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Partial purification and characterisation of dipeptidyl peptidase II from porcine skeletal muscle

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Abstract

The purification and study of biochemical properties of dipeptidyl peptidase II (DPP II; EC 3.4.14.2) from porcine skeletal muscle have been carried out in the present work. The purification included ammonium sulphate fractionation and two HPLC chromatographic separations using a Resource-Q anion exchange column. The enzyme was purified 1270 fold, with a 1.6% recovery and was completely separated from DPP IV activity. The pure enzyme displayed one main protein band with a Mr of 58 kDa on SDS-PAGE. Maximum activity was reached at pH 5.5 and 65°C. Those synthetic substrates containing Pro in *N*-penultimate position were the most efficiently hydrolysed, whereas in the case of peptides, DPP II efficiently hydrolysed both X-Pro- and X-Ala-peptides. The serine peptidase inhibitors PMSF and Pefabloc SC suppressed DPP II activity in a high degree, whereas 3, 4-DCI and cysteine peptidase inhibitors exerted little effect. Alkaline metal salts inhibited the enzyme activity according to the size of the cation, and among the assayed divalent cations, only Cu^{2+}, Fe^{2+} and Hg^{2+} showed significant inhibition of the activity. This is the first time that porcine muscle DPP II has been purified and its biochemical characteristics studied. So, these results contribute to improve the knowledge in relation with the proteolytic chain and the generation of flavour characteristics in meat products.

Keywords: Dipeptidyl peptidase; Muscle proteolysis; Peptide; Enzyme purification; Meat; Protease

1. Introduction

Dipeptidyl peptidases (DPP) are enzymes capable to hydrolyze different dipeptide sequences from the amino termini of peptides. There are actually four different DPP activities, 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), which are well defined and classified according to their general biochemical characteristics (McDonald & Barrett, 1986). Traditionally, dipeptidyl peptidases have been purified and studied for medical purposes such as the regulation of peptide hormones, the protein turnover or its implication in some diseases (McDonald & Schwabe, 1977). However, little is known about the contribution of these enzymes to the postmortem changes occurring during meat storage or even during the curing of processed meat products like Spanish dry-cured ham (Blanchard & Mantle, 1996; Toldrá, Flores & Sanz, 1997). Those changes are mainly related with the extensive degradation of the myofibrilar structure by endopeptidases (cathepsins, calpains and proteasome) giving rise to large polypeptides. These polypeptides would be degraded, in further stages, to smaller fragments (Toldrá & Flores, 1998). The first stage of this process, the initial breakdown of myofibrils by endopeptidases, has been deeply studied in relation to the increase of meat tenderness (Koohmaraie, 1994; Valin & Ouali, 1992). More recently, the last stage of the proteolytic chain has got importance with the study of muscle aminopeptidases, since they constitute one of the main enzyme groups directly related with the high increase of free amino acids occurring during meat aging (Flores, Aristoy & Toldra, 1993, 1996; Nishimura, Kato, Rhyu, Okitani & Kato, 1992; Nishimura, Rhyu & Kato, 1991) and dry-curing of hams (Toldrá, Aristoy & Flores, 2000). However, the enzymes responsible for the intermediate stages of this proteolytic chain still remain obscure. Large polypeptides are degraded in those intermediate stages giving rise to small peptides. These generated peptides could be further degraded by other exopeptidases, generating mainly dipeptides and free amino acids, forming part of the non-volatile compounds that are responsible of flavour characteristics in cured meat products. DPP I and DPP III have been recently purified and characterised

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from porcine skeletal muscle (Sentandreu & Toldrá, 1998, in press). Regarding DPP II, this is the first time in which this peptidase is purified and fully characterised from muscle. DPP II, like DPP I, is located in the lysosomes. It was first reported in 1968 (McDonald, Leibach, Grindeland & Ellis, 1968; McDonald, Reilly, Zeitman & Ellis, 1968) and along this time it has been known with different names like dipeptidyl arylamidase II, dipeptidyl aminopeptidase II, brain dipeptidyl aminopeptidase A or carboxi-tripeptidase. DPP II is widely distributed in tissues, although not especially abundant in muscle (Bury & Pennington, 1975; Gossrau & Lojda, 1980; McDonald & Schwabe, 1977). High levels of DPP II activity have been reported in cases of muscular dystrophies and polymyosites, probably due to an increase of lysosomal activation observed in such diseases (Kar & Pearson, 1978). The complete primary structure of DPP II is not yet available; only the sequence of the first 41 amino acid residues of the amino termini has been determined (Huang, Takagaki, Kani & Ohkubo, 1996), but this has seem to be enough to observe a significant homology with lysosomal prolyl carboxypeptidase (EC 3.4.16.2). Such homology agrees with the numerous biochemical similarities occurring between both enzymes. So, it is assumed that the amino acid residues of the catalytic triad may be those of lysosomal prolyl carboxypeptidase, Ser/Asp/His (Rawlings & Barrett, 1996), so that both peptidases have been classified into the same family, in the serine peptidase class. The objective of the present work is to increase the knowledge on the possible role of DPP II in the intermediate stages of the proteolytic chain, generating proline and alanine dipeptides. In addition, the conditions for the assay of DPP II activity directly in muscle extracts have been revised in order to improve their sensitivity.

2. Experimental procedures

2.1. Materials

Muscles *Longissimus dorsi* and *Biceps femoris*, coming from 6-month-old Landrace×Large White pigs, were used as enzyme source. Muscles were cut and vacuum-packed between 12 and 24 h post mortem, and immediately frozen at −20°C until their utilisation. Peptides, synthetic substrate derivatives of 7-amido-4-methyl-coumarin (AMC) and *p*-nitroanilide (*p*-NA), chemical agents, inhibitors and cations were purchased from Sigma (St. Louis, MO), except Gly-Arg-AMC, Ala-Arg-AMC and Arg-Arg-AMC, which were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Protein standards for SDS-PAGE were from Bio-Rad (Richmond, VA).

2.2. Enzyme assays

The standard determination for DPP II activity was performed by using 0.5 mM of Lys-Ala-AMC or Gly-Pro-AMC as substrate in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 0.04 mM bestatin. To 250 μl of substrate solution, 50 μl of each enzyme preparation were added. The reaction mixture was incubated in a multiwell plate at 37°C for 20 min. The generated fluorescence was determined in a multiscan fluorometer (Fluoroskan II, Labsystems, Finland), using excitation and emission wavelengths of 355 and 460 nm, respectively. The substrate concentration was reduced to 0.25 mM when monitoring the enzyme activity of the fractions obtained during the different chromatographic separations. Three replicates were done for each experimental point. DPP IV activity was determined by using 0.25 mM of Gly-Pro-AMC as substrate in 50 mM Tris-base buffer, pH 8.0, containing 5 mM DTT, in the same way as DPP II.

Aminopeptidase activity was determined as described by Flores et al. (1993, 1996) by using 0.1 mM Arg-AMC as substrate in 100 mM sodium phosphate, pH 6.5, containing 200 mM NaCl, in the same way as DPP II. In all cases, one unit of enzyme activity (U) was defined as the amount of enzyme which hydrolyses 1 μM of substrate per h at 37°C.

2.3. Purification of DPP II from porcine skeletal muscle

2.3.1. Enzyme extraction

Unless indicated, all steps were performed at 4°C. Sixteen g of thawed pork muscle, with no visible fat or connective tissue, were homogenised in 160 ml of 100 mM sodium phosphate buffer, pH 7.0, by using a polytron (three strokes, 10 s each at 27 000 r.p.m. with cooling in ice) homogenizer (Kinematica, Switzerland). The homogenate was then centrifuged at 17 000 *g* for 20 min and the supernatant filtered through glass wool.

2.3.2. Ammonium sulfate fractionation

The soluble extract was fractionated with ammonium sulfate, collecting the precipitated protein in the range 30–50% saturation after centrifugation at 12 000 *g* for 20 min. The pellet was then gently redissolved in 10 ml of 20 mM phosphate buffer, pH 6.5, containing 25 mM NaCl. The redissolved protein was dialysed overnight against the same buffer. The dialysed protein was subjected to centrifugation at 1000 *g* for 5 min and the supernatant was collected. It was further clarified by filtering first through a 0.45 μm and then through a 0.22 μm membrane filter (Millipore, Bedford, MA).

2.3.3. HPLC anion exchange chromatography

The 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 millilitres of the filtered protein were injected in a Resource-Q anion exchange column (6.4 × 30 mm; Pharmacia LKB,

Uppsala, Sweden), previously equilibrated with 10 ml of 10 mM sodium phosphate buffer, pH 6.5, containing 25 mM NaCl. Elution was performed at a flow rate of 1 ml/min, consisting in an isocratic gradient with the equilibration buffer for 10 min and then a linear salt gradient from 25 to 260 mM NaCl in 50 min. Fractions of 1 ml were collected and assayed for both DPP II and DPP IV activity, together with aminopeptidase activity. The two fractions with maximal DPP II activity (fractions 21–22) that were not coincident with maximal aminopeptidase activity were pooled. A total of four separations were carried out in such conditions, pooling the active DPP II fractions and dialyzing them against 20 mM sodium phosphate buffer, pH 7.0, containing 25 mM NaCl. The dialysed fractions were filtered through a 0.22 μm membrane filter and concentrated to a final volume of 2 ml with a 15 ml centrifugal filter, 10 kDa cutoff (Millipore, Bedford, MA). The concentrated protein was reinjected in the anion exchange column, which was equilibrated with 10 ml of 10 mM phosphate buffer, pH 7.0, containing 25 mM NaCl. Elution was first with 10 min of isocratic gradient with the equilibration buffer, then with a linear salt gradient from 25 to 500 mM NaCl in the same buffer for 50 min at a flow rate of 1 ml/min. Thirty-six fractions of 1 ml were collected and assayed for DPP II and aminopeptidase activity. Fractions containing maximum DPP II activity were pooled, dialysed against bidistilled water and stored at 4°C, constituting the enzyme solution for further studies.

2.4. Determination of protein concentration

Protein concentration in extracts and eluted fractions was determined using the 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.

2.5. SDS-PAGE

The monitoring of each purification step, together with the determination of purity and molecular weight of DPP II, were estimated by sodium dodecil sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% gels and silver staining. 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.

2.6. Optimal pH and temperature

The activity of porcine muscle DPP II in the hydrolysis of Gly-Pro-AMC was assayed in the pH range from 3.0 to 6.5 by using 100 mM citric acid/200 mM disodium phosphate buffer. Fluorescence at each pH value was expressed as percentage of activity at optimum pH.

The effect of temperature on DPP II activity against Gly-Pro-AMC was studied in the range 5–75°C. The substrate solution (250 μl) was previously equilibrated in Eppendorf tubes, and then the reaction initiated by the addition of the enzyme solution (50 μl). After incubation for different times, according to each temperature, 75 μl of 0.6 M acetic acid solution were added to stop the reaction. Samples were then transferred to a multiwell plate and the generated fluorescence measured. Activity was expressed as percentage of activity at optimum temperature.

2.7. Effect of chemical agents

The effect of different chemical compounds on DPP II activity was studied, through the standard enzyme assay, in the presence of the respective compound. The compounds and concentration ranges were: LiCl, NaCl and KCl: 5–100 mM; ammonium sulfate: 40–380 mM (0.5–5%); *trans*-epoxysuccinyl-*L*-leucylamido-(4-guanidino)butane (E-64), *p*-chloromercuribenzoic acid (*p*-CMB), iodoacetic acid, phenylmethylsulfonylfluoride (PMSF), 3,4-dichloroisocoumarin (3, 4-DCI), 4-(2-aminoethyl)-benzosulfonylfluoride hydrochloride (Pefabloc SC), puromycin, bestatin, leupeptin, diprotin A, and pepstatin A: 0.05 and 0.5 mM; Dithiotreitol (DTT), β-mercaptoethanol (β-ME), cysteine, EDTA, EGTA and *o*-phenantroline: 1 and 5 mM; $ZnSO_4$, $FeSO_4$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$, $HgCl_2$ and $MgCl_2$: 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.

2.8. Substrate specificity

The exopeptidase activity of porcine muscle DPP II was tested, through the standard enzyme assay, by using the synthetic substrates Gly-Pro-AMC, Lys-Ala-AMC, Gly-Arg-AMC, Arg-Arg-AMC, Ala-Arg-AMC, Gly-Gly-AMC, Pro-AMC, Ala-Ala-Phe-AMC, Gly-Pro-pNA, Arg-Pro-pNA and Ala-Ala-pNA, in a final concentration of 0.5 mM in the reaction mixture.

In addition, DPP II activity was assayed against different peptide substrates (see Table 5). In this case, the 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 shaken-plate incubator and aliquots (40 μl) were taken at different times (up to 20 h). Ten μl of 1 M citric acid solution were added to each aliquot in order 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 an 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 hexanosulfonic acid. The peptide cleavage was expressed as the amount of peptide hydrolysed per hour and was referred as a percentage of the hydrolysis of Met-Ala-Ser, which was given a value of 100%.

3. Results and discussion

3.1. Purification of the enzyme

The purification of DPP II from skeletal muscle is a complicated process due to its low activity in the crude extract (Table 1), as previously reported by other authors (Bury & Pennington, 1975; Gossrau & Lodja, 1980). The effect of freezing/thawing the muscle sample is not responsible for this reduced activity, since these values are similar to those obtained with meat samples assayed at just 2 h postmortem (Toldrá & Flores, 2000). This low initial activity influenced the low final yield obtained (1.6%) after full purification, although it has been possible to purify the enzyme more than 1200-fold. However, this value is below the purification of DPP II from other sources like the porcine ovary, 1400 fold (Eisenhauer & McDonald, 1986), porcine seminal plasma, 1700-fold (Huang et al., 1996), rat brain, 2600-fold (Imai, Hama & Kato, 1983) and 10 500-fold (Mentlein & Struckhoff, 1989), rat, 3700-fold (Fukasawa, Hiraoka & Hirada, (1983) or human kidney, 5200-fold (Sakai, Kojima & Nagatsu, 1987). On the contrary, the yield of DPP II was generally low, normally ranging between 1 and 10% (Eisenhauer & McDonald, 1986; Fukasawa et al., 1983; Huang et al., 1996; Imai et al., 1983; Mentlein & Struckhoff, 1989; Sakai et al., 1987), as in our case (see Table 1). Though DPP II better hydrolysed Gly-Pro-AMC than Lys-Ala-AMC (see Table 4), the latter substrate was preferred during all purification steps (except the second HPLC separation) in order to avoid the interfering action of DPP IV, since Lys-Ala- derivatives do not appear to be as good substrates for DPP IV as Gly-Pro- derivatives (McDonald & Barrett, 1986).

During the first chromatographic step (see Fig. 1a)., DPP II activity eluted at 82 mM NaCl, corresponding with fractions 21–22–23. DPP II activity was completely separated from DPP IV activity, achieving the main objective of this purification step, the separation of the two dipeptidyl peptidase activities capable to liberate dipeptides from the amino termini of peptides with a proline residue in penultimate position (Mentlein, 1988), which is decisive for the right characterisation of both enzymes. Apart from these dipeptidyl peptidase activities, aminopeptidase activity was also determined by using Arg-AMC as substrate. According to Flores et al. (1993, 1996), it is possible to determine both arginyl and alanyl aminopeptidase activities with this substrate. In fact, the hydrolysis of Arg-AMC revealed the presence of two peaks of aminopeptidase activity (see Fig. 1a). The first peak eluted at 91 mM NaCl, being maximal in fractions 23 and 24, very close to the DPP II activity peak (Fig. 1a.). In the presence of bestatin, these fractions did not hydrolysed Arg-AMC, indicating that possibly this activity corresponded to arginyl aminopeptidase (Flores et al., 1993). The second Arg-AMC hydrolysing activity peak appeared at higher NaCl concentration (205 mM). The aminopeptidase activity of this second peak, which probably corresponded with alanyl aminopeptidase (Flores et al, 1996), was also inhibited by the presence of bestatin.

The second chromatographic separation was performed at pH 7.0 with the aim to eliminate aminopeptidase activity and other contaminating proteins, as checked by polyacrylamide gel electrophoresis (Fig. 2, lane D). DPP II activity in fraction 20 (see Fig. 1b) was effectively concentrated and notably higher than in the fractions of the first separation (Fig. 1a). Despite this, an efficient separation between DPP II and aminopeptidase activity was not possible, since the latter activity appeared in fraction 21. None of the different assayed conditions succeeded in a better separation than those reported here (Fig. 1a and b). Mentlein and Struckhoff (1989)

Table 1
Purification of DPP II from porcine skeletal muscle[ab]

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2153.57	1.967	0.001	100	1
Soluble extract	721.55	2.087	0.003	106.1	3.17
Ammonium sulphate fractionation	72.77	0.592	0.008	30.08	8.9
Strong anion exchange:					
A. 25–260 mM NaCl; pH 6.5	0.79	0.095	0.120	4.85	131.4
B. 25–500 mM NaCl; pH 7.0	0.03	0.031	1.16	1.6	1270

[a] Enzyme activity was express as μmol of released AMC per hour at 37°C.
[b] Enzyme assays and protein determinations were performed as described in Experimental.

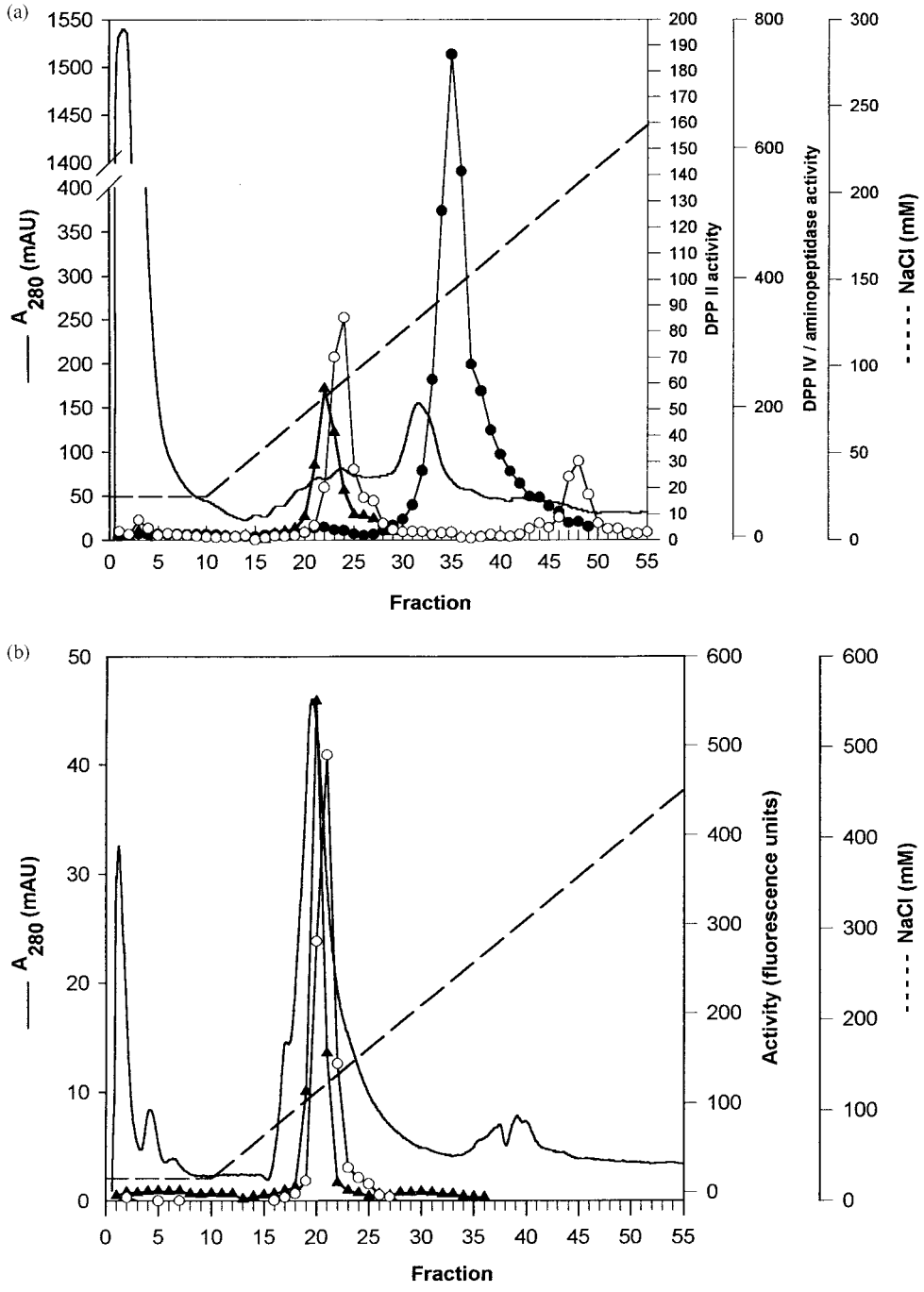

Fig. 1. Chromatographic separation of the ammonium sulphate precipitate (30–50% fraction) on HPLC strong anion exchange column (Resource-Q 1 ml) for: (a) 25–260 mM NaCl linear gradient at pH 6.5 and (b) 25–500 mM NaCl linear gradient at pH 7.0 (–) $A_{280\ nm}$; ▲ DPP II activity; o aminopeptidase activity;● DPP IV activity.

observed something similar in the purification of rat brain DPP II, though in that case DPP II activity was higher and overlapped with a lower aminopeptidase activity peak. In our case, data of specific activity revealed an important increase in the purification fold after the second chromatography (Table 1). In order to avoid any possible interference, 0.04 mM of bestatin was included in the reaction buffer when using DPP II active fractions in the characterisation studies. Once purified, DPP II proved to be stable during more than 3 months at 4°C (data not shown).

3.2. Molecular mass

The SDS-PAGE corresponding to the different stages of the purification process is shown in Fig. 2. The last

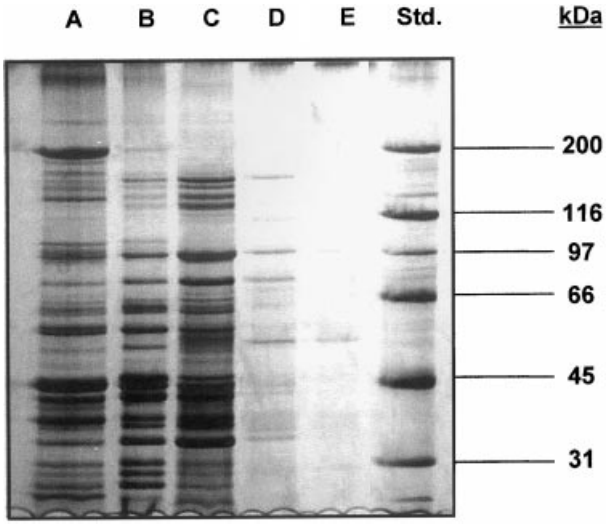

Fig. 2. 10% SDS-PAGE with silver staining of the different purification steps for DPP II from porcine skeletal muscle: (A) crude extract; (B) soluble extract; (C) 30–50% ammonium sulphate fractionation; (D) first anion exchange separation, pH 6.5; (E) second anion exchange separation, pH 7.0, and Std, protein standards).

step showed a protein band with an electrophoretic mobility corresponding to a Mr of 58 kDa, attributable to the subunits conforming DPP II (see Fig. 2, lane E). Regarding the dimeric structure generally proposed for this enzyme, DPP II from porcine skeletal muscle would display a native Mr around 116 kDa. This result agrees with the general characteristics reported for DPP II isolated from other sources like rat (Fukasawa et al., 1983) and human kidney (Sakai et al., 1987), rat brain (Mentlein & Struckhoff, 1989) or porcine ovary (Eisenhauer & McDonald, 1986), where the Mr value of the subunits was reported to be 52–64 kDa, with a Mr value for the native enzyme of 110–130 kDa. A slightly lower Mr value of 96–98 kDa was reported for DPP II from porcine spleen (Lynn, 1991). One exception to the general proposed dimeric structure for DPP II was proposed by Huang et al. (1996) for DPP II isolated from porcine seminal plasma. These authors obtained a native Mr of 185–200 kDa and a Mr value of 61 kDa on SDS-PAGE, so they proposed a structure composed of three subunits.

3.3. pH and temperature

Maximum DPP II activity was observed at pH 5.5 (data not shown), as previously reported (Eisenhauer McDonald, 1986; Fukasawa et al., 1983; Imai et al., 1983; Mentlein & Struckhoff, 1989). This pH value coincided with that reported for DPP I from porcine muscle (Sentandreu & Toldrá, in press), according with the lysosomal origin of these two dipeptidyl peptidases.

DPP II activity increased with incubation temperature until 65°C, where the enzyme reached maximum activity (data not shown). Higher temperatures resulted in an important decrease of the activity. In addition, DPP II showed a low percentage of activity at 5 and 15°C (1 and 3% of optimum temperature, respectively), as was the case of dipeptidyl peptidase III from porcine muscle (Sentandreu & Toldrá, 1998).

3.4. Effect of chemical agents

The sulphonyl fluorides, PMSF and Pefabloc SC, exerted an important inhibition of DPP II activity (Table 2), which is in accordance with the classification of this enzyme as a serine peptidase (Mentlein & Struckhoff, 1989; Rawlings & Barrett, 1996). The degree of inhibition due to the presence of PMSF is comparable to that obtained for DPP II from rat brain (Imai et al., 1983; Mentlein & Struckhoff, 1989) or porcine ovary (Eisenhauer & McDonald, 1986). For DPP II isolated from porcine seminal plasma (Huang et al., 1996) or rat kidney (Fukasawa et al., 1983), lower inhibition degrees in the presence of PMSF were observed. For DPP II isolated from human kidney different inhibition degrees have been reported, since Sakai et al. (1987) observed a 85% inhibition at 1 mM PMSF, while Mantle (1991) observed for the same enzyme only a 50% inhibition at a concentration of 2 mM.

On the other hand, 3,4-DCI did not significantly inhibit DPP II activity (see Table 2), despite to be considered a reference inhibitor of the serine peptidase class (Barrett, 1994; Harper, Hemmi & Powers, 1985). The effect of this inhibitor on DPP II had not been previously reported in the literature. The low effectiveness of 3,4-DCI in suppressing DPP II activity could evidence that it does not inhibit to all serine peptidases. In that case, the use of this substance as reference inhibitor of serine peptidases should be taken with some care. DPP II seems to possess a catalytic triad integrated by Ser/Asp/His, different to that of the peptidases belonging to the classical families of serine peptidases like the chymotrypsin family, having a catalytic triad integrated by His/Asp/Ser (Rawlings & Barrett, 1993). Deeper

Table 2
Effect of different peptidase inhibitors on the activity of porcine muscle DPP II

Substance	0.05 mM	0.5 mM
Control[a]	100	100
PMSF	72	27
Pefabloc-SC	75	24
3,4-DCI	86	89
p-CMB	83	78
E-64	74	77
Iodoacetic acid	94	89
Puromycin	91	85
Bestatin	94	80
Leupeptin	95	99
Diprotin A	66	20
Pepstatin A	101	94

[a] Control activity, with no added compound, was taken as 100%.

investigation is necessary in order to conclude if 3,4-DCI is a general inhibitor of serine peptidases or only of some families with a certain catalytic triad. The resistance of subtilisin (EC 3.4.21.62) to the presence of 3,4-DCI (Powers & Kam, 1994) would support this idea, since this enzyme gives name to a serine peptidase family possessing a catalytic triad integrated by Asp/His/Ser, also different to the classical one (Rawlings & Barrett, 1989). Contrary to 3,4-DCI, the presence of the 7-amino-4-methyl-coumarin group (AMC) at 0.05 and 0.1 mM concentrations exerted an inhibition of 53 and 83% of DPP II activity, respectively.

The cysteine peptidase inhibitors E-64, *p*-CMB and iodoacetic acid did not exert a significant inhibition on the activity of DPP II (Table 2), indicating that there is no –SH group involved in the catalytic mechanism of this peptidase. From the rest of the tested compounds shown in Table 2, only diprotin A (Ile-Pro-Ile) exerted a significant inhibition on muscle DPP II, inhibiting 80% of its activity at 0.5 mM. This apparent competitive inhibition of diprotin A seems to be an interesting kinetic artifact which is due to the substrate-like structure of tripeptides with a penultimate proline residue. The results of the present work can be considered an evidence of this phenomenon, previously observed and commented for DPP IV (Rahfeld, Schierhorn, Hartrodt, Neubert & Heins, 1991). This is logical because these two enzymes have common specificities. So, DPP II proved to hydrolyse two substrates at the same time, diprotin A and Gly-Pro-AMC, so that one of them acted as the competitive inhibitor for the other substrate.

To determine the effect of bestatin on DPP II activity, arphamenin B was used as an alternative inhibitor, since it efficiently inhibits the activity of muscle arginyl aminopeptidase (Flores et al., 1993). Bestatin did not exert significant inhibition of DPP II activity (only 6% inhibition at 0.05 mM), and made possible the addition of this compound in the DPP II reaction buffer with the aim to inhibit the possible interference of any aminopeptidase activity. According with the effect of cysteine peptidase inhibitors, the presence of reducing agents in the reaction mixture did not affect the enzyme activity. The same was observed with the chelating agents EDTA and EGTA (Table 3). So, it is concluded that DPP II from porcine muscle does not need the presence of cations for its peptidase activity. However, the presence of *o*-phenantroline in the mixture caused a confusing inhibitory effect that might be related with the ability of this compound to link, in a non specific way, with the active site of some enzymes different to metallopeptidases (Barrett, 1994). The presence of ammonium sulphate in the reaction mixture at 40, 75, 150 and 380 mM caused a progressive 46, 62, 70 and 83% reversible inhibition of DPP II activity, respectively, as happened with porcine muscle DPP I and DPP III (Sentandreu & Toldrá, 1998, in press). Dialysis after fractionation with ammonium sulphate is then required during the purification procedure in order to recover maximal enzyme activity.

Table 3
Effect of reducing and chelating agents on DPP II activity

Substance	1 mM	5 mM
Control[a]	100	100
DTT	105	100
β-mercaptoethanol	108	102
Cysteine	111	100
EDTA	112	108
EGTA	106	97
o-Phenantroline	96	56

[a] The activity without addition of any agent was taken as 100%.

3.5. Effect of cations

To determine if DPP II from porcine skeletal muscle can be inhibited in the presence of cations, as was reported for DPP II from pituitary (McDonald, Reilly, et al. 1968), human serum (Vanha-Perttula & Kalliomäki, 1973) or dental pulp (McDonald & Schwabe, 1980), the activity of porcine muscle DPP II was assayed against increasing concentrations of LiCl, NaCl and KCl. The results obtained (shown in Fig. 3) confirmed that the cations of the salts are really responsible of the inhibition. The inhibitory effect of the three salts is low, and more or less of the same intensity, at concentrations up to 10 mM. From this value, the inhibition increased according to the salt concentration, being different and proportional to the size of the cation. Fig. 3 shows that KCl is the salt with a stronger inhibitory action (more than 50% inhibition at 100 mM), while LiCl only exerted a moderate inhbition. According with its atomic radium, NaCl displayed an intermediate inhibition between LiCl and KCl. The inhibition results

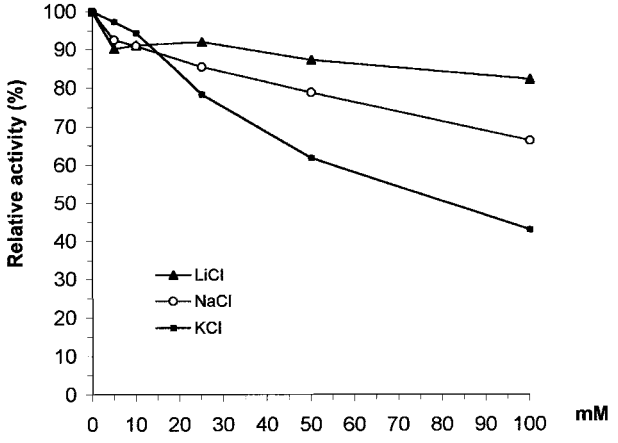

Fig. 3. Influence of different alkaline metal salts on the activity of DPP II from pork muscle. Activity with no salt added was taken as 100% value.

obtained with these three salts on muscle DPP II are comparable to those obtained for DPP II purified from brain (Imai et al., 1983).

Different divalent cations were tested in their capacity to inhibit the activity of porcine muscle DPP II. Of the nine assayed cations (Fig. 4) only three of them, Cu^{2+}, Fe^{2+} and Hg^{2+}, performed significant inhibition of the enzyme activity. Cu^{2+} exerted the strongest effect, 85% inhibition at 0.5 mM, whereas Fe^{2+} and Hg^{2+} showed a more moderate effect, 52 and 38% inhibition at 0.5 mM, respectively. This is comparable to data obtained for DPP II isolated from other sources (Huang et al., 1996; Lynn, 1991; Mantle, 1991). DPP II from rat brain was also inhibited by Cu^{2+}, though in a lower degree (Imai et al., 1983). On the other hand, DPP II from rat kidney (Fukasawa et al., 1983) and porcine ovary (Eisenhauer & McDonald, 1986) were completely inhibited by the presence of 1 mM Hg^{2+}. Zn^{2+} caused low inhibition, irrespective of its concentration (Fig. 4). The rest of the assayed cations, Ca^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+} and Mg^{2+}, did not affect the peptidase activity of DPP II. However, Co^{2+} and Cd^{2+} were inhibitors of DPP II from human kidney (Mantle, 1991).

3.6. Substrate specificity

The affinity of porcine muscle DPP II against several synthetic substrates with different N-terminal peptide sequences is shown in Table 4. DPP II from porcine skeletal muscle preferentially hydrolysed the synthetic substrates of type X-Pro-, better than those of type X-Ala-, both in fluorescent (dipeptidyl-AMC) and colorimetric (dipeptidyl-pNA) derivatives. This is in agreement with the observations of Eisenhauer and McDonald (1986) about the different substrate specificity of DPP II from porcine tissues, in relation with other species where the enzyme better hydrolyzes Lys-Ala- and Ala-Ala- derivatives (Fukasawa et al., 1983; Imai et al., 1983; Mentlein & Struckhoff, 1989; Sakai et al., 1987). The hydrolysis of Lys-Ala-AMC by porcine muscle DPP II, the substrate normally employed in determining DPP II activity in muscle extracts (Blanchard, Ellis, Maltrin, Falkovs, Harris & Mantle, 1993; Blanchard & Mantle, 1996; Toldrá, Flores, Aristoy, Virgili & Parolari, 1996) represented only 37% of the hydrolysis obtained with Gly-Pro-AMC (Table 4), the substrate normally employed to determine DPP IV activity (McDonald & Barrett, 1986). Instead of using traditional Lys-Ala-derivatives, it would be more appropriate the use of X-Pro- substrates like Gly-Pro-AMC, Gly-Pro-pNA or Arg-Pro-pNA, to determine DPP II activity. Substrates containing Pro in *N*-penultimate position will provide maximum sensibility and higher resistance to the possible sequential hydrolysis by aminopeptidases, especially if pH is kept below 5.5 to avoid the interfering action of DPP IV.

Gly-Arg-AMC and Arg-Arg-AMC, normally employed as substrates for DPP I and DPP III, respectively (Smyth & O'Cuinn, 1994), proved to be resistant to DPP II action. Ala-Arg-AMC, Gly-Gly-AMC, Pro-AMC and Ala-Ala-Phe-AMC were also resistant to DPP II action (Table 4), in agreement with the general characteristics reported for this peptidase (McDonald & Barrett, 1986; Mentlein, 1988). The *p*-nitroanilide derivatives Gly-Pro-pNA, Arg-Pro-pNA and Ala-Ala-pNA were also assayed, observing that DPP II preferentially hydrolysed Gly-Pro-pNA and Arg-Pro-pNA in relation with Ala-Ala-pNA. In this case, the differences in the hydrolysis rates were lower compared with that of the AMC derivatives, because Ala-Ala-pNA represented 85% of Gly-Pro-pNA hydrolysing activity (Table 4).

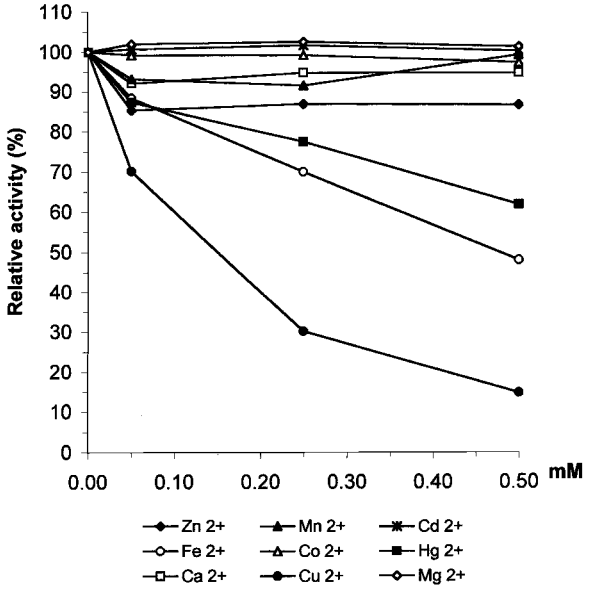

Fig. 4. Effect of divalent cations on the activity of muscle DPP II. Activity with no cation added was taken as 100%.

Table 4
Specificity of porcine muscle DPP II on various fluorescent and colorimetric synthetic substrates

Substrate	Activity (U/ml)	Relative activity[a] (%)
Gly-Pro-AMC	0.021	100
Lys-Ala-AMC	0.08	37.7
Gly-Arg-AMC	0.0	0.0
Arg-Arg-AMC	0.0	0.0
Ala-Arg-AMC	0.0	0.0
Gly-Gly-AMC	0.0	0.0
Pro-AMC	0.0	0.0
Ala-Ala-Phe-AMC	0.0	0.0
Gly-Pro-pNA	0.116	561.6
Arg-Pro-pNA	0.122	591
Ala-Ala-pNA	0.099	480.9

[a] Expressed as a percentage of Gly-Pro-AMC hydrolysing activity, which was taken as value 100%

It was clearly observed that porcine muscle DPP II hydrolysed more efficiently *p*-nitroanilide substrates in relation with AMC derivatives, as shown in Table 4. For instance, the same N-terminal sequence (Gly-Pro-) was hydrolysed 5.6 times more in the *p*-nitroanilide derivative compared with the respective fluorescent substrate. This greater activity on *p*-nitroanilide derivatives would make feasible the future development of commercial kits for the determination of DPP II activity, together with other peptidases, in the field of meat industry (Flores, Sentandreu & Toldrá, 1999).

In addition to synthetic substrates, the ability of DPP II to hydrolyze different peptide sequences was studied. The best hydrolysed sequences by porcine muscle DPP II were tripeptides (see Table 5), coinciding with the general characteristics reported for this enzyme (McDonald, Leibach et al., 1968; McDonald & Schwabe, 1977). The tripeptide Met-Ala-Ser was the peptide better hydrolysed, even better than Gly-Pro-Ala, though the differences were minimal. It is, however, a remarkable aspect because the preference for substrates of type X-Pro- is not maintained, contrary to synthetic derivatives (Table 4). Gly-Pro-Ala would constitute the basis of a model collagen chain, and its hydrolysis by porcine muscle DPP II supports the idea of McDonald, Hoisington and Eisenhauer, (1985) and Eisenhauer and McDonald (1986) about the existence of a coupled mechanism between tripeptidyl peptidase I (EC 3.4.14.9) and DPP II from porcine ovary capable to degrade collagen. Mentlein and Struckhoff (1989) also proposed the existence of this enzymatic tool in rat brain cells, so that the coupled action of the two peptidases seems to be basic inside lysosomes. Diprotin A (Ile-Pro-Ile) has been considered until recently as a reversible inhibitor of DPP IV (Beynon & Salvesen, 1989; Umezawa, Aoyagi, Ogawa, Naganawa, Hamada & Takeuchi, 1984), and a similar apparent action on DPP II has been observed in this work (see Table 2). However, diprotin A acts as a substrate of both DPP II (see Table 5) and DPP IV, as observed with other tripeptides of analogous structure (Rahfeld et al., 1991).

The influence of the chain length was studied by comparing the hydrolysis rates of peptides integrated by three, four or five alanine amino acids. The results obtained clearly showed that tripeptides are the best substrates for muscle DPP II. The enzyme was also capable to hydrolyze tetrapeptides, though in a lower degree (Table 5), according to the observations of Fukasawa et al. (1983) for DPP II isolated from rat kidney. The tetrapeptide Gly-Pro-Gly-Gly was hydrolysed in a very low rate, whereas Arg-Pro-Lys-Pro was resistant to hydrolysis, despite it contained the N-terminal dipeptide sequence better hydrolysed between the assayed synthetic substrates (Table 4). Those sequences containing proline in N-antepenultimate position (Arg-Pro-Pro-Gly-Phe and Val-Ala-Pro-Gly) were also resistant to DPP II action, despite it has been reported that DPP II from other sources was able to hydrolyse peptides with proline in *N*-antepenultimate position, including Arg-Pro-Pro-Gly-Phe (Mentlein & Struckhoff, 1989). Further research is necessary to elucidate if this different specificity is due to organ differences, skeletal muscle, or differences in the porcine species. Penta-alanine was also resistant to hydrolysis.

The present work contributes to improve knowledge about the proteolytic chain in relation with development of the characteristic flavor of processed meat products. In addition, the knowledge of DPP II biochemical properties allows a reproducible, sensible and accurate determination of the enzyme activity directly in muscle extracts, improving the potential of this enzyme determination to be used as predictor of meat quality, or during the processing of meat.

Table 5
Specificity of porcine muscle DPP II on different N-terminal peptide sequences

Peptide	Relative activity[a](%)
Met-Ala-Ser	100
Gly-Pro-Ala	95.6
Ala-Ala-Ala	54.0
Ile-Pro-Ile (Diprotin A)	39.7
Ala-Ala-Ala-Ala	38.7
Gly-Pro-Gly-Gly	15.6
Arg-Pro-Lys-Pro (substance P 1-4)	0
Arg-Pro-Pro-Gly-Phe (Bradikinin 1-5)	0
Val-Ala-Pro-Gly	0
Ala-Ala-Ala-Ala-Ala	0

[a] Expressed as a percentage of activity against Met-Ala-Ser, which was taken as 100%.

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