[a] Expressed as a percentage of the activity against Val-Tyr-Ile-His-Pro-Phe, which was given a value of 100%.

1986; Blanchard et al., 1993), were not split by DPP I. These results are logical because this enzyme preferentially hydrolyzes N-terminal peptides with a penultimate basic residue such as Arg, whereas those containing a basic amino acid in the ultimate position are resistant to attack, as are those containing a proline residue in any side of the peptide bond (McDonald et al., 1972a; McGuire et al., 1992). For this reason, DPP I has been used as a sequencing reagent of proteins and peptides (Callahan et al., 1972; Caprioli and Seifert, 1975).

Some recent works have reported an additional endopeptidase activity consisting in the hydrolysis of Z-Phe-Arg-AMC by DPP I from bovine spleen (Kuribayashi et al., 1993), rat liver (Nikawa et al., 1992), and human kidney (Popovic et al., 1996). On the other hand, it has been generally assumed that DPP I lacks any endopeptidase activity (McDonald et al., 1969a; McDonald and Schwabe, 1977), cathepsins B and L being the main enzymes capable of hydrolyzing Z-Phe-Arg-AMC (Popovic et al., 1996). Several N-blocked synthetic substrates, including *N*-Cbz-Phe-Arg-AMC, were assayed, but none of them was hydrolyzed by muscle DPP I (Table 2), so that no endopeptidase activity could be attributed to porcine muscle DPP I.

DPP I is reported to have a broad substrate specificity in the degradation of a good variety of polypeptide hormones with free N termini (McDonald et al., 1969a, 1972a). In our case, des-Asp-Arg angiotensin II was the most rapidly hydrolyzed peptide (see Table 3). On the contrary, the hydrolysis rate of angiotensin II was the lowest, and this may be the reason we have not observed the sequential hydrolysis of Asp-Arg and Val-Tyr from this peptide as expected and as reported for DPP I from beef spleen (McDonald et al., 1972a). Angiotensin III was resistant to attack (Table 3). The bioactive hormone Leu-enkephalin proved to be also well hydrolyzed by DPP I from pork muscle, as was the case of DPP I from goat brain (Pal et al., 1993). Leu-enkephalin was also a good substrate for porcine muscle DPP III. This enzyme hydrolyzed des-Tyr1-Leu-enkephalin even better than Leu-enkephalin (Sentandreu and Toldrá, 1998a), whereas DPP I was unable to hydrolyze des-Tyr1-Leu-enkephalin (Table 3). Therefore, DPP I and DPP III from porcine muscle share some common substrate specificities. This offers the possibility of hydrolyzing some substrates over a wide range of physiological conditions in the muscle because these peptidases have shown complementary action in the pH range, reducing/oxidizing conditions, and subcellular location (McDonald and Barrett, 1986). The tripeptides Met-Leu-Phe and Met-Ala-Ser were hydrolyzed to a lower degree (22 and 9%, respectively). Tyr-Gly was not removed from Tyr-Gly-Gly, as happened in Leu-enkephalin. Thus, even if they are not the best substrates, DPP I from pork muscle was able to hydrolyze tripeptides (McDonald et al., 1972a), in opposition to DPP III, which was unable (Sentandreu and Toldrá, 1998a). The sequences Gly-Arg-Gly-Asp and Gly-Phe-Leu were not hydrolyzed, although the liberation of Gly-Arg and Gly-Phe from peptides with different amino acids in the N-antepenultimate position has been reported (McDonald and Barrett, 1986). This would indicate the importance of the DPP I source and the nature of the amino acid on the other side of the peptide bond, apart from the N-terminal dipeptide sequence.

Kinetic Studies. The kinetic parameters K_m and V_{max} for the hydrolysis of Gly-Arg-AMC were found to be 0.148 mM and 3.98 units/mg, respectively (data not shown). A much higher K_m value (0.5 mM) was reported for DPP I from human kidney with the same substrate (Mantle, 1991), whereas a similar K_m value (0.14 mM) was obtained using Gly-Arg-4mβ-NA for DPP I from goat brain, although in this case V_{max} was much higher (Pal et al., 1993). For the hydrolysis of Ala-Arg-AMC we obtained a K_m of 0.017 mM and a V_{max} of 5.39 units/mg (data not shown). This low K_m value was similar to that reported for the hydrolysis of His-Ser-2-NNap and glucagon by rat liver DPP I (McDonald et al., 1969b), but apart from this, K_m values obtained for DPP I substrates were normally much higher (McDonald and Schwabe, 1977; Barceló et al., 1980). According to our kinetic data in the assayed concentration range (0−500 μM), we obtained an apparent Michaelis−Menten kinetics for the hydrolysis of Gly-Arg-AMC (data not shown). This could agree with the findings of Gorter and Gruber (1969), who reported the allosteric nature of DPP I and concluded that an apparent Michaelis−Menten kinetics could be observed only when DPP I was fully activated. The main difference was that they found an absolute requirement of halide ions for DPP I activity from beef spleen, whereas we found necessary a reducing agent such as 5 mM DTT (see Figure 4A), as will be described below. For the hydrolysis of Ala-Arg-AMC we observed a remarkable substrate inhibition above 250 μM in the assay mixture (data not shown). This inhibition was not observed, in the assayed concentration range, for the hydrolysis of Gly-Arg-AMC, probably due to its quite higher K_m value compared with Ala-Arg-AMC. To explain this substrate inhibition, a model of DPP I was proposed, consisting of four substrate molecules that can bind to the enzyme but only the binary enzyme−substrate complex is active (Dolenc et al., 1995).

Effect of Chemical Agents. The thiol inhibitors E-64 and iodoacetic acid exerted a drastic reduction of DPP I activity (see Table 4), which is in accordance with the early classification of this enzyme as a cysteine peptidase (Fruton and Myceck, 1956) and with previous reports of DPP I from other sources (Huang and Tappel, 1971; Barceló et al., 1980; Paszkowsky et al., 1983;

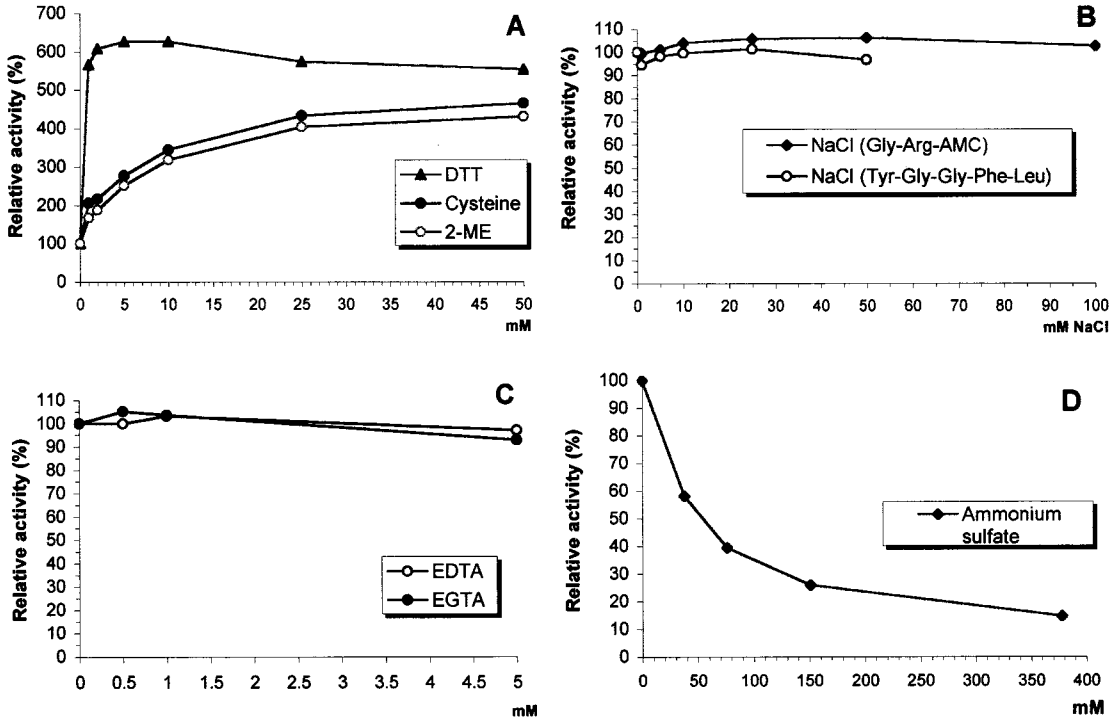

Figure 4. Effect of different chemical compounds on the activity of porcine muscle DPP I: (A) reducing agents; (B) NaCl; (C) chelating agents; (D) ammonium sulfate. The activity without addition of any chemical agent was taken as 100%.

Table 4. Residual Activity of DPP I from Porcine Muscle Expressed as a Percentage of Control after Incubation with Various Protease Inhibitors

substance	0.05 mM	0.5 mM
none	100	100
E-64	18	0
iodoacetic acid	12	0
p-CMB	98	98
PMSF	92	93
Pefabloc-SC	108	103
3,4-DCI	21	0.5
leupeptin	50	12
pepstatin A	84	83
diprotin A	97	95
puromycin	91	90
bestatin	95	97

McGuire et al., 1992; Nikawa et al., 1992; Krepela et al., 1996). However, no inhibition with the cysteine protease inhibitor *p*-CMB was detected, as was reported for DPP I from beef spleen (Myceck, 1970) and rabbit lungs (Paszkowsky et al., 1983). The latter authors observed an inhibition with *p*-CMB only when DPP I was incubated in the absence of any reducing agent. On the contrary, for DPP I from human placenta (Lalu et al., 1987), chicken liver (Barceló et al., 1980), and porcine kidney (Vanha-Perttula et al., 1965), this compound proved to be a very strong inhibitor. The sulfonyl fluorides, inhibitors of serine proteases, PMSF and Pefabloc-SC, had negligible effect on DPP I activity (Table 4) as reported previously for DPP I from other sources (Nikawa et al., 1992; McGuire et al., 1992). DPP I from rabbit lungs was, however, very sensitive against PMSF (Paszkowsky et al., 1983). The coumarin-based compound 3,4-DCI, considered to be a specific serine peptidase inhibitor (Salvesen and Nagase, 1989), exerted a very strong inhibition of DPP I activity, almost complete at 0.5 mM (see Table 4), this being the first time this has been reported for DPP I. In some way, a compound having a coumarin ring in its structure might react with the −SH groups, inhibiting the enzyme activity. 3,4-DCI and related compounds would not be so specific inhibitors of serine proteases because, apart from the present results, there is evidence that this substance is able to inhibit other cysteine peptidases such as calpains and interleukin 1β-converting enzyme (Powers and Kam, 1994). An important inhibitory effect of DPP I activity was also observed in the presence of the 7-amido-4-methylcoumarin group (AMC), the fluorescent group of many synthetic substrates for determining enzyme activities of peptidases. We observed 60 and 84% inhibition on DPP I activity at 0.05 and 0.1 mM, respectively. Thus, this byproduct inhibition has to be taken into account by reducing the incubation times when using this kind of substrate for determining the enzyme activity.

The effect of reducing agents on the activity of DPP I from porcine muscle is shown in Figure 4A. DTT was clearly the most powerful activator of the enzyme activity, with a >5-fold increase even at low concentrations such as 1 mM. Other authors, although reporting more activation with DTT than with cysteine or β-ME, did not find such a great activation (Barceló et al., 1980; Lalu et al., 1987). This can be explained by the lower oxidation−reduction potential of DTT, and its resistance to air oxidation, compared with those of β-ME and cysteine, which have similar oxidation−reduction potentials (Konigsberg, 1972). This would explain the parallel evolution of their activating effect along the assayed concentration range. To reach the activation levels of DTT, a much higher concentration of β-ME and cysteine would be required (Figure 4A). Therefore, DTT, at a concentration of 5 mM, was the preferred activator for DPP I activity. Together with the thiol requirement, DPP I is considered to have an absolute halide requirement for the enzyme activity (Gorter and Gruber, 1970; McDonald and Schwabe, 1977; Paszkowsky et al., 1983; McDonald and Barrett, 1986). Surprisingly, in our case there was no halide requirement for the activity of DPP I from porcine muscle. Furthermore, its presence was

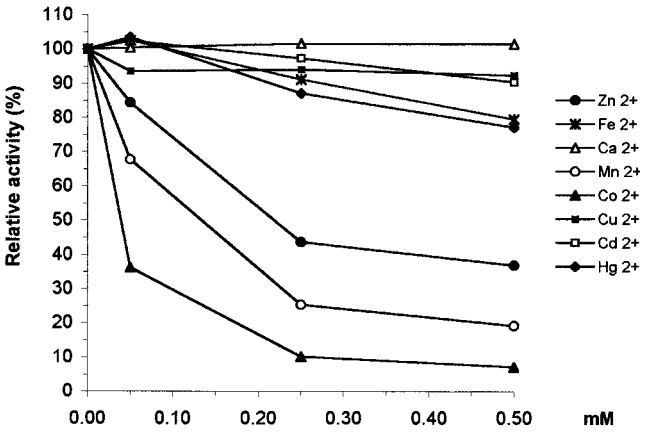

Figure 5. Effect of divalent cations on the activity of DPP I from porcine muscle. The activity with no cation added was taken as 100%.

damaging for the enzyme activity because it favored the gradual oxidation of the −SH groups (Barceló et al., 1980). This is the reason fractions with maximal enzyme activity were dialyzed against a buffer devoid of Cl^-. The effect of NaCl was assayed in the hydrolysis of Gly-Arg-AMC (0−100 mM) and Leu-enkephalin (0−50 mM), with 5 mM DTT in the assay mixture. In such assay conditions, DPP I activity remained unchanged for all of the NaCl concentration values (see Figure 4B). Thus, there is no need for NaCl addition when DPP I activity from porcine muscle is assayed in the presence of DTT.

The chelating agents EDTA and EGTA exerted no action on DPP I activity (see Figure 4C), as normally reported (McDonald and Schwabe, 1977; Paszkowsky et al., 1983; Mantle, 1991). Ammonium sulfate was a strong inhibitor of the activity of pork muscle DPP I (see Figure 4D). This explained the high reduction of the total activity after the ammonium sulfate fractionation during the purification procedure and its recovery after the gel filtration chromatography (see Table 1). Therefore, ammonium sulfate caused a reversible inhibition that could be easily neutralized by the elimination of the salt, giving no interferences with the activity of the final enzyme preparation. Both the SO_4^- and NH_4^+ groups inhibited the enzyme activity (data not shown). DPP I from chicken liver, however, was not affected by the SO_4^- group (Barceló et al., 1980). Other peptidases from pork muscle such as alanyl aminopeptidase, aminopeptidase B, and DPP III are also reversibly inhibited by ammonium sulfate (Flores et al., 1993, 1996; Sentandreu and Toldrá, 1998a). The reversible nonspecific inhibitor leupeptin also suppressed DPP I activity in a considerably high proportion, as happened for DPP I isolated from other sources (Lalu et al., 1987; Mantle, 1991; Nikawa et al., 1992). The aspartic peptidase inhibitor pepstatin A had little effect on DPP I activity (see Table 4), and even lower was the effect of bestatin, puromycin, and diprotin A, an apparent inhibitor of DPP IV (Rahfeld et al., 1991).

Effect of Divalent Cations. The most powerful inhibitory action among the assayed cations was by Co^{2+}, with 93% inhibition at 0.5 mM (Figure 5). This cation also inhibited DPP I activity from human kidney (Mantle, 1991), whereas it slightly affected that from human placenta (Lalu et al., 1987). DPP III from porcine skeletal muscle, on the contrary, has been recently reported to be greatly activated by Co^{2+} (Sentandreu and Toldrá, 1998a). The opposite action of Co^{2+} on the activity of DPP I and III reinforces the idea, mentioned above, that both enzymes are capable of hydrolyzing some common substrates under different physiological conditions in the muscle. Mn^{2+} and, to a lower degree, Zn^{2+} also exerted a strong inhibitory effect on DPP I activity (Figure 5), as reported previously (Paszkowsky et al., 1983). A slight inhibition was observed with Hg^{2+} and Fe^{2+}, and negligible or no inhibition was observed with Cu^{2+}, Cd^{2+}, and Ca^{2+} (Figure 5).

In summary, DPP I from porcine muscle fits many of the characteristics described for DPP I isolated from other sources, such as its classification as a cysteine peptidase, optimum pH and temperature, thermal stability, substrate specificity, and effect of some compounds on the enzyme activity or molecular mass. To the contrary, some other aspects of porcine muscle DPP I have been proved to be quite different from data previously reported for DPP I from other sources. This is of special importance when the role of DPP I during meat aging and/or the processing of meat products is considered. In addition, DPP I has shown good thermal stability and could be active in cooked meat products such as cooked ham, which is exposed to relatively high temperatures during processing (Flores and Toldrá, 1993). The dipeptides generated by DPP I action, which are poorly hydrolyzed by porcine muscle aminopeptidases (Flores et al., 1996), would accumulate in meat and/or processed meat products. Therefore, the presence of dipeptides in meat products is the object of further research in our laboratory.

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