# Degradation of several high molecular weight muscle proteins by calpains I and II mimics changes occurring throughout lamb meat aging.

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SUMMARY: Experiments have been carried out to investigate the proteolityc degradation of lamb skeletal myofibrils throughout postmortem storage and following incubation with calpains I and II.

Polyacrylamide gel electrophoresis showed the ability of calpain I and II to degrade to a varying extent several high molecular weight muscle proteins, two of them likely to be titin and nebulin. The same proteins were found to be hydrolysed following conventional postmortem conditions, but their rate of degradation was much slower.

INTRODUCTION: Many studies have been carried out on the changes undergone by low molecular weight myofibrillar proteins during postmortem storage, their causes and the relationship between these alterations and meat tenderization (Olson et al., 1976, 1977; Penny & Dransfield, 1979). Two neutral $Ca^{++}$-activated proteases, currently named calpains, which are activated by μM (calpain I) and mM (calpain II) $Ca^{++}$ concentrations, have been indeed reported to be responsible for specific degradation of tropomyosin, C-protein, troponin T and troponin I, during the meat aging process (Azanza et al., 1980; Goll et al., 1983; Zeece et al., 1986).

However, although changes of those proteins can be considered as an indicator of meat tenderization, they are not likely to be directly related to the postmortem changes involved in tenderness (Ducastaing et al., 1985), which are to be accounted for by degradation of high molecular weight muscle proteins (Goll et al., 1983; Ouali, 1990) Previous studies have shown that cytoskeletal proteins titin and nebulin were degraded during postmortem storage (Lusby et al., 1983). Calpains have been found to be involved in the postmortem decrease of these two high molecular weight proteins (Zeece et al., 1986), while no titin degradation by calpain was found by Maruyama et al. (1981). Bechtel and Parrish (1983) found little proteolysis of the major contractile proteins when muscles were stored at postmortem conditions.

We present here the study of the proteolysis brought about by calpains I and II on lamb muscle proteins showing a molecular size larger than that of myosin heavy chain, as well as their degradation following conventional storage conditions.

MATERIAL and METHODS : a) Myofibrillar protein isolation. Muscle samples were obtained from Longissimus dorsi muscle stored at 4°C from three month-old lambs. Myofibrillar proteins were isolated by the MFI method (Olson et al., 1976) at 1, 2, 4 and 8 days after slaughter, boiled in SDS samples buffer (Porzio & Pearson, 1977) and stored at -20 °C.

b) Calpains isolation. Calpain I and calpain II were isolated from lamb Longissimus dorsi according to Koohmaraie et al. (1988).

c) Myofibrillar proteins incubation with calpains Myofibrillar proteins isolated by the MFI method two hours after slaughter were incubated with either calpain I or calpain II, using incubation with EDTA as control, at optimum pH and $Ca^{++}$ concentration (pH:7.5 and 2.5 mM $Ca^{++}$) and 25°C (calpain/ myofibrillar protein ratio 1/200). At 0, 5 and 24 hours of incubation aliquots were taken to assess protein degradation, according to the method followed by Dayton et al. (1976).

d) SDS-Polyacrylamide gel electrophoresis. SDS-PAGE was performed according to Porzio & Pearson (1977) using 6.5% acrylamide. Approximately 8-10 mg of protein was loaded on each gel and the samples were run for 1h 30min at 4 mA, then 5h at 13 mA and 14h at 4 mA at room temperature, to get a separation enough of high molecular weight myofibrillar proteins.

e) Scans of gels. The gels were preserved in destaining solution (25% methanol, 8% acetic acid and 2% glycerine) where they became straight and suitable for scanning.

RESULTS: SDS-PAGE gels obtained using 6.5% acrylamide (Figure 1) revealed that both calpain I and II were capable of degrading several high molecular weight muscle proteins when these were incubated with the enzymes at optimum conditions of pH 7.5, 2.5mM Ca++ and 25°C. At least seven different proteins showing a molecular size larger than that of myosin heavy chain were degraded to a varying extent by effect of incubation with either calpain I or II.

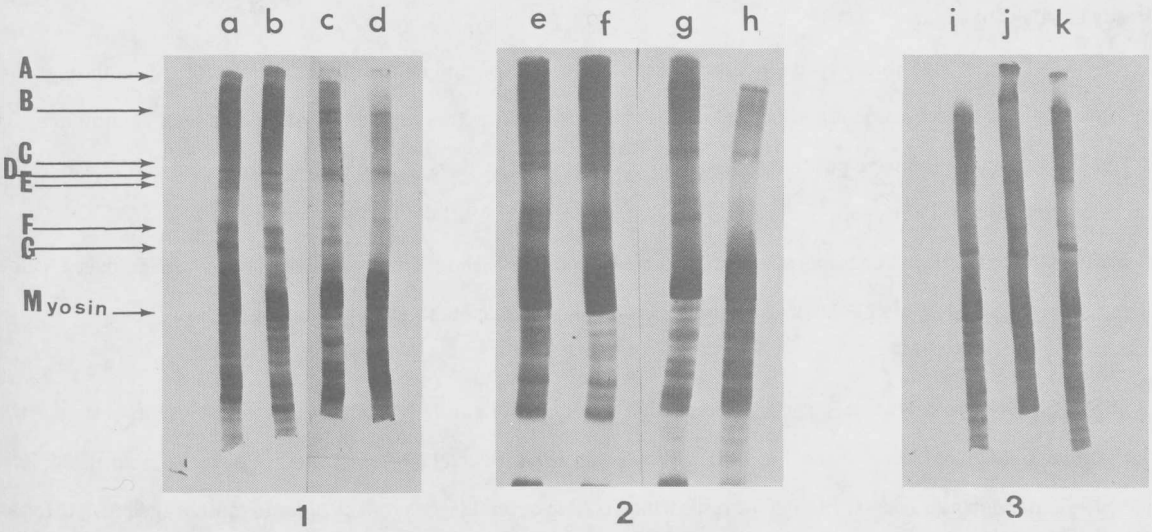


Figure 1.- SDS 6.5%, pH 8.8, polyacrylamide gels of myofibrils prepared from lamb Longissimus dorsi: 1) incubated with calpain I; 2) incubated with calpain II; 3) throughout aging at 4°C. Incubation or storage times: **a**, **e**: controls 0h; **b**, **f**: incubated with EDTA 5h; **c**, **g**: incubated with enzyme 5h; **d**, **h**: incubated with enzyme 24h; **i**: prerigor; **j**: 4 days of aging; **k**: 7 days of aging.

It is noteworthy that calpains I and II have been found to degrade the same proteins, namely those referred to in Figure 1 as A, B, C, D, E, F and G. Proteins found to be hydrolysed more extensively were those corresponding to peaks D, E, F and G; these bands lose at least 40% and 70% of their initial amount after five and 24 hours of incubation, respectivetly (see Table 1). Hydrolysis of proteins corresponding to peaks B and C, likely to be titin and nebulin, was much less intense since these proteins lose 40-60% of their initial amount only after 24 hours of incubation at the same conditions that the former.

Proteolysis of the top gel proteins (peak A) was also evident by effect of incubation with calpain I and II. Although their degradation was difficult to quantify by differences between the gels, a dramatic loss of 80% and 89% by calpain I and II, respectivetly, was found after 24 hours of incubation.

Table 1.- Gels scans peak areas decrease (as percentage of initial area) by effect of myofibril incubation with calpains I and II or by meat aging at 4°C.

| PEAK | DISTANCE* | CALPAIN I | | CALPAIN II | | AGING | |
|---|---|---|---|---|---|---|---|
| | | 5h | 24h | 5h | 24h | 4d | 7d |
| A | 0 | 40 | 80 | 50 | 89 | 28.6 | 62 |
| B | 24 | 12.3 | 40.5 | - | 50 | 4 | 12.3 |
| C | 51 | - | 44.16 | - | 55.76 | 10 | 18 |
| D | 54.76 | 43.51 | 46.18 | 50.25 | 80.12 | 24.3 | 35.07 |
| E | 59.52 | 64.66 | 73.39 | 64.54 | 85.42 | 48 | 50 |
| F | 70 | 41.53 | 75.8 | 49.25 | 67 | 3.2 | 17.97 |
| G | 82 | 41.98 | 89.1 | 62.32 | 78.6 | 4 | 15.3 |

* As a percentage of the distance run by myosin heavy chain.

The same proteins were found to be naturally degraded throughout postmortem meat ageing (Figure 1.3). Nevertheless, the proteolytic effect was significantly slower than direct incubation with calpains; so that seven days were neccessary to achieve a loss of about 15% in peaks B, C, F and G as related to their initial area. A major degradation was found for top gel proteins (peak A), though less extensive than that following incubation with calpains. The rate of proteolysis was faster for D and E protein peaks which lose a 24% and 48% of initial area already at 4 days of storage.

At the same time that proteins were degraded, several bands appeared evident by effect of proteolysis throughout aging and by incubation with calpains. Two unidentified peaks became prominent below titin but with a larger size than nebulin after incubation with calpain I, and a new peak in the same position was also observed after seven days of aging in samples stored at 4°C. However, no new bands appeared below titin by effect of calpain II.

Various unidentified minor bands appeared also between peaks E and F either after incubation with calpain I, calpain II or during aging. They were evident only after 24 hours of incubation with the enzyme or seven days of aging.

DISCUSSION: Proteolytic degradation of some high molecular weight muscle proteins during postmortem storage has been reported by a large number of authors (Maruyama et al., 1981; Lusby et al., 1983; Bechtel & Parrish, 1983; Locker & Wild, 1984; Zeece et al., 1986; Troy et al., 1987; Bandman & Zdanis, 1988). Our results showed that several large proteins of lamb muscle aged at 4°C for seven days were degraded at a slow but significant rate. Proteolysis of those proteins by effect of incubation with calpains I and II at optimum conditions showed a highly increased rate, although its intensity was not the same for the different proteins. This effect is supported by a number of studies in which proteolysis of the largest muscle proteins was little at low temperature for long time while they showed a much larger degradation at higher temperature for a short time (Bechtel & Parrish, 1983; Lusby et al., 1983; Bandman & Zdanis, 1988).

Our results showed that exactly the same proteins (peaks A to G) were degraded to a high extent by both calpains I and II and to a limited but significant extent also after seven days of storage at 4°C. Cytoskeletal titin (peak B) and nebulin (peak C) are most likely to be included among these proteins.

Titin was shown to be degraded by CAF when the purified enzyme was added directly to myofibrils at neutral pH (Zeece et al., 1986) and nebulin has been found to be very susceptible to proteolytic degradation by CAF (Maruyama et al., 1981). Though on the contrary, Maruyama et al. (1981) reported that titin was resistant to CANP. Both proteins were found to be degraded during postmortem storage (Lusby et al., 1983; Locker & Wild, 1984; Bandman & Zdanis, 1988).

In this study four as yet unidentified proteins (peaks D through G) have been demonstrated to be extensively degraded by calpains I and II, their degradation being very intense after 24 hours of incubation. However, these proteins exhibited little proteolysis during postmortem storage; though no explanation has been found for this behaviour. Neither peak F nor G are likely to be the filamin protein observed by Bechtel & Parrish (1983) and Troy et al. (1987) because their retaining times in scans were higher than that of filamin in gels obtained by these authors. Peaks D and E are likely to be the band 4 (doublet band) of Maruyama et al. (1981), but no proteolysis of these proteins by CANP was found by these authors.

Our results also evidentiated the appearance of several peaks as a result of proteolysis. The pattern of fragments generated by incubation with calpains and during postmortem storage paralleled fragment patterns generated in ox muscle aged at 15°C (Locker and Wild, 1984) or high pH beef (Troy et al., 1987). These new peaks are not likely to proceed from titin proteolysis because the fragments originated from its degradation during aging were shown to appear below myosin heavy chain by Bandman & Zdanis (1988). These bands could proceed from top gel protein hydrolysis.

CONCLUSIONS: Calpains I and II are capable to degrade extensively several large proteins of lamb muscle when myofibrils are incubated at optimum conditions; two of these proteins are likely to be titin and nebulin. The same proteins were shown to be degraded following conventional postmortem storage, though proteolysis found after seven days of storage was much less intense.

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