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Development of cathepsin B, L and H activities and cystatin-like activity during two different manufacturing processes for Spanish dry-cured ham

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Dry-cured ham is a high-quality meat product, and interest in the study of the different technological and biochemical parameters involved in its manufacture is high. Lysosomal cysteine proteinase activity and cystatin-like activity were determined in the *Semimembranosus* and *Biceps femoris* muscles during two ham-manufacturing processes of different length, and dependence of these activities on curing time was found. Results also showed that cathepsin L lost its activity more rapidly than cathepsin B and that the contribution of cathepsin H to the proteolysis produced in these curings was very low. Cystatin-like activity decreased in both processes, although the ratio between proteinase activity and cystatin-like activity depended on the length of the curing process.

INTRODUCTION

Dry-cured ham is a traditional meat product of great commercial value. The length of the curing process and its complexity are the reasons for the high prices of hams and for the demand for high quality by the consumer.

Various parameters are involved in the production of dry-cured ham, which can be summarized in two general aspects: the quality of the raw material and the curing process (Arnau, 1991).

Several studies have shown intense proteolysis during the curing with different technologies in Italian Parma ham (Cantoni *et al.*, 1972; Bellati *et al.*, 1983), Spanish dry-cured ham (Flores *et al.*, 1974; Astiasaran *et al.*, 1988; Cordoba, 1990; Hortos & Garcia Regueiro, 1991), and American country-style ham (McCain *et al.*, 1968).

The ultrastructural and biochemical changes observed during post-mortem ageing of meat have been attributed to some extent to muscle proteinases, especially calpains and lysosomal proteinases, and their contribution to meat tenderization. These two proteolytic systems have been widely studied in fresh and aged meat from different species (Goll *et al.*, 1983; Ouali *et al.* 1987; Ouali, 1992; Koohmaraie *et al.* 1988; Koohmaraie, 1992; Zeece & Katoh, 1989; Etherington *et al.*, 1990). The lysosomal cysteine proteinases, cathepsins B, H, and L, are active at acidic pH and have the ability to degrade myofibrillar proteins. These cathepsins are regulated *in vivo* by protein inhibitors, called cystatins. These inhibitors are cytosolic proteins that bind tightly and reversibly to the papain-like cysteine proteinases. They may protect the cells from inappropriate endogenous or external proteolysis and could be involved in the control mechanism responsible for protein breakdown (Barrett *et al.*, 1987; Turk & Bode, 1991).

However, there are few studies on the role of these proteinases in cured ham. It has been suggested that cathepsins could be one of the factors responsible for the proteolysis that takes place during the curing of hams, since their activity is maintained throughout the process (Toldrá & Etherington, 1988; Gil *et al.*, 1991), whereas calpain activity is not detected after the first stages of curing (Parreño *et al.*, 1990; Sarraga *et al.*, 1993). Other studies focusing on the influence of the

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curing agents (salt, nitrate), and other parameters of the process (water activity, temperature, pH) in muscle proteinases have been reported (Sarraga *et al.*, 1989; Rico *et al.*, 1990; Toldrá *et al.*, 1992). But the contribution of these proteinases during the dry-curing process to the final characteristics of the ham is still unknown.

In this paper, the development of cathepsins B, L, and H and their endogenous inhibitors, cystatins, was monitored and related to the length of the curing process.

MATERIALS AND METHODS

Selection of raw material

Hams from female pigs were selected for normal meat quality to avoid problems derived from PSE and DFD meat (Arnau *et al.*, 1990). The selection was made by measuring pH at 45 min and 24 h post-mortem (pHmeter Crison with Ingold 406 penetration electrode), electric conductivity (Quality Meater, Digi 550, Wissenschaftlichtechmishe, Weilheim, Germany), and light-scattering (Fiber Optic Probe, McDougall & Jones, 1985).

Manufacturing Technologies

Two series of hams of different weight were manufactured according to two different curing processes.

After 24 h post-mortem, hams were covered with curing salt at 0–5°C for 10 days (salting stage). Hams were washed and then hung for 30 days at 2–5°C while the salt diffused into them (salt-equalization stage). Finally, they were dried under different conditions, temperature and time, in the curing chamber for the development of the texture and flavour (ripening stages) for three months in the short process or seven months in the long process. The two processes are summarized in Table 1.

Sampling

Muscle samples were taken from three fresh hams, three hams after the salting stage (post-salting) and three hams every month until the end of each curing process. Thus, 12 and 24 hams were analyzed for the short and long processes, respectively.

The muscles studied were *Semimembranosus* (SM) and *Biceps femoris* (BF). Anatomically, SM and BF muscles are both located at the surface of the ham; however, BF is covered with subcutaneous fat, thus making diffusion of the salt more difficult, whereas in SM salt diffuses faster (Arnau, 1991).

The dissected muscles were trimmed of fat and connective tissue and then ground. Portions of about 100 g of ground muscle were vacuum-packed in dark bags and then frozen until use (to ensure the homogeneity and stability of the samples).

Table 1. Manufacturing conditions in the curing processes

Short process		
Length of time	4 months	
Mass of fresh hams	8 kg	
Stages	Temperature (°C)	Time of curing (days)
Salting*	0–5	10
Salt-equalization	2–5	30
Ripening	8–10	30
	14–16	30
	24–26	30
Long process		
Length of time	8 months	
Mass of fresh hams	10 kg	
Stages	Temperature (°C)	Time of curing (days)
Salting*	0–5	10
Salt-equilization	2–5	30
Ripening	8–10	30
	10–12	30
	14–16	60
	18–20	60
	24–26	30

* The period of salting was determined by the mass of the fresh hams and the length of the process: 1·25 days/kg in the short process and 1 day/kg in the long process.

Preparation of muscle extracts

A portion of frozen muscle was homogenized at 4°C with a Polytron in 10 vol of ice-cold 50 mM sodium citrate buffer, pH 5·0, containing 1 mM EDTA and 0·2% (v/v) Triton X-100. The homogenate was stirred for 1 h at 4°C and then centrifuged at 30000 *g* for 20 min (Etherington *et al.*, 1987). The supernatant was filtered through glass wool and used for the assay of cathepsins B, L, and H. A second portion of the muscle was homogenized (1/4, w/v) in 10 mM Tris–HCl buffer, pH 7·5, containing 3mM EDTA. The homogenate was centrifuged at 25000 *g* for 20 min, and the supernatant was filtered. A portion of this extract was adjusted to pH 10–11 with 5N NaOH, left for 60 min at 40°C, and then adjusted to pH 6·8 with 5N HCl. The precipitate was removed by centrifugation at 3000 *g* for 30 min. The supernatant did not present cathepsin B, L, or H activity and was kept frozen with 20% (v/v) glycerol until it was used to titrate the cystatin-like cysteine proteinase inhibitors.

Assay of enzyme activities

Cathepsins B, L and H were assayed fluorimetrically by using fluorescent binding peptides as substrates (Barrett, 1980).

Cathepsins B and L were assayed with their common substrate N-CBZ-L arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NH Mec) (Bachem) at a final concentra-

tion of 10 μm. Cathepsin B was assayed with N-CBZ-L arginyl-L-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NH Mec) (Bachem) at a final concentration of 10μM, and cathepsin H was assayed with L-arginine 7-amido-4-methylcoumarin (Arg-NH Mec) (Bachem), at a final concentration of 5μM.

The 7-amino-4-methylcoumarin (NH Mec) released in the assays was monitored continuously by using a record attached to the fluorescence spectrometer with a thermostatically controlled cell holder. The NH Mec produced was determined at 350–360 nm (excitation) and 460 nm (emission). An excitation scan was performed before the assay. The instrument was calibrated by using pure N Mec (0·005–0·15 μM) (Etherington & Wardale, 1982).

One unit of enzymatic activity was defined as that amount of enzyme that hydrolyses 1 μmol of substrate per minute at 37°C. Specific activities were given in microunits of enzyme per mg of protein.

Determination of cystatin-like activity

Cysteine proteinase inhibitors were assayed on papain by using a micro-scale titration based on the method of Anastasi *et al.* (1983).

The molar concentration of active papain was previously determined by titration with E-64, as proposed by Barrett *et al.* (1982), but after the micro-scale titration of cystatin. Hence, the total papain concentration was about 6·25nM in the preincubation. In order to avoid the inactivation of papain due to high dilution, the enzyme was diluted with 0·2M potassium sodium phosphate buffer, pH 5·8, containing 2mM EDTA, 2mM DTT, and 25mM NaCl.

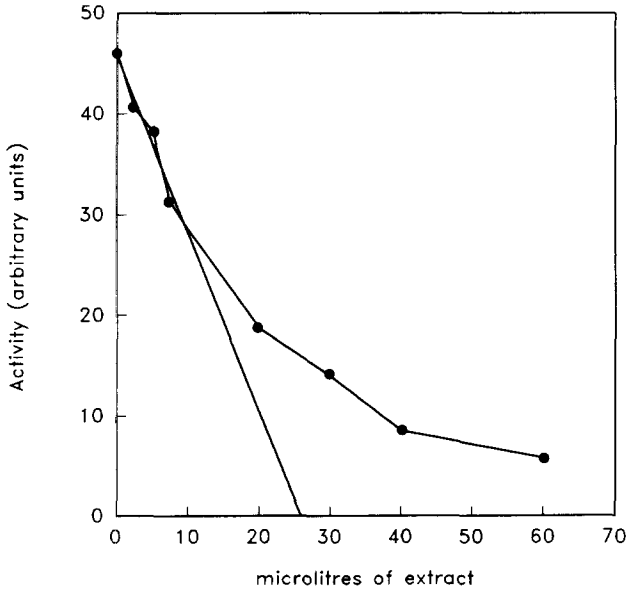

Fig. 1. Titration of cystatins in muscle extract. Different volumes of muscle extracts (μl) were pre-incubated with the same concentration of titrated papain. In the plot, the measured fluorescence shows residual activity against the substrate (Z-Phe-Arg-NH Mec). Results in the linear part of dose-response curve were extrapolated to give the molar concentration of cystatin.

The quantification of cystatin-like cysteine proteinase inhibitors was performed by preincubation with 1·87nM active papain and by using 5μM Z-Phe-Arg-NH Mec as substrate to determine residual-papain activity. The results in the linear part of the dose-response curve were extended to give the molar concentration on the assumption of a 1:1 (mol:mol) binding ratio of cystatin with papain (Fig. 1).

The quantification of cystatins in the samples was expressed as pmol of cystatin per mg protein.

Protein determination

Protein concentration was determined by the method of Bradford (1976), bovine serum albumin being used as standard.

RESULTS AND DISCUSSION

With the aim of obtaining deeper knowledge of the role of muscle proteases in the proteolysis that takes place during the curing process of ham, the activities of cathepsins B, L, and H in two processes of different length were studied.

Cathepsin B activities in SM and BF muscles is shown in Fig. 2. In the four-month process, the activity was maintained throughout the curing and was 84% at the end of the short process in both muscles (Fig. 2A). In contrast, activity in the eight-month process decreased slowly and only 27% and 45% of the initial values remained in the SM and the BF muscles, respectively (Fig. 2B). An increase in activity in post-salting (about 35% with respect to fresh muscle) was observed in both processes, and after six months of the long curing process (52% in SM, 82% in BF with respect to the fifth month). The specific activities in BF were 5–25% higher than in the SM muscle in the short process and 3·8–28·8% higher in the long process.

The gradual development of cathepsin L activity during the two processes was studied together with that of cathepsin B, since they utilize a common substrate (Fig. 3). Cathepsin B+L activity in both muscles had decreased by about 34% at the end of the short process (Fig. 3A), and 15% (SM) and 27% (BF) at the end of the long one (Fig. 3B). These data may indicate that cathepsin L loses its activity more rapidly than cathepsin B, which is more stable throughout curing.

Although cathepsin B+L activity was similar in both fresh muscles, these activities were higher in the BF than in the SM muscle throughout both processes, being 2·5–39·5% for the short process and 10·9–39·5% for the long process. This could be explained by a lower inhibitory effect by NaCl (Sárraga *et al.*, 1989; Rico *et al.*, 1990) in the BF muscle than SM as a result of the slower diffusion of the salt in BF, owing to its anatomical location (Arnau, 1991).

Cathepsin H activity found in the fresh muscle fell sharply in the post-salting, and it was practically negligible (less than 4%) throughout the rest of the curing

Table 2. Cathepsin H activity during a short curing process of ham, expressed as μ units per mg of protein

Curing process	SM*	BF*
Fresh muscle	39·9 ± 10·5 (100%)	36·1 ± 7·6 (100%)
Post-salting	1·7 ± 0·35 (4·3%)	11·4 ± 1·1 (31·6%)
3 months	1 ± 0·4 (2·5%)	2 ± 0·6 (5·5%)
4 months	0·7 ± 0·2 (1·8%)	1 ± 0·1 (2·8%)

*SM—Semimembranosus muscle
BF—Biceps femoris muscle
Mean ± SD data refer to three separate hams.
Values in parentheses are the percentages of remaining activity at each curing step with respect to the activity in fresh muscle.

process (Tables 2 and 3). From our data we can conclude that cathepsin H has little participation in the curing, possibly owing to its instability. These data differ from those reported by Toldrá and Etherington (1988), who found a remaining activity of 20% for cathepsin H in the SM muscle from only one sample of an eight-month-cured Serrano ham. The differences could be due to the curing process and to the quality of the raw material.

Studies on meat-ageing showed that acidification and high temperatures help to cause the rupture of lysosomal membranes (Weisman, 1964; Stagni & de Bernard, 1968), thus favouring the release and subsequent action of cathepsins on the proteins of the muscle cell (Bandman & Zdanis, 1988). These findings may be extrapolated to the curing process of ham, in which temperatures up to 26°C are reached in the curing chamber.

Table 3. Cathepsin H activity during a long curing process of ham, expressed as μunits per mg protein.

Curing process	SM*	BF*
Fresh muscle	39·9 ± 10·5 (100%)	36·1 ± 7·6 (100%)
Post-salting	1·7 ± 0·35 (4·3%)	11·4 ± 1·1 (31·6%)
3 months	1 ± 0·4 (2·5%)	1·4 ± 0·3 (3·9%)
4 months	0·8 ± 0·2 (2%)	1 ± 0·5 (2·8%)
5 months	0·5 (1·3%)	0·8 ± 0·1 (2·2%)
6 months	0·4 ± 0·2 (1%)	1 ± 0·2 (2·8%)
7 months	0·3 (0·8%)	0·5 ± 0·2 (1·4%)
8 months	0·6 ± 0·2 (1·5%)	0·4 ± 0·1 (1·1%)

*SM—Semimembranosus muscle
BF—Biceps femoris muscle
Mean ± SD data refer to three separate hams.
Values in parentheses are the percentages of remaining activity at each curing step with respect to the activity in fresh muscle.

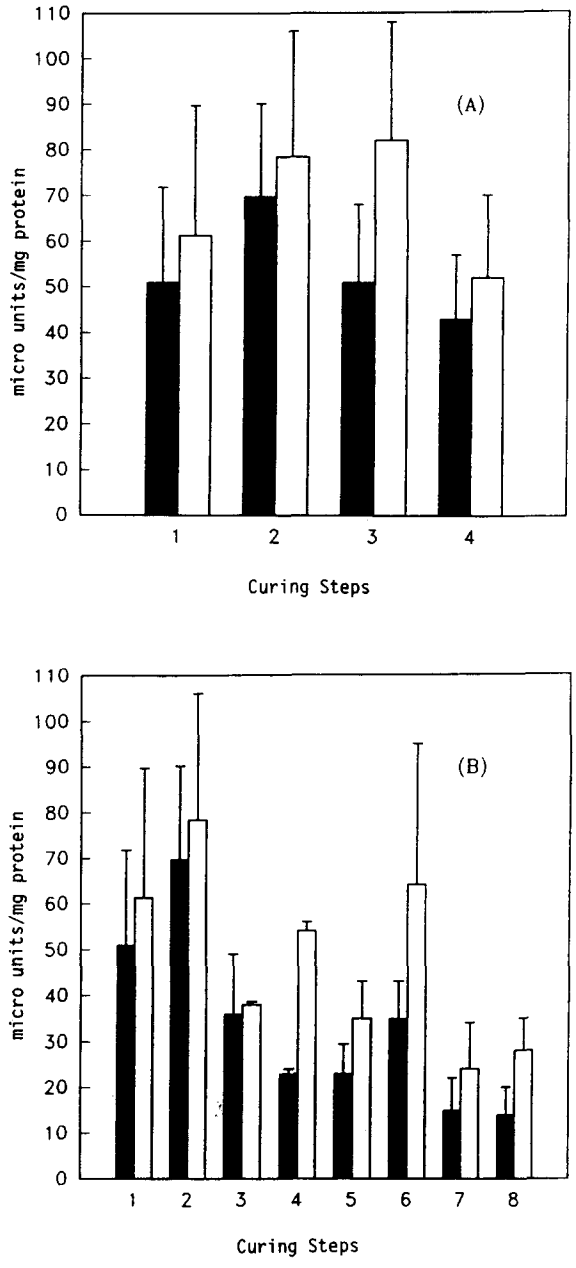

Fig. 2. Cathepsin B activity during two curing processes of ham: (A) short curing process and (B) long curing process; 1, fresh muscle; 2, post-salting; 3–8, months of curing. SM muscle (■); BF muscle (□). Results are the averages from three separate hams.

Studies on the enzyme activities were performed by using whole homogenates with the lysated lysosomes, which could result in underestimation of cathepsin activities owing to the presence of their endogenous inhibitors. We quantified these inhibitors in order to obtain more information about the proteolytic process that takes place during curing. Fig. 4 shows that the profile of cystatin-like activity decreased slowly and progressively during the curing process. The remaining activities at the end of the process were 57% in the short process (Fig. 4A) and about 40% in the long one (Fig. 4B).

It has been shown that the binding of cystatins to cysteine proteinases is strong and reversible and that they are stable to heat and over a wide pH range

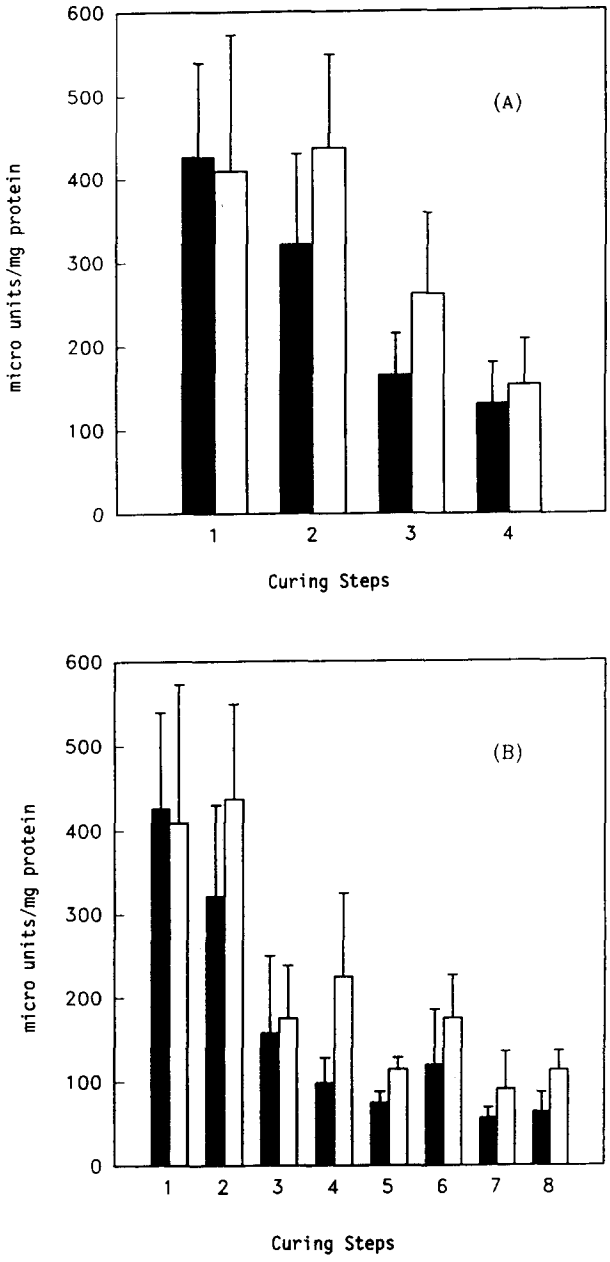

Fig. 3. Cathepsin B + L activity during two curing processes of ham: (A) short curing process and (B) long curing process; 1, fresh muscle; 2, post-salting; 3–8, months of curing. SM muscle (■); BF muscle (□). Results are the averages from three separate hams.

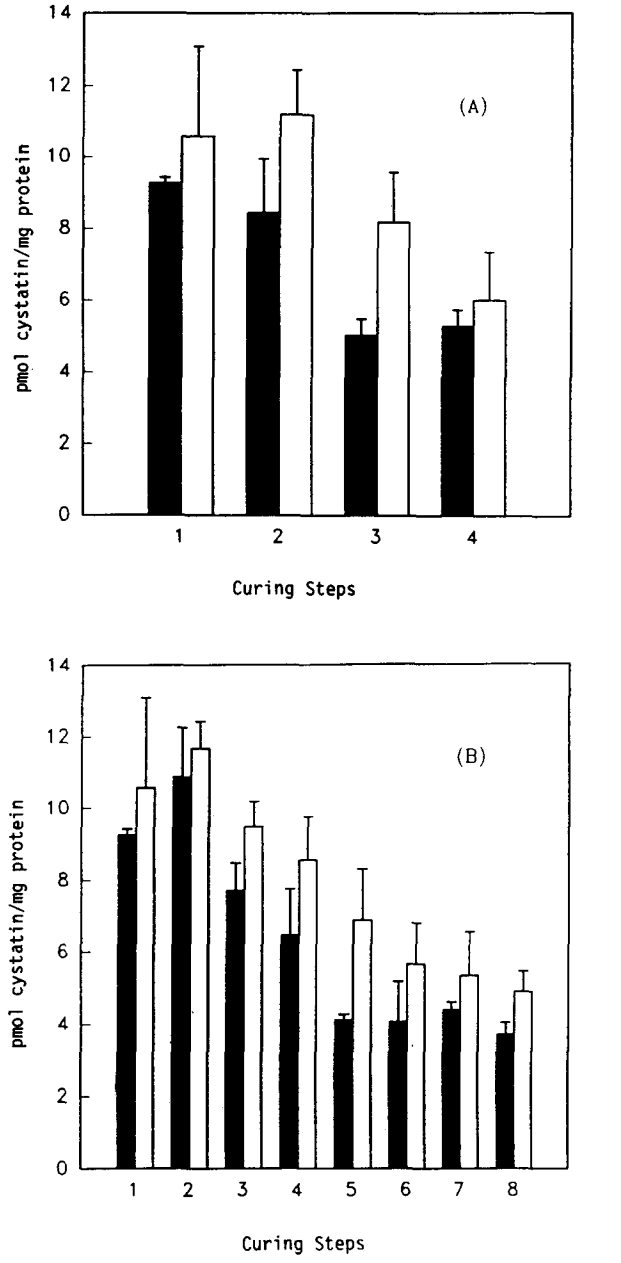

Fig. 4. Cystatin-like activity during two curing processes of ham: (A) short curing process and (B) long curing process; 1, fresh muscle; 2, post-salting; 3–8, months of curing. SM muscle (■), BF muscle (□). Results are the averages from three separate hams.

(Turk & Bode, 1991). Cystatins also bind more specifically to cathepsin L than to cathepsin B (Barrett, 1987), so the level of cathepsin L in the muscle extract is underestimated as compared with cathepsin B by the inhibitory effect of cystatins.

The ratio cathepsin B+L: cathepsin B decreased by 50% from fresh muscle to post-salting in SM and from fresh muscle to three months in BF, being maintained the same values until the end of both processes (Tables 4 and 5). This may indicate that cathepsin L lost a great amount of its activity at the beginning of the curing process. When B+L:B was related to cystatins, the ratio (B+L:B): C decreased in the post-salting, whereas it maintained the values during the rest of the processes (Tables 4 and 5). However, in the long process, the SM muscle showed a gradual increase in the ratio after post-salting (Table 5). These results suggest that cathepsin L decreases faster than cystatins in the first stage, and then levels of cathepsin L and inhibitors decrease in parallel with the time of curing for both processes although, in the long process in SM muscle, the cystatin decreases slightly faster than the enzyme.

In the short process (Table 4), the ratio B:C increased, which indicated that the cystatins fell faster than cathepsin B activity and promoted its proteolytic activity. In the long process (Table 5), the ratio B:C decreased slightly except for the six month, when there was a marked increase, together with an increase in cathepsin B activity (Fig. 2B). The differences in B:C

Table 4. Relationship of cathepsin B+L with cathepsin B and with cystatins in the short curing process*

Curing process	B + L:B		(B + L:B):C		B:C	
	SM	BF	SM	BF	SM	BF
Fresh muscle	8·3	6·7	0·9	0·63	5·5	5·8
Post-salting	4·6	5·6	0·54	0·50	8·2	7·0
3 months	3·2	3·2	0·64	0·39	10·1	10
4 months	3	2·9	0·57	0·48	8·1	8·6

* Cathepsin B + L activity (B + L).
Cathepsin B activity (B).
Cystatin-like activity (C).

ratio in the two processes could explain why the same amount of amino acids is produced at the end of the processes (Hortos & Garcia-Regueiro, 1991). The data suggest that cathepsin B activity could be modified according to the curing process used.

Electrophoretic studies on the effect of these proteinases on myofibrillar proteins show that cathepsin L affected most contractile proteins, cathepsin H hardly degraded any myofibrillar proteins, and cathepsin B mainly degraded the low-Mr protein fractions and, to a less extent, myosin (Ouali *et al.*, 1987; Dufour *et al.*, 1989). In ultrastructural studies, it was found that both the Z-line and the dense edges of the A-band were extensively degraded by cathepsin B, as well as by cathepsin L, whereas cathepsin H hardly affected the myofibrillar ultrastructure (Davey & Dickson, 1970; Penny, 1980; Ouali *et al.*, 1987).

The proteolysis that takes place during ham-curing could be produced in the first stage (from fresh muscle to post-salting) by cathepsin L and calpain (Parreño *et al.*, 1990; Sárraga *et al.*, 1993). Cathepsin B could have an intermediate role in degrading low-Mr protein fractions into amino-acids.

The addition of the curing salt is one of the most characteristic factors in the manufacture of cured ham. The salt seems to have a double role. Firstly, it produces changes in the myofibrillar structures (Aranda *et al.*, 1991), owing to the high osmotic pressure, which weakens the myofibrillar structure. This action could improve the affinity of proteinase towards the myofibrillar structure and other muscle proteins. Secondly it inhibits muscle proteinases, delaying the proteolytic process, and stabilizes the enzymes (Toldrá *et al.*, 1991).

Table 5. Relationship of cathepsin B+L with cathepsin B and with cystatins in the long curing process*

Curing process	B + L:B		(B + L:B):C		B:C	
	SM	BF	SM	BF	SM	BF
Fresh muscle	8·3	6·7	0·89	0·63	5·5	5·7
Post-salting	4·6	5·6	0·42	0·47	6·4	6·7
3 months	4·4	4·7	0·57	0·49	4·6	4
4 months	4·3	4·2	0·66	0·48	3·5	6·3
5 months	3·2	3·3	0·77	0·40	5·6	5·1
6 months	3·4	2·7	0·86	0·44	8·6	11·3
7 months	3·8	2·4	0·86	0·44	3·4	4·5
8 months	4·5	4·1	1·2	0·83	3·7	5·7

* Cathepsin B+L activity (B+L).
Cathepsin B activity (B).
Cystatin-like activity (C).

Our results show that the activity of the lysosomal cysteine enzymes in relation to cystatins depended on the length of the curing process. Since cathepsin activities contribute only partially to the general proteolysis that occurs during curing, there is a clear gap in our knowledge, which makes it difficult to relate these changes with the quality of the ham. Moreover, several factors other than proteolysis are involved in the sensorial development of the final product, and these should all be considered to explain the quality achieved by the cured hams.

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