GENERATION OF ACE INHIBITORY DIPEPTIDES BY PORK MUSCLE DIPEPTIDYL PEPTIDASES I AND III

Miguel Ángel Sentandreu and Fidel Toldrá*

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

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Introduction

Dipeptidyl peptidases (DPP) are a group of enzymes belonging to different families and catalytic classes but that have in common the ability to liberate dipeptides from the N-terminus of larger peptides. Their action in the living cells has been related with many physiological processes such as regulation of bioactive peptides, protein turnover or implication in some pathological conditions [Rawlings et al., 2004]. Concerning the biochemistry of postmortem muscle, DPP are assumed to contribute to the intense proteolysis of muscle proteins occurring during meat aging but specially during the processing of meat products. This phenomenon gives rise to an intense breakdown of the myofibrilar structure and the generation of important amounts of free amino acids and small peptides (lower than 1200 Da), which are directly related with the development of the typical flavor characteristics of these products [Toldrá and Flores, 1998]. However, little is known about the physiological function of these peptides generated during the ripening of dry-cured ham. 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. One of the most interesting tertiary functions is the property of some peptides to inhibit the activity of angiotensin-I converting enzyme (ACE; EC 3.4.15.1), an ectoenzyme implicated in the renin-angiotensin system. ACE catalyzes the conversion of inactive angiotensin I into angiotensin II, which is a potent vasoconstrictor and also inactivates the vasodilator bradykinin, giving rise to a hypertensive effect [Houston, 2002]. By this way, substances inhibiting ACE activity are able to reduce blood pressure.

Objectives

The present work has the objective to investigate if the activity developed by dipeptidyl peptidases I and III along the processing of dry-cured ham could contribute to the generation of small peptides having antihypertensive activity, which could be especially positive for helping in the regulation of blood pressure through diet. For that purpose, some dipeptide sequences known to be products of the activity of DPP I and III on different substrates were assayed for the inhibition of ACE activity.

Methodology

Preparation of enzyme solutions.

Stock solution: Commercial angiotensin-I converting enzyme (0.51 mg) was diluted in 3.3 mL of a 50 % glycerinated solution with 0.15 M tris buffer, pH 8.3, containing 1 μM $ZnCl_2$. This solution was kept at –20 ºC until use.

ACE working solution. Stock solution was diluted 1/20 with 0.15 M tris buffer, pH 8.3, having a concentration of 7.2 μg mL^{-1} of ACE corresponding to 3 mU mL^{-1} of enzyme activity.

Inhibition of ACE activity by addition of dipeptides: Inhibition of angiotensin-I converting enzyme activity by dipeptides shown in figure 2 at different concentrations, ranging from 0.5 to 200 μM, was determined through the development of a fluorimetric assay using *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz-Gly-Phe(NO_2)-Pro) as substrate. The assay was optimized 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 ACE working solution. The reaction was initiated by the 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 (300 μL/well) were immediately shaken and incubated at 37ºC for 45 minutes and the generated fluorescence was measured in a multiscan fluorometer (Fluoroskan Ascent, Labsystems, Finland) using excitation and emission wavelengths of 355 and 405 nm, respectively. ACE activity obtained in the presence of each assayed peptide concentration was referred to controls, which were simultaneously measured in the absence of any added peptide, taking the value of 100 %.

Assay of DPP I and DPP III enzyme activity on different peptide substrates. Study of substrate specificity of DPP I and III on the release of N-terminal dipeptides from both

synthetic and natural peptides, together with determination of optimum pH, was described in previous works [Sentandreu and Toldrá, 1998 and 2000].

Results & Discussion

Table 1 shows some of the N-terminal dipeptide sequences generated due to proteolytic action of DPP I and III on both synthetic and natural peptides. DPP I hydrolyzed well peptide substrates having alanine or arginine in N-penultimate position under acidic conditions. On the contrary, peptides having a basic amino acid in N-terminal position, or those containing a proline residue in any side of the peptide bond were resistant to attack. According to this, Met-Ala, Gly-Arg, Ala-Arg and Ala-Ala could be split from different substrates due to porcine muscle DPP I. These two latter dipeptides were also generated due to DPP III activity at basic pH, together with Arg-Arg, Arg-Phe, Phe-Gly and Arg-Gly. Even if DPP I and DPP III share some common substrate specificity, each one differs from the other in terms of optimum pH, reducing conditions, concentration of some divalent cations and subcellular location. A remarkable difference is the fact that DPP I was able to hydrolyze peptides as short as tripeptides, whereas DPP III was unable, being tetrapeptides the minimum length required for activity [Sentandreu and Toldrá, 1998 and 2000].

It has been shown that dipeptidyl peptidases I and III remain active during the whole processing period of dry-cured ham. In the case of DPP I its optimum pH range is coincident with muscle pH during dry-curing, and so an important dipeptide generation due to DPP I activity would be expected from the early stages of dry-curing. In the case of DPP III its optimal pH is higher than muscle pH and consequently a relevant dipeptide generation would be expected mainly after a long time of action, at the end of the process [Sentandreu and Toldrá, 2001]. We considered of interest, from the nutritional aspects of dry-cured ham intake, to evaluate if dipeptides that can be generated by the proteolytic action of DPP I and III during the whole processing of Spanish dry-cured ham could be effective angiotensin-I converting enzyme inhibitors. For that purpose, we have developed a fluorimetric assay for a rapid and accurate determination of ACE activity which is based on the hydrolysis of the internally quenched fluorescence substrate Abz-Gly-Phe(NO_2)-Pro developed by Carmel and Yaron (1978). Hydrolysis of this substrate by the action of ACE generates the fluorescent product *o*-aminobenzoylglycine (Abz-Gly), which can be continuously monitored in a microtiter-plate fluorescent reader, using excitation and emission wavelength of 355 and 405 nm, respectively. Then, we have established the optimal conditions for its linearity, sensitivity and precision. This assay has several advantages with respect to other methods actually in use such as the simplicity to carry on and the possibility to process a high number of samples in a short time. As can be appreciated in figure 1, the method showed a very good correlation (r = 0.9925) with that of Cushman and Cheung (1971), which is the assay commonly employed for the study of ACE inhibitors in foods. As can be observed, a higher hydrolysis rate of Abz-Gly-Phe(NO_2)-Pro is obtained with respect to hydrolysis of hippuryl-His-Leu (Hip-His-Leu) at a given enzyme concentration (figure 1).

Different concentrations of the dipeptides generated by the activity of DPP I and III (see table 1) were incubated with angiotensin-I converting enzyme before the addition of the substrate in order to evaluate if these dipeptides can be effective ACE inhibitors able to positively influence the control of blood pressure. As can be seen in figure 2, Ala-Ala, which can be liberated by the action of both DPP I and DPP III, exerted an important ACE inhibitory activity since 50 % inhibition was achieved at 50 µM peptide concentration. Ala-Arg, also generated by the two exopeptidases, and Arg-Phe, generated by DPP III, proved to be also effective ACE inhibitors. Met-Ala and Gly-Arg, both of them generated by DPP I activity, were less inhibitory, requiring a concentration of 150 µM to achieve 50 % ACE inhibition. The rest of the assayed dipeptides, Arg-Gly, Phe-Gly and Arg-Arg, did not exert a remarkable inhibition of ACE activity (figure 2).

Dry-cured ham processing can vary depending on the different types and qualities of hams but, in all cases, an intense proteolysis takes place on both sarcoplasmic and myofibrillar proteins during this long period. As it has been reported, this intense protein degradation is mainly due to the coordinated action of endogenous muscle enzymes [Toldrá and Flores. 1998]. So, muscle endopeptidases such as calpains, cathepsins and proteasome would be responsible of the initial breakdown of proteins to generate large polypeptides, a phenomenon directly related with the development of meat tenderness [Sentandreu, Coulis and Ouali, 2002]. Then, these polypeptides would serve as substrates for the action of different groups of exopeptidases like DPP I and III. The ability of these enzymes to generate dipeptides able to inhibit ACE activity (see figure 2), together with their good stability during dry-curing [Sentandreu and Toldrá, 2001] and the long

duration of the process, would make feasible the generation of sufficient amounts of antihypertensive peptides that, in some way, would help to balance the effect of NaCl content of these products on hypertension.

Conclusions

The proteolytic action of dipeptidyl peptidases I and III along the processing of dry-cured ham can generate dipeptides which, some of them, are able to considerably inhibit the activity of angiotensin-I converting enzyme. This could help to maintain and adequate systolic blood pressure even if dry-cured ham is present in the diet, which it would be specially positive for hypertensive population from countries like Spain, having great tradition in consumption of this traditional food.

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Tables and Figures

Table 1: Proteolytic action of Dipeptidyl peptidases I and III on different natural and synthetic peptides

Enzyme[1]	**Classification**[2]	**Location**	**pH range (Optimum pH)**	**Released dipeptides due to their enzyme activity**[3]
DPP I (EC 3.4.14.1)	Cystein peptidase Family C1, clan CA (Papain family)	Lysosome	5 – 6.5 (5.5)	Gly-Arg-↓AMC Ala-Arg-↓AMC Ala-Ala-↓pNa Met-Ala-↓Ser
DPP III (EC 3.4.14.4)	Metallopeptidase Family M49, clan MA	Cytosol	7.5 –8.5 (8.0)	Arg-Arg- ↓AMC Ala-Arg-↓AMC Ala-Ala-↓pNa Arg-Arg-↓Lys-Ala-Ser-Gly-Pro Arg-Phe-↓Arg-Ser Phe-Gly-↓Gly-Phe Arg-Gly-↓Asp-Ser

[1]: Data from NC-IUMB (http:\www.chem..qmw.ac.uk)
[2]: Data from MEROPS database (http:\www.merops.co.uk)
[3]: Red arrow indicate cleavage of the corresponding peptides bond

Figure 1: Correlation between the fluorimetric assay using Abz-Gly-Phe(NO_2)-Pro as substrate for ACE (ordinate) with that of Cushman and Cheung (abscissa).

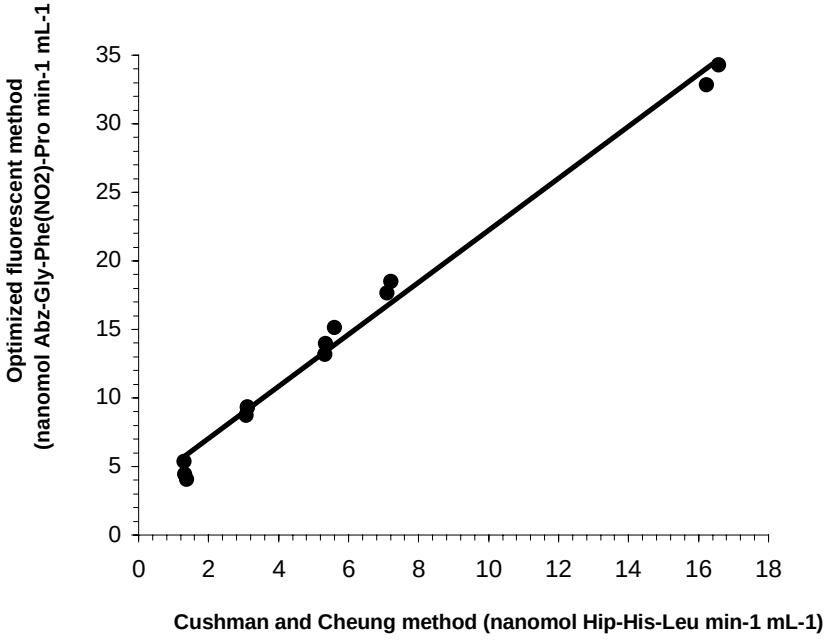

Figure 2: Effect of dipeptides generated by proteolytic action of DPP I and DPP III on the activity of angiotensin-I converting enzyme. Activity in the absence of added peptide was taken as 100 %.