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Contribution of muscle aminopeptidases to flavor development in dry-cured ham

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Abstract

The activity of muscle aminopeptidases (alanyl, arginyl, leucyl and pyroglutamyl aminopeptidases) have been assayed along the processing of dry-cured ham. The generation of free amino acids resulting from aminopeptidase action on N-terminal of proteins and peptides has been also analyzed. The assayed aminopeptidases, except pyroglutamyl aminopeptidase, showed good stability. Alanyl and arginyl aminopeptidases have optimal neutral pH near the pH in ham and, in addition, their spectrum of activity against terminal amino acids is in coincidence with the observed release of free amino acids in ham. So, both aminopeptidases appear to be the main contributors to the generation of free amino acids during the processing of dry-cured ham.

Keywords: Ham; Flavor; Aminopeptidases; Amino acids

1. Introduction

Dry-cured hams constitute a typical food product from the Mediterranean area, some of the most important and well-known being Iberian, Serrano, Parma and Bayonne hams. The quality of dry-cured hams depends on many factors although the raw materials (Armero, Baselga, Aristoy & Toldrá, 1999) and the ripening/drying conditions (Toldrá, 1998; Toldrá, Flores & Sanz, 1997) are the most important. The generation of the final characteristic aroma of dry-cured ham is reached after several months of process as a result of previous enzymatic reactions such as proteolysis and lipolysis (Toldrá & Flores, 1998). The volatile compounds found in the headspace of Spanish Serrano dry-cured ham, derived from oxidative decomposition of lipids, are aliphatic hydrocarbons, aliphatic alcohols and aldehydes, esters, ketones and furans (Flores, Grimm, Toldrá & Spanier, 1997). However, there are other volatile compounds derived from the degradation or reaction of amino acids that also contribute to the aroma of dry-cured ham (Flores, Spanier & Toldrá, 1998). This is the case of methyl branched alcohols and aldehydes, sulphur compounds are important contributors to meat flavour because of their low flavour thresholds, pyrazines that contribute to roasted aromas and furans (Toldrá & Flores).

The objective of this study is to elucidate the role of muscle aminopeptidases in the generation of free amino acids and thus understand its contribution to the flavor of dry-cured ham

2. Materials and methods

2.1. Dry-cured hams

Thirty hams from 6-month-old pigs (Landrace×Large White) were submitted to the dry-curing process which consisted of the traditional stages of salting (14 days), post-salting (40 days) and ripening-drying until the 12th month of final processing time. Six hams were taken at each sampling time (0, 40, 100, 240 and 365 days). The muscle *Biceps femoris* was excised and used for the assay of aminopeptidases activity and analysis of free amino acids as described below.

2.2. Measurement of enzyme activity

The enzyme crude extracts were prepared as described by Lauffart and Mantle (1988) with slight modifications: 10 g of muscle *Biceps femoris*, with no visible fat or connective tissue, was homogenized in 50 ml of 50 mM phosphate buffer containing 5 mM ethylene glycol

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tetraacetic acid (EGTA), pH 7.5 by using a Polytron (three strokes, 10 s each at 27 000 rpm with cooling in ice) homogenizer (Kinematica, Switzerland). The extract was centrifuged at 10 000 g for 20 min at 4°C and the supernatant, filtered through glass wool, (soluble fraction) and used for further purification. Alanyl, arginyl, leucyl and pyroglutamyl aminopeptidases were assayed, as previously described by Toldrá et al. (1992), by using the fluorescent substrates 0.1 mM alanyl-AMC, 0.1 mM arginyl-AMC, 0.25 mM leucyl-AMC and 0.1 mM pyroglut amyl-AMC, respectively. The reaction buffer was 0.1 M disodium phosphate at pH 6.5 for arginyl, and containing 2 mM 2-mercaptoethanol when measuring the activity of alanyl. In the case of leucyl and pyroglutamyl aminopeptidases, the reaction buffer was 0.05 M sodium borate at pH 9.5 and 8.5, respectively. The reaction mixture consisted in 50 μl of enzyme extract and 250 μl of reaction buffer and was incubated at 37°C for 15 min. Four replicates were carried out for each enzyme assay and the fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan II multiscanning fluorimeter (Labsystems, Finland). One unit (U) of aminopeptidase activity is defined as the amount of enzyme capable of hydrolysing 1 μmol of substrate in 1 h at 37°C.

2.3. Analysis of free amino acids

Samples for the amino acid and dipeptides analysis were extracted and deproteinised following the method described by Aristoy and Toldrá (1991). Samples were homogenised with 0.01 N HCl (dilution 1:4) in a Stomacher during 8 min at 4°C and centrifuged in cold at 10 000 g for 20 min. Supernatant was filtered through glass wool and stored at −80°C until use. 250 μl of thawed samples plus 50 μl of an internal standard solution (hydroxyproline, 0.325 mg/ml), were deproteinised with 750 μl of acetonitrile. 200 μl of the supernatant were derivatised to their phenylthiocarbamyl derivatives according to the method of Bidlingmeyer, Cohen, Tarvin, and Frost (1987). The derivatised aminoacids and dipeptides were analysed by reverse-phase HPLC in a Nova Pak C18 (300×3.9 mm) column (Waters Corporation, MA). The separation was achieved in 65 min at 52°C, using a gradient between two solvents: 70 mM sodium acetate at pH 6.55 with 10% acetic acid, containing 2.5% of acetonitrile (solvent A) and water–acetonitrile–methanol, 40:45:15 v/v (solvent B) as described by Flores et al. (1997). The detection was monitored at 254 nm.

3. Results and discussion

The evolution of aminopeptidases activities along the processing of dry-cured ham is shown in Fig. 1. Alanyl aminopeptidase shows the highest exopeptidase activity along the full process. In fact, this enzyme is considered as the major aminopeptidase in skeletal muscle (Flores, Aristoy & Toldrá, 1996). Arginyl and leucyl aminopeptidases also show some activity left at the end of the process while pyroglutamyl aminopeptidase shows a rather poor stability since its activity is negligible at about 40 days of process. It must be taken into account that muscle aminopeptidases do not have the same degree of preference depending on which amino acid is found in the aminus terminal. So, alanyl aminopeptidase shows a broad range of specificity, especially hydrolysing phenylalanine, lysine, methionine, alanine and leucine (Flores et al., 1996) while arginyl aminopeptidase is more restricted to a few terminal amino acids mainly of basic nature such as arginine and lysine (Flores, Aristoy & Toldrá, 1993).

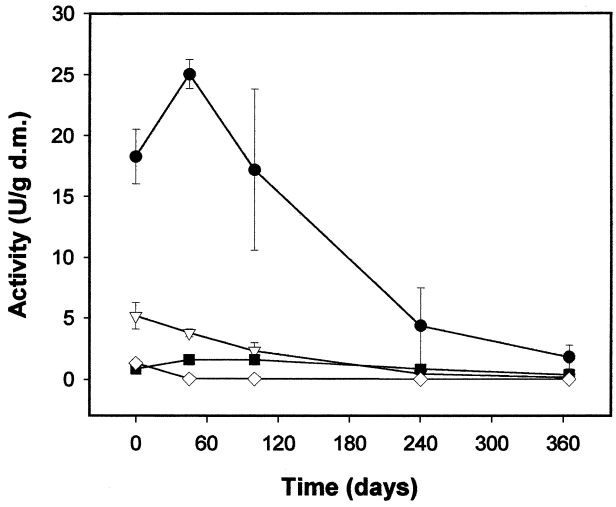

Fig. 1. Evolution of muscle aminopeptidases along the processing of Serrano type dry-cured ham: (●) alanyl, (▽) arginyl, (■) leucyl and (◇) pyroglutamyl aminopeptidases.

Several circumstances reduce aminopeptidases activity along the dry-curing process. Salt is an effective inhibitor of most of the muscle proteases (Flores, Aristoy & Toldrá, 1997). So, alanyl and pyroglutamyl aminopeptidases are inhibited, leucyl aminopeptidase is not affected while arginyl aminopeptidase, which is a chloride activated enzyme, experiences a sensible activation. Water activity is also an important factor since it decreases as drying progresses reaching values as low as 0.85 such as those find after mid-process or even below 0.80 towards the end of the process. In general, all the enzymes are significantly affected by the decrease in water activity (Toldrá, Rico & Flores, 1992). pH is perhaps the variable with lower effect due to its narrow range of variation during the process, from around 5.6–5.8 to 6.4 at the end of the process. So, leucyl and pyroglutamyl aminopeptidases are those muscle enzymes with more unfavourable pH for their activity. Finally, the accumulation of free amino acids into the hams also

produce a feedback inhibition on aminopeptidases as reported by Flores, Aristoy and Toldrá (1998) resulting in a decreased activity. Thus, taking into account all these facts, the good stability of both alanyl and arginyl aminopeptidases during the process, as shown in Fig. 1, and their optimal neutral pH, it appears that both enzymes, especially alanyl aminopeptidase may be the main responsible for the generation of free amino acids during the dry-curing process.

The generation of free amino acids along the processing of dry-cured ham is shown in Fig. 2A to F. As can be observed, the highest rates of generation of free amino acids take place between 0 and 240 days. The release of free amino acids is incredibly high, which is logical due to the initial higher activity levels of alanyl aminopeptidase, that is capable to hydrolyze a wide range of terminal amino acids. Then, the generation trend still went on but at a slower rate, probably due to the reduced enzyme activity as well as to further degradation reactions to any other compounds such as volatile molecules. The evolution of taurine and natural dipeptides (carnosine and anserine) is shown in Fig. 2F. Taurine and anserine remain constant while carnosine slightly decreases after 100 days of process.

Glutamic acid, lysine, alanine, leucine and arginine are the amino acids generated in highest amounts along the process. In this sense, the generation of free amino acids is important because they directly contribute to taste (Kato, Rhue & Nishimura, 1998; Nishimura & Kato, 1988). In dry-cured ham, some relationships have been reported such as glutamic acid, aspartic acid, leucine, isoleucine, phenylalanine, tryptophan and lysine with

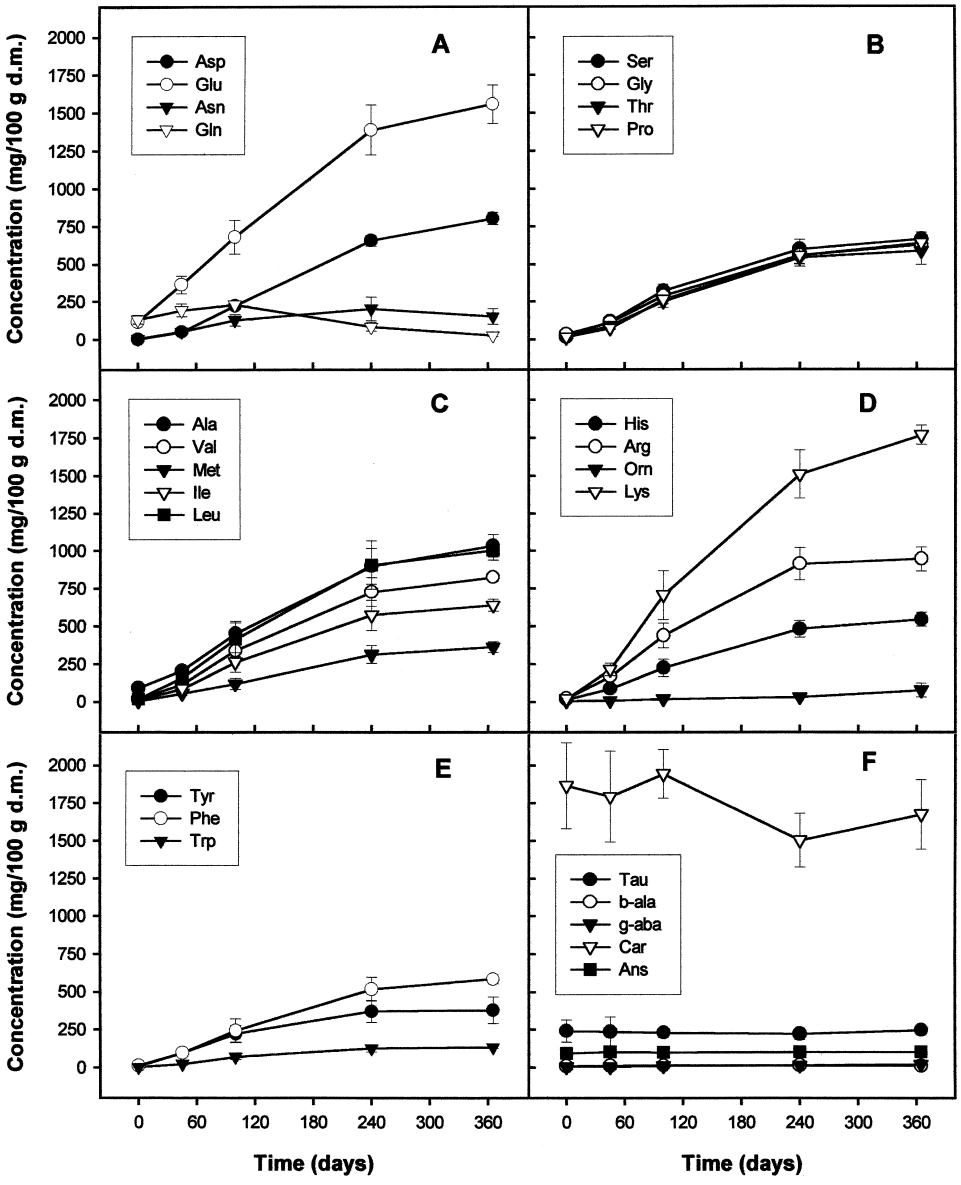

Fig. 2. Evolution of free amino acids (A–F) and natural dipeptides (F) along the processing of Serrano type dry-cured ham.

Table 1
Main volatile compounds isolated in Spanish dry-cured ham (Serrano type) resulting from amino acids

Amino acid	Compound	Reaction	Aroma[a]
Val	2-Me-Propanal	Strecker	
Ile	2-Me-Butanal	Strecker	
Leu	3-Me-Butanal	Strecker	Cheesy green
Other	3-Me-butanol	Strecker	Penetrating green
	3-Me-2-hexanol	Strecker	Potato–wheat
Met, Cys, Cis	Sulphur comps.:		
	DiMe-Disulphide	Strecker	Dirty socks
Other	Pyrazines:		
	Me-pyrazine	Maillard	Nutty
	2,6-DiMe-pyrazine	Maillard	Toasted nuts
Met, Cys, Cis	Furans:		
	2-penthyl-furan	Aac + carbohydrate or lipid oxidation	Ham-like
	2,5-DiMe-furan	Aac + carbohydrate	Sulphury-fishy

[a] Aroma compound detected and identified in Spanish dry-cured ham (adapted from Flores et al., 1997; Toldrá & Flores, 1998).

length of the drying process (Flores, Aristoy, Spanier & Toldrá, 1997), tyrosine and lysine with aged taste, phenylalanine and isoleucine with acidity and glutamic acid with saltiness (Careri, Mangia, Barbieri, Bolzoni, Virgili & Parolari, 1993). Free amino acids also constitute a potential source of volatile compounds (see Table 1): (i) through Strecker degradation of valine, isoleucine and leucine giving 2-methyl-propanal, 2-methyl-butanal and 3-methylbutanal, (ii) generation of dimethyl disulfide compounds from sulfur-containing amino acids such as methionine, cysteine and cystine, and (iii) the generation of a several pyrazines from Maillard reactions. Their respective aromas, described or found in the headspace of Spanish dry-cured ham, are also indicated in Table 1. So, these compounds also have an influence on the final flavor of dry-cured ham (Flores, Spanier & Toldrá, 1998).

4. Conclusions

According to the obtained results, it appears that protein fragments and peptides generated by cathepsins and calpains at the early stages of dry-curing (Rico, Toldrá & Flores, 1993) would have strong importance as new substrates for the action of aminopeptidases and subsequent generation of free amino acids as taste-active compounds but also as aroma precursors. Thus, alanyl and arginyl aminopeptidases, which show good stability and have an optimal neutral pH, would have a sensible effect during the entire process and would justify the flavour development in the latter stages of the process.

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