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Activity of cathepsin B, D, H and L in Spanish dry-cured ham of normal and defective texture

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Abstract

Thirty-six specimens of Spanish dry-cured ham from 18 different batches were studied to determine the influence of endogenous enzymes on the textural quality of the end product. It was shown that, the residual enzyme activity of cathepsins B + L is a reliable indicator of textural defects associated with strong proteolysis in Spanish dry-cured ham. In contrast, the activity of cathepsin B, which varied widely among samples, was less influential. Salt levels can have a moderate effect on the residual enzyme activities. Other compositional parameters also influence the properties of the end product; the amount of protein in *semimembranosus* muscle was found to be less and the moisture greater in texturally defective specimens. Also, the efficiency of the drying-curing process was less in hams yielding poor textures.

1. Introduction

Spanish dry-cured ham accounts for nearly one half of all pork products manufactured each year in Spain. This product is widely accepted by consumers because of its peculiar sensory properties (basically texture and flavour), which it acquires via a complex sequence of chemical and biochemical reactions during manufacturing (Timón, Barandiaran, De la Vega & Ventanas, 1995; Timón, Barandiaran & Ventanas, 1995; Timón, Barandiaran, De la Vega & Ventanas, 1995; Toldrá, 1998).

Both the raw material and the technological process used in making dry-cured ham influence the sensory quality of the end product. Specially important among the factors in the raw material are the thickness of the subcutaneous fat layer, which influences the amount of intramuscular fat present and the green ham weight. These last parameters are used as criteria in choosing raw materials and dictate the type of processing to be used (Arnau, Guerrero & Gou, 1993; Diestre, 1992). The pH of the meat is another significant parameter as it affects the sensory quality of the end product. In this respect, most authors recommend not using green hams with a pH above 6.2 to avoid both microbial growth (Newton & Gill, 1981) and problems related to soft textures (Guerrero, Arnau, Maneja & Gou, 1992). Another factor to be considered is the age of the animal at slaughter, which can affect the ham colour, its content of intramuscular fat and the extent of proteolysis (Sárraga, Gil & García-Regueiro, 1993).

Some authors also recommend measuring proteolytic activity in the fresh Parma ham to assess its suitability for making the cured products (Virgili, Parolari, Schivazappa, Soresi-Bordini & Born, 1995a; Virgili, Parolari, Schivazappa, Soresi-Bordini & Volta, 1995b). Spanish ham is cured using a salting, desiccation procedure involving increasing temperatures and decreasing relative humidity. Salt plays a central role in the microbiological stabilization of the product (Pineda & Carrascosa, 1993) and appears to have some influence on sensory properties as a result of its influence on the formation of tyrosine crystals in the ham, the appearance of a white film on the surface in contact with the atmosphere and changes in textural properties (Arnau, Guerrero & Gou, 1997; Martin, Córdoba, Antequera, Timón & Ventanas, 1998). Some authors have examined the effect of temperature and found that the extent of proteolysis and the ocurrence of defects such as brightness, pastiness and piquantness increase with increasing

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temperature at the late stages of the manufacturing process (Martín et al, 1998; Arnau et al).

The sensory quality of Spanish dry-cured ham is judged on its texture, flavour and appearance. Consequently, texture-related (e.g. poor mechanical strength, pastiness, stickiness), flavour-related (e.g. piquantness, bitterness) and appearance-related problems (e.g. formation of a white film or white spots) frequently result in major economic losses to the manufacturer and severely detract from the quality of the product (Arnau, 1998). Thus, sensory-related defects are the subject of increasing research that suggests a relationship with uncontrolled proteolysis of the muscle proteins (Parolari, Rivaldi, Leoneli, Bellati & Bovis, 1988; Toldrá, Flores & Voyle, 1990). The mechanism by which proteolysis takes place is still not fully known; however, it is believed that texture development in the fresh meat (tenderization) is partly a result of the action of one or more proteolytic systems present in the muscle tissue (Etherington, Taylor & Dransfield, 1987; Ouali, 1992; Roncales, Geesink, van Laack, Jaime, Beltrán, Barneir et al, 1995).

While calcium-dependent neutral proteases, calpains, play a central role in conditioning meat (Koohmaraie, 1992), they exhibit virtually no activity at the end of the salting step (Córdoba, Antequera, Ventanas, López-Bote, Garciá & Asensio, 1994; Sárraga, Gil, Arnau, Monfort & Cussó, 1989). Hence it has been suggested that lysosomal enzymes (cathepsins N, B + L, H and D) might be wholly or partly responsible for proteolysis during curing as their activity is preserved throughout the process (Parreño, Cussó, Gil & Sárraga, 1994; Sárraga et al., 1993; Toldrá & Etherington, 1988; Toldrá, Rico & Flores, 1993), the level at the end is still 5–15% of the starting value. In most cases, proteolytic activity in ham has been related to endogenous systems (Toldrá & Etherington, 1988). However, several studies have suggested some microbes contribute to the proteolytic process (Francisco, Gutiérrez, Menes, Garciá, Díez & Moreno, 1981; Núñez, Rodríguez, Martin, Córdoba, Bermúdez & Asensio, 1988).

The purpose of this work was to determine whether the presence of the defect called "pastiness" is related to the residual enzyme activity of cathepsins B, B + L, H and D in Spanish dry-cured ham.

2. Materials and methods

2.1. Raw material and curing

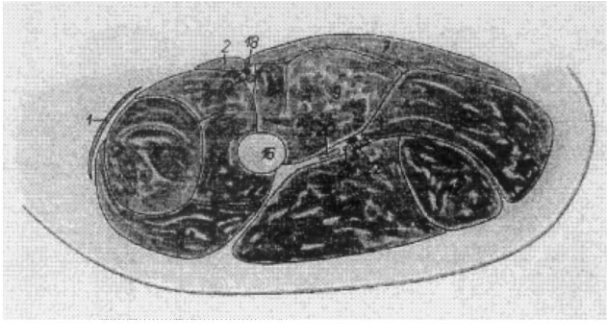
Refrigerated hams weighing 10–12 kg from different suppliers were processed 3–5 days after slaughter. The curing procedure was typical of that used to produce Spanish dry-cured ham. Briefly, following addition of the curing salts ($NaNO_2$ and KNO_3) in appropriate amounts (characteristic of each manufacturer), the hams were stored salt-coated at 2–3°C for about 1 day per kilogram of green ham. They were then rinsed and hung from stainless steel trolleys at 4°C and 80–90% relative humidity for about 2 months to allow absorbed salt to equilibrate. This was followed by the ripening step, which was developed at a gradually increasing temperature (up to 25°C) and decreasing relative humidity (to 65%) for at least 9–10 months.

2.2. Sampling

Homogeneous batches of about 300 hams from different suppliers and ready for market were sampled over a 10 month period. Approximately 10% of the hams in the batch were randomly selected for boning and a cross-sectional cut was made immediately below the hip bone to obtain samples for sensory assessment (Fig. 1); the cut contained the *adductor, graci!is, semimembranosus, biceps femoris* and *semitendinosus* muscles, among others (Arnau, Guerrero, Casademont & Gou, 1995). Samples for analysis were selected for the presence or absence of the defect pastiness. Thus, each batch was used to obtain a sample exhibiting no such defect and another markedly defective in this respect (i.e. two samples, one qualified as 1 and the other as 4, were taken from each batch), the samples spanned the whole cross-section of the ham and were 5–10 cm thick. Analytical determinations were carried out on the *semimembranosus* and *biceps femoris* muscles.

2.3. Sensory assessment

As stated above, the hams were assessed for sensory quality in order to detect the presence or absence of



1. *M. tensor faciae latae*
2. *M. sartorius*
3. *M. vastus medialis*
4. *M. rectus femoris*
5. *M. vastus lateralis*
6. *M. vastus intermedius*
7. *M. gracilis*
8. *M. pectineus*
9. *M. adductor*
10. *M. semimembranosus*
11. *M. biceps femoris*
12. *M. semitendinosus*
13. *Caput mediale mi. gastrocnemii*
14. *M. flexor digitorum superficialis*
15. *Caput laterale mi. gastronemii*
16. *Os femoris*
17. *Patella*
18. *N. saphenus*
19. *A. et v. femoralis*
20. *N. ischiadicus*
21. *N. cutaneus surae caudalis*
22. *Ramus a. et v. circumflexae*
23. *A. et v. poplitea*
24. *N. tibialis*
25. *N. peroneus communis*

Fig. 1. Ham slice used to perform the sensory assessment and the sampling for chemical analyses.

pastiness, which was encountered mainly in the *biceps femoris* muscle and to a smaller extent, in the *semimembranosus, semitendinosus, vastus intermedius, vastus lateralis* and *vastus medialis* muscles. As regards texture, a pasty ham is softer to the touch, more easily deformed and stickier than a non-defective ham. Regarding flavour, pasty ham is bitter, somewhat piquant, with a metallic taste, less salty with a weaker cured ham flavour. Finally, the aroma is also stronger than in non-pasty ham.

The presence of the defect was judged by a group of three experts working for the manufacturer that gave a score of 4 to the absence of the defect, 3 to the presence of a slight defect, 2 to a more marked defect and 1 to an extremely strong defect. The final score was the arithmetic mean of the individual scores given by each expert.

2.4. Analytical methods

2.4.1. Compositional analysis

pH, moisture, protein and salt contents, were all determined by the official methods of analysis of the Spanish Ministry of Food, Agriculture and Fisheries (1994).

2.4.2. Non-protein nitrogen

Non-protein nitrogen (NPN) was determined using the method of Keresse (1984), with slight modifications. Briefly, 5 g of sample was homogenized with 6 vols of cold $HClO_4$. After 1 h at 4°C, the extract was filtered through Whatman No. 6 paper, adjusted to pH 6.00 with 30% w/v KOH and made up to 100 ml. Twenty ml of this solution was used to determine the nitrogen content by the Kjeldahl method, using a Büchi digester. The distillate was collected in 100 ml of 4% w/v H_3BO_3. Finally, the ammonia thus formed was titrated with 0.1 M HCl.

2.4.3. Enzyme activities

Lysosomal enzyme extracts were obtained by homogenizing an accurately weighed amount of 5 to 6 g of sample containing no fat or connective tissue with 7 vol of 50 mM sodium acetate buffer pH 5.00 ± 0.01 containing 1 mM Na_2EDTA and 0.2% v/v Triton X-100 at 4°C. The extract was magnetically stirred at 4°C for 1 h and centrifuged on a Sigma 3K30 ultracentrifuge at 32 000 *g* for 30 mm, followed by filtration through glass wool that was previously de-ionized by successive washings with ultrapure water (Etherington et al., 1987; Koohmaraie & Kretchmar, 1990).

The enzymes tested were the lysosomal proteases called cathepsin B, B + L, H and D. The activity of cathepsin D was determined at pH 3.50 ± 0.01 45°C, using 10% w/v hemoglobin as substrate (Rico, Toldrá & Flores, 1991). The activities of cathepsin B, B + L and H were determined following isolation from their physiological inhibitors, cystatins, by affinity chromatography. The activities of cathepsin B, B + L and H were determined at 37°C and pH 6.00 ± 0.01 for cathepsin B and B + L, and pH 6.80 ± 0.01 for cathepsin H; the fluorescent substrates employed were NCBZ-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec) (Bachem), N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) (Bachem) and NCBZ-L-arginine-7-amido-4-methylcoumarin (Z-Arg-NHMec) (Sigma), respectively (Barret, 1980; Koohmaraie & Kretchmar, 1990). All reagents used were analytical grade.

2.5. Statistical analysis

Experimental data were statistically processed using the SPSS version 8.0 software suite (SPSS, Inc., 1998).

3. Results and discussion

Following sensory assessment (pastiness), the hams were classified into two groups. Group 1 comprised the specimens given a score of 4 (no defect) and Group 2 those that were given a score of 1 to 3 (i.e. the defective ones). As noted in previous work (García-Garrido et al., 1999), pasty hams also exhibit a higher incidence of other defects such as white spots, crusting or a colourless core.

As can be seen from Table 1, splitting the data set into two groups according to the presence or absence of the defect pastiness showed differences in enzyme activity between the two groups. Multivariate analysis of var-

Table 1
Comparison of the enzyme activities of cathepsins B, B + L, H and D in samples with normal and defective texture

	Normal texture		Defective texture	
Enzyme	Mean[a]	CV[b]	Mean[c]	CV[d]
Cathepsin B_{sm}[e]	1.00	39.7	1.25	37.6
Cathepsin B_{bf}	0.97a[f]	38.8	1.36b	43.21
Cathepsin $B + L_{sm}$	2.76c	7.90	3.36d	2.02
Cathepsin $B + L_{bf}$	2.89c	5.20	3.59f	4.73
Cathepsin H_{sm}	0.48	19.10	0.47	24.09
Cathepsin H_{bf}	0.50	18.03	0.47	23.21
Cathepsin D_{sm}	2.91	9.54	3.03	10.65
Cathepsin D_{bf}	2.97	7.61	2.98	12.19

[a] Mean enzyme activity for samples of normal texture, in U/g wet matter ($n = 18$).

[b] Coefficient of variation for the values of the 18 samples studied.

[c] Mean enzyme activity for samples of defective texture, in U/g wet matter ($n = 18$).

[d] Coefficient of variation for the 18 samples studied.

[e] *sm*, *Semimembranous* muscle; *bf*, *biceps femoris* muscle.

[f] a,b,c,d,e,f, Different letters denote significant difference ($P < 0.05$).

Table 2
Compositional analysis of normal and defective texture hams[a]

		Normal texture				Defective texture			
Variable	%CV[d]	Mean[e]	%CV[f]	Minimum	Maximum	Mean[g]	%CV[h]	Minimum	Maximum
$\%H_{sm}$[bc]	0.33	44.48a[i]	5.87	40.6	52.33	49.68b	5.25	41.11	53.86
$\%H_{bf}$	0.33	57.64	2.82	54.8	60.64	58.07	4.03	52.32	61.89
$\%PROT_{sm}$	1.23	43.3c	4.85	37.24	46.56	36.19d	6.52	29.68	41.29
$\%PROT_{bf}$	1.23	30.63	10.38	26.49	38.61	29.12	5.98	26.62	36.09
$\%SALT_{sm}$	0.71	7.91e	10.24	6.29	9.05	6.35f	10.87	4.78	7.56
$\%SALT_{bf}$	0.71	8.92g	8.74	7.71	10.27	7.09h	11.42	5.48	8.14
NPN_{sm}	2.79	1.34i	6.71	1.12	1.55	1.76	7.39	1.51	2.1
NPN_{bf}	2.79	1.38k	5.80	1.2	1.59	1.78l	6.18	1.4	2.08

[a] Results expressed as % wet sample.
[b] %H, % moisture; %PROT, %protein; %SALT, %NaCl.
[c] *sm*, *semimembranosus* muscle; *bf*, *biceps femoris* muscle.
[d] Coefficient of variation from 22 control samples analyzed during the interval of the ham analyses.
[e] $n=18$.
[f] Coefficient of variation from the results obtained from samples with normal texture.
[g] $n=18$.
[h] Coefficient of variation from the results obtained from samples with defective texture.
[i] a,b,c,d,e,f,g,h,i,j,k,l Different letters denote significant difference ($P<0.05$).

iance (MANOVA) to which the activities were subjected revealed significant differences between the two groups ($F=28.723$, $P<0.001$). Univariate analysis also revealed significant differences between the hams in Groups 1 and 2, particularly regarding cathepsin B + L in both the *semimembranosus* and *biceps femoris* muscle ($P<0.001$), and to a lesser extent, in cathepsin B in *biceps femoris* muscle ($P<0.05$). No significant differences between in the activities of cathepsin H or D, in any of the muscles studied was found between the groups.

As can be seen from the results of the compositional analysis (Table 2), there were significant differences ($P<0.05$) in all parameters between the two groups, except for the moisture and protein contents in the *biceps femoris* muscle.

The activity of cathepsin B in *biceps femoris* is closely related to textural defects in Parma ham, where it was found to be strongly correlated to NPN (Parolari et al., 1994; Virgili et al., 1995a). However, we found very high variability in the activity of this enzyme in our samples, even between values for the same textural group and our cathepsin B activities in the two muscles were not correlated to NPN. However, the NPN contents in our samples were highly correlated to the activity of cathepsin B + L in both types of muscle (Table 3).

Regarding residual enzyme activities and compositional parameters, only the salt content and the activity of cathepsin B + L were found to be related (Table 3). Although NaCl was previously found to influence proteolytic activity in in vitro tests involving these enzymes (Sãrraga et al., 1989; Toldrá, 1992), we found no correlation between the activities of cathepsins B, H or D and the salt contents of the hams. We did find a

Table 3
Relationships between the salt and non protein nitrogen contents with the residual enzymatic activities in dry-cured ham

Enzyme	$\%NaCl_{sm}$[a]	$\%NaCl_{bf}$[b]	NPN_{sm}	NPN_{bf}
Cathepsin B	r[c] = 0.113	$r=0.010$	$r=0.198$	$r=0.258$
Cathepsin B + L	$r=-0.510$[d]	$r=-0.721$[d]	$r=0.840$[d]	$r=0.888$[d]
Cathepsin H	$r=0.388$	$r=0.400$	$r=0.035$	$r=-0.145$
Cathepsin D	$r=-0.207$	$r=-0.207$	$r=0.200$	$r=0.084$

[a] *SM*, *Semimembranosus* muscle.
[b] *BF*, *Biceps femoris* muscle.
[c] *r*, Pearson's coefficient of correlation.
[d] Correlation is significant at the 0.01 level.

relationship between the salt content and NPN ($r_{sm}=-0.534$, $P=0.01$; $r_{BF}=-0.700$, $P=0.01$), which indicates that NaCl may play a role in regulating endogenous proteolysis during curing.

We carried out a principal component analysis of the variables significantly contributing to the overall variance between the groups. Two principal components (PCs) were found to account for 82.9% of the overall variance. Each PC was a linear combination of the original variables. The loadings obtained upon a Varimax rotation (Forina, Armanino, Lanteri & Leardi, 1998; Malinowski & Howery, 1980) are shown in Table 4. These rotated components allow one to interpret the data as follows: the first PC shows the influence of total protein and the enzyme activities in *semimembranosus* muscle, whereas the second shows the influence of salt in the same muscle. As can be seen from Fig. 2, the samples belonging to Group 1 (normal texture) show salt levels higher in both muscles and also a higher protein content in the *semimembranosus* compared to

Table 4
Loadings of the first two "rotated" principal components

Principal component	Variables						
	Cat. B_{bf}	Cat. $B+L_{sm}$	Cat. $B+L_{bf}$	$\%HUM_{sm}$	$\%PROT_{sm}$	$\%NaCl_{sm}$	$\%NaCl_{bf}$
1	0.72052	0.85622	0.81001	0.64299	−0.87700	−0.31787	−0.24434
2	0.55269	−0.28302	−0.36519	0.55020	0.32769	0.88995	0.91021

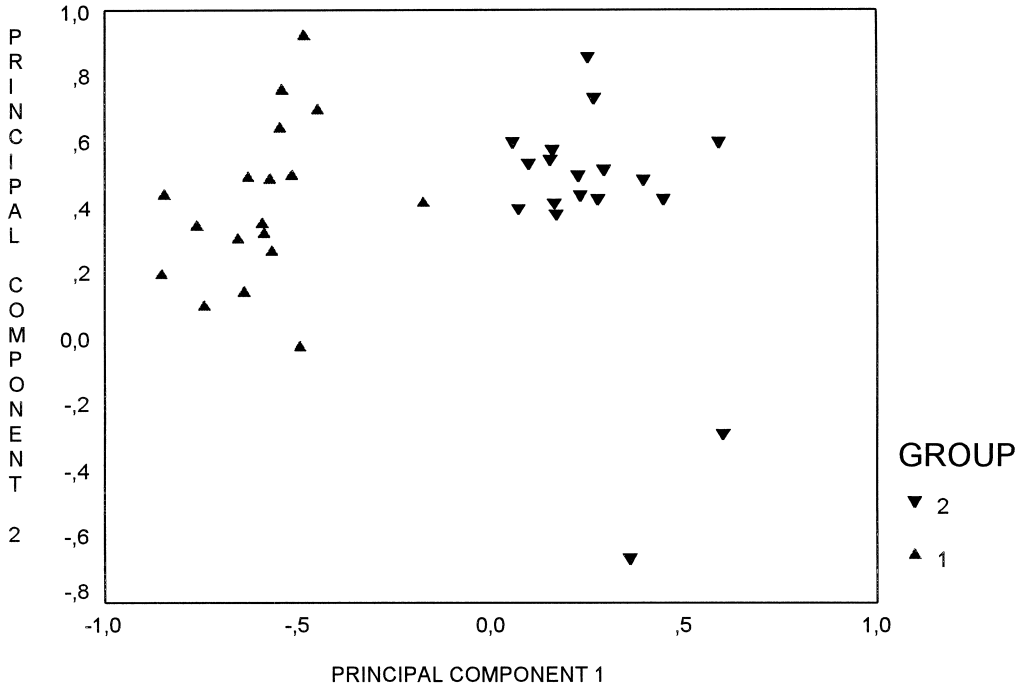


Fig. 2. Projection of principal components for the samples studied.

Group 2 (defective texture). The groups are clearly differentiated (Fig. 2).

4. Conclusions

The residual enzyme activity of cathepsin B + L is a reliable indicator to identify textural defects in ham due to strong proteolysis during curing. The residual activity levels of cathepsin B in pasty ham are similar to those in non-pasty ham; also, such levels vary widely between samples. Thus this parameter is not suitable for predicting ham texture.

Salts levels are only weakly correlated to residual (B + L) enzyme activities, so their effect on proteolysis during harm curing must be limited and the process must be controlled by other factors. Prominent among these are the compositional characteristics of the end product; thus the amount of protein in *semimembranosus* muscle is lower and the moisture higher in texturally defective hams. The efficiency of the drying process is also lower in defective hams.

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