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Muscle lipolysis phenomena in the processing of dry-cured ham

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Muscle lipases and esterases were assayed for activity at different stages (0–15 months) in the processing of dry-cured hams. The evolution of volatile and nonvolatile free fatty acids, during processing, was also determined. All the assayed enzymes were found to be quite stable and active even after 15 months of processing. Most lipolysis seemed to occur during the first 5 months when maximal generation of free fatty acids was detected. Neutral and basic lipases are more active at the beginning of the process, while lysosomal acid lipases are active through the entire process. These enzymes seem to be the main ones responsible for the observed lipolysis. Muscle esterases showed excellent stability, but the amounts of volatile free fatty acids were very low, suggesting only a minor role for these enzymes. The study indicates that lipolysis plays a role in the ripening of Spanish dry-cured ham.

INTRODUCTION

Spanish dry-cured ham is a nonsmoked meat product manufactured according to ancient traditions: stabilization through salt diffusion, a decrease in water activity and development of the typical flavour through a long period of maturation. Proteolysis and lipolysis increase during the ageing of Country-style hams (Ockerman *et al.*, 1964), Parma hams (Chizzolini *et al.*, 1984), French hams (Berdagué & García, 1990) and Spanish hams (Flores *et al.*, 1987, 1988; García-Regueiro *et al.*, 1989; López-Bote *et al.*, 1990). The phenomena undoubtedly contribute to flavour development. Due to the high level of fattening, variation in the flavour of dry-cured Spanish Serrano ham has been attributed to the lipid composition of fatty tissues and also to the degree of lipid breakdown during processing, resulting from an increase in the free fatty acid (FFA) content in the muscle (Flores *et al.*, 1988; López-Bote *et al.*, 1990). The autoxidation of fatty acids, especially of the unsaturated ones (oleic, linoleic, linolenic and arachidonic), gives rise to a number of different hydroperoxides which, in conjunction with the many different decomposition pathways involved, leads to a large number of volatile compounds, such as aliphatic aldehydes, ketones, lactones, fatty acids, alcohols and esters (Hayman & Acton, 1978; Lillard, 1978; Schrödter *et al.*, 1986; Shahidi *et al.*, 1986; Welsh *et al.*, 1989; Berger *et al.*, 1990; Yasuhara & Shibamoto, 1990; Matheis, 1991; Ramarathnan *et al.*, 1991). Many different flavour compounds have been identified in Spanish Serrano and Iberian hams and French hams (Berdagué & García, 1990; Berdagué *et al.*, 1991; García *et al.*, 1991; López *et al.*, 1992).

There is evidence that skeletal muscle contains lipases which could be responsible for the lipolysis that occurs during the dry-curing process. Lysosomal acid lipase (EC 3.1.1.3) is involved in the hydrolysis of neutral lipids, such as cholesterol esters and triacylglycerols, introduced into cells as parts of lipoproteins with an optimum pH of 4 to 5 (Imanaka *et al.*, 1981, 1984; Nègre *et al.*, 1985; Sando & Rosenbaum, 1985; Tackeuchi *et al.*, 1985). Lipoprotein lipase (EC 3.1.1.34) is responsible for the hydrolysis of the di- and tri-acylglycerol constituents of very low density lipoproteins and chylomicrons, and has an optimum pH of 8 to 9 for triacylglycerol substrate (Miller *et al.*, 1987; Gorski *et al.*, 1988; Young *et al.*, 1988; Okuda, 1991). Some authors (Hülsmann *et al.*, 1982) have reported the presence of a neutral lipase (pH 7–7·5), but its intracellular localization is still not clear. Oscai *et al.* (1982) and Miller *et al.* (1987) have observed that it has characteristics similar to those of lipoprotein lipase. However, other authors (Holm *et al.*, 1987) have proposed that this activity could correspond to hormone-sensitive lipase (EC 3.1.1.3), which is located in intramuscular adipose tissue with optimum activity at neutral pH.

Several nonspecific, nonlysosomal esterases can be detected in cells (Lake & Patrick, 1970; Kasama *et al.*, 1976; Young *et al.*, 1978); however, in contrast to lipases

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their physiological function is still obscure (Tsujita *et al.*, 1990).

In previous work, important lipase and esterase activities have been detected in pork muscle (Motilva *et al.*, 1992), as well as the effect that the curing agents and other process parameters may exert on those activities (Motilva & Toldrá, 1993).

Very little research has been carried out on the biochemical mechanisms involved in lipolysis occurring during the dry-curing process, which constitutes a preliminary stage in flavour development. This paper reports the studies on the evolution of the lipase and esterase activities in muscle and their relation to lipolysis during processing of dry-cured ham.

MATERIALS AND METHODS

Sampling

Three samples of *Biceps femoris* muscle were removed from hams at different stages in the processing of Spanish Serrano dry-cured ham: raw ham (t = 0 days), after salt equalization (t = 50 days), and at different drying intervals (t = 3·5, 5, 7, 10 and 15 months).

Nonvolatile fatty acids analysis

Lipids were extracted from the muscles according to the method described by Folch *et al.* (1957), using a chloroform–methanol mixture (2:1).

Nonvolatile free fatty acids were separated from the rest of the lipids through amino-propyl bonded phase (Bond Elut, Varian, Harbor City, CA, USA) columns (Kaluzny *et al.*, 1985; Nourooz-Zadeh, 1990). Fifty milligrams of lipids were dissolved in 1 ml of hexane–1,2-dichloroethane (1:1) and then applied on a NH_2 cartridge (Bond-Elut LCR™). The column was washed with 17 ml of hexane and 25 ml of hexane–1,2-dichloroethane–2-propanol (55 : 30 : 15, v/v) to remove cholesterol esters as well as tri-, di- and mono-glycerols. Finally, the column was washed with 8 ml of 2% acetic acid in diethyl ether to elute the nonvolatile fatty acids. The solvent was removed by rotary evaporation at 35°C, and the residue was dissolved in 0·5 ml of 1,2-dichloroethane containing arachidic acid ($C_{20:0}$) (2·248 mg/ml) as internal standard (IS). The fatty acids were analysed without derivatization. The chromatographic analysis was performed in a Perkin Elmer (Norwalk, CT, USA) 8320 gas chromatograph using a capillary column of bonded FFAP-CB (25 m × 0·32 mm × 0·3 μm film thickness, Chrompack). The temperature was programmed to increase from 170 to 225°C at a rate of 1·5°C/min with a final isothermal of 2 min. Two microlitres of standards and samples was split-injected (175°C) into the system. Helium (flow rate 24 cm/s) was used as the carrier gas. The compounds were detected at 275°C by flame ionization. Fatty acids were identified by comparing their retention times with those of standard compounds (Perkin Elmer LCI-100 integrator). The standard fatty acids (Sigma, St. Louis, MO) were: myristic acid (14 : 0), myristoleic acid (14 : 1), pentadecanoic acid (15 : 0), palmitic acid (16:0), palmitoleic acid (16 : 1), heptadecanoic acid (17:0), stearic acid (18 : 0), oleic acid (18 : 1), linoleic acid (18:2), linolenic acid (18 : 3), arachidic acid (20 : 0; IS) and arachidonic acid (20 : 4). The results were expressed as percentages of total lipids.

Short-chain fatty acids analyses

Fatty acid extraction was partially based on methods described by Gerrant *et al.* (1982) and Yeo (1986). Muscle (10 g) was homogenized in 30 ml of distilled water with a Polytron (Kinematica GmbH, Lucerne, Switzerland) (27 000 rpm for 30 s). Extracts were deproteinized by addition of 5 ml of 1N H_2SO_4 and 8 ml of 20% (w/v) phosphotungstic acid (Aristoy & Toldrá, 1991) with agitation for 20 min. Samples were then centrifuged at 4000 rpm for 10 min. The supernatant was filtered through glass wool, neutralized to pH 9·5 to 10 by adding 1N NaOH and evaporated to dryness in a rotary evaporator. The pellet was dissolved in 5 ml of distilled water, and the solution was saturated with NaCl and acidified to pH 1 to 2 by adding 20% (v/v) HCl. Finally, 1 ml of diethyl ether with IS (cyclopropanecarboxylic acid, 0·487 mg/ml) was added and the mixture was centrifuged to break any emulsions. The fatty acids remaining in the ether layer were then ready for chromatographic analysis.

Samples were analysed in the same chromatograph and column as for nonvolatile fatty acids. In this case, the carrier gas (helium) flow rate was 23 cm/s, and the injection and detection temperature was kept at 250°C. The temperature of the column was programmed from 85 to 200°C at 9°C/min with initial isothermal of 3 min. Three microlitres of standards or samples were split-injected into the system. Fatty acids were identified by comparing their retention times with those of standard compounds (Perkin-Elmer LCI-100 integrator). The standard fatty acids (Sigma) were: acetic acid (2 : 0), propionic acid (3 : 0), butyric acid (4 : 0), isobutyric acid (4 : R), valeric acid (5 : 0), hexanoic acid (6 : 0) and octanoic acid (8 : 0). The results were expressed in mg of fatty acid/100 g of dry matter.

Preparation of muscle extracts for enzyme assays

Muscle extracts were prepared as previously described by Motilva *et al.* (1992). Muscle (10 g) was homogenized in 100 ml of 100 mM Tris–HCl, pH 7·0, containing 1 mM EDTA, 0·2% (v/v) Triton X-100, 0·1 μg/ml pepstatin and 1 μg/ml leupeptin. A Polytron homogenizer (Kinematica GmgH, Switzerland) was used (3 × 10 s with cooling on ice). After centrifugation at 10 000g at 4°C for 20 min, the supernatant was filtered through glass wool and collected for the enzyme assays. All operations were carried out at 4°C.

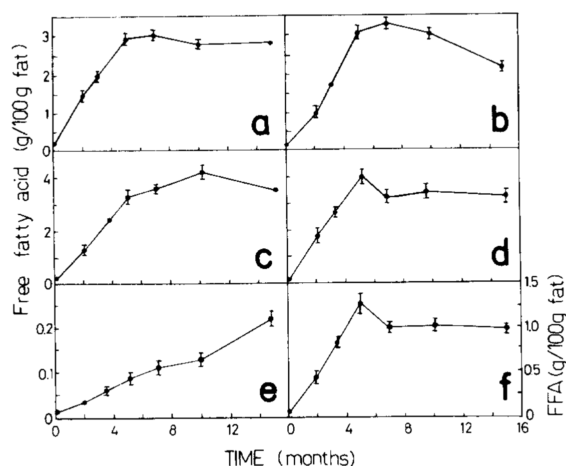

Fig. 1. Evolution of free palmitic (a), stearic (b), oleic (c) linoleic (d), palmitoleic (e) and arachidonic (f) acids generated during the complete processing of dry-cured ham.

Assay of lipids and esterase activities

The assays were carried out as previously described by Motilva *et al.* (1992) The reaction mixture contained 0·2 ml of enzyme extract, 0·1 ml of substrate and 2·7 ml of reaction buffer. The substrates were 10 mM 4-methylumbelliferyloleate and 4-methylumbelliferylpropionate (Sigma, St. Louis, MO) for lipase and esterase activities assays, respectively. The reaction buffer was 100 mM citric acid–200mM disodium phosphate, 0·8 mg/ml bovine serum albumin and 0·05% (v/v) Triton X-100 at pH 5·0 for the assay of acid lipase and acid esterase and at pH 7·5 for the assay of the neutral esterase activity. In the case of the neutral and basic lipase, the reaction buffer was 220mM Tris–HCl, 5 mg/ml BSA and 10 μg/ml heparin at pH 7·0 and 8·0, respectively. The reaction mixture was incubated at 37°C for 45 min and stopped by adding 0·5 ml of 1N HCl solution. The fluorescence was measured at λ_{ex} = 328 nm and λ_{em} = 470 nm using a Shimadzu (Kyoto, Japan) RF-5000 spectrofluorophotometer. One unit of activity (U) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate/h at 37°C. Activities were expressed as U/g of protein. Protein content in muscle was previously determined by an Official Standard method based on the Kjeldahl semimicro method (Presidencia del Gobierno, 1979).

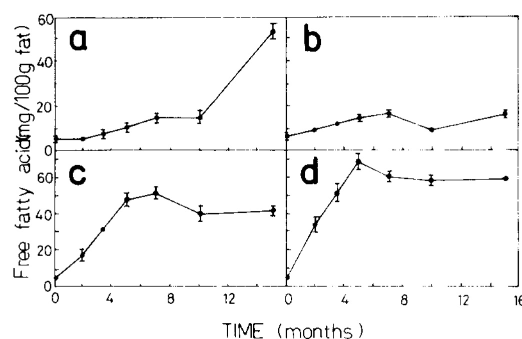

Fig. 2. Evolution of free myristic (a), pentadecanoic (b), heptadecanoic (c) and linolenic (d) acids generated during the complete processing of dry-cured ham.

Statistics

Three replicates were tested for each parameter. Conventional statistical methods were used to calculate mean values and standard deviations.

RESULTS

Lipolysis during the dry-curing process

Generation of FFAs was shown to occur during the processing of dry-cured hams, especially during the initial 5 month period. The evolution of the main nonvolatile fatty acids is shown in Fig. 1. Palmitoleic acid (Fig. 1(e)) increased uniformly during the complete process while palmitic and oleic acids (Fig. 1(a) and (c), respectively) reached a maximum concentration at 5 months. Stearic, linoleic and arachidonic acids (Fig. 1(b), (d) and (f), respectively), however, decreased after reaching a maximum at 5 months storage. The other nonvolatile fatty acids did not noticeably increase during storage. Their evolution is shown in Fig. 2. The main increase in heptadecanoic and linolenic acids (Fig. 2(c) and (d), respectively) was observed during the 5 months, whereas myristic acid (Fig. 2(a)) increased during storage (from 10 to 15 months). On the other hand, pentadecanoic acid (Fig. 2(b)) was almost constant during storage.

The concentration of volatile fatty acids generated during the process was low and variable, as indicated in

Table 1. Evolution of volatile fatty acids in muscle *Biceps femoris* along the dry-curing process of ham

Time (months)	Volatile fatty acid[a]						
	Acetic C2:0	Isobutyric c4:R	Butyric C4:0	Isovaleric C5:R	Valeric C5:0	Hexanoic C6:0	Octanoic C8:0
0	12·5 ± 0·1	3·7 ± 1·1	1·6 ± 0·0	4·1 ± 1·0	TR	TR	TR
2	9·0 ± 0·1	TR	2·1 ± 0·0	TR	TR	0·7 ± 0·4	TR
3·5	18·4 ± 1·6	TR	1·7 ± 0·5	0·9 ± 0·3	TR	0·4 ± 0·1	0·3 ± 0·0
5	29·4 ± 5·9	0·4 ± 0·1	1·7 ± 0·1	2·9 ± 0·7	0·4 ± 0·1	0·5 ± 0·2	0·3 ± 0·1
7	3·0 ± 0·4	TR	1·5 ± 0·1	TR	TR	0·6 ± 0·2	0·3 ± 0·1
10	18·5 ± 5·1	0·3 ± 0·1	1·0 ± 0·2	1·3 ± 0·5	0·4 ± 0·0	0·9 ± 0·1	0·4 ± 0·1
15	2·6 ± 0·8	0·4 ± 0·1	1·1 ± 0·1	2·5 ± 0·4	0·4 ± 0·1	0·3 ± 0·0	0·3 ± 0·0

[a] The results are expressed as mg fatty acid/100 g dry matter (mean ± standard deviation).
TR = traces.

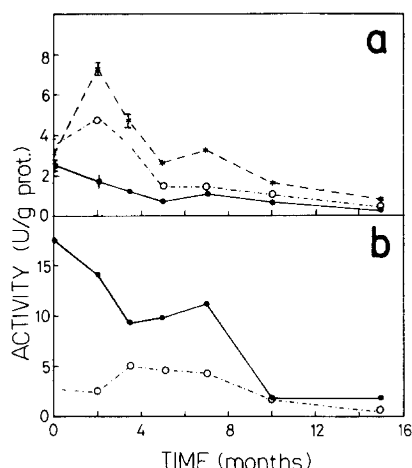

Fig. 3. Evolution of the lipolytic enzyme activity during the complete processing of dry-cured ham: (a) lysosomal acid lipase (●); neutral lipase (○); basic lipase (*); (b) acid esterase (●), neutral esterase (○).

Table 1. Acetic acid was the major volatile fatty acid (see Table 1). The concentration of the other volatile fatty acids was very low (below 2 mg/100 g) or negligible.

Enzyme lipolytic activity during the dry-curing process

Muscle lipases and esterases seem to be quite important in the processing of dry-cured ham. In fact, these enzymes remained active during the complete process, even after 15 months storage, showing good stability (see Fig. 3(a)). The activity of the acid lipase decreased progressively during the process and reached a final activity at 15 months, which was only 10% of the initial value (see Fig. (3(a)). The activities of the neutral and basic lipases increased during the first 2 months (see Fig. 3(a)), but then sharply decreased when drying began (2 months in advance), although their remaining activities at the end of the process (15 months) was still measurable (10 to 20% of the initial activity).

Muscle esterases also showed an extremely good stable activity during the entire process (see Fig. 3(b)). The decrease in the activity of acid esterase was greater in the last 5 months, while the neutral esterase, which showed lower activity, remained fairly constant throughout the process.

DISCUSSION

There is a progressive increase in the concentration of free nonvolatile fatty acids during the first 5 months of storage (see Figs 1 and 2). This increase is in agreement with the maximal lipolytic enzyme activity and with the fat composition of the *Biceps femoris* muscle (Sweeten *et al.*, 1990; Lesigneur-Meynier & Gandemer, 1991) (see Fig. 3(a)). From this point, the generation of most of the free nonvolatile fatty acids did not further increase (Figs 1(a)–(c) and 2(b)–(d)), while palmitoleic and myristic acids (Figs 1(e) and 2(a) respectively) were still being generated. However, the polyunsaturated fatty acids (linoleic and arachidonic acids) decrease (Fig. 1(d) and (f)) due to their double bonds and probable oxidation (Hayman & Acton, 1978; Lillard, 1978; Schrödter *et al.*, 1986; Shahidi *et al.*, 1986; Welsh *et al.*, 1989; Berger *et al.*, 1990; Yasuhara & Shibamoto, 1990; Matheis, 1991; Ramarathnan *et al.*, 1991). In fact, all these FFAs constitute a first step as precursors for secondary reactions (oxidations, interactions with proteins, etc.) for flavour development (Moody, 1983; Gray & Pearson, 1984).

It is surprising that the volatile fatty acids (C_2 to C_8) are only detected at ppm levels (Table 1) in spite of the high esterase activity detected in muscle during the entire process (Fig. 3(b)). This is a consequence of the absence of an adequate substrate in muscle (short chain fatty acid glycerides) for esterase action, since the muscle glycerides and phospholipids are esterified to long-chain fatty acids (Sweeten *et al.*, 1990; Leseigneur-Meynier & Gandemer, 1991).

The lipase and esterase activities in muscle indicate that they are active (Tombs, 1985) although the muscle conditions (concentration of curing agents and process parameters) found at each stage could somehow limit enzyme functionality. In fact, the salting and post-salting stages are characterized by a progressive increase in salt (2–6%) and low temperatures (2–4°C), which, in a previous study by Motilva *et al.* (1992), was demonstrated to affect neutral and basic muscle lipases. As the process continues (drying/curing stage), the temperature increases and the water activity decreases, which may inactivate the neutral and basic lipases and favour the lysosomal acid lipase, which is increased by salt concentration and a decrease in A_w (Motilva & Toldrá, 1993). In view of these circumstances, neutral and basic lipases would be more active at the beginning of the process, while lysosomal acid lipase would be active through the entire process, being almost unique through the drying/curing stage.

The activity of muscle esterases could be limited by salt and low temperatures although their role in the process seems not to be relevant in view of the absence of adequate substrates and the low amounts of volatile FFAs being generated.

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