CTROPHORETIC AND IMMUNOLOGICAL ASSESSMENT OF DEGRADATION OF LARGE MUSCLE TEINS OF LAMB BY EFFECT OF CALPAINS I AND II AND POSTMORTEM AGING.

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SUMMARY. Densitometric scans of SDS-PAGE, using 6.5% acrylamide, of lamb myofibrillar proteins showed that eight ent proteins of a molecular size amongst that of α-actinin and myosin heavy chain were extensively degraded by incubation at optimum ions with both calpain I and II, and significantly slower by effect of postmortem aging. Three of these proteins were likely to be C n, M protein and α-actinin. By effect of proteolysis several degradation peptides appeared also in this region; three of them have been n by means of immunoblotting to originate from an as yet unidentified protein of approximately 300,000 Da named G protein by lves (Ceña et al., 1991), which could correspond to filamin.

INTRODUCTION. Postmortem changes undergone by low molecular weight muscle proteins may be considered as an index of tenderisation, though most of them are not likely to have a direct relationship with myofibrillar structural changes involved in tenderness ge (Ducastaing et al., 1985). These are to be accounted for presumably by degradation of structural proteins of higher molecular weight tonan & Zdanis, 1988). Calpains I and II have been shown to be responsible for specific proteolysis of tropomyosin, troponin T, in I, C protein, M protein, titin and nebulin, and have been involved too in the postmortem degradation of these proteins (Goll et al., Koohmaraie et al., 1986). However, there is still controversy about which proteases are responsible for high molecular weight proteins dation during postmortem storage. Ceña et al. (1991) recently showed that a number of muscle proteins larger that 200,000 dalton were ded either by effect of aging or by in vitro incubation under optimum conditions with calpains I and II; titin, nebulin and two as yet ntified proteins possibly corresponding to filamin among them.

The aim of this work was to study which myofibrillar proteins showing a molecular size within the range of α-actinin (about ,00 Da) to myosin heavy chain (about 200,000 Da) are degraded during conventional postmortem storage, as well as to determine if eins are capable in vitro to degrade to the same extent those proteins. Proteins showing a molecular size above that of MHC have been dy investigated (Ceña et al., 1991). We focussed also on the appearance of peptides within the same region following protein dation. Immunological techniques were used in an attempt to relate degraded proteins to appearing peptides.

MATERIALS and METHODS. **Myofibrillar proteins isolation.** Muscle samples were obtained from Longissimus dorsi scle, stored at 4°C, from three month-old lambs. Myofibrillar proteins were isolated by the MFI method (Olson et al., 1976) at 2 hours 7 days after slaughter, boiled in SDS samples buffer (Porzio & Pearson, 1977) and stored at -20°C. **Calpains and calpastatin** ation. Calpain I and II and calpastatin were isolated from lamb Longissimus dorsi by ion-exchange chromatography according to ohmaraie et al. (1988). **Myofibrillar proteins incubation with calpains and calpastatin.** Myofibrillar proteins isolated by the I method two hours after slaughter were incubated with either calpain I, calpain II or calpastatin, using incubation with EDTA as control, timum pH (7.5) and Ca^{2+} concentration (2.5 mM) and 25°C (calpain/myofibrillar protein ratio 1/200). At 1 and 5 hours of incubation ions were taken to assess protein degradation, according to the method followed by Dayton et al. (1976). **SDS-Polyacrylamide gel trophoresis.** SDS-PAGE was performed according to Porzio & Pearson (1977) using 6.5% acrylamide. Approximately 0.5 mg of tein was loaded on each gel and the samples were run for 1 h 30 min at 4 mA, then 5 h at 13 mA and 14 h at 4 mA at room temperature, in er to get a convenient separation of high molecular weight myofibrillar proteins (over 100,000 Da). **Scans of gels.** The gels were served in destaining solution (25% methanol, 8% acetic acid and 2% glycerine), where they became straight and suitable for scanning. **munogens and immunization procedures.** Proteins corresponding to electrophoretic bands named by ourselves in a previous report ed G (Ceña et al., 1991) were obtained from ninety unstained disc gels from prerigor lamb Longissimus dorsi samples. Antibodies were ed in a rabbit by injecting subcutaneously 0.7 mg of each protein emulsified with complete Freund´s adjuvant. Injections were distributed s several sites on rabbit back, according to Towbin et al. (1979). Booster injection of the same formulation but with incomplete Freund´s vant was given on day 30. Rabbit blood was extracted by cardiac punction on day 40. **Western blotting procedure.** Samples of ofibrillar proteins either incubated with calpains or stored postmortem at 4°C were first subjected to SDS-PAGE. Proteins were then nsferred to nitrocellulose sheets for 30 min, according to the procedure followed by Towbin et al. (1979). **Immunological detection of teins on nitrocellulose.** Nitrocellulose sheets were soaked in 5% ovoalbumin in physiological solution for 4 h at room temperature to urate additional protein binding sites. They were incubated with antiserum appropriately diluted into 5% ovoalbumin in physiological serum v 4:1) for 2 h and rinsed with physiological solution. Rabbit peroxidase-conjugated IgG preparations were used at a 1/200 dilution in siological solution containing 3% ovoalbumin. Sheets were incubated for 2 h at room temperature and washed in physiological solution. the color reaction, the sheets were soaked in a freshly prepared solution of 0.6 mg/ml 4-Cl-1-naphtol, 20% methanol, 0.1% H_2O_2 in siological solution for 15 min. Sheets were then dried using filter paper and stored protected from light.

RESULTS AND DISCUSSION. SDS-PAGE using 6.5% acrylamide revealed that both calpain I and II were able to extensively degrading several proteins showing a molecular size between that of myosin heavy chain and α-actinin. As shown in Table densitometric peaks named I, II, III, IV, V (probably M protein), VI, VII (likely to be C protein) and VIII (α-actinin) were greatly reduced when myofibrils were incubated with the enzymes at optimum conditions of pH 7.5, 2.5 mM Ca^{2+} and 25°C. These proteins were degraded faster by effect of calpain I than by calpain II; in the presence of the former, one hour of incubation was enough to result in a intense degradation of peaks II, IV and VI (77.78%, 95% and 84.62%, respectivetly). The same proteins appeared to be naturally degraded also throughout postmortem meat aging. Nevertheless, this proteolytic effect was significantly slower than direct incubation with calpains; 7 days were necessary to evidentiate peak area reductions by far lower than that obtained with calpains. However, in both cases subsequent changes and even results presented here were difficult to quantify since new electrophoretic bands corresponding most probably to degradation

TABLE 1. Decrease of peak areas of lamb myofibrillar proteins SDS-PAGE densitometric scans (expressed as percentage of initial area) by effect of either incubation (1 h) with calpains I and II or aging (7 days).

PEAK	RELATIVE MOBILITY*	CALPAIN I	CALPAIN II	AGING
I	122-124	37.50	50.63	27
II	125-127	77.78	24.73	15
III	130-134	59.21	52.07	—
IV	141-143	95	35.8	12
V	146-150	58.68	11.76	17
VI	154-156	84.62	41.10	11
VII	159-163	44.37	29.55	—
VIII	191-197	61.2	34.06	10

* Related to myosin heavy chain mobility (100).

peptides emerged at gel positions close to that of proteins being degraded, thus interfering correct densitometer readings. The slower rate of protein degradation throughout aging as opposed to incubation with calpains is supported by a number of studies in which proteolysis of the largest muscle proteins was scarce at low temperatures even after long times of storage, while they showed a much more intense and rapid degradation at higher temperatures (Bandman & Zdanis, 1988). Dransfield et al. (1980) reported indeed that an increase of only 5°C affected positively meat tenderness.

M protein is a component of the M line, whose structure has been shown to be disrupted during postmortem storage (Hay et al. 1973) and degraded by CAF activity (Dayton et al., 1976; Merdaci et al., 1983). C protein has been reported to be degraded during meat aging too (Etherington, 1981). Dayton et al. (1976) showed that C protein of porcine skeletal muscle was also degraded by calpains. Alfa-actinin is located at the Z line, and it has been shown to be removed from this structure during aging (Young et al., 1981). Furthermore, Takahashi & Saito (1979) reported that removing of α-actinin caused the weakening of Z disks thus allowing their proteolytic degradation during postmortem storage. In agreement with our results, several researchers showed previously in other species that calpains were responsible for α-actinin degradation and Z line disruption (Merdaci et al., 1983; Takahashi et al., 1987). Hwan & Bandman (1985) demonstrated that Z line was also degraded throughout meat storage at 4°C, although very slowly. These facts have been highly correlated with meat tenderness (Parrish, 1977; Takahashi et al., 1985).

As stated before, several unidentified bands appeared within the same molecular weight region at the same time that proteins were being degraded. Table 2 shows changes of peak densitometric areas brought about by either calpain I, calpain II or postmortem storage. Four of them (f, g, h and j) were coincident or very close to the position of former peaks I, II, III and VII; furthermore, a new peak (i) was also evident between peaks IV and V. These peaks were clearly evident after 5 h of incubation with calpains, except peak g for calpain II, or after 7 days of aging, except peaks f and g. With a view to know if any of these emerging peptides originated from some of the high molecular weight proteins previously reported to be extensively degraded by calpains and during postmortem storage (Ceña et al., 1991), we studied F and G proteins (about 300,000 Da, possibly filamin) degradation using immunological techniques. F Protein, contrary to G protein, seemed unfortunately to possess very little immunogenic ability, at least following the methods used, since no antibodies were detected in rabbit serum.

A Western blotting (Figure 1) of a SDS-PAGE gel of myofibrillar proteins incubated 24 hours with both calpains I and II using anti-G protein antibodies clearly demonstrated the appearance of three peptide bands resulting from degradation of G protein, as well as a decrease

E 2. Peak areas of lamb myofibrillar proteins SDS-PAGE densitometric scans (expressed as the ratio peak area/MHC area) at selected s of incubation with calpains I and II or aging.

PEAK	RELATIVE MOBILITY*	CALPAIN I 1 h	CALPAIN I 5 h	CALPAIN II 1 h	CALPAIN II 5 h	AGING 2 h	AGING 7 d
f							
g	122-124	0.035	0.135	0.039	0.113	0.131	0.096
h	125-127	0.014	0.071	0.070	0.051	0.153	0.130
i	130-134	0.031	0.169	0.058	0.127	0.124	0.240
j	144-146	0	0.141	0	0.073	0	0.128
	159-163	0.079	0.502	0.093	0.393	0.125	0.220

ed to myosin heavy chain mobility (100).

FIGURE 1: Immunoblot of lamb Longissimus dorsi myofibrils incubated with calpain I and II. Western blot prepared from a 6.5% SDS-polyacrylamide gel reacted with anti-G protein antibody. Times of incubation: a) control with EDTA 5 h; b) sample with calpain I 5 h; c) sample with calpain II 5 h.

amount of intact protein after incubation with either calpain I or II. These bands could well correspond, according to their relative ity, to appearing peaks previously named h, i and j (Table 2), that is to approximate molecular weights of 178,000, 158,500 and 0 Da, respectively. A reactive band in the region of 17-20,000 Da was also evident in both control and samples; it is possible that this originated from G protein during myofibrils extraction of SDS treatment. Some of these bands could well correspond to peptides of 00 and 127,000 Da reported by Merdaci et al. (1983) to originate following the degradation of rabbit myofibrils either incubated with at 30°C, pH 7.5, or aged at 5°C. Elgasim et al. (1985) found, too, several degradation peptides in the region of 140,000 Da by effect F on ovine myofibrils at optimum conditions of incubation. The appearance of some peaks in the region of 130-140,000 Da by effect bovine myofibrils at 37°C was also reported by Troy et al. (1986,1987). It is then demonstrated that all these degradation peptides seem nate from what we named G protein, possibly corresponding to filamin. However, no relationship of the appearance of these peptides eat tenderness has been as yet reported.

CONCLUSIONS. Calpains I and II were found to be able to degrade extensively several large proteins (100,000 to 200,000 Da) muscle when myofibrils were incubated at optimum conditions; some of these proteins are likely to be C protein, M protein and α- . The same proteins were shown to be degraded following conventional postmortem storage, though proteolysis found after 7 days of e was much less intense. The appearance of a number of degradation peptides in the same molecular weight region has been also three of them have been demonstrated to originate from the degradation of an as yet unidentified protein (possibly filamin) of an inate molecular weight of 300,000.

ACKNOWLEDGEMENTS. The authors wish to thank the University of Zaragoza for the partial support of this research (grant 69-11/1987).

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