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THE COMBINED EFFECTS OF THE CALCIUM ACTIVATED FACTOR AND CATHEPSIN D ON SKELETAL MUSCLE

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Abstract

Myofibrils were isolated from at-death ovine longissimus muscles and incubated with crude calcium activated factor prepared from the same muscle and with purified cathepsin D. Myofibrils were incubated with these enzymes separately (first incubation) and successively (second incubation). The major changes induced by cathepsin D first incubation include degradation of myofibrillar proteins with molecular weight > 200 K, myosin, actin, troponin-T and troponin 1. Also new bands appeared at the 140-160 K, 80 K, 68 K and 30 K regions. Similar changes were obtained when myofibrils were incubated first with CAF then with cathepsin D (second incubation). On the other hand CAF first incubation resulted in the degradation of the high molecular weight proteins (> 200 K), desmin, troponin T, troponin I and it released α-actinin. Also new bands appeared immediately below C-protein (140 K), 95 K and 30 K. Unlike cathepsin D, CAF did not affect myosin or actin. However, when myofibrils were first incubated with cathepsin D then with CAF (second incubation) the latter was able to degrade actin to a much greater degree than cathepsin D. Both enzymes were able to affect the Z-lines of the myofibrils.

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Introduction

In spite of the efforts by muscle biologists and meat researchers to solve the puzzle of muscle protein degradation, some parts of it have yet to be solved. The calcium-activated factor (CAF) and cathepsins, particularly cathepsins B and D, are two of the major groups of enzymes most involved in muscle protein degradation. Cathepsin D is capable of degrading myosin, actin and high molecular weight (> 200 K) proteins (Schwartz and Bird, 1977; Robbins et al., 1979; Elgasim et al., 1985). In addition, cathepsin D was shown to degrade the Z-lines of intact myofibrils (Eino and Stanley, 1973; Robbins and Cohen, 1976).

After the calcium-activated factor (CAF) was identified by Huston and Krebs (1968), Busch et al.(1972) and purified and characterized by Dayton et al. (1976), catheptic proteases were no longer considered to be the sole causative factor for the observed improvement in meat tenderness with post-mortem aging. CAF was reported by several investigators (Busch et al., 1972; Goll et al., 1974;Dayton et al., 1975; Olson et al., 1977; Penny, 1980; Koohmaraie et al., 1984)to degrade Z-line, M-line, to cause a gradual disappearance of troponin T and a gradual appearance of the 30,000 dalton component. Recently we (Koohmaraie et al., 1984) observed that although the intensity of myosin light chain-I is reduced by CAF, CAF does not affect the major contractile proteins (Myosin and actin).

Since the skeletal muscle is a heterogeneous system, the objectives of the current work were to study the individual and combined effects of the CAF and cathepsin D on the muscle structure and on myofibrillar protein degradation

Materials and Methods

Sample source

Samples for myofibril isolation and calcium activated factor (CAF) extraction were obtained from 4 ewes slaughtered at the Oregon State University Meat Science Laboratory. The longissimus muscle (last lumbar vertebrae to the 8th thoracic) was removed immediately, i.e., at-death, from both sides of each carcass.

Calcium activated factor preparation and assay

A portion of at-death longissimus muscle was trimmed of fat and connective tissues, immediately chilled in ice and ground in a prechilled meat grinder. Two-hundred grams of the ground muscles were suspended in 500 ml of 4 mM EDTA, pH 7.6, homogenized in a Waring blender, isoelectrically precipitated and clarified according to the procedures of Busch et al. (1972). After dialysis and the final clarification at 105,000 G for 1 hr at 2°C, the resulting supernatant was designated crude CAF and protein concentration was determined with the biuret method (Gornall et al., 1949) as modified by Robson et al. (1968). Crude CAF activity was assayed at 25°C for 30 min in 100 mM tris-acetate (pH 7.5), 100 mM 2-MCE, 1 mM NaN_3, 0.1 mM EDTA, 5 mM $CaCl_2$, 5 mg/ml casein and 0.33 mg/ml crude CAF (CAF to casein ratio 1:15) in a total assay volume of 2 ml (Olson et al., 1977). Controls for enzyme and substrate accompanied each assay.The reaction was started by addition of CAF and stopped by addition of 2 ml of 5% TCA (Dayton et al., 1976). The assay tubes were then centrifuged at 1000 G for 20 min and the absorbance of the supernatant was measured at 278 nm. The specific activity was expressed as absorbance unit per mg of crude CAF solution.

Myofibrils isolation

Myofibrils were prepared from at-death muscle according to the procedure described by Goll et al. (1974) using 100 mM KCl, 50 mM Tris-HCl, pH 7.6 and 5 mM EDTA as the isolating medium. Finally myofibrils were suspended in 100 mM KCl, 1 mM NaN_3 and protein concentration was determined by the biuret method (Gornall et al., 1949).

Myofibrils incubation

Isolated myofibrils (10 mg/ml) were incubated with (a) Crude CAF (Myofibril to CAF ratio 10:1) according to the procedure described for measuring the crude CAF activity except that casein was placed with myofibrils and the incubation period was 1 hr. (b) 0.25 units/ml of purified cathepsin D (Sigma Chemical, St. Louis, MO) in 200 mM sodium-acetate pH 5.0. Myofibrils were incubated with the enzyme at 37°C for 1 hr (long incubation) and 5 min (short incubation). A ratio of 1:250 of cathepsin D to myofibrils was used. Incubations (a) and (b) above were called first incubation. (c) At the end of the incubation period, the mixture from (a) was centrifuged at 2000 G for 15 min at 2°C. The supernatant was discarded and the pellet was washed three times with 100 mM NaCl and centrifuged as before. Pellet from the last centrifugation was suspended in a medium suitable for cathepsin D and treated as in (b) above. (d) At the end of the incubation period, the mixture from (b) was centrifuged at 2000 G for 15 min at 2°C. The supernatant was discarded and the pellet was washed three times with 100 mM NaCl and centrifuged as before. The pellet from the last centrifugation was suspended in a medium suitable for CAF and treated as in (a) above.

Incubations (c) and (d) were called second incubation. First and second incubations were carried out in triplicate per sample (n = 12). Treated myofibrils from the first incubation were examined with phase contrast microscope (Zeiss - UEM).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the procedures described by Porzio and Pearson (1977) using 10% gel. Each gel was loaded with 60 µg of protein. Myosin, B-galatosidase, phosphyrylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were used as protein standards. SDS-PAGE was performed in duplicate per sample (n = 8).

Results and Discussion

Beside CAF, our crude enzyme preparation may contain other proteases in minute quantities. As the incubation conditions employed were optimum for CAF activity, it is hoped that other proteases will exhibit minor activity, if any.

The SDS-PAGE of myofibrillar proteins from control (CON) and enzymes treated (first incubation) myofibrils is presented in Fig. 1. When myofibrils were incubated with cathepsin D for longer, 1 hr, (Fig. 1b) or shorter (Fig. 1c) durations, the high molecular weight (> 200 K) proteins were extensively degraded. This finding supports our previous contention (Elgasim et al., 1985) that the proteins of this region are very vulnerable to cathepsin D. Connectin, nebulin and filamin are some of the proteins in this region identified by other researchers (Maruyama et al., 1976; Wang, 1981; Bechtel, 1979). Obviously, the myosin heavy chain (200 K) was affected by cathepsin D but a distinctive feature

in Fig. 1b is the appearance of a heavy band that occupies the region between 140-160 K. Such a band was not observed in the CON (Fig. 1a) or CAF (Fig. 1d) treated samples. It is not known whether the C-protein (140 K) was degraded with cathepsin D or was just masked by this band. This heavy band is a degradation product of proteins with molecular weight greater than 140 K and it is likely to be made of a number of closely migrating bands. Schwartz and Bird (1977) observed a major degradation product at 150 K when myosin heavy chain was treated with purified cathepsin D. Under the conditions employed in this experiment, α-actinin (100 K) was slightly affected (Fig. 1b) with cathepsin D. Similar observation was reported by Matsumoto et al., (1983). It is not clear whether this slight effect is a result of a release or degradation of α-actinin. Some of the notable changes between 100 K and 45 K region was the appearance of a doublet band at 80 K and 68 K and that actin band (45 K) was affected by cathepsin D. The latter observation is in agreement with Schwartz and Bird (1977). The 80 K component could have originated from the degradation of myosin heavy chain (Robbins et al., 1979). Below 45 K cathepsin D affected troponin T and tropomyosin resulting in the appearance of a band at 30 K. This band could be a degradation product of any protein with molecular weight greater than 30 K. According to Olson et al. (1977) this band is a degradation product of troponin T. Other changes in this region include a reduced intensity of myosin light chain, troponin C and a complete disappearance of troponin 1. Shorter period (5 min) of cathepsin D treatment (Fig. 1c) showed that only the high molecular weights (>200 K) proteins were appreciably degraded. Apparently cathepsin D effects on other myofibrillar proteins is a time dependent reaction.

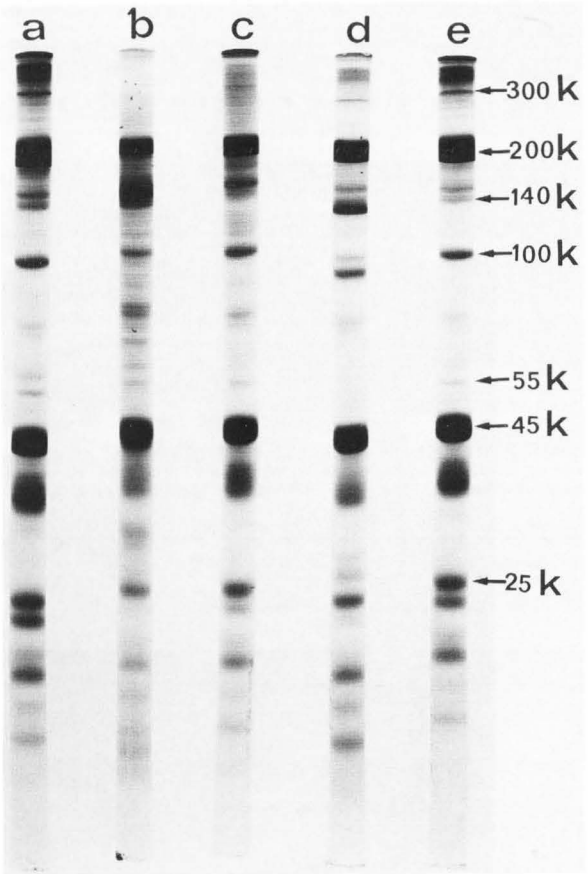

Fig.1. SDS-Polyacrylamide gel electrophoresis of myofibrils from at-death longissimus muscle incubated with CAF or cathepsin D(first incubation): a) CON,undigested myofibril, b) cathepsin D-treated (1 hr), c) same as (b) except shorter incubation (5 min), d) CAF treated (1 hr) e) same as (d) except that the incubation mixture contained 5 mM EDTA instead of the 5 mM $CaCl_2$. Note the effects of both enzymes on the high molecular weight (> 200 K) proteins.

On the other hand, incubation of myofibril, with CAF (Fig. 1d) resulted in reduced intensities of the high molecular weight proteins (> 200 K) but did not affect myosin heavy chain. CAF also resulted in the appearance of a band immediately below the C-protein (140 K) and other bands at 95 K, 30 K and 27 K. With the exception of the band below C-protein, all other changes observed here were reported previously by other researchers (Dayton et al., 1976; Olson et al., 1977; Koohmaraie et al. 1984). In addition to the above changes CAF effectively removed desmin (Fig. 1d). The effect of CAF on desmin (55 K) is very significant since desmin surrounds the myofibrils at the Z-line location.

Comparison of the effects of cathepsin D and CAF (Fig. 1b and Fig. 1d) reveal that both of them affect the high molecular weight proteins (> 200 K), however, cathepsin D is more complete in its effect than CAF. The major difference between the two enzymes is at the desmin level where CAF is more effective than cathepsin D.

Results of the second incubation are presented in Fig. 2. When myofibrils were incubated with CAF first then cathepsin D, the electrophoretic pattern (Fig. 2b) resembled that of cathepsin D particularly the appearance of bands at 80 K (doublet band) and 68 K. It is to be noted that the disappearance of desmin is not due to the cathepsin D second incubation, rather it is due to the CAF treatment that preceded it.

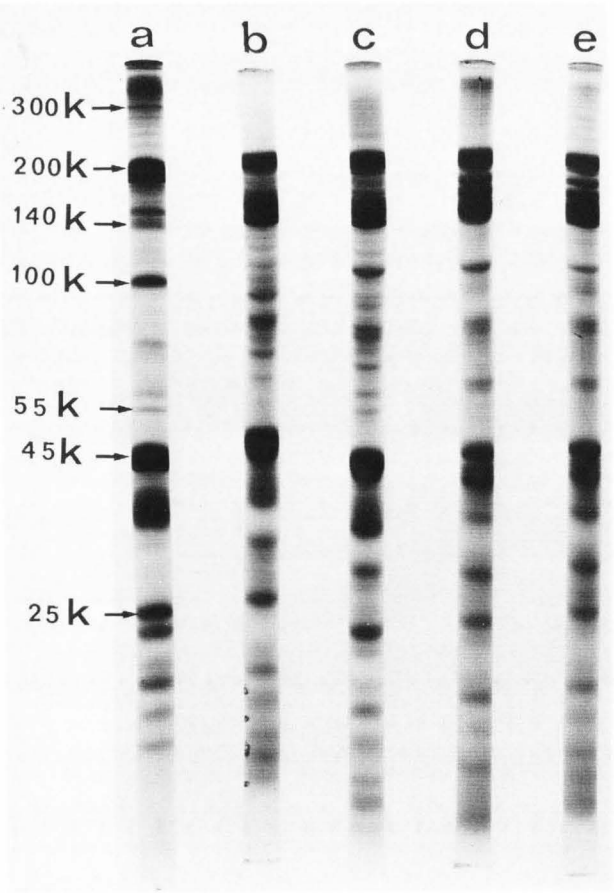

Fig.2. SDS-Polyacrylamide gel electrophoresis of myofibrils from the second incubation: a) CON, undigested myofibrils, b) myofibrils incubated first with CAF then with cathepsin D (1 hr), c) myofibrils incubated with CAF in the absence of Ca^{2+} then incubated with cathepsin D for a short period (5 min), d) myofibrils incubated with cathepsin D for a short period (5 min), then with CAF, e) same as (d) except that cathepsin D first incubation was for a longer period, (1 hr), Note the ability of CAF to affect actin.

When myofibrils were incubated (for long or short periods) with cathepsin D then with CAF, the latter was able to further degrade some of the degradation products of cathepsin D particularly the 80 K bands (Fig. 2d and 2e). Two interesting features of cathepsin D-CAF second incubation were the appearance of a 58 K band (this is not a characteristic feature of either enzyme alone) and the obvious degradation of actin. Since CAF is known for its inability to degrade actin it is reasonable to suggest that effect of cathepsin D on actin has resulted in substantial conformational changes that enabled CAF to attack actin.

The loss of Z-line from myofibrils incubated with CAF or cathepsin D can be seen upon examination with phase contrast microscope (Fig. 3).

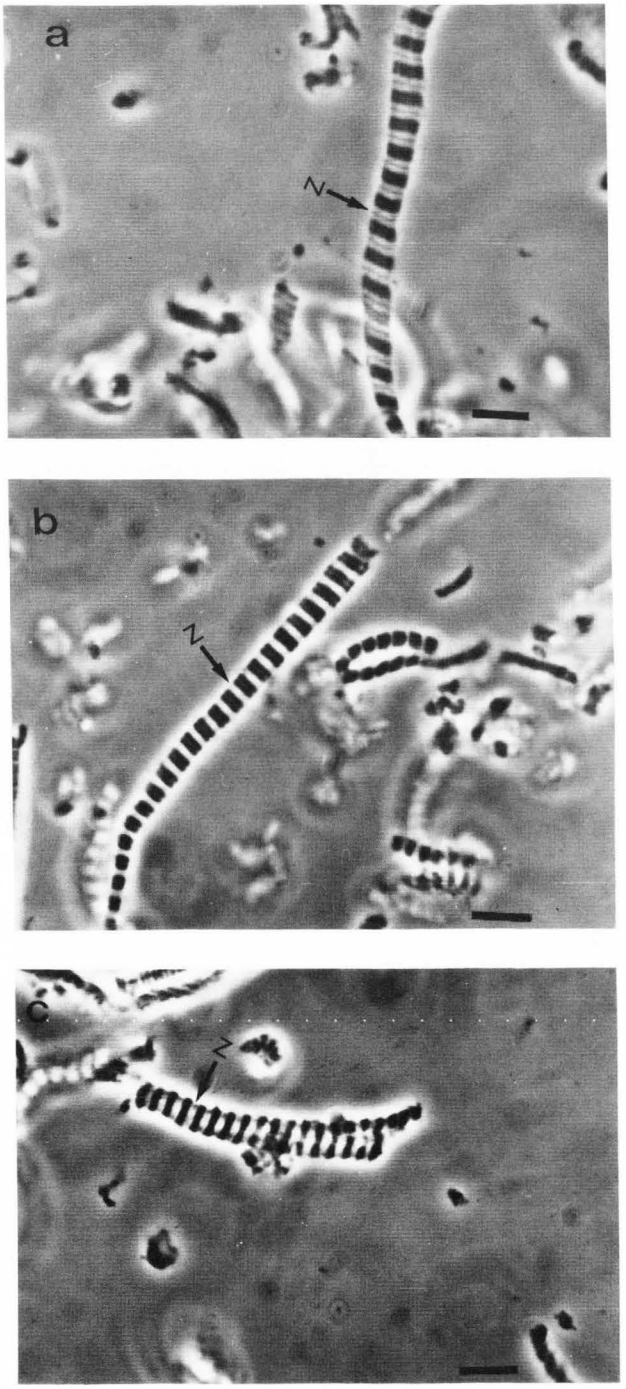

Fig.3.Phase micrographs of myofibrils from at-death ovine longissimus muscle; a) CON, b)CAF-treated (1 hr), c) cathepsin D-treated, (1 hr). Note the effects of both enzymes on the Z-lines. Bar = 5.6 μm

In the case of CAF the loss is complete (Fig. 3b) compared to cathepsin D treatment (Fig. 3c) where remnants of Z-lines can be observed. Robbins et al. (1979) reported that cathepsin D effectively removed the Z-lines of bovine myofibrils. For CAF our finding is in complete agreement with the observations of Busch et al. (1972), Dayton et al. (1976) and Olson et al. (1977). As presented earlier, the Z-line proteins (α-actinin, desmin and actin) were degraded or released by the two enzymes to varying degrees. CAF was capable of degrading desmin and releasing α-actinin with no effect on actin, whereas cathepsin D was capable of affecting actin with only a slight effect on α - actinin and none on desmin. Therefore it is reasonable to suggest that different mechanisms were employed by the two enzymes to bring about their observed effects on the Z-lines. The nature of these mechanisms is beyond the scope of this study.

This study did not establish conclusively that the two enzymes act in a cooperative fashion to degrade myofibrillar proteins; however the tendency to do so is presented. The study was extended to incorporate several variables that were not fully investigated here e.g. temperature, pH, duration of incubation and substrate and enzyme concentrations.

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Discussion with Reviewers

Bechtel PJ: In a number of instances in the manuscript, the authors refer to specific bands as being a protein i.e., desmin as 55 K. Can the authors explain how these positive identifications were made? If control proteins were not used, the identification is tenuous and should be treated as such in the manuscript.

Authors: Protein standards (of known molecular weights) e.g. myosin, bovine serum albumin, phosphorylase B etc, accompanied each electrophoresis run of the samples. The relative mobility (RF value) of the bands in question was calculated and compared with that of the standards for tentative identification.

Reviewer IV: In view of published evidence that at least 7-8 neutral and alkaline proteases are typically found in muscle homogenates and that these proteases usually sediment with myofibrils, what evidence do the authors have that the proteolytic activity in their crude CAF fraction is not due to one or more of the other proteases?

Authors: We are reasonably sure that the activity in crude CAF is due to CAF. First the three controls described by Dayton et al., (1976) accompanied each assay for enzyme activity. Secondly so far only two of the neutral and alkaline proteases were known to be activated with calcium. These two enzymes are CAF and myosin light-chain proteinase, and the latter is not found in muscle cells (Goll et al., 1983).

Reviewer IV: Why does the MHC band in Fig. 1b and 1c appear to be decreased after cathepsin D treatment but the actin band seems to be unaffected?

Reviewer III: Gels 1b and 1c do not show a decrease in the amount of actin. What evidence do the authors have that the actin band was affected by cathepsin D?

Authors: Careful examination of Fig. 1b reveals that actin was affected by cathepsin D treatment especially the bottom of the band. In Fig. 1c actin was not affected by cathepsin D probably due to the short incubation (5 min) period employed.

Bechtel PJ: Do the authors know how much protein was degraded during the proteolytic digestions?

Authors: Unfortunately no.

Reviewer IV. Because of the longer incubation times and higher cathepsin D to protein ratio used by Schwartz and Bird (1977) to digest native rabbit myosin, what indications do the authors have that the amount of cathepsin D and/or time may have been factors which limited the changes seen after cathepsin D treatment in their experiment?

Authors: In an earlier study (Elgasim et al., 1985) we used a higher purified cathepsin D to protein ratio (0.25 unit/ml cathepsin D to 1 mg/ml myofibrillar protein) for two hours and the digestion of myosin and actin in this case was almost complete. In the current study we elected to use a shorter incubation time and lower cathepsin D to protein ratio to leave some of the proteins undigested for the second incubation.

Reviewer IV: Cathepsin D cleaves MHC and actin into smaller peptides but CAF has no known ability to utilize actin as a substrate. Why then, has the actin band in Fig.2b and 2c not been diminished after cathepsin D treatment?

Authors: In Fig. 2b the actin was treated first with CAF then with cathepsin D. The nature of the substrate (actin) might have been altered by the CAF treatment in such a way that the points of interaction with the enzyme on the substrate are totally or partially blocked. Actin was not affected in Fig. 2c probably due to the inadequate incubation time employed. It should be emphasized that these are only tentative explanations.

Bechtel PJ: Would the authors expect a pH effect on degradation? CAF is often referred to as protease active at neutral pH, whereas the lysosome has a lower pH.

Authors: Both CAF and lysosomal enzymes have different optimum pH at which they are maximally active. As such, yes we do expect a pH effect on degradation.

Cohen SH, Robbins FM and Walker JE: Cathepsins are most active at post-mortem pH (5.5), whereas CAF is most active at physiological pH. Therefore, the authors should have compared both enzymes at post-mortem and physiological pH's.

Cohen SH, Robbins FM and Walker JE: Why were not both the CAF and cathepsins incubated at exactly the same temperature? Perhaps the appearance of the myofibrils would have been different. In any case samples must be treated under precisely the same conditions pH, temperature etc.

Authors: Unfortunately the two enzymes have different optimum conditions (pH and temperature), and we elected to compare their effects at these conditions. As we stated at the end of the manuscript our future work on this problem will cover these points and others.

Cohen, SH, Robbins FM and Walker JE: How pure is Sigma cathepsin D? There is a possibility that the authors may be looking at cathepsin B or L under these conditions,since the Sigma preparation is from spleen.

Authors: We did not run any purity test on the enzyme obtained from Sigma. We took it for granted that it was pure.

Discussion References

Goll DE, Oltsuka Y, Nagainis PA, Shannon JD, Sathe SK, Muguruma M. (1983). Role of muscle proteinases in maintenance of muscle integrity and mass. J. Food Biochem. 7, 137-177.