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Effect of Low-Calcium-Requiring Calcium Activated Factor on Myofibrils under Varying pH and Temperature Conditions

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ABSTRACT

The effect of low-calcium-requiring calcium-activated factor (μM CAF) on the myofibrils under varying pH at 5°C and 25°C was examined spectrophotometrically (absorbance at 278 nm), electrophoretically (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and microscopically (phase microscopy and transmission electron microscopy). Results indicated that at conditions similar to those of postmortem storage (i.e., pH 5.5–5.8 and 5°C), μM CAF retained 24–28% of its maximum activity (pH 7.5 at 25°C). This 24–28% of maximum activity was sufficient to reproduce most of the known changes associated with the tenderization process during postmortem aging. It was concluded that because of the activity of μM CAF under postmortem conditions, it seems reasonable to suggest that μM CAF may be responsible, in part, for some of the postmortem changes observed.

INTRODUCTION

AGING OF CARCASSES after slaughter for 8–14 days at 0–4°C, to improve their tenderness, has been practiced for many years and still remains an important procedure for producing tender meat. Although the increase in meat tenderness is measurable both subjectively and objectively, the mechanism(s) of this tenderization process still remains an unresolved issue for meat scientists.

Tables 1 and 2 summarize the changes observed in myofibrils as a result of postmortem storage and the effects of calcium-activated factor (CAF) on myofibrils, respectively. Comparison of these two tables clearly indicates that CAF is capable of producing many changes that are associated with aged muscle. In spite of a remarkable resemblance between the effect of postmortem aging and CAF treatment on myofibrils, several legitimate questions have been raised suggesting that CAF may not be responsible for the changes observed as a result of postmortem storage. First, high-Ca^{++} requiring form of CAF (mM CAF) is maximally active at pH 7.0–8.0 (Dayton et al., 1976b) and, therefore, has very little activity at pH 5.5–5.8 (postmortem condition). The question arising at this point is, whether CAF must be maximally active to cause sufficient damage to the myofibrils to produce the observed changes during postmortem again. Marsh (1981) reported that if only one sarcomere in every 250 was broken, such a change (even though undetectable) could result in a significant improvement in tenderness. Therefore, CAF does not need to be maximally active to bring about postmortem changes in muscle. After all, if CAF was maximally active almost all of the Z-disks would be degraded (Koohmaraie, 1984a), a change that is never associated with postmortem aging.

Table 1—Changes associated with postmortem tenderization at 2–4°C

1. Z-disk weakening (Henderson et al., 1970; Olson et al., 1976) and myofibril fragmentation.
2. Disappearance of troponin-T and appearance of a 30,000-dalton component (MacBride and Parrish, 1977; Olson et al., 1977; Koohmaraie et al., 1984a, b, c).
3. Disappearance of desmin (Young et al., 1981; Koohmaraie et al., 1984a, b, c).
4. Appearance of a 95,000-dalton component (Koohmaraie et al., 1984a, b, c).
5. Disappearance of titin (Takahashi and Saito, 1979).
6. The major contractile proteins, myosin and actin, are not affected (Olson et al., 1977; Penny, 1980; Koohmaraie et al., 1984a, b, c,).

Table 2—Effects of CAF on myofibrils

1. Z-disk degradation (Dayton et al., 1975, 1976a, b; Koohmaraie et al., 1984a).
2. Disappearance of troponin-T and appearance of a 30,000-dalton component (Dayton et al., 1975; Olson et al., 1977; Koohmaraie et al., 1984a).
3. Appearance of a 95,000-dalton component (Koohmaraie et al., 1984a).
4. Loss of desmin (O'Shea et al., 1979; Zeece et al., 1983; Koohmaraie et al., 1984a).
5. Titin is quickly degraded (Penny et al., 1984).
6. The major contractile proteins, myosin and actin, are not affected (Dayton et al., 1975; Olson et al., 1977).

Secondly, mM CAF is maximally active at 1–5 mM concentration of free Ca^{++} (Dayton et al., 1976b). The reason for concern in this instance is that free intracellular Ca^{++} concentrations would never reach this level and therefore CAF cannot be activated during postmortem storage. This argument was correct until the discovery of the second form of CAF, low-Ca^{++} requiring CAF (μM CAF). Although intracellular free Ca^{++} concentration is thought to be only 1–10 μM, the Ca^{++} concentration increases gradually with increasing time of postmortem storage and may reach levels at which μM CAF can be activated (Goll et al., 1983).

On the basis of these arguments as well as others (Goll et al., 1983), it would seem unlikely that mM CAF is responsible for postmortem changes. If CAF is involved, it must be the low-Ca^{++} requiring form of CAF, due to its low Ca^{++} requirements and also its broader range of pH activity (Dayton et al., 1981). In view of the lack of any experimental data regarding roles of μM CAF in postmortem aging, this study was conducted to examine the effects of μM CAF on myofibrils at conditions that exist during postmortem aging of meat (i.e., pH 5.5–5.8 and 5°C).

MATERIALS & METHODS

Extraction and purification of low-calcium-requiring CAF

Low-calcium-requiring CAF was prepared according to the procedure described by Dayton et al. (1981). Briefly, chilled muscle (4,000g) was ground, suspended in 2.5 volumes of 50 mM Tris-acetate, pH 8.0, 4 mM ethylene-diaminetetraacetate (EDTA), homogenized and centrifuged at 15,000 × g_{max} for 30 min. The resulting supernatant was salted out between 30–40% saturation with ammonium sulfate. The precipitate was dissolved in 20 mM Tris-acetate, pH 7.5, 1 mM

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EDTA, 10 mM 2-mercaptoethanol (MCE) and dialyzed against the same buffer for 12 hr. This solution was clarified at 105,000 × g_{max} for 60 min and chromatographed on DEAE-Sephacel that had been equilibrated against dialysis buffer. The bound protein was eluted with a continuous gradient of 10–400 mM KCl in dialysis buffer. Fractions with calcium-activated proteolytic activity at 100 μM Ca^{++} (Peak I of Dayton et al., 1981) were pooled and salted-out between 0–45% saturation with ammonium sulfate. The precipitate was dissolved in 20 mM Tris-acetate, pH 7.5, 1 mM EDTA, 1 mM NaN_3 and chromatographed on Ultrogel AcA-34 (LKB Instruments, Inc.). Fractions containing calcium-activated proteolytic activity at 100 μM Ca^{++} were pooled and then subjected to a second DEAE-Sephacel column. The bound protein was eluted with a continuous gradient from 0–200 mM KCl in dialysis buffer.

Protein concentration

Protein concentrations were determined by the biuret method (Gornall et al., 1949) or by the protein-dye binding method (Bradford, 1976).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the procedure described by Porzio and Pearson (1977) using 10% gels. Myosin, β-galactosidase, phosphorylase B, bovine albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were used as protein standards.

Myofibril isolation

Myofibrils were prepared immediately after slaughter from sternomandibularis 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.

Assay for μM CAF activity

Calcium-activated proteolytic activity was assayed using either casein (Hammerstein) or purified myofibrils as the substrate at 25°C (or stated temperature) in 100 mM KCl, 50 mM Tris-acetate, pH 7.5 (or stated pH), 10 mM MCE, 100 μM $CaCl_2$ and 5 mg/mL casein or myofibrils. Total reaction volume was 2 mL. Control for enzyme as substrate accompanied each assay. The reaction was initiated by addition of μM CAF, and stopped by addition of 2 mL of 5% trichloroacetic acid (TCA) when casein was used, or 0.22 mL of 100 mM EDTA when myofibrils were used. The assay tubes were then centrifuged at 1,000 × g_{max} for 30 min (when myofibrils were used, centrifugation was 14,000 × g_{max} for 30 min), and the absorbance of the supernatant was measured at 278 nm. To assess the effects of pH and temperature on the substrates alone, each treatment (pH × temperature) was accompanied by its own control. This control tube was used as a blank for the spectrophotometric readings to measure the activity of the corresponding treatment.

Incubation of myofibrils with purified μM CAF

Myofibrils isolated from at-death sternomandibularis muscle were incubated with chromatographically purified μM CAF at the ratio of 1:200 (μM CAF:myofibrils) for 90 min according to the procedure described for measuring μM CAF activity. The reaction was initiated by addition of the μM CAF fraction and stopped by addition of 0.22 mL of 100 mM EDTA. Three sets of tubes were prepared for analysis of all treatments by SDS-PAGE, phase microscopy and electron microscopy. For analysis of the results by SDS-PAGE, myofibrils were sedimented at 2,000 × g_{max} at 4°C and washed three times at 4°C by suspension in 2 mL of 100 mM NaCl and sedimented at 2,000 × g_{max}. The sedimented myofibrils were then dissolved in 2 mL of 1% SDS and boiled in a water bath for 15 min (Dayton et al., 1975).

Electron microscopy

Upon completion of CAF incubation of myofibrils (90 min), the reaction was stopped by the addition of 0.22 mL of 100 mM EDTA. Myofibrils were then pelleted at 3,000 × g_{max} for 10 min. The supernatant was discarded and cold 2% glutaraldehyde, 0.1M cacodylate, pH 7.2, was layered gently over the pellet and allowed to stand undisturbed for 10 min. This step and all subsequent steps prior to addition of the embedding resins were carried out on ice. After 10 min the pellet was gently dislodged, fresh glutaraldehyde was added and samples were cubed (2 mm) with a razor blade. After 1 hr, samples were rinsed three times in 0.1M cacodylate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide, 0.1M cacodylate buffer, pH 7.2 for 1 hr. Sample tubes were then dehydrated in a graded series of acetone and were embedded in an Epon-Araldite mixture and cured overnight at 56°C. Silver-gold sections were cut, using a diamond knife on a LKB Ultratome-5, stained with uranyl acetate and Reynold's lead citrate and examined with a Zeiss EM-10A transmission electron microscope.

RESULTS & DISCUSSION

FIGURE 1 SHOWS the effect of pH on μm CAF activity at 5 and 25°C with casein or myofibrils used as substrate for the protease. Based on Fig. 1, it can be concluded that: (1) μM CAF is more active when myofibrils are used as substrate and (2) μM CAF is, indeed, active at pH 5.5–5.8 and 5°C (24–28% of pH 7.5 and 25°C). The difference in the activity of μM CAF with casein and myofibrils as substrate could partially be explained by a decrease in casein solubility at the lower pH and 5°C. This loss of solubility could, therefore, affect availability of casein as the substrate for the protease (isoelectric point of casein 4.6).

Penny (1980), based on the findings of Dayton et al. (1976b) and Reddy et al. (1975) with high-Ca^{++}-requiring form of CAF, suggested that CAF retained 15–25% of maximum activity between pH 5.5–5.9. Results of this experiment are in

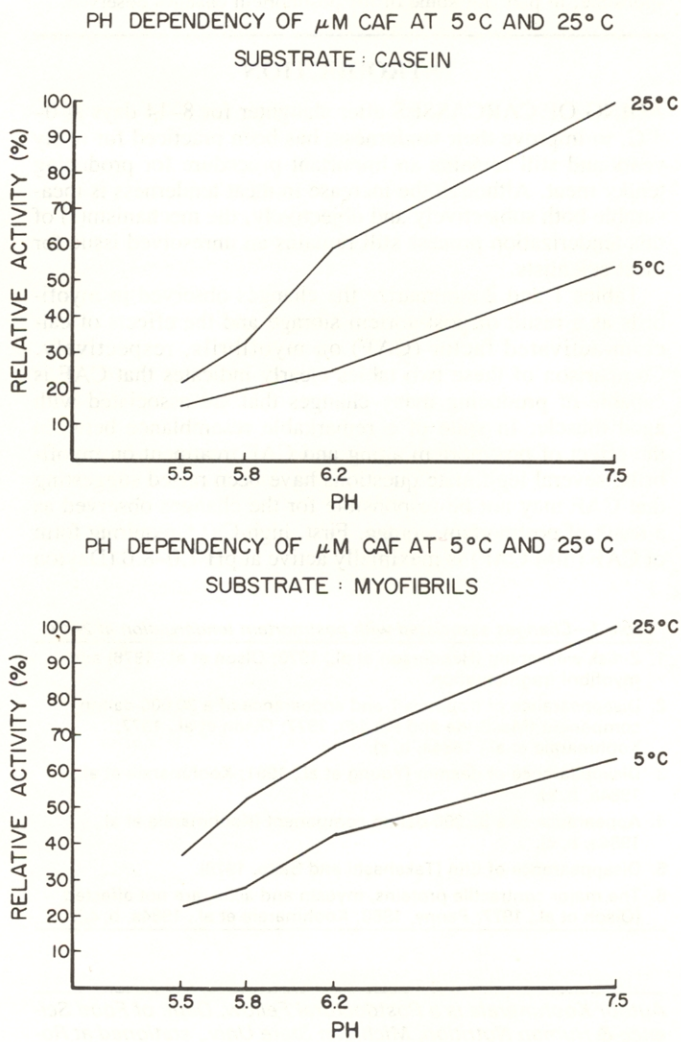

Fig. 1—Effect of pH on μm CAF activity at 5 and 25°C with casein or bovine skeletal myofibrils as substrate. Assay conditions: 100 mM KCl, 50 mM Tris-acetate, pH 7.5 (or stated pH), 10 mM 2-mercaptoethanol, 100 μM $CaCl_2$, 5 mg/mL of casein or myofibrils and 25 μg of purified μM CAF/mL, 90 min at 5 or 25°C.

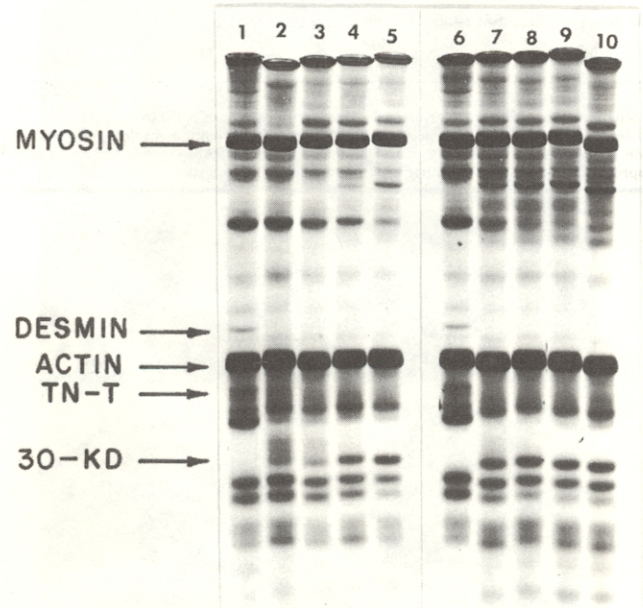

Fig. 2—SDS-PAGE of μM CAF-treated myofibrils isolated from at-death bovine sternomandibularis muscle under varying pH conditions at 5 and 25°C using 10% acrylamide with 0.1% bis-acrylamide (100:1) w/w at 400 mM Tris-glycine, pH 8.8, 5% glycerol and 0.10% SDS. Gels 1, 2, 3, 4, and 5 represent control, pH 5.5, 5.8, 6.2, and 7.5 at 5°C, respectively. Gels 6, 7, 8, 9, and 10 represent control, pH 5.5, 5.8, 6.2, and 7.5 at 25°C, respectively.

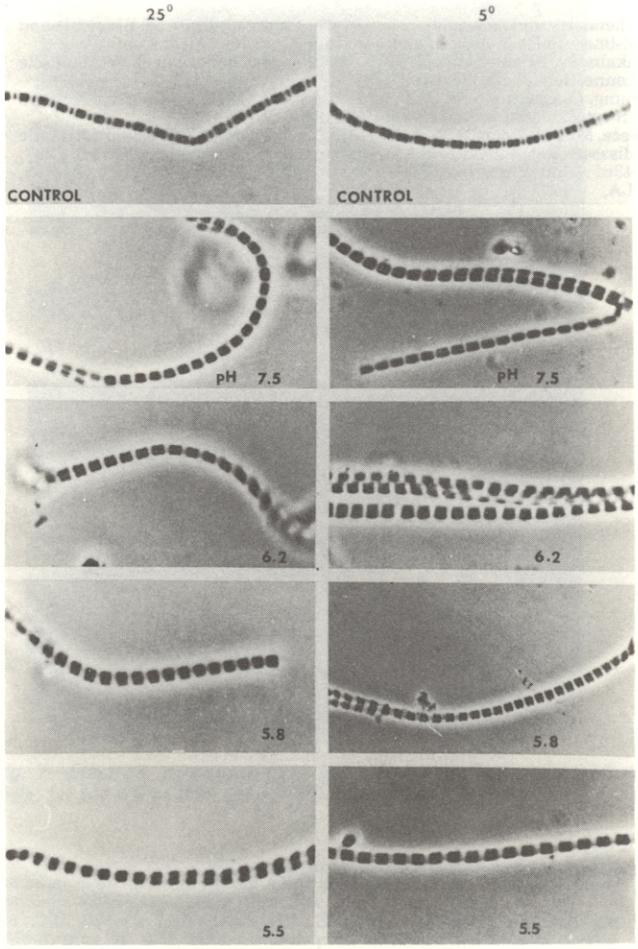

Fig. 3—Phase micrographs of μM CAF-treated myofibrils from at-death bovine sternomandibularis muscle (×2000). Note that the Z-disk-removing ability of μM CAF is not effected by pH at 25°C, while the Z-disk-removing ability decreases with decreasing pH at 5°C.

agreement with the conclusions of Penny (1980). As mentioned above, μM CAF at 5°C, retained 24 and 28% of its maximum activity at pH 5.5 and 5.8, respectively. The question arising at this point is whether this level of activity would be sufficient to bring about the changes occurring in muscle during postmortem aging. In order to answer this question, myofibrils were extracted from sternomandibolaris muscle immediately after slaughter and were incubated with chromatographically purified μM CAF and the effects were analyzed by SDS-PAGE, phase microscopy and electron microscopy.

Figure 2 shows the effect of μM CAF on myofibrillar protein under varying pH conditions at 5° and 25°C. Clearly, μM CAF can reproduce most of the changes observed during postmortem storage (pH 5.5–5.8 and 5°C), namely, (1) disappearance of desmin; (2) disappearance of troponin-T and appearance of a 30,000 dalton component; (3) removal of α-actinin, although incomplete, has begun; (4) no detectable effect on myosin and actin. A new band also appeared at about 130,000–150,000-dalton pH 6.2 and 7.5 at 5°C and at every pH at 25°C. Comparison of these results with the SDS-PAGE of myofibrils extracted from postmortem muscle (Koohmaraie et al., 1984a, b, c) strongly suggests that μM CAF could be responsible for the changes observed during postmortem storage. However, it appears that at pH 5.5–5.8 and 5°C these changes are incomplete (particularly the disappearance of troponin-T and appearance of a 30,000 dalton component). An inherent flaw in this experiment and any experiment of its kind is the inability to exactly mimic the behavior of postmortem muscle in an *in vitro* setting. Immediately after slaughter the pH and temperature of muscle are 7.0 and 37°C, respectively, and final conditions (pH 5.5–5.8 and 5°C) are achieved gradually over the first 24 hr of postmortem storage. Therefore, it is quite possible that the minor differences between electrophoretic patterns of CAF-treated myofibrils (at pH 5.5–5.8 and 5°C) and those of aged muscle (Koohmaraie et al., 1984a, b, c) are due to a gradual decrease in pH and temperature of muscle during postmortem storage. During this gradual change in pH and temperature, CAF would certainly be more active than at final conditions, pH 5.5–5.8 and 5°C (i.e., our experimental procedure).

Another similarity between postmortem aging and CAF treatment of myofibrils is the effect on desmin. Desmin appears to be the most susceptible protein to both these treatments. while there was a gradual increase in the degradation of troponin-T with increase in pH at 5°C, the removal of desmin is completed at pH 5.5 and 5°C. The same effect is observed during postmortem aging. By day three of postmortem storage, desmin is completely degraded while degradation of troponin-T is incomplete (Koohmaraie et al., 1984a).

Since the discovery of the degradation of troponin-T and simultaneous appearance of a 30,000 dalton component with postmortem aging (Olson et al., 1977), there has been considerable effort to correlate this change with the degree of meat tenderness (MacBride and Parrish, 1977; Penny and Dransfield, 1979). Indeed, Penny and Dransfield (1979) reported that this change was responsible for 60% of the variation in the toughness. These same workers also reported that in one of their samples (stored at 0°C at pH 6.3, conditions which cause muscle to cold-shorten) there was a loss of troponin-T without a corresponding increase in tenderness. One troublesome aspect of this hypothesis is a logistical one in that troponin-T is a constituent of the thin filaments of myofibrils and not of the Z-disks (Ebashi et al., 1968) and the first changes seen in myofibrils during postmortem storage in the loss of structural integrity of the Z-disks. On the other hand, desmin forms a proteinaceous network surrounding the Z-disks of each

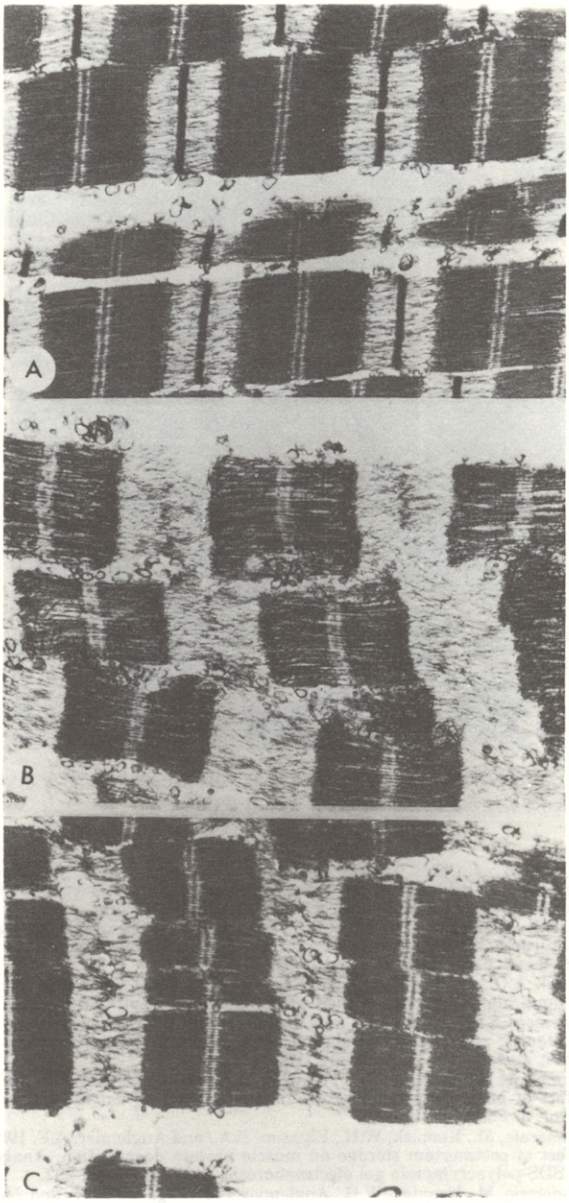

Fig. 4—Electron micrographs of μM CAF-treated bovine myofibrils. (a) Control at 25°C; (b) pH 7.5 at 25°C; (c) pH 6.2 at 25°C.

myofibril and is thought to interlock myofibrils at the Z-disk level (Robson et al., 1981). Because of the relative location of troponin-T versus desmin and also their degree of susceptibility to the conditions of postmortem storage and CAF treatment, it is proposed that the rate of the disappearance of desmin may be a better indicator of the degree of structural integrity of myofibrils at the Z-disk level and the subsequent increase in meat tenderness.

Figure 3 indicates the effect of pH on Z-disk-removing-ability of μM CAF at 5 and 25°C. These results clearly support those obtained by SDS-PAGE (Fig. 2). It demonstrates that μM CAF is clearly capable of completely removing Z-disks under all pH treatments at 25°C. However, its Z-disk-removing-ability gradually decreases with the decrease in pH at 5°C. At pH 5.5 and 5°C, Z-disks, although not completely removed, have a very faint appearance as compared to those of control myofibrils. As a result of postmortem storage, Z-disks are weakened, and this weakening of Z-disks is revealed by their faint appearance when examined by the light microscope (Davey and Gilbert, 1967, 1969). Based on the morphological changes

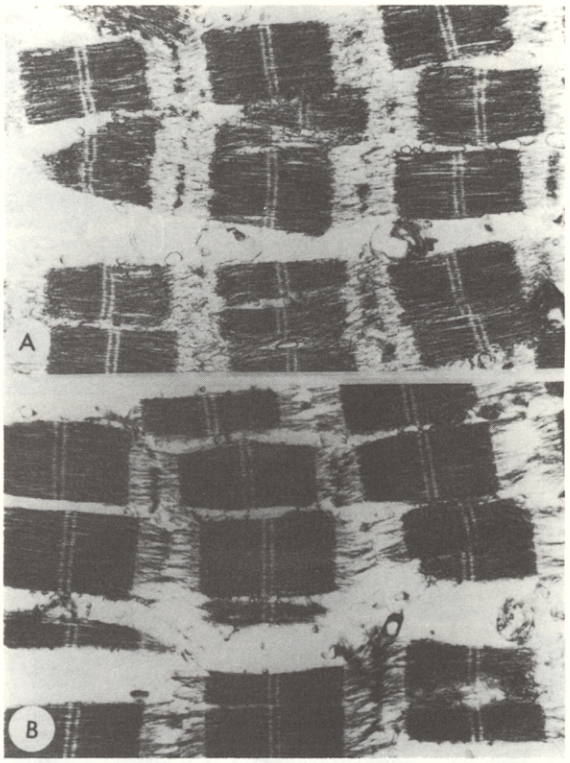

Fig. 5—Electron micrographs of μM CAF-treated bovine myofibrils. (a) pH 5.8 at 25°C; (b) pH 5.5 at 25°C.

seen in the Z-disks, therefore, again it can be concluded that μM CAF is capable of producing the changes observed in the muscle during postmortem storage. It is interesting to note that although CAF is not fully active at pH 5.5, 5.8, and 6.2 at 25°C, its Z-disk removing ability has not changed. Therefore, CAF does not need to be fully active to completely remove Z-disks.

Figures 4 through 7 show electron micrographs of control and μM CAF-treated myofibrils isolated from at-death bovine sternomandibularis muscle at varying pH and temperatures. These results clearly document the effect of pH on the Z-disk-removing ability of μM CAF at 5° or 25°C and confirm those results obtained by SDS-PAGE (Fig. 2) and phase microscopy (Fig. 3). As before, the Z-disk-removing ability of μM CAF was not affected by pH at 25°C as evidenced by complete removal of the Z-disk at all pH values examined. At conditions of interest (pH 5.5–5.8 at 5°C), although Z-disks are not completely removed, the Z-disks have clearly been affected. Based on the comparison between these results (pH 5.5–5.8 at 5°C) and those of aged myofibrils (Penny, 1980), it can be concluded that μM CAF is quite capable of reproducing observed changes in the muscle during postmortem aging.

In summary, the results of this present experiment demonstrated that although μM CAF was maximally active at pH 7.5, 24–28% of its maximum activity was retained at pH 5.5 to 5.8 and 5°C and, more importantly, this level of activity was sufficient to reproduce the changes observed during postmortem aging. Therefore, although the original questions which were raised concerning the involvement of CAF (the mM form) in postmortem aging may have been valid because of the pH and Ca^{++} concentrations required for the activity of that protease, it seems reasonable to suggest that with the identification of μM CAF these questions can no longer be of legitimate concern. We conclude that the low-Ca^{++}-requiring form of CAF is active at postmortem conditions and is capable of producing changes observed in the myofibrils during postmortem storage.

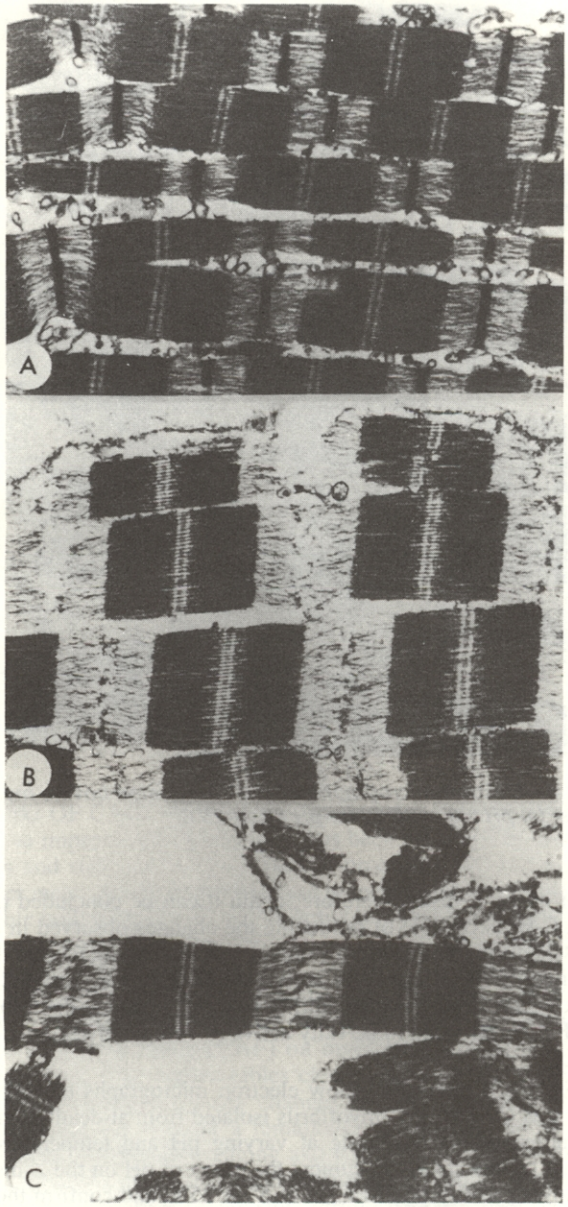

Fig. 6—Electron micrographs of μM CAF-treated bovine myofibrils. (a) Control at 5°; (b) pH 7.5 at 5°C; (c) pH 6.2 at 5°C.

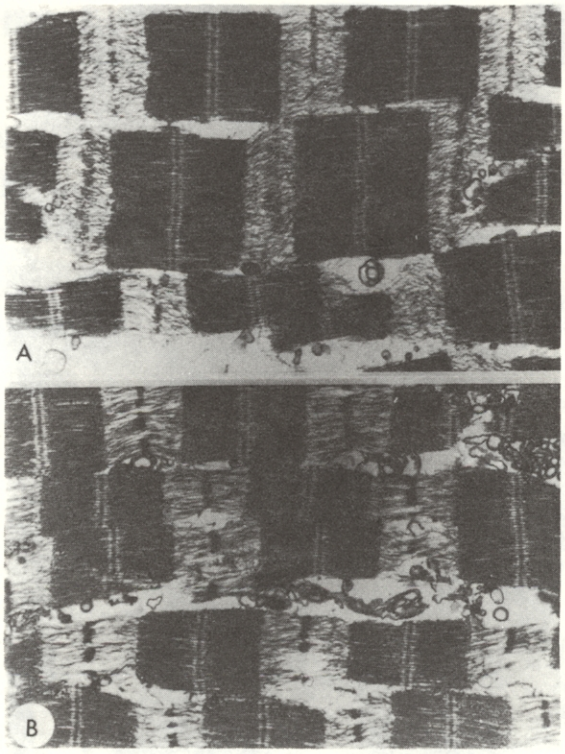

Fig. 7—Electron micrographs of μM CAF-treated bovine myofibrils. (a) pH 5.8 at 5°C; (b) pH 5.5 at 5°C.

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