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Evaluation of ACE inhibitory activity of dipeptides generated by the action of porcine muscle dipeptidyl peptidases

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Abstract

Dipeptidyl peptidases (DPP) constitute a group of proteolytic enzymes able to release a good number of dipeptides from the N-terminus of both synthetic substrates and natural polypeptides. Specific sequences generated by the action of DPP purified from porcine skeletal muscle have been assayed to evaluate their capacity to inhibit the activity of angiotensin-I converting enzyme (ACE; EC 3.4.15.1). A fluorimetric assay based on the hydrolysis of the internally quenched fluorescent substrate *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline by the action of ACE was used for this purpose. The generated fluorescence of the product (the *o*-aminobenzoylglycine group) was continuously monitored in a microtiter-plate multiscan fluorometer.

Among the assayed dipeptides, Arg-Pro showed the strongest ACE inhibitory activity, being able to suppress more than 60% of initial enzyme activity at a concentration of 25 μM. Dipeptides Lys-Ala, Gly-Pro and Ala-Ala also demonstrated to be effective ACE inhibitors, although at a lower degree. Dipeptides Ala-Arg and Gly-Arg caused a more moderate inhibition of ACE activity, and even lower was the inhibition exerted by Arg-Arg. From the data obtained, it is suggested that the proteolytic action of DPP along the ripening period of dry-cured meat products could contribute to the generation of antihypertensive peptides.
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Keywords: Dipeptidyl peptidases; Angiotensin-I converting enzyme; Dipeptides; Fluorescence; Dry-cured meat products; Hypertension

1. Introduction

Numerous biochemical processes take place in postmortem skeletal muscle. One of them, proteolysis of both sarcoplasmic and myofibrillar proteins by the action of endogenous muscle enzymes has a major role in the development of an adequate meat texture (Sentandreu, Coulis, & Ouali, 2002) and the generation of typical flavour characteristics of dry-cured meat products (Toldrá & Flores, 1998). Different groups of endopeptidases such as calpains, cathepsins or proteasome have been studied in relation with meat ageing for being directly implicated in the weakening of the myofibrillar structure, the increase in meat tenderness and the generation of large polypeptides. During the processing of dry-cured meat products, these large polypeptides will serve as substrates for the action of exopeptidases, which will generate important amounts of free amino acids and small peptides either directly implicated in the generation of the typical flavour characteristics of these products or indirectly related for being precursors of other flavour components. Dipeptidyl peptidases (DPP) constitute a group of exopeptidases that are able to liberate dipeptides from the N-terminus of longer peptides. They have been purified and characterised from porcine skeletal muscle with the aim to better know the contribution of these enzymes to the generation of peptides related to flavour development in meat products (Sentandreu & Toldrá, 1998, 2000, 2001a, 2001b). In fact, Sentandreu et al. (2003) and Sforza et al. (2001) have confirmed the presence of dipeptides in the savoury fractions of dry-cured ham extracts. This would indicate the importance of DPP action along this process and how these enzymes can contribute to reach its final characteristic flavour.

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In relation to health, however, nothing is known about the physiological function of dipeptides generated by the action of DPP. This physiological, or tertiary function, can be defined as the property of some food components to exert direct beneficial effects on health by regulating different biochemical, biological and/or physiological processes. Some peptides resulting from different food sources have shown remarkable positive effects on health. For that reason they are known as "biologically active peptides" or "functional peptides". In Japan, some food products containing functional peptides have been already commercialised under the label of Foods for Special Health Use (Yamamoto, Ejiri, & Mizuno, 2003). One of the most important physiological functions exerted by certain peptides is their ability to reduce blood pressure by inhibiting the activity of angiotensin-I converting enzyme (ACE, EC 3.4.15.1), which is a key enzyme of the renin–angiotensin system. ACE is a carboxypeptidase responsible for the generation of the potent vasoconstrictor angiotensin II by releasing the C-terminal dipeptide His-Leu from angiotensin I, being also responsible for the inactivation of the vasodilator bradykinin and then giving rise to a net hypertensive effect. For that reason, compounds inhibiting ACE activity are able to generate the opposite effect, a reduction of systolic blood pressure (Houston, 2002). Peptides derived from different protein sources such as milk (Korhonen & Pilanto, 2003), soybean (Lo & Li-Chan, 2005), maize (Miyoshi et al., 1991) or fish (Yokoyama, Chiba, & Yoshikawa, 1992) have been shown to posses antihypertensive properties. Interestingly, hydrolysis of meat proteins by digestive enzymes has been also reported to generate peptides able to considerably inhibit ACE activity (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Jang & Lee, 2005).

The present work aims at better know the potential inhibitory effect on ACE activity of dipeptides generated by the action of DPP along the curing process of meat products. It has been reported that dipeptidyl peptidases remain active during either a great part or the whole processing period of dry-cured ham (Sentandreu & Toldrá, 2001c). During this long period their proteolytic action would give rise to some peptides whose physiological function is not known. We considered of interest, from the point of view of health related to dry-cured ham intake, to investigate the potential contribution of DPP activity to the generation of peptides able to decrease blood pressure.

2. Materials and methods

Angiotensin-I Converting Enzyme from rabbit lung was obtained from Sigma (St. Louis, Mo, USA). Dipeptides Gly-Arg, Ala-Arg, Arg-Arg, Ala-Ala, Gly-Pro, Arg-Pro and Lys-Ala were from Bachem Feinchemikalien (Bubendorf, Switzerland). Dipeptidyl peptidases I, II, III and IV were purified from porcine skeletal muscle as previously described (Sentandreu & Toldrá, 1998, 2000, 2001a, 2001b): DPP I was obtained by preparation of a muscle soluble extract followed by a thermal shock, which was able to precipitate great part of the protein content but not DPP I. In subsequent steps, the supernatant was subjected to a selective fractionation with ammonium sulphate, size exclusion and anion exchange chromatography. The final DPP I preparation was purified 115-fold with respect to the initial extract, having a specific activity of 3.1 U/mg protein. The other three enzymes were purified from different muscle soluble extracts by selective precipitation with ammonium sulphate, followed by two successive anion exchange chromatographies. The final enzyme preparations of DPP II, III and IV were enriched 1270-, 245- and 2329-fold with respect to their initial extracts, having specific activities of 1.16, 96.1 and 2.0 U/mg protein, respectively. One Unit of enzyme activity was defined as the amount of hydrolysed substrate per hour at 37 °C.

2.1. Assay of DPP enzyme activity on different peptide substrates

Study of DPP substrate specificity on the release of N-terminal dipeptides was determined as follows: 50 μl of each enzyme solution were added to 250 μl of assay buffer at pH 5.5 (DPP I and II) or pH 8.0 (DPP III and IV) containing 0.5 mM of each one of the synthetic substrates illustrated in Tables 1 and 2. The reaction mixture was incubated in a multiwell plate at 37 °C for 20 min. For 7-amido-4-methylcoumarin (AMC) derivatives, hydrolysis was determined by measuring the generated fluorescence at 355 and 460 nm excitation and emission wavelength, respectively. For *p*-nitroanilide (*p*-Na) derivatives, peptide hydrolysis was followed by measuring the colour development at $\lambda = 410$ nm.

Table 1
Substrate specificity of muscle dipeptidyl peptidases I and III

Enzyme	Substrate	Relative activity (%)[a]
DPP I	Gly-Arg-↓AMC[b]	100
	Ala-Arg-↓AMC	124
	Ala-Ala-↓pNa[c]	13
	Arg-Arg-AMC	0
	Lys-Ala-AMC	0
	Gly-Pro-AMC	0
DPP III	Arg-Arg-↓AMC	100
	Ala-Arg-↓AMC	69
	Ala-Ala-↓pNa	50
	Gly-Pro-↓AMC	9
	Lys-Ala-↓AMC	3
	Gly-Arg-↓AMC	2

(↓): Arrows indicate cleavage of the peptide bond.
[a] Enzyme activity expressed as percentage of the hydrolysis against Gly-Arg-AMC (DPP I) or Arg-Arg-AMC (DPP III), which was taken as reference (100%).
[b] AMC: 7-amido-4-methylcoumarin.
[c] pNa: *p*-nitroanilide.

Table 2
Substrate specificity of muscle dipeptidyl peptidases II and IV

Enzyme	Substrate	Relative activity (%)[a]
DPP II	Gly-Pro-↓AMC[b]	100
	Lys-Ala-↓AMC	38
	Gly-Pro-↓pNA[c]	562
	Arg-Pro-↓pNA	591
	Ala-Ala-↓pNA	481
	Gly-Arg-AMC	0
	Arg-Arg-AMC	0
	Ala-Arg-AMC	0
DPP IV	Gly-Pro-↓AMC	100
	Lys-Ala-↓AMC	13
	Gly-Pro-↓pNA	144
	Arg-Pro-↓pNA	216
	Ala-Ala-↓pNA	29
	Gly-Arg-AMC	0
	Arg-Arg-AMC	0
	Ala-Arg-AMC	0

(↓): Arrows indicate cleavage of the peptide bond.
[a] Enzyme activity expressed as percentage of the hydrolysis against Gly-Pro-AMC, which was taken as reference (100%).
[b] AMC: 7-amido-4-methylcoumarin.
[c] pNa: *p*-nitroanilide.

2.2. Inhibition of ACE activity by addition of dipeptides generated by DPP action

Inhibition of angiotensin-I converting enzyme activity by dipeptides Gly-Arg, Ala-Arg, Arg-Arg, Ala-Ala, Gly-Pro, Arg-Pro and Lys-Ala at different concentrations, ranging from 0.5 to 200 μM, was determined through a fluorimetric assay using *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz-Gly-Phe(NO_2)-Pro) as substrate. The assay was optimised in a previous work (Sentandreu & Toldrá, 2006) until the following conditions: 50 μL of each peptide concentration were added to wells of a microtiter plate, then adjusting to 100 μL by addition of a solution containing 7.2 μg mL^{-1} of ACE. The reaction was initiated by addition of 200 μL of 0.45 mM Abz-Gly-Phe(NO_2)-Pro dissolved in 150 mM tris buffer, pH 8.3, containing 1.125 M NaCl. The reaction mixtures were incubated at 37 °C for 45 min and the generated fluorescence due to the liberation of the Abz-Gly group was measured in a multiscan fluorometer (Fluoroskan Ascent, Labsystems, Finland) using excitation and emission wavelengths of 355 and 405 nm, respectively. ANOVA treatment of IC_{50} values (Table 3) was done using the Statgraphics® Plus version 5.1 (Manugistics Inc., Rockville, MD, USA).

Table 3
IC_{50}[a] values of dipeptides generated by the action of muscle DPP

Peptide	IC_{50} (μM) ± s.d.
Arg-Arg	267.1 ± 11.3[*]
Gly-Arg	162.2 ± 4.3
Ala-Arg	95.5 ± 0.4
Gly-Pro	66.0 ± 3.1
Ala-Ala	51.4 ± 5.1
Lys-Ala	31.5 ± 3.1
Arg-Pro	15.2 ± 0.2

Results expressed as the mean of three replicates ±standard deviation.
[a] The concentration inhibiting 50% of angiotensin-I converting enzyme activity, determined as described in Section 2.
[*] Obtained values for the different assayed peptides were all significantly different. $P < 0.001$.

3. Results and discussion

The four DPP existing in skeletal muscle belong to different peptidase families and catalytic classes, having also quite different biochemical characteristics. DPP I and DPP II are cystein and serin peptidases, respectively, both of them located into lysosomes in the living cell and so having acid optimum pH (McDonald & Ohkubo, 2004; Turk, Turk, Dolenc, & Turk, 2004). DPP III is a cytosolic metallopeptidase with basic optimum pH, being its activity greatly enhanced by the presence of cobalt ions (Chen & Barret, 2004). DPP IV is a serin peptidase located in the outer part of the membrane cell with slightly basic optimum pH (Misumi & Ikehara, 2004). An important difference between the basic enzymes is that, under the acid conditions found in postmortem muscle, the activity of DPP III is reduced more drastically than in the case of DPP IV which is able to express an important percentage of maximal activity. Temperature for maximal activity is higher in the case of lysosomal enzymes than in the case of DPP III and DPP IV. However, it is more relevant the activity of these enzymes at lower temperatures because during a long part of processing time, dry-cured meat products are kept at low temperatures (between 4 and 15 °C). In these conditions, DPP I and DPP IV retain an important percentage of their activity, whereas the activity of DPP II and III is considerably lower (Sentandreu & Toldrá, 2001c).

Tables 1 and 2 illustrate the substrate specificity of porcine muscle DPP on different synthetic peptide substrates. DPP I and DPP III are able to hydrolyse substrates having Arg or Ala in N-penultimate position (Table 1). On the other hand, DPP II and DPP IV hydrolyse preferentially substrates containing proline in N-penultimate position, but they can also accept Ala in this position (Table 2). Contrary to that, DPP I and DPP III had negligible or no effect on peptide bonds containing a proline residue. When a basic residue is located in the N-terminal position, DPP I is unable to liberate dipeptides, contrary to the other three enzymes.

To study the physiological function of dipeptides generated by the action of DPP on the regulation of blood pressure, a rapid and sensitive assay to determine with high precision the inhibitory effect of such dipeptides on the activity of angiotensin-I converting enzyme has been recently developed (Sentandreu & Toldrá, 2006). The assay is based on the hydrolysis of the internally quenched fluorescent substrate Abz-Gly-Phe(NO_2)-Pro developed by Carmel and Yaron (1978). Optimal conditions were established for linearity, sensitivity and precision of the assay.

ACE activity was found maximal within the pH range 8.0–8.5 having 0.5–0.75 M NaCl and 0.3 mM substrate concentration in the final assay mixture (Sentandreu & Toldrá, 2006).

The effect of dipeptides generated by DPP action on ACE activity is shown in Figs. 1 and 2. Of the assayed dipeptides, Arg-Arg exerted a weak inhibition of ACE activity. Gly-Arg, generated by DPP I action, exerted a moderate inhibition since a peptide concentration of 150 μM almost inhibited 50% of ACE activity. Nevertheless, in our case, the ACE inhibitory effect of Gly-Arg when using Abz-Gly-Phe(NO_2)-Pro as substrate resulted to be higher than in the case of Cheung, Wang, Ondetti, Sabo, and Cushman (1980), where they obtained lower inhibitory values using Hippuril-His-Leu as substrate for ACE. Inhibition caused by Ala-Arg, a product of DPP I and DPP III action, was more important because 100 μM of peptide concentration suppressed more than 50% of ACE activity (Fig. 1). Ala-Ala, which can be generated by the four DPP, together with Gly-Pro, a product of DPP II and IV, showed an important ACE inhibitory activity since a concentration of 100 μM was able to inhibit more than 60% of initial ACE activity (Fig. 2). In the case of Ala-Ala, our results are comparable to those reported by Soffer (1976). On the contrary, we obtained in the present work an IC_{50} value of 66 μM for Gly-Pro (see Table 3) whereas in the study of Cheung et al. (1980) a concentration of 450 μM of Gly-Pro was necessary to inhibit 50% of ACE activity. In accordance to previous observations (Soffer, 1976), a strong inhibition of ACE activity was achieved with Lys-Ala, generated by the action of DPP II and DPP IV, which suppressed 70% of ACE activity at a concentration of 100 μM (Fig. 2) and displayed an IC_{50} of 31.5 μM (Table 3).

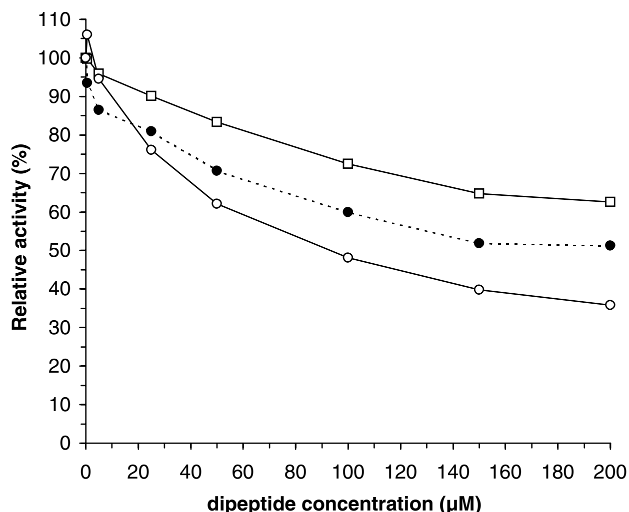

Fig. 1. Effect of dipeptides generated by the action of porcine muscle dipeptidyl peptidases I and III on ACE activity. ACE activity against Abz-Gly-Phe(NO_2)-Pro in the absence of any peptide was taken as 100%. (□): Arg-Arg; (–●–): Gly-Arg; (○): Ala-Arg.

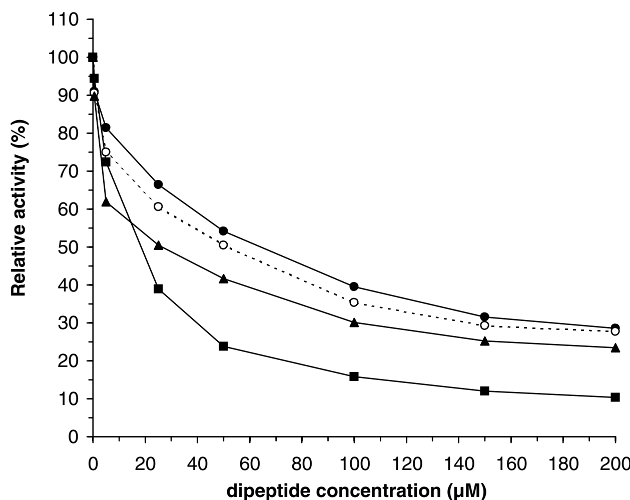

Fig. 2. ACE inhibitory activity of dipeptides, generated by the action of porcine muscle DPP, containing either Proline or Alanine at the C-terminus. ACE activity against Abz-Gly-Phe(NO_2)-Pro in the absence of any peptide was taken as 100%. (●): Gly-Pro; (–○–): Ala-Ala; (▲): Lys-Ala; (■): Arg-Pro.

Of all the assayed dipeptides, Arg-Pro showed the strongest inhibition of ACE activity, as shown in Fig. 2. This dipeptide, generated by both DPP II and DPP IV action, inhibited more than 60% of initial ACE activity at a concentration of 25 μM, whereas at a concentration of 200 μM only 10% of initial ACE activity remained. As in the case of Gly-Pro, the ACE inhibitory action of this dipeptide when using Hippuril-His-Leu as substrate (Cheung et al., 1980) was lower ($IC_{50} = 180$ μM) than the inhibition observed by us on ACE activity using Abz-Gly-Phe(NO_2)-Pro as substrate (see Table 3). This is not surprising considering the fact that IC_{50} values are difficult to compare when ACE activity was determined using different substrates and/or assay conditions. Despite of this, the importance of the inhibition rate between the peptides should be maintained, as it was the case between our results and those of Cheung et al. (1980).

4. Conclusion

According to the results of the present work, the proteolytic action of pork muscle DPP typically generates a good number of dipeptides, some of them with a relevant ACE inhibitory activity. Those dipeptides generated by the action of DPP II and DPP IV showed the strongest inhibitory action, although some of the peptides produced by DPP I and DPP III activity were also important ACE inhibitors. The natural generation of antihypertensive peptides by DPP action along the curing process could play an important role in dry-cured meat products with respect to blood pressure regulation. Research is actually being carried out in our laboratory to advance our knowledge in this point by identifying DPP dipeptide products in dry-cured ham able to inhibit ACE activity.

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