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Dipeptidyl peptidase IV from porcine skeletal muscle: purification and biochemical properties

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Abstract

Dipeptidyl peptidase IV (EC 3. 4. 14. 5) from porcine skeletal muscle has been purified to homogeneity by selective protein fractionation with ammonium sulfate and HPLC separations with strong anion-exchange chromatography. Pure DPP IV showed a single band with Mr about 70 kDa by SDS-PAGE and optimum activity at pH 8.0 and 45°C. Substrates best hydrolyzed were those containing a proline residue in the penultimate position at the N-terminal, but it was also possible to hydrolyze (in lower amounts), X-Ala- synthetic and peptide substrates. The presence of diprotin A, puromycin, Co^{2+} and Fe^{2+} considerably inhibited DPP IV activity. A contribution of DPP IV action to the total proteolytic activity occurring in postmortem muscle during meat storage and ripening of meat products is feasible and could contribute to flavor generation in those products.

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

1. Introduction

Dipeptidyl peptidases (DPP) constitute a group of enzymes capable of releasing different dipeptide sequences from the N-termini of peptides. Four different DPP activities have been described in mammalian tissues, these being DPP I (EC 3. 4. 14. 1) and DPP II (EC 3. 4. 14. 2) located in the lysosomes, DPP III (EC 3. 4. 14. 4) in the cytosol and DPP IV (EC 3. 4. 14. 5) in the cell membrane (McDonald & Barrett, 1986). Although they have been purified and studied for medical purposes (McDonald & Schwabe, 1977), little is known about the role of these enzymes in the biochemistry of postmortem muscle during meat storage and especially during the processing of meat products, such as Spanish dry-cured ham (Toldrá & Flores, 1998). Changes occurring during meat storage are mainly related to the degradation of the myofibrillar structure by muscle endopeptidases, giving rise to an increase in meat tenderness (Goll, 1991; Valin & Ouali, 1992). The polypeptides generated during this protein degradation would be further degraded to smaller peptides and free amino acids in subsequent steps, especially during the ripening of meat products. DPP are one of the peptidase groups supposed to be implicated in this process, giving rise to a dipeptide generation that could contribute, together with free amino acids and other non-volatile compounds, to flavour development in processed meat products (Toldrá & Flores, 1998, 2000). The current state-of-the-art in this field prompted us to carry out research in the study of muscle DPP, with the aim of knowing more about the contribution of these enzymes in the proteolytic chain of postmortem muscle.

DPP IV was discovered in 1966 by Hopsu-Havu and Glenner (1966) and it has been known under different names, such as "Dipeptidyl aminopeptidase IV", "Post-proline dipeptidyl aminopeptidase IV", "X-Pro dipeptidyl aminopeptidase" or "Gly-Pro naphthylamidase" (McDonald & Barrett, 1986). DPP IV is present in most vertebrate tissues, and typical sources for its purification and study have been kidney (McNair & Kenny, 1979), intestine (Bella, Erickson, & Kim, 1982), submaxillary gland (Kojima, Hama, Kato, & Nagatsu, 1980), liver (Ikehara, Ogata, & Misumi, 1994) or placenta (Püschel, Mentlein & Heymann, 1982). As with DPP I and DPP II, in cases of muscular dystrophies and polymyositis,

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levels of DPP IV activity are abnormally elevated, indicating that, apart from lysosomes, other cell parts contribute to the hydrolytic potential of diseased muscles (Kar & Pearson, 1978).

Due to its sensitivity to diisopropylfluorophosphate, DPP IV was classified as a serine peptidase. This was later confirmed by numerous sequencing studies of its primary structure by cloning and sequencing of the respective cDNA. The reported catalytic triad (Ser/Asp/His) is characteristic of some families of serine peptidases (Kiyama et al., 1998; Marguet, Bernard, Vivier, Darmoul, Naquet, & Pierres, 1992; Rawlings & Barrett, 1993). In peptide hydrolysis, DPP IV from human placenta has proved able to hydrolyze different bioactive peptides and proteins such as substance P, casomorphin-5, prolactin, aprotinin or chorionic gonadotropin (Nausch, Mentlein, & Heymann, 1990; Püschel et al., 1982). It has also been proved that DPP IV peptidase activity is essential for the digestion of β-casein, which represents 25% of the total protein content in milk, suggesting the important role of DPP IV in the digestion of proline-rich proteins (Heymann & Mentlein, 1986). Previous reports concerning the study of DPP IV in muscle have been limited to the measurement of DPP IV activity directly in muscle soluble extracts (Blanchard & Mantle, 1996; Rosell & Toldrá, 1998), without either purification or study of its biochemical properties.

The present work reports the purification and main biochemical characteristics of DPP IV. This is the first purification of this enzyme from skeletal muscle.

2. Materials and methods

2.1. Materials

Muscles *Longissimus dorsi* and *Biceps femoris*, from 6-month-old Landrace × Large White pigs, were used as enzyme source. Muscles were cut and vacuum-packed between 12 and 24 h postmortem, 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 for 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). The Resource-Q 1 ml anion-exchange column (6.4×30 mm) was purchased from Pharmacia LKB (Uppsala, Sweden).

2.2. Enzyme assays

The standard determination for DPP IV activity was performed by using 0.25 mM of Gly-Pro-AMC as substrate in 50 mM Tris–base buffer, pH 8.0, containing 5 mM DTT. 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. Three replicates were done for each experimental point. DPP II activity was determined by using 0.5 mM of Lys-Ala-AMC as substrate in 50 mM sodium acetate/acetic acid buffer, pH 5.5, containing 0.04 mM bestatin, in the same way as DPP IV. One unit of enzyme activity (U) was defined as the amount of enzyme which hydrolyses 1 μM of substrate per hour at 37°C.

2.3. Purification of DPP IV from porcine skeletal muscle

2.3.1. Enzyme extraction

Unless indicated, all steps were performed at 4°C. Sixteen grams of pork meat, 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 27000 rpm. 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 protein precipitated in the range 30–50% saturation by 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, being further clarified by filtering it 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 milliliters of the filtered protein were injected onto the Resource-Q 1 ml anion-exchange column, 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 the same buffer for 50 min. Fifty fractions of 1 ml were collected and assayed for both DPP IV and DPP II activities. The six fractions with maximal DPP IV activity from a total of

two separations, carried out under the conditions described above, were pooled and dialysed against 10 mM Tris–HCl buffer, pH 7.0, containing 5 mM β-mercaptoethanol and 50 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 injected again onto the anion exchange column, equilibrated now with 10 ml of 10 mM Tris–HCl buffer, pH 7.0, containing 50 mM NaCl. Elution was performed by developing a 10 min isocratic gradient with the equilibration buffer, then a linear salt gradient from 50 to 500 mM NaCl in the same buffer for 50 min at a flow rate of 1 ml/min. 33 fractions of 1 ml were collected and assayed for DPP IV activity. Fractions containing maximum DPP IV activity were pooled and stored at 4°C, constituting the enzyme solution for further studies.

2.4. Determination of protein concentration

Protein concentrations in extracts and eluted fractions was determined according to Smith et al.'s (1985) method using bicinchoninic acid as reagent and bovine serum albumin as standard. The eluted fractions from the chromatographic separations were also monitored at $\lambda = 280$ nm.

2.5. SDS-PAGE

The monitoring of each purification step, and with the determination of purity and molecular mass of DPP IV, were achieved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), using 10% gels and silver staining. Standard proteins, myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme and aprotinin, were simultaneously run for molecular mass estimation.

2.6. Optimal pH and temperature

The activity of porcine muscle DPP IV in the hydrolysis of Gly-Pro-AMC was assayed over the pH range 4.5 to 9.5 by using 100 mM citric acid/200 mM disodium phosphate buffer (pH 4.5–8.0) and 50 mM sodium tetraborate/100 mM monopotassium phosphate buffer (pH 8.5–9.5). Activity at each pH value was determined through the standard enzyme assay and expressed as percentage of activity at optimum pH.

The effect of temperature on DPP IV activity against Gly-Pro-AMC was studied over the range 5–60°C. The substrate solution (250 μl) was previously equilibrated at the desired pH in Eppendorf tubes, and then the reaction initiated by the addition of the enzyme solution (50 μl). After incubation for different times, at each temperature, 75 μl of 0.6 M acetic acid solution were added in order 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 agents on DPP IV activity was studied through the standard enzyme assay. The compounds and concentrations employed were: *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)-benzosulfonyl-fluoride hydrochloride (Pefabloc-SC), puromycin, bestatin, leupeptin, diprotin A, and pepstatin A: 0.05 and 0.5 mM; dithiotreitol (DTT), β-mercaptoethanol (β-ME) and cysteine: 1–10 mM; EDTA, EGTA and *o*-phenanthroline: 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; ammonium sulfate: 40–380 mM (0.5–5%). Activity, at each concentration assayed, was referred to controls, which were simultaneously measured in the absence of any chemical agent.

2.8. Substrate specificity

The activity of porcine muscle DPP IV was studied, through the standard enzyme assay, by using the synthetic substrates Gly-Pro-AMC, Lys-Ala-AMC,

Table 1
Purification of DPP IV from porcine skeletal muscle. Enzyme activity was expressed as μmol of released AMC per hour at 37°C[a]

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1897	15.5	0.008	100	1
Soluble extract	636	11.1	0.018	72.0	2.15
Ammonium sulfate fractionation	71.6	7.29	0.102	47.1	12.5
Ion-exchange chromatography					
(A) 25–260 mM NaCl; pH 6.5	1.32	1.51	1.15	9.75	141
(B) 25–500 mM NaCl; pH 7.0	0.14	0.28	2.0	1.8	245

[a] Enzyme assays and protein determinations were performed as described in Materials and methods.

Table 2
Effect of different chemical agents on the activity of porcine muscle DPP IV[a]

Substance	0.05 mM	0.5 mM
Control	100	100
PMSF	86	88
Pefabloc-SC	97	94
3,4-DCI	95	91
p-CMB	86	88
E-64	89	83
Iodoacetic acid	98	96
Puromycin	20	20
Bestatin	94	106
Leupeptin	98	103
Diprotin A	58	16
Pepstatin A	99	109
Chelating agents	1 mM	5 mM
EDTA	120	117
EGTA	124	113
o-Phenanthroline	111	66

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

Table 3
Specificity of porcine muscle DPP IV on various fluorescent and colorimetric synthetic substrates

Substrate	Activity (U/mL)	Relative activity[a] (%)
Gly-Pro-AMC	0.044	100
Lys-Ala-AMC	0.006	13.3
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	0.063	144
Arg-Pro-pNA	0.095	216
Ala-Ala-pNA	0.003	29.2

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

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, at final concentrations of 0.5 mM in the reaction mixture.

In addition, DPP IV activity was assayed against different peptide substrates (see Table 4 below). 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 reported as a percentage of the hydrolysis of Arg-Pro-Lys-Pro, which was given a value of 100%.

Table 4
Specificity of porcine muscle DPP IV on different N-terminal peptide sequences

Peptide	Relative activity[a] (%)
Arg-Pro-Lys-Pro (substance P 1–4)	100
Arg-Pro-Pro-Gly-Phe (Bradikinin 1-5)	43
Met-Ala-Ser	17.9
Gly-Pro-Gly-Gly	10
Ile-Pro-Ile (Diprotin A)	3.9
Ala-Ala-Ala	0
Ala-Ala-Ala-Ala	0
Ala-Ala-Ala-Ala-Ala	0
Gly-Pro-Ala	0
Val-Ala-Pro-Gly	0

[a] Expressed as a percentage of activity against Arg-Pro-Lys-Pro, which was taken as 100%.

3. Results and discussion

3.1. Purification of the enzyme

The final enzyme preparation of DPP IV from porcine muscle gave a specific activity of 2.0 U/mg, which is 245-fold higher than in the crude extract and similar to that obtained by Brownlees, Williams, Brennan, and Halton (1992) in the purification of DPP IV from bovine kidney. However, higher purification degrees, about 1000–1300-fold, were obtained for DPP IV isolated from rat liver (Hartel, Hanski, Kreisel, Hoffmann & Mauck, 1987), human submaxillary gland (Kojima et al., 1980) and rat intestine (Erickson, Bella, Brophy, Kobata, & Kim, 1982). Even higher were purification levels obtained for DPP IV from human placenta (Püschel et al., 1982), lymphocytes (De Meester, Vanhoof, Hendriks, Demuth, & Yaron, 1992) and porcine seminal plasma (Ohkubo, Huang, Ochiai, Takagaki, & Kani, 1994), reaching values of 3000-, 5300- and 5700-fold, respectively. Our final yield was 1.8% (Table 1), similar to that obtained in the purification of DPP II from porcine skeletal muscle (Sentandreu & Toldrá, 2000). As shown in Fig. 1a, DPP IV was efficiently separated from DPP II activity after the first chromatography on the anion-exchange column at pH 6.5. DPP IV and DPP II are the two dipeptidylpeptidases capable of releasing dipeptides from the N-termini of peptides with a proline residue in the penultimate position (IUBMB, 1992). Because of their common specificity,

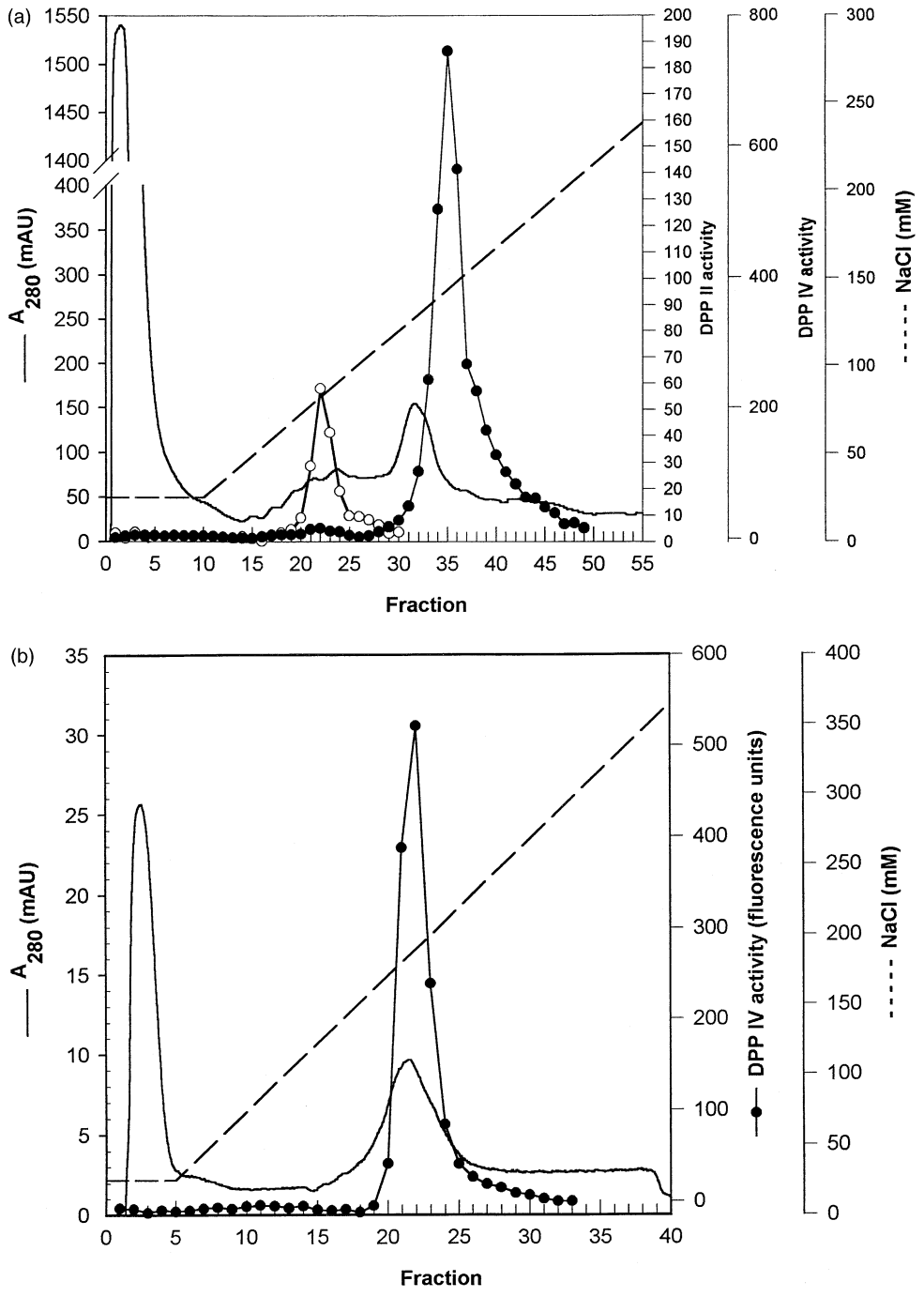

Fig. 1. Separation of the 30–50% ammonium sulfate precipitate on an HPLC anion-exchange column (Resource-Q 1 mL) with: (a) 25–260 mM NaCl linear gradient at pH 6.5 and (b) 50–500 mM NaCl linear gradient at pH 7.0; (___): $A_{280\ nm}$; (●): DPP IV activity; (○): DPP II activity.

the separation of DPP IV and DPP II from each other is necessary for an adequate characterisation of both peptidases. Apart from DPP II, we also observed that, during this first chromatography, DPP IV was efficiently separated from fractions having aminopeptidase activity (data not shown). DPP IV eluted at 143 mM NaCl, the maximum activity being located in fractions 34-35-36 (Fig. 1aA). The second chromatographic separation was carried out at pH 7.0 by developing a steeper salt gradient in order to avoid the dispersion of DPP IV activity and tailing. The result was the elution of a well-defined DPP IV activity peak, coincident with a protein peak eluting at 195 mM NaCl (see Fig. 1b). Fractions with maximal DPP IV activity were pooled together and stored at 4°C, where DPP IV was stable for up to 4 weeks, constituting the enzyme preparation for the characterisation studies. Longer storage caused a gradual loss of activity (data not shown).

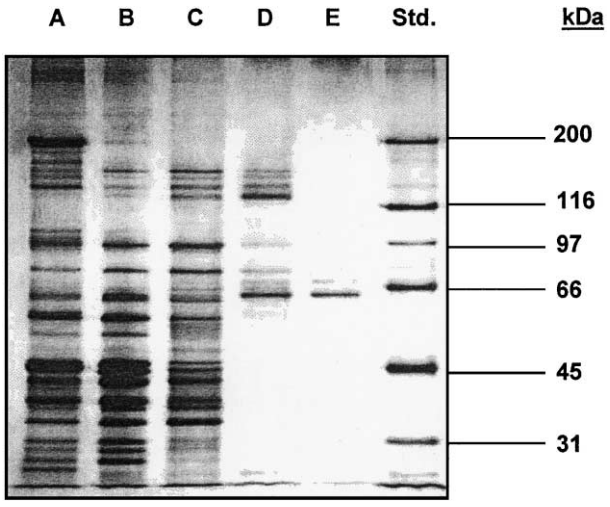

Fig. 2. 10% SDS-PAGE of the different purification steps for DPP IV from porcine skeletal muscle. Silver staining: (A) crude extract; (B) soluble extract; (C) ammonium sulfate fractionation; (D) first anion-exchange separation, pH 6.5; (E) second anion-exchange separation, pH 7.0 and (Std) protein standards.

During our purification procedure, DPP IV activity was monitored by using the substrate Gly-Pro-AMC without the addition of any aminopeptidase inhibitor in the assay mixture, since Gly and Pro are not hydrolysed, or at a very low rate (5% or less), by the main muscle aminopeptidases such as alanyl (Flores, Aristoy, & Toldrá, 1996) and arginyl aminopeptidases (Flores et al., 1993). So, the possible sequential hydrolysis of these two amino acids from Gly-Pro-AMC by aminopeptidase activity was not a problem, especially since, after the first chromatographic separation, DPP IV activity was completely separated from aminopeptidase activity. To confirm this, fractions from this separation containing maximum DPP IV activity (fractions 34–36) were assayed for the hydrolysis of Gly-Pro-AMC in the presence and absence of bestatin. The results obtained showed that differences among the two determinations were negligible (data not shown).

3.2. Molecular mass

Polyacrylamide gel electrophoresis, under denaturing and reducing conditions (SDS-PAGE), showed the presence of one main protein band after the last purification step, proving enzyme homogeneity, as can be seen in Fig. 2 (lane E). This band had an electrophoretic mobility corresponding to a M_r of 70 kDa, attributed to the DPP IV subunits. This value is in contrast to data obtained for DPP IV isolated from different mammalian tissues because in all cases, the M_r value obtained for each of the two *N*-glycosylated mature subunits constituting DPP IV was 100 kDa or higher, usually around 110 kDa (Hartel et al., 1987; Ikehara et al., 1994; Iwaki-Egawa, Watanabe, Kikuya, & Fujimoto, 1998; Kojima et al., 1980; Kyouden, Himeno, Ishikawa, Ohsumi, & Kato, 1992; Marguet et al., 1992; Püschel et al., 1982). A dimeric structure, generally proposed for this enzyme (McDonald & Barrett, 1986), porcine muscle DPP IV would display a native M_r at about 140–150 kDa, lower than the values generally obtained for DPP IV isolated from other sources (McDonald & Barrett; McDonald & Schwabe, 1977). Those differences in the M_r value are possibly due to the different glycosilation degrees of the polypeptide chain. The results of the present work are more consistent with the characteristics of DPP IV isolated from some microorganisms. Thus, DPP IV from *Flavobacterium meningosepticum* was described as an enzyme composed of two subunits of 75 kDa (Yoshimoto & Tsuru, 1982). For DPP IV, purified from *Lactobacillus helveticus*, the size was reported to be 72 kDa (Khalid & Marth, 1989), whereas, for this enzyme isolated from *Lactobacillus casei ssp. casei* LLG, a value of 79 kDa was described (Habibi-Najafi & Lee, 1994), though authors suggested a monomeric structure in these two cases. Some studies carried out in mammalian cells have also reported an enzyme structure other than the dimeric structure generally established for DPP IV (McDonald & Barrett). The enzyme from porcine seminal plasma was reported to have a native molecular weight of 310 kDa, with a M_r of 105 kDa for each of the subunits, suggesting the existence of a trimeric structure (Ohkubo et al., 1994). Similar results were reported for DPP IV from bovine kidney (Brownlees et al., 1992), whereas DPP IV from human fibroblasts was described as an enzyme with an M_r of 400 kDa and composed of subunits of different size (Saison, Verlinden, Van Leuven, Cassiman, & Van Den Berghe, 1983).

3.3. pH and temperature

DPP IV from porcine muscle displayed maximum activity at pH 7.0–8.0, in agreement with characteristics generally reported for this enzyme (McDonald & Schwabe, 1977; Mentlein, 1988). DPP IV retained an important percentage of activity over the pH range 6.5–8.5, and at pH 6.0 the enzyme retained more than 20% of maximum activity (data not shown).

Optimum temperature for muscle DPP IV was reached at 45°C. Higher temperatures resulted in a rapid decrease of the enzyme activity. At temperatures of 5 and 15°C, DPP IV retained an important percentage (20%) of maximum activity (data not shown).

3.4. Effect of chemical agents on DPP IV activity

Of the assayed serine peptidase inhibitors, both the sulphonyl fluorides, PMSF and Pefabloc-SC, and 3,4-DCI showed low effectiveness in the suppression of pork muscle DPP IV activity (Table 2). This is in accordance with previous work reporting the ineffectiveness of the

serine peptidase inhibitors, other than diisopropylfluorophosphate (DFP), in the inhibition of DPP IV activity (Ikehara et al., 1994; Mantle, 1991; Ohkubo et al., 1994). However, it was precisely due to its sensitivity to DFP that DPP IV was classified as a serine peptidase (McDonald & Barrett, 1986; Rawlings & Barrett, 1993), confirmed later with the numerous sequencing studies of its primary structure (see Introduction). The low effectiveness of 3,4-DCI in the suppression of DPP IV activity is an interesting result in view of the fact that this compound is considered to be a reference inhibitor of the serine peptidase class (Barrett, 1994; Harper, Hemmi, & Powers, 1985). 3,4-DCI was previously assayed on DPP IV from human lymphocytes (De Meester et al., 1992), showing in this case, 50% inhibition at 1 mM concentration. Like DPP IV, it has also been observed that porcine muscle DPP II was unaffected by the presence of 3,4-DCI (Sentandreu, 2000). DPP II has been also classified as a serine peptidase (Rawlings & Barrett, 1996). It is interesting to note that both enzymes, DPP II and DPP IV, seem to possess the same catalytic triad Ser/Asp/His, but different from that of the enzymes belonging to the classical serine peptidase families, eg. the chymotrypsin family, which possess the catalytic triad His/Asp/Ser (Rawlings & Barrett, 1993). From the present observations, deeper research is necessary in order to conclude whether 3,4-DCI is really a general inhibitor of all serine peptidases or only of some families with a specific catalytic triad. The high sensitivity of DPP I and DPP III against 3,4-DCI (Sentandreu) suggests that this compound may not be a specific inhibitor of serine peptidases, since DPP I is a cysteine peptidase (Rawlings & Barrett, 1993) and DPP III was recently classified as a metallopeptidase (Fukasawa, Fukasawa, Kanai, Fujii, Hirose, & Harada, 1998). The presence of the 7-amino-4-methyl-coumarin group (AMC) exerted a 33 and 59% inhibition at the concentrations of 0.05 and 0.1 mM, respectively (data not shown). This would explain why product inhibition was observed in the hydrolysis of Gly-Pro-AMC, and not in the colorimetric equivalent Gly-Pro-pNA, by DPP IV activity (data not shown). As the dipeptide Gly-Pro was released from both substrates, the fluorescent group AMC must be responsible of such inhibition, showing the importance of establishing optimal reaction conditions in relation to enzyme and substrate concentrations, together with appropriate incubation times.

Like the serine peptidase inhibitors assayed, the cysteine peptidase inhibitors E-64, p-CMB and iodoacetic acid exerted little effect on DPP IV activity (Table 2), as previously reported (Ikehara et al., 1994; Ohkubo et al., 1994). Despite this, a remarkable increase of DPP IV activity due to the presence of reducing agents in the assay mixture was observed (Fig. 3). This activation has already been described by Lalu, Lampelo, and Vanha-Perttula (1987), though in a lower degree, for DPP IV from human placenta, although DPP IV has been reported not to require any activator for full activation (Ikehara et al., 1994; McDonald & Schwabe, 1977). From the results obtained, we decided to add 5 mM DTT in the assay buffer to determine maximum activity.

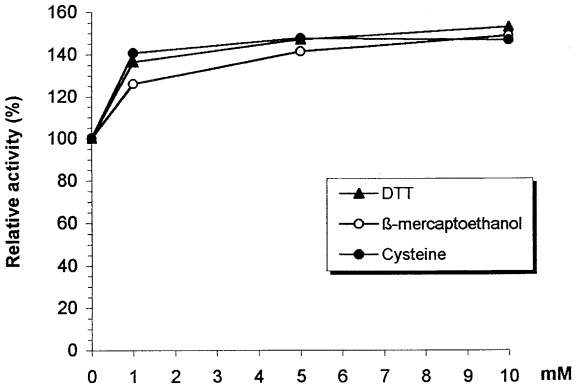

Fig. 3. Effect of reducing agents on the activity of DPP IV from porcine skeletal muscle. Activity without reducing agent was taken as 100%.

The chelating agents EDTA and EGTA did not affect the activity of DPP IV, whereas *o*-phenanthroline induced a slight inhibition (Table 2). The microbial inhibitors pepstatin A, leupeptin and the aminopeptidase inhibitor bestatin did not affect DPP IV activity, as was reported previously (Lalu et al., 1987; Mantle, 1991; McDonald & Barrett, 1986; Ohkubo et al., 1994). In contrast, puromycin, another typical aminopeptidase inhibitor (Flores et al., 1996), suppressed 80% of muscle DPP IV activity at both 0.05 and 0.5 mM. This sensitivity towards puromycin establishes a difference among DPP IV and DPP II from porcine skeletal muscle, because DPP IV was strongly inhibited by the presence of this substance (Table 2), whereas DPP II proved to be rather insensitive to its presence (Sentandreu & Toldrá, 2001). DPP IV from human kidney was also inhibited by the presence of puromycin, though in a lower degree (Mantle), whereas Lalu et al. (1987) did not observe any inhibition in the case of DPP IV from human placenta.

Diprotin A (Ile-Pro-Ile) also strongly suppressed DPP IV activity against the hydrolysis of Gly-Pro-AMC, giving 42 and 84% inhibition at 0.05 and 0.5 mM, respectively (Table 2). As diprotin A has proved to be a substrate of muscle DPP IV (Table 4), the decrease of Gly-Pro-AMC hydrolysis in the presence of this substance (Table 2) was considered to be due to competitive inhibition, in accordance with the observations of Rahfeld, Schierhorn, Hartrodt, Neubert, and Heins (1991) for this enzyme. According with the common substrate specificity between DPP II and DPP IV in the hydrolysis of tripeptides with a penultimate proline residue, this was also observed for DPP II from porcine muscle (Sentandreu, 2001) that also hydrolyzed tripeptides with a penultimate proline residue.

3.5. Effect of divalent cations

The effect of different divalent cations on the activity of pork muscle DPP IV is shown in Fig. 4. Co^{2+} exerted the highest inhibitory effect. This inhibition has previously been observed for DPP IV from porcine seminal plasma (Ohkubo et al., 1994), human lymphocytes (De Meester et al., 1992) and placenta (Lalu et al., 1987). The effect of Fe^{2+} was less marked, yielding only 33% inhibition at the concentration of 0.5 mM. This effect of Fe^{2+} has not been observed previously because its assay has not been usual in the characterisation of this enzyme. In the case of DPP IV from porcine skeletal muscle, we considered the study of this cation to be of interest because of the content of Fe^{2+} in the myoglobin structure, one of the most abundant proteins in muscle (Rosell, Flores, & Toldrá, 1996). The rest of the assayed cations (Zn^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+} and Mg^{2+}) exerted negligible effect on DPP IV activity (Fig. 4), in contrast to data obtained for DPP IV isolated from other sources, since it was generally observed that Zn^{2+}, Hg^{2+} and Cd^{2+} acted as potent inhibitors of DPP IV activity (De Meester et al., 1992; Lalu et al., 1987; Püschel et al., 1982).

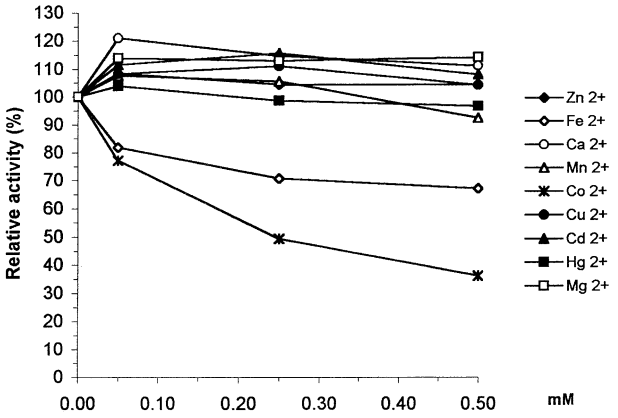

Fig. 4. Effect of divalent cations on the activity of porcine muscle DPP IV. Activity with no cation added was taken as 100%.

Table 5
Kinetic parameters of DPP IV from porcine skeletal muscle on several synthetic substrates

Substrate	K_m (μM)	V_{max} (U/mg)	V_{max}/K_m U/(mg μM)
Gly-Pro-AMC	46.2	1.33	2.88×10^{-2}
Lys-Ala-AMC	100.1	0.196	1.95×10^{-3}
Arg-Pro-pNA	66.1	2.12	3.2×10^{-2}
Gly-Pro-pNA	272	1.85	6.8×10^{-3}

3.6. Substrate specificity

In accordance with the general characteristics described for DPP IV (McDonald & Barrett, 1986; Mentlein, 1988), the enzyme from porcine skeletal muscle hydrolysed both X-Ala- and X-Pro- sequences in different substrates, with a marked preference for the latter, as shown in Table 3. It can be seen that, of all the fluorescent substrates assayed, DPP IV only hydrolysed Gly-Pro-AMC and, more slowly, Lys-Ala-AMC (13% of that for Gly-Pro-AMC). In the hydrolysis of *p*-nitroanilide derivatives, the preference for substrates with Pro in next to the N-terminal residue was maintained. The hydrolysis of Arg-Pro-pNA was 1.5-fold higher than Gly-Pro-pNA hydrolysis (Table 3). This preference was also observed for DPP IV isolated from other mammalian cells (Püschel et al., 1982) and microorganisms (Khalid y Marth, 1990). Values of enzymatic activity for the hydrolysis of Gly-Pro-AMC and Gly-Pro-pNA in Table 3 showed that DPP IV hydrolysed p-nitroanilide substrates more efficiently than AMC derivatives.

The exopeptidase action of DPP IV on different peptide sequences is shown in Table 4. As in the hydrolysis of synthetic substrates, peptides containing Arg-Pro- in N-terminal position, substance P (1–4) and bradikinin (1–5), were hydrolyzed to a greater extent, even if they contained (in the case of bradikinin) a proline residue also in the third position from the N-terminus. This is in contrast to the general substrate specificity characteristics described for this enzyme, because it has been assumed that DPP IV was unable to hydrolyze peptides with Pro in this position (McDonald & Barrett, 1986; Mentlein, 1988). The hydrolysis of these peptides establishes a clear difference in the substrate specificity between porcine muscle DPP II and DPP IV, because DPP II was unable to hydrolyze either of these two peptides (Sentandreu & Toldrá, 2001). DPP IV also hydrolysed the tripeptide Met-Ala-Ser, although in lower amounts, and the hydrolysis of Gly-Pro-Gly-Gly was even lower. Diprotin A (Ile-Pro-Ile), according to Rahfeld et al. (1991) is a substrate of DPP IV, but the hydrolysis rate appeared to be very low (Table 4). This would explain our finding that the presence of diprotin A in the assay mixture gave rise (Table 2) to a decrease in the hydrolysis of Gly-Pro-AMC, displaying an apparent competitive inhibition. Tri-, tetra and penta-alanine were resistant to DPP IV action, as were the peptides Gly-Pro-Ala and Val-Ala-Pro-Gly (Table 2).

3.7. Kinetic studies

The kinetic parameters K_m and V_{max} were determined for the hydrolysis of Gly-Pro-AMC, Lys-Ala-AMC, Arg-Pro-pNA and Gly-Pro-pNA (Table 5). The highest V_{max} was obtained for the colorimetric substrate Arg-Pro-pNA and also the value V_{max}/K_m was the highest for this substrate. This is in agreement with the hydrolysis rates obtained in Table 3. Gly-Pro-pNA was the second most rapidly hydrolyzed substrate. The K_m value in this case, however, was much higher, so the affinity for this substrate is lower than for the corresponding

fluorescent substrate, Gly-Pro-AMC (Table 5). The V_{max} values obtained for these substrates were considerably lower than those obtained for the hydrolysis of different synthetic derivatives by DPP IV isolated from other sources, such as human placenta (Püschel et al., 1982), porcine seminal plasma (Ohkubo et al., 1994) or bovine kidney (Brownlees et al., 1992), where values of 20 μmol mg^{-1} min^{-1} have been reported. In contrast, the values for DPP IV activity, directly assayed in muscle extracts (without any enzyme purification) under reaction conditions extrapolated from other sources, gave similar values (Blanchard, Ellis, Maltrin, Falkous, Harris, & Mantle, 1993; Blanchard & Mantle, 1996). Lys-Ala-AMC, had the lowest V_{max} and affinity (V_{max}/K_m) of the four assayed substrates (Table 3). The K_m values obtained in the present work were similar to data reported for DPP IV isolated from other sources (Brownlees et al., 1992; Ohkubo et al.), with the exception of the value obtained for the hydrolysis of Gly-Pro-pNA, which is more similar to data obtained with Gly-Pro- naphthylamide derivatives in the case of DPP IV from porcine kidney (Hopsu-Havu, Rintola, & Glenner, 1968). Comparing the results obtained in the present work with the kinetic studies carried out for the main muscle aminopeptidases, it can be concluded that the hydrolytic activity of aminopeptidases against fluorescent substrates of the aminoacyl-AMC type (Flores et al., 1993, 1996) is notably higher that hydrolytic activity of dipeptidylpeptidases against dipeptidyl-AMC derivatives. In the concentration range assayed, 15–400 μM, substrate inhibition was only observed for the hydrolysis of Gly-Pro-AMC at concentrations higher than 250 μM. So, it was concluded that the use 0.25 mM of Gly-Pro-AMC in the assay mixture, as proposed by Blanchard et al. for the determination of DPP IV activity in muscle extracts, was adequate to obtain maximum activity avoiding product inhibition. In all cases, the activity of porcine muscle DPP IV, under the assayed conditions, followed Michaelis–Menten kinetics (data not shown).

The results presented here reveal that DPP IV isolated from muscle possessed many biochemical characteristics in common with DPP IV isolated from other sources (McDonald & Barrett, 1986). However, some other relevant aspects of the muscular enzyme have proved to be different from data previously reported for non-muscular DPP IV. Thus in order to study the contribution of this peptidase to flavor development in meat and meat products, the availability of a pure muscle DPP IV preparation is required. Data obtained here in relation to the relevant percentage of activity that DPP IV retained at acidic pH, and also at the temperatures of 5 and 15°C, would suggest that DPP IV remains active during meat storage and the ripening of processed meat products, contributing to the hydrolysis of proline bonds in the polypeptides coming from muscle proteins. The oligopeptides generated might be present in the final product, as part of the total components contributing to taste. The study of such components will be dealt with in subsequent work.

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