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Calpain and cathepsin activities, and protein extractability during ageing of *longissimus* porcine muscle from normal and PSE meat

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Proteolytic activity on commercial substrates and protein extractability in solutions of different ionic strength were compared for normal and exudative *longissimus* porcine muscles during ageing at 4°C. No effect of meat quality on calpain and cathepsin activities was observed, whereas ageing time had a statistically significant effect on calpain activity and cathepsin B+L activity. Protein extractability depended on meat quality and it was higher in normal meat than in exudative meat. Differences in accessibility of the myofibrils, according to meat quality, can determine the differences in the action of proteolytic enzymes and in protein extractability. It is suggested that myofibrillar protein extractability could be a suitable meat quality indicator. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The PSE (pale, soft, exudative) condition in pork meat was first described by Ludvigsen (1954) as muscle degeneration. The PSE condition results from a rapid *post-mortem* glycolytic rate causing a drop in pH while muscle temperature is still high (Briskey and Wismer-Pedersen, 1961). Breeding programs designed to achieve higher muscular development of the animals with a concomitant decrease in fat content have led to a higher incidence of PSE porcine meat (Oliver *et al.*, 1993; Webb *et al.*, 1987) and problems with regard to acceptance by consumers and manufacture of derived meat products (Arnau, 1991). Considerable research has been conducted to describe the changes that occur and the causes of the PSE condition (for review see: Briskey, 1964; Cassens *et al.*, 1975; Honikel and Kim, 1986).

Muscle protein solubility is a major factor affecting the juice-retaining properties of the muscle. Muscle protein solubility is significantly altered by temperature and pH conditions at the onset of *rigor mortis* or during the first hours after death (Sayre and Briskey, 1963). PSE muscle protein solubility declines to a much greater extent than that of normal muscle (Park *et al.*, 1975; Penny, 1969; Sayre and Briskey, 1963). Some authors have suggested the possibility of using this biochemical characteristic as a meat quality indicator (López-Bote *et al.*, 1989; Murray and Johnson, 1994).

The rate of tenderization that takes place during *post-mortem* ageing of meat is influenced by many factors and the calpain system (EC 3.4.22.17) has been shown to play a major role in the tenderization process (Ouali, 1990; Koohmaraie, 1992; Roncalés *et al.*, 1995). Some of the biochemical and structural changes which have not been attributed to calpain action can be explained by the action of cathepsins (EC numbers: B-3.4.22.1; L-3.4.22.15; D-3.4.23.5) (Goll *et al.*, 1983; Ouali, 1990; Roncalés *et al.*, 1995). To our knowledge, few studies comparing proteinase activities from N and PSE porcine meat during ageing are available (Hortós *et al.*, 1994).

The problems regarding acceptance and manufacturing, which arise from PSE meat, show the importance of studying the biochemical differences between meat qualities carefully. The present paper is concerned with the *post-mortem* development of the levels of calpains and cathepsins, together with changes in muscle protein solubility during ageing of N and PSE muscles.

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MATERIAL AND METHODS

Selection and ageing of the samples

Longissimus porcine muscles of N (n = 10) and PSE (n = 10) meat were selected by measuring electric conductivity with a Quality Meater (Digi 550, Wissenschaftlichtechmishe, Weilheim, Germany) at 45 min post mortem (PSE > 10, normal < 7.0).

Muscles were stored at 4°C and samples were taken after slaughter (t = 0) and at 1 day, 7 days and 14 days *post mortem*. The following procedures were carried out at each sampling time.

Preparation of the muscle extracts

Extraction of calpains

Calpains were extracted as described by Etherington *et al.* (1987). After extraction, the calpastatin—endogenous inhibitor of these enzymes—was removed using a Phenyl Sepharose (Pharmacia LKB) hydrophobic column.

Obtention of lysosomal enzymes (Etherington et al., *1990)*

A portion of muscle was homogenized with an Ultra-Turrax T25 (13 500 rpm, 15 s) in 4 parts (w/v) of cold 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 2ml litre^{-1} Triton X-100. The homogenate was stirred for 1 h at 4°C and centrifuged at 25 000 **g** for 15 min. The supernatant was filtered through glass wool and used as the cathepsin source (cathepsin extract, CE).

Preparation of the lysosomal fraction

The lysosomal fraction was obtained according to Obled *et al.* (1984). The muscle was homogenized (Ultra-Turrax T25 at 13 000 rpm for 15s) in 7 vol of 250 mM sucrose, 150 mM KCl, 1 mM EDTA, pH 7.2 solution and the homogenate was centrifuged at 1000 **g** for 10 min. The resulting pellet was extracted again and the supernatants of the two extractions were pooled and centrifuged at 3000 **g** for 10 min, and then at 25 000 **g** for 20 min. The pellet of the last centrifugation was extracted with acetate buffer containing Triton X-100, as described in the above paragraph, and the extract was used as the lysosomal-enriched fraction, LEF.

Assay of calpain activity

Total calpain activity, mainly due to calpain II, was determined according to Ceña *et al.* (1990) in 50 mM Tris HCl, pH 7.5 buffer containing 100 mM KCl, 10 mM 2-ME, 2.5 mM $CaCl_2$ and 2.5 mg casein according to Hammarsten (Merck) ml^{-1} in an incubation volume of 2 ml. Controls without enzyme accompanied the assay. The reaction was held at 30°C for 60 min. It was initiated by the addition of calpains and stopped with 2.0 ml of 5% TCA. Absorbance of the supernatants of centrifugation was measured at 278 nm. One unit of activity was defined as the amount of enzyme which caused a change in absorbance of 0.001 units min^{-1}.

Assay of the lysosomal enzymes

Cysteine proteinases from the cathepsin extract (CE) and the lysosomal-enriched fraction (LEF) were assayed fluorimetrically with commercial substrates (Etherington and Wardale, 1982). Cathepsin B was assayed with N-CBZ-L-arginyl-L-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec) and cathepsins B and L with the common substrate N-CBZ-L-phenylalanyl-L-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) (Bachem). The glycosidase β-glucuronidase (EC 3.2.1.31) was determined using 4- methylumbelliferyl-β-D-glucuronide (Sigma) (Etherington *et al.*, 1987).

One unit of activity was defined as the amount of enzyme hydrolysing 1 nmol of substrate per min at 37°C.

The aspartyl proteinase cathepsin D was assayed against denatured bovine haemoglobin (Sigma) (Etherington, 1972) and the TCA-soluble peptides were measured by the method of Lowry *et al.* (1951) using L-tyrosine as standard. One unit of activity was defined as the amount of enzyme releasing 1 g of tyrosine per min at 45°C.

Activities were given in enzyme units per gram of muscle.

Muscle protein extractability

Three extracts of protein were obtained in solutions of low ionic strength (1:20, w:v) directly from the ground muscle:

A portion of ground muscle was homogenised with an Ultra-Turrax T25 (13 500 rpm, 15 s) in ice-cold distilled water and allowed to stand for 1 h at 4°C. Water soluble proteins were recovered in the supernatant after a centrifugation at 10 000 **g** for 30 min at 4°C.

1% salt-extractable proteins were obtained from a second portion of ground muscle in the same way but with 1% NaCl instead of water.

The third portion of ground muscle was homogenised in 30 mM phosphate buffer, pH 7.4, allowed to stand for 1 h at 4°C and centrifuged under the same conditions as above. The supernatant was called the sarcoplasmic extract. The pellet resulting from the centrifugation process was resuspended in 100 mM phosphate buffer, pH 7.4, 1.1 M KI (Helander, 1957), allowed to stand for 3h and then centrifuged again at 10 000 **g** for 30 min at 4°C. The filtered supernatant contained the myofibrillar proteins.

The total protein of the extracts was determined by the Kjeldahl method (Presidencia del Gobierno, 1979). The results were given as percentages of extracted protein (Nx6.25) to total protein.

Protein determination

The protein concentration of the enzyme-enriched extracts was measured according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Statistical analysis

Analysis of data was performed with the Statistical Analysis System (SAS, 1985) using the following model from the General Linear Models procedure:

$$\text{variable} = \text{time} + \text{meat quality} + \text{loin (meat quality)} + \text{time*meat quality}$$

in which A(B) means 'A nested within B'.

RESULTS AND DISCUSSION

Calpains are relatively unstable enzymes, especially calpain I (Dransfield *et al.*, 1992; Koohmaraie *et al.*, 1987). Accordingly, several authors have reported that calpain activity decreases during ageing in beef (Dransfield *et al.*, 1992), chicken (Etherington *et al.*, 1990) and lamb (Koohmaraie *et al.*, 1991). The results obtained from the statistical analysis of our data confirmed this finding (Table 1). On the other hand, no significant differences were found between meat qualities (the values of activity were 0.719–0.723 ± 0.039 units g^{-1} of muscle, irrespective of meat quality). Previous results from our group show that, when calpain activity is measured on myofibrils from N or PSE meat, the activity of this enzyme depends on the meat quality (Hortós *et al.*, 1994). Thus, the 'level' of calpain activity—that is, measured with casein—appeared to be independent of the type of meat, in contrast to the different susceptibilities of myofibrils, from N and PSE meat, to the action of these proteinases. Indeed, myofibrils from different species and different muscles have been reported to have different sensitivities to proteolysis (Dufour *et al.*, 1989; Mikami *et al.*, 1987).

The other group of enzymes likely to be involved in meat tenderisation are the lysosomal cysteine proteinases, cathepsins B and L, and the aspartyl proteinase cathepsin D. The former are regulated by the cystatins (Barrett, 1987), endogenous inhibitors for cysteine proteinases, which are located in the cytoplasm. β-glucuronidase has been measured as a typical lysosomal enzyme during studies of subcellular fractionation (Stahl and Touster, 1971) and it is not known to be inhibited by any cellular components. We have extracted the lysosomal enzymes from whole muscle extracts (CE) and from lysosomal-enriched fractions (LEF). As the muscle extracts include cystatins, cathepsin B and L activities could be underestimated in CE. From the ratio β-glu (LEF)/ β-glu (CE) we obtained the lysosomal-enriched fraction recovery, which was found to be significantly higher in N meat than in PSE meat (26% and 17%, respectively) and independent of storage time, probably because lysosome disruption takes place early in *post mortem* and does not modify during ageing.

The activities of the lysosomal enzymes studied were affected neither by time nor by meat quality. These results agreed with the ones obtained for myofibrils (Hortós *et al.*, 1994) and also with those of Dransfield *et al.* (1992), who reported that cathepsin B and B + L activity and β-glucuronidase activity from bovine meat were unaffected by storage. The only variable which depended on time ($p < 0.05$) was cathepsin B + L activity from CE (Table 2). As this extract contained cystatins, it would appear that cystatin action on the enzymes became either more enhanced or less enhanced during the *post-mortem* storage because cathepsin B + L activity from the fraction that does not include cystatins (LEF) showed no significant dependence on time (0.429–0.564 ± 0.060 units g^{-1} of muscle). Moreover, cystatin affinity for cathepsin L is higher than for cathepsin B (Anastasi *et al.*, 1983; Gauthier *et al.*, 1986), so that the effect of the changes in the action of these inhibitors would presumably be higher on cathepsin B + L activity than on cathepsin B activity.

Meat protein solubility is influenced by physiological conditions (Sayre and Briskey, 1963). Some authors suggest that protein solubility in PSE muscles is lower than in N muscles (Park *et al.*, 1975; Penny, 1969; Sayre and Briskey, 1963), since in PSE muscles the temperature/pH/time conditions result in protein denaturation, which usually means a loss of extractability

Table 1. Least squares means of calpain activity[1] from *longissimus* muscle during ageing time at 4°C

Time	Calpain activity
0	0.757[a]
1	0.838[a]
7	0.820[a]
14	0.470[b]

Least squares means within the same parameter with different superscripts differ ($p < 0.05$). Standard error of least squares means was 0.056.
[1]Activities are given as units per gram of muscle.

Table 2. Least squares means of cathepsin B + L activity[1] from the cathepsin extract from *longissimus* muscle during ageing time at 4°C

Time	Cathepsin B + L activity
0	0.566[a]
1	0.568[a]
7	0.441[b]
14	0.505[a,b]

Least squares means within the same parameter with different superscripts differ ($p < 0.05$). Standard error of least squares means was 0.036.
[1]Activities are given as units per gram of muscle.

of the denatured proteins. López-Bote *et al.* (1989) report that sarcoplasmic protein concentration, extracted according to Helander (1957), is the best potential quality index in populations with PSE, DFD and N carcasses. Murray and Johnson (1994) suggest that 1M NaCl is a suitable extraction medium to use for porcine meat quality prediction.

During meat processing technologies, the physicochemical properties of muscle proteins are modified and these modifications affect the sensorial properties of the final product. Changes in the concentration of salt or in the pH of meat, for instance, would modify the characteristics of the muscle and enhance the solubilization of some myofibrillar proteins (Dilber-Van Griethuysen and Knight, 1991).

Given the great importance of the cured ham industry in Spain and other Mediterranean countries, it is worth finding out more about the basic biochemical and physical phenomena that take place during curing, as well as about the influence of the raw material on the final product (Guerrero *et al.*, 1996). In cured ham, the sarcoplasmic protein fraction cannot be obtained under the same conditions as the sarcoplasmic fraction from fresh meat because the presence of the curing salt affects the muscle itself as well as the ionic strength of the extract. Hortós (1995) suggests a methodology for protein extraction in hams which yields 95–100% of protein and makes it possible to assess the degrading and denaturing phenomena undergone by muscle proteins throughout the ham-curing process. In this study we analyzed the extractability of sarcoplasmic proteins in water (water-soluble protein), in 1% NaCl solution (salt-extractable protein) and in 30 mM phosphate buffer, pH 7.4 (Helander, 1957) (sarcoplasmic protein) in order to establish reference values for the studies on cured ham. The water extracts gave us the soluble nitrogen of the muscle resulting from the intrinsic ionic strength of the meat. The 1% salt extracts provided us with information about the effect of the salt as an extracting agent; the ionic strength in this medium is fixed and variation of pH is very little. Extracts in the Helander buffer were taken as a reference. We compared the extractabilities of N and PSE meat during ageing at 4°C, and also with the myofibrillar protein extractability.

A summary of the statistical analysis of the results is shown on Table 3. The protein extractability of the four types of extracts were all affected by meat quality, and water-soluble protein and myofibrillar protein were also affected by the ageing time. The development of water-soluble protein and myofibrillar protein with time is presented in Table 4. The amount of protein extracted from the muscle using water decreased as the ageing time increased. On the other hand, myofibrillar protein values were higher at longer ageing times, showing that the solubilization of myofibrils became easier as the post-mortem time went on. The values of extractability for salt-extractable protein (33.0–31.3 ± 1.14 g extracted protein/g total protein×100) and sarcoplasmic protein (34.1–32.8 ± 0.94 g extracted protein/g total protein×100) were independent of time. The controlled extraction conditions (ionic strength and pH) of these two fractions minimized the effect of ageing time on protein extractability. In contrast, water-soluble protein was highly influenced by the development of the intrinsic pH and ionic strength of the meat during ageing. Therefore, the effect of the interaction time*meat quality was only significant for water-soluble protein. The analysis of the development of water-soluble protein extractability with time, and also according to meat quality, showed that the decrease with ageing time was clear in PSE meat (Table 5).

Finally, Table 6 reports the values of protein extractability in the different extracts according to meat quality. Differences in the protein solubility of the different types of meat increased with the ionic strength of the media so that the greatest differences were found among the myofibrillar protein extracts. These results agreed with the qualitative ones observed in the SDS-PAGE ferograms of post-mortem degradation of myofibrillar proteins from N and PSE meat during ageing at 4°C (Hortós *et al.*, 1994). This degradation is the result of the action of proteases and it is quantitatively related to the protein solubility.

The results reported in this paper add evidence to the differences between myofibrillar proteins from N and PSE muscles, which possibly explain the differences in protein extractability and in the susceptibility of myofibrils to proteolysis depending on meat quality, and also suggest that myofibrillar protein extractability

Table 3. Effect of time, meat quality (N,PSE), loin and their interactions on the protein extractability in *longissimus* muscle

	Time	Meat quality	Loin	Time*meat quality
Water-soluble protein	**	***	**	*
Salt-extractable protein	NS	**	NS	NS
Sarcoplasmic protein	NS	***	NS	NS
Myofibrillar protein	***	***	*	NS

*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, NS = non-significant.

Table 4. Least squares means of water-soluble protein and myofibrillar protein extractability[1] from *longissimus* muscle during ageing time at °4C

Time	Water-soluble protein	Myofibrillar protein
0	25.73[a]	33.84[a]
1	26.22[a]	35.29[a]
7	23.80[b]	36.76[a]
14	22.94[b]	46.61[b]

Least squares means within the same parameter with different superscripts differ ($p < 0.05$). Standard errors of least squares means were 0.70 and 1.54 for water soluble protein and myofibrillar protein extractability, respectively.

[1]Values of protein extractability are given as percentages of extracted protein to total protein.

Table 5. Least squares means of water-soluble protein extractability[1] for *longissimus* from N and PSE meat during ageing time at 4°C

Time	Water soluble protein	
	N	PSE
0	25.5[a]	26.0[a,b]
1	28.4[b]	24.0[a,d]
7	25.4[a]	22.2[c,d]
14	24.7[a,d]	21.2[c]

Least squares means with different superscripts differ ($p < 0.05$). Standard errors of least squares means was 0.96.
[1]Values of protein extractability are given as percentages of extracted protein to total protein.

Table 6. Least squares means of protein extractability[1] for *longissimus* from N and PSE meat

	Meat quality		SE
	N	PSE	
Water-soluble protein	26.0[a]	23.3[b]	0.48
Salt-extractable protein	34.5[a]	29.8[b]	0.80
Sarcoplasmic protein	36.3[a]	30.5[b]	0.74
Myofibrillar protein	44.9[a]	31.3[b]	1.2

Least squares means within the same parameter with different superscripts differ ($p < 0.05$).
[1]Values of protein extractability are given as percentages of extracted protein to total protein.

might be a suitable meat quality indicator. Moreover, the results show that, even if the calpain system is responsible for most of the changes occurring during meat tenderization, a minor contribution of cathepsins, especially of cathepsin L activity, cannot be ignored. In this sense, Taylor *et al.* (1995) report that cathepsins would act mainly from the 6th day *post mortem*, when muscle pH is low. The role of cystatins in meat tenderization, as well as in meat processing, would appear to be of great importance. Studies on the control of proteolysis by cystatins in meat products are under way in our laboratory.

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