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Peptide generation in the processing of dry-cured ham

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High-performance liquid chromatography (HPLC) was used to separate small water-soluble peptides extracted from dry-cured ham at different processing times (from 0 to 15 months). The elution profile of the deproteinised extracts after gel filtration HPLC showed five ranges of molecular mass: I (4500–2700 Da), II (2700–1200 Da), III (1200–500 Da), IV (500–375 Da) and V (375–160 Da). A clear increase of those peptides below 2700 Da was observed as dry-curing progressed confirming an intense proteolysis especially up to 3·5 months of process. Peptide profiling of deproteinized extracts by reverse phase HPLC and free solution capillary electrophoresis showed substantial changes in peptide patterns along the dry-curing. These mappings could provide valuable information regarding time of processing.

INTRODUCTION

Many biochemical changes are involved in the processing of dry-cured hams. An increase in the non-protein nitrogen has been reported for Italian Parma ham (Bellatti *et al.*, 1983), Spanish Serrano ham (Aristoy & Toldrá, 1991) and American country-style ham (McCain *et al.*, 1968) as a result of muscle protein breakdown (Toldrá *et al.*, 1992*a*, 1993). Toldrá (1992) suggested that muscle cathepsins B, D, H and L, proteinases located in lysosomes (Goll *et al.*, 1983), which remain active during the entire process even after 15 months (Toldrá & Etherington, 1988; Toldrá *et al.*, 1993), could be responsible for these changes. The last proteolytic step, consisting in the conversion of peptides to free amino acids, would be the result of muscle aminopeptidases action (Toldrá, 1992) which are also stable during the process and still keep some activity even after 15 months of processing (Toldrá *et al.*, 1992*b*). In fact, a very high increase in the concentration of free amino acids at the end of the process has been previously reported (Toldrá & Aristoy, 1993). However, there is a lack of information regarding the evolution of generated or hydrolysed peptides along the process. This information would be very important to understand the path of proteolysis mechanisms during dry-curing as well as for future studies on flavour development. In other foods such as cheese manufacture, the proteolysis indices proved very useful for fast indication of the degree of ripening, objective age and maturation (Christensen *et al.*, 1989; Gonzalez de Llano *et al.*, 1991; Kaiser *et al.*, 1992). Furthermore, the peptides present in the water-soluble extract proved to have a wide range of flavours from bitterness to more desirable savoury flavours (Champion & Stanley, 1982; Aston & Creamer, 1986; Mojarro-Guerra *et al.*, 1991; Cliffe *et al.*, 1993).

The objective of this study was to study the evolution of peptides throughout the complete processing of high-quality dry-cured ham and from those peptide mappings to try to characterise the course and degree of proteolysis.

MATERIALS AND METHODS

Ham samples

Three samples of the muscle *biceps femoris* were removed from three hams at different stages in the processing of Spanish Serrano dry-cured ham: raw ham (t = 0), after salt equalisation (t = 2 months) and after different drying times (t = 3·5, 5, 7·5, 10 and 15 months). All hams were obtained from 6-month-old porks (mother: Large White × Landrace; father: White Belgium).

Extraction procedure

The extraction was performed according to Aristoy and Toldrá (1991). Samples (5 g) were homogenised with 25 ml

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of 0·1 M HCl in a stomacher homogeniser for 8 min and centrifuged at 10 000 × *g* for 20 min. The supernatant was filtered through glass wool and 300 μl deproteinised for the high-performance liquid chromatography (HPLC) separations according to Toldrá and Aristoy (1993) by adding 2·5 volumes of acetonitrile and centrifuging at 10 000 × *g* for 5 min. It was then evaporated at 30°C under vacuum to dryness and resuspended in 300 μl of 0·1 M HCl. In the case of capillary electrophoresis, deproteinisation was carried out by ultrafiltration in Microsep tubes (Filtron, MA, USA) with nominal molecular mass cut-off of 10 kDa and centrifugation at 3000 × *g* for 60 min at 2–4°C.

Gel filtration HPLC

Deproteinised ham extracts were filtered through a Millipore membrane filter (0·45 μm) and assayed (30 μl) directly in a biocompatible 1050 Hewlett–Packard chromatograph equipped with multiwavelength UV detector (280 nm). The column was a TSK-2000 SW (Tosoh Corp., Japan), 300 × 7·8 mm (4 μm particle size). The eluent was 50 mM phosphate buffer, pH 7, with 50 mM potassium sulphate and 0·05% (w/v) sodium azide. Flow rate was 0·4 ml/min. The column was calibrated by injecting the following standards with known molecular mass: bovine serum albumin (68 kDa), egg albumin (45 kDa), chymotrypsinogen A (25 kDa), myoglobin (18 kDa), cytochrome C (12·5 kDa), aprotinin (6·5 kDa), ristocetin A sulphate (2·5 kDa), pepstatin (686 Da) and tryptophan (204 Da).

Reverse-phase-HPLC (RP-HPLC)

The chromatographic analysis was performed according to Toldrá and Aristoy (1993). Deproteinised ham extracts were filtered through a membrane filter (0·45 μm) and assayed (50 μl) directly in the same HPLC equipment at a wavelength of 214 nm. The column was a Lichrospher 100 RP-18, 250 × 4 mm (5 μm particle size) protected with a guard column (4 × 4 mm) packed with the same material. The temperature was controlled to 35 ± 1°C. The eluant system consisted of solvent A, water with 0·1% (v/v) trifluoroacetic acid (TFA) and solvent B, acetonitrile with 0·07% TFA. Separation was performed at 0·8 ml/min with a linear gradient 0–25% B (25 min) and 25–100% B (25 min).

Free solution capillary electrophoresis (FSCE)

Deproteinised samples (by ultrafiltration) were assayed directly on an Applied Biosystems Model 270A capillary electrophoresis apparatus. Samples were injected for 1·5 s by vacuum and run at 20 kV for 35 min at 35°C in a standard 72 cm capillary (50 μm internal diameter and 50 cm to detector) in 60 mM sodium phosphate buffer, pH 2·5 and 60 mM zinc sulphate. UV absorbance was monitored at 200 nm.

RESULTS AND DISCUSSION

Gel filtration

The dry-cured ham extracts obtained at different processing times were fractionated by gel filtration HPLC and shown in Fig. 1(a)–(g). The chromatograms clearly showed five peaks which could be assigned to five ranges I–V of molecular mass previously established by column standardisation: range I (4500–2700 Da), range II (2700–1200 Da), range III (1200–500 Da), range IV (500–375 Da) and range V (375–160 Da). Due to the low molecular mass, these peptides would fit the category of presumptive flavour principles (Spanier & Edwards, 1987). Peak areas, expressed as percentage against total area, for ranges I–V in Fig. 1(a)–(g) are represented as a function of processing time in Fig. 2. There is a general increase for ranges III–V and a decrease for range I up to 3·5–5 months of process arriving then to a kind of steady-state situation.

These results show that there is a clear increase in peptides with molecular mass below 2700 Da reflecting an intense proteolysis as previously observed for muscle proteins (Toldrá *et al.*, 1992*a*, 1993), especially during the first 3·5 months of process where temperature of drying increases and water activity in hams has not decreased excessively yet.

RP-HPLC

Deproteinised samples from hams after 0, 2, 3·5, 5, 7, 10 and 15 months dry-curing were also fractionated by RP-HPLC in order to quantify the relative levels of

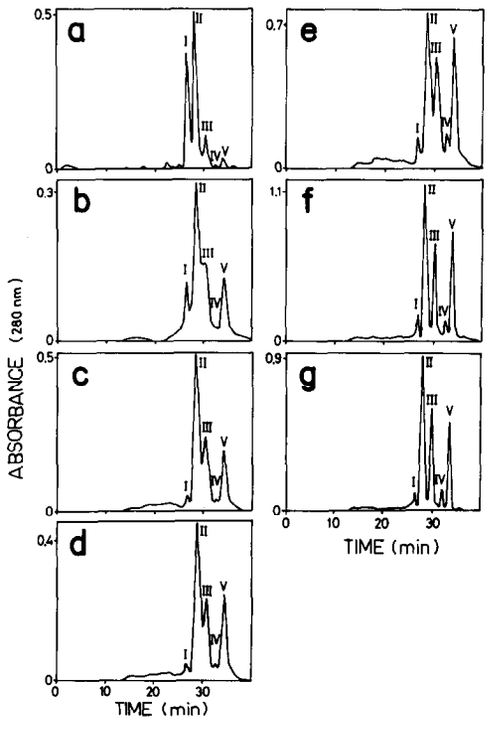

Fig. 1. Gel filtration HPLC chromatograms of ham extracts during dry-curing: (a) 0, (b) 2, (c) 3·5, (d) 5, (e) 7, (f) 10 and (g) 15 months.

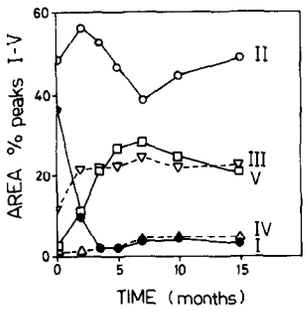

Fig. 2. Peptide evolution plotted as sum of area percent of ranges I–V versus dry-curing processing time. Ranges I–V indicate the subsequent order of ranges of molecular mass. Range I (4500–2700 Da), range II (2700–1200 Da), range III (1200–500 Da), range IV (500–375 Da) and range V (375–160 Da).

hydrophilic and hydrophobic peptides. The peptides in each sample were different because the multiple degradation processes from muscle proteins into peptides and further into amino acids all occurred simultaneously (Toldrá, 1992; Toldrá *et al.*, 1993). Peptide mappings obtained at each of the stages of the dry-curing process are shown in Fig. 3(a)–(g). The peaks or groups of peaks were numbered in the order of elution. There were about 11 major peaks and numerous minor ones. Peaks 6, 7 and 8, which correspond to tyrosine, phenylalanine and tryptophan, respectively, and peak 4 experienced a sensible increase as dry-curing progressed. In the hydrophobic zone there was a noticeable decrease of peaks 9 and 11 in the first 2 months although peak 11 increased up to its initial area after 7 months of process. Something similar happened with peak 2 in the hydrophilic zone. These peptide patterns could provide valuable information about the time of processing of the dry-cured ham.

FSCE

Ham extracts obtained at different processing times were deproteinised by ultrafiltration at a nominal 10 000 Da molecular mass cut-off and analysed by FSCE. The electropherograms are shown in Fig. 4(a)–(g) and showed substantial changes in the peptide mappings. In fact, the high resolution of capillary electrophoresis allows a better observation of differences among peptide patterns. The peaks were assigned to three ranges I–III, corresponding to peptides with higher to lower net positive charge. There was a progressive increase in the first peak of range I and in the late eluting peptides from range I and the peptides eluting in range III as dry-curing progressed up to 7 months while the major peptide in range II progressively decreased from the initial ham samples to 10 months dry-curing. Thus, peptide patterns obtained through capillary electrophoresis could be helpful in determining the approximate time of processing in dry-cured ham.

CONCLUSIONS

Peptide mappings obtained through RP-HPLC and FSCE at different stages of the processing of dry-cured ham have shown an intense proteolysis. The following peptide patterns could provide valuable information about the time of processing. Further research is currently underway in the authors' laboratory in order to determine the flavour characteristics of the different peptide fractions and have a better knowledge of flavour development along the processing of dry-cured ham.

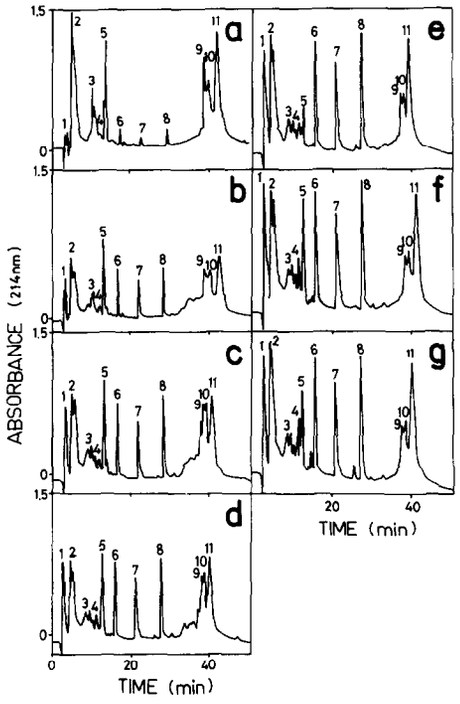

Fig. 3. Peptide mapping by RP-HPLC of acetonitrile-deproteinised ham extracts during dry-curing: (a) 0, (b) 2, (c) 3·5, (d) 5, (e) 7, (f) 10 and (g) 15 months.

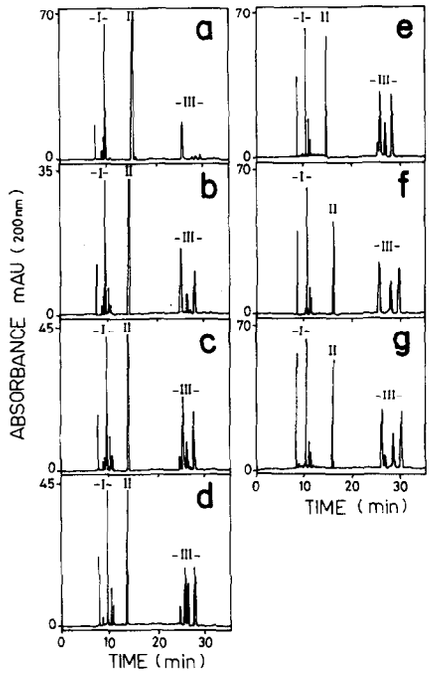

Fig. 4. Peptide mapping by free solution capillary electrophoresis of ultrafiltered (through 10 000 Da molecular mass cut-off) ham extracts during dry-curing: (a) 0, (b) 2, (c) 3·5, (d) 5, (e) 7, (f) 10 and (g) 15 months.

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