The Role of Endogenous Proteases in Meat Tenderness

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Introduction

Tenderness is the predominant quality determinant and probably the most important organoleptic characteristic of red meat. It has clearly been documented that meat tenderness increases gradually as a result of postmortem storage. The first scientific report (Penny, 1980) was that of Lehmann, who in 1907 reported that there was a 30% increase in the tenderness of meat stored for 8 days postmortem. Although this increase in meat tenderness with postmortem storage is measurable both subjectively and objectively, the exact mechanism(s) of the postmortem tenderization has remained as an unresolved issue. A clear understanding of the mechanism responsible for this tenderization process or development of capabilities to reproduce this effect would have at least two major impacts on the animal industry: a) storage of carcasses for 8 to 14 days would no longer be necessary and b) perhaps would eliminate the toughness associated with meat from special groups of animals, e.g. mature animals and animals fed some β-adrenergic-agonists (see Tarrant, 1987 and Merkel, 1988). The purpose of this presentation is to review recent work in this area; it is by no means a comprehensive review of literature. The reader is referred to excellent reviews written on this subject during the past several years (Penny 1980; Davey, 1983; Dutson, 1983; Goll et al., 1983a; Dutson and Pearson, 1985; Greaser, 1986; Pearson, 1986; Asghar and Bhatti, 1987).

Background Information

In order to determine the factor(s) responsible for postmortem tenderization, one must determine the changes in skeletal muscle associated with this improvement in tenderness. To provide background for discussion in the causes of postmortem tenderization, some of the changes associated with postmortem tenderization will be reviewed. For details of these changes, the reader is referred to review papers on this subject (Penny, 1980; Goll, et al., 1983a; Greaser, 1986; Asghar & Bhatti, 1987). The first and possibly the most noticeable change that occurs in the myofibrils during postmortem storage is the weakening of Z-disks. Because of the weakening of the Z-disks, aged meat yields a higher proportion of smaller fragments upon homogenization than unaged meat (Davey and Gilbert; 1969; Moller et al., 1973). This degree of fragmentation is now determined by a spectrophotometric method and has been named Myofibril Fragmentation Index (MFI), by researchers at Iowa State University (for review, see Parrish, 1977). Although a measure of structural change in the native myofibrils, MFI correlates significantly with the shear force values of cooked meat (Moller et al., 1973; Olson et al., 1976). The second and perhaps most reported change in the myofibrillar proteins is the gradual disappearance of troponin-T with simultaneous appearance of a 30,000 dalton component (MacBride and Parrish, 1977; Olson and Parrish, 1977; Penny 1980; Penny and Ferguson-Pryce, 1979; Koohmaraie et al., 1984a,b,c). Other changes as related to this discussion include degradation of desmin (Robson et al., 1980, 1981, 1984; Robson and Huiatt, 1983, Koohmaraie et al., 1984a,b,c) and of titin (Takahashi and Saito, 1979; Young et al., 1981; Lusby et al., 1983; Robson and Huiatt, 1983). Perhaps the most important observation is that the major contractile proteins, myosin and actin, are not degraded during postmortem storage at refrigerated temperatures (Arakawa, et al., 1976; Olson et al., 1977; Penny, 1980, Koohmaraie et al., 1984a, b,c; Bandman and Zdanis, 1988).

Because all of the changes mentioned in the previous paragraph are the result of proteolysis (Dutson 1983, Goll et al., 1983a), the question now becomes what protease(s) is (are) responsible for postmortem tenderization of meat? It is generally agreed that at least two proteolytic systems could be responsible for bringing about the postmortem changes and, consequently, the increase in meat tenderness. These two proteolytic systems are catheptic enzymes and the Ca^{2+}-dependent proteases (Penny, 1980; Dutson, 1983; Goll et al., 1983a; Etherington, 1984; Dutson and Pearson, 1985; Greaser, 1986; Asghar and Bhatti, 1987).

Lysosomal Proteases

Lysosomes contain a variety of proteolytic enzymes including cathepsins. The lysosomal cathepsins are present in all mammalian cell types, with the exception of enucleated red blood cells (Bond and Butler, 1987). The name "cathepsin" is derived from a Greek term meaning "to digest" (Willstatter and Bamann, 1929). The concentration of lysosomes, and hence the proteases, varies in different cells and tissues and is particularly high in liver, spleen, kidney and macrophages. The lysosomal cathepsins from different species and cell types have very similar properties. In general, the lysosomal enzymes are small proteins (20,000-40,000

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Table 1. Lysosomal Enzymes Indigenous to Skeletal Muscle Fiber Capable of Degrading Myofibrils and/or Myofibrillar Proteins.

Enzyme	*Molecular weight*	*Functional group at active site*	*Approximate pH optimum*	*Muscle Protein Cleaved*
Cathepsin A	100,000	–OH	5.0	Myosin, myoglobin
Cathepsin B_1	25,000	–SH	5.0	Myosin, actin, intact myofibrils collagen
Cathepsin D	42,000	–COOH	5.0	Myosin, actin, intact myofibrils
Cathepsin H	28,000	–SH	5.0	Actin, Myosin
Cathepsin L	24,000	–SH	5.0	Actin, myosin, α-atactinin troponin-T, troponin-I, collagen

Adapted from Goll et al. (1983a)

daltons), optimally active at acidic pH values, and unstable at neutral or alkaline pH values (Goll et al., 1983a; Bond and Butler, 1987).

Of the 13 lysosomal proteases identified thus far, seven have been shown to exist in skeletal muscle cells (Goll et al., 1983a). These include cathepsin A, B_1, C, D, H, L and lysosomal carboxypeptidase B (Table 1); of these, the best-characterized are cathepsin B, D, H and L.

Cathepsin B_1 (Table 1) was discovered over 30 years ago (Barrett 1977a,b) and is a glycoprotein composed of 252 amino acid residues. (Katunuma and Kominami, 1983). It is an endopeptidase with PI of 5.0 to 5.2 and is active within the pH range of 4.0 to 6.5 (Goll et al., 1983a). Cathepsin B_1 requires EDTA and reduced SH groups for full activity and it is inhibited by thiol reagents (Bond and Butler, 1987). Its presence in skeletal muscle cells was demonstrated by direct cytochemical procedures (Bird and Carter, 1980; Bird et al., 1977, 1978) and immunofluorescence (Stauber and Ong, 1981).

Cathepsin D is a well-characterized lysosomal endopeptidase of the aspartic acid type, distinguished from most other proteases by its pH optimum and sensitivity to pepstatin (Barrett, 1977b). Among catheptic enzymes, cathepsin D was discovered first (for review, see Ashgar and Bhatti, 1987). It is a glycoprotein that resolves into several forms of similar molecular weight and different isoelectric point upon purification. The purified cathepsin D from rat skeletal or cardiac muscle, for instance, contains three forms when subjected to isoelectric focusing (Barrett and Heath, 1977; Samaral et al., 1984). Cathepsin D requires at least five amino residues in the substrate to express its activity and preferentially attacks peptide bonds flanked by hydrophobic amino acids, e.g., phe-phe, phe-tyr, leu-phe (Bond and Butler, 1987). Its pI is 5.5 to 6.5 and range of pH activity is 2.5 to 5.0 (Goll et al, 1983a). The presence of cathepsin D in skeletal muscle has been demonstrated by direct cytochemical (Bird and Carter, 1980; Bird et al., 1977, 1978) and by immunocytochemical procedures (Whitaker et al., 1983).

In 1976, Kirschke and co-workers isolated a new thiol endopeptidase with a molecular weight of 28,000 daltons from rat liver lysosomes and named it "cathepsin H." Cathepsin H shows both aminopeptidase and endopeptidase activities (Kirschke et al., 1977), and it exists in two multiple forms with pIs of 7.1 and 7.3 (Locnikar et al., 1981). The range of pH activity is 5.5 to 6.5. Cathepsin H is designated as a "endoaminopeptidase" because of the combination of endo- and amino-peptidase activities (Kirschke et al., 1977). It is estimated that cathepsin H has five times greater specific activity against myosin than cathepsin B (Bird and Carter, 1980). Its presence in skeletal muscle was demonstrated by Stauber and Ong (1982).

Cathepsin L was also discovered by Kirschke and co-workers (see Kirschke et al., 1981; 1982 for summary). Cathepsin L has approximately the same molecular weight as cathepsin B and like cathepsin B and H is a thiol protease with endopeptidase activity. Cathepsin L is considered to be one of the most effective lysosomal proteases when assayed against protein substrates. Bird and Carter, (1980) estimated that cathepsin L, when compared to cathepsin B, has 10 times greater specific activity against myosin. However, studies with purified cathepsin L have shown that it has very little activity on small peptides, including peptide substrates (see Goll et al., 1983a). Its range of pH activity is between pH 3.0 and 6.5 with pI of 5.8 to 6.1 (Kirschke et al., 1981). It is routinely assayed with azocasein as substrate; however, Z-phe-arg-NMec (benzyloxycarbonyl-phe- arg-4-methyl 7-coumarylamide) has been recommended as an alternate substrate (Barrett and Kirschke, 1981). A potent inhibitor of this protease is Z-phe-phe-cHN_2 (benzyloxycarbonyl-phe-phe-deazmethyl ketone), which has little effect on cathepsin B and H. A cathepsin L-like protease was purified from rabbit skeletal muscle by Okitani et al. (1980). Using an immunohistochemical approach, Taylor et al. (1987), positively identified cathepsin L in muscle fibers from rabbit skeletal muscle.

Calcium-Dependent Proteases

The first report linking Ca^{2+} to the postmortem tenderization process is perhaps that of Davey and Gilbert (1969). They indicated that weakening and disappearance of Z-disks during postmortem aging were inhibited by EDTA. They also speculated that EDTA may exert its effect by chelating Ca^{2+} ions. Busch et al. (1972) demonstrated that myofibril frag-

Table 2. Effects of Postmortem Storage at 2° to 4°C and Calcium-Dependent Protease on Myofibrils.

1. Z-disk degradation.
2. The major contractile protein, myosin and actin, are not degraded.
3. Degradation of troponin-T to several closely related peptides of approximately 30,000 dalton.
4. Appearance of a 95,000 dalton component.
5. Degradation of the intermediate filament protein, desmin.
6. Degradation of titin (connectin).
7. Increases the activities Mg^{2+}-modified and Ca^{2+}-modified ATPase of myofibrils.

Adapted from Goll et al. (1983a) and Koohmaraie et al. (1986).

mentation was inhibited by EDTA but the presence of Ca^{2+} ion induced myofibril fragmentation, which led to their successful isolation of calcium-dependent protease from skeletal muscle. The protease was later purified and characterized by Dayton et al. (1976a,b). The protease referred to as calcium-dependent protease (CDP) in this paper has a variety of other names, including calcium-activated factor (Busch et al., 1972; Olson et al., 1977; Goll et al., 1983a; Koohmaraie et al., 1984c; Koohmaraie et al., 1986); calcium-dependent neutral protease (Vidalenc et al., 1983; Ducastaing et al., 1985); calcium-activated protease (Wheelock, 1982); calpain (Murachi, 1985) and others (for details, see Goll et al., 1985).

The first documentation on the existence of CDP is perhaps that of Guroff (1964) who reported the existence of a protease in rat brain which required both calcium ions and a sulfhyl-reducing agent. Its presence in skeletal muscle was first documented by Huston and Krebs (1968) in the course of their studies on activation of phosphorylase kinase. It has now become evident that CDP is a ubiquitous protease which is present in the cytosol of all mammalian cells and tissues thus far examined (Goll et al., 1983b; Murachi, 1985). Two different forms of the enzyme have been isolated and are referred to a CDP-I and CDP-II, based on the sequence of elution from a DEAE-cellulose column (Murachi, 1985). Both forms of the protease are heterodimers of 110,000 daltons composed of an 80,000 dalton catalytic subunit and a 30,000 dalton subunit of unknown function. The 30,000 dalton subunit is the same in both enzymes, while the larger subunits are the products of different genes (Wheelock, 1982). There are no differences between CDP-I and CDP-II in optimum temperature and optimum pH. But CDP-I requires a very low concentration of calcium for 50% activation, when compared to CDP-II (for review, see Goll et al., 1985; Murachi, 1985). Also, CDP-I is activated by Mn^{2+} and Ba^{2+}, whereas CDP-II is not (Otsuka and Tanka, 1983; Croall and DeMartino, 1984; Otsuka, et al., 1988).

Another important component of the calcium-dependent protease system is a specific heat-stable protein which inhibits the activity of both CDP-I and CDP-II. This protein does not inhibit a variety of the other proteases including papain and clostripain which, like the CDPs, have thiol active sites (Mellgren et al., 1985). The precise mode of action of the CDP-inhibitor is not known, but the following observations have been reported: 1) The interaction between CDP and the CDP-inhibitor is reversible and both CDP and the CDP-Inhibitor retain full activity after dissociation (DeMartino and Croall, 1985); 2) The binding of CDP and CDP-inhibitor requires Ca^{2+} but the calcium dependence of the inhibition differs for CDP-I and CDP-II, being the same as the calcium-concentration required for catalytic activity of the respective protease (DeMartino and Croall, 1985); 3) In bovine skeletal muscle, one molecule of inhibitor appears to inhibit about six molecules of the protease and when greater quantities of protease are present, the inhibitor is hydrolyzed (Shannon and Goll, 1985).

In 1982, DeMartino and Blumenthal, while examining the role of calmodulin on the activities of CDPs in brain, identified a protein which was capable of stimulating the activities of both CDP-I and CDP-II (DeMartino and Blumenthal, 1982). This protein was called "calcium-dependent protease regulator" (CDPR) by DeMartino and Blumenthal (1982). It stimulates the activities of both CDP-I and CDP-II up to 25 folds but does not alter their calcium-requirement for activity. Whether or not CDPR exists in skeletal muscle remains to be determined.

In late 1987, an activator of CDP called "isovalerycarnitine" (IVC), a product of leucine metabolism, was discovered by Pontremoli et al. (1987). IVC increased the affinity of human neutrophil CDP for calcium so that full activity was expressed at lower calcium concentrations. Perhaps the most important characteristic of IVC is that it reverses the effect of the endogenous inhibitor of human neutrophil CDP. Whether IVC will have the same effects on CDP of skeletal muscle and whether it plays any role in the regulation of CDP activity during postmortem storage remains to be determined.

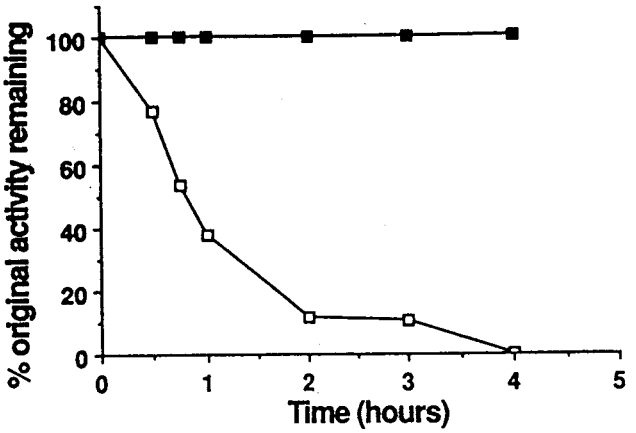
Another significant characteristic of CDP-I and CDP-II is that these proteases will undergo autolysis in the presence of Ca^{2+} (Guroff, 1964). Although Guroff (1964) believed that the presence of substrate seems to prevent autolysis of the protease, it is now clear that upon exposure to Ca^{2+} the enzyme will undergo autolysis even in the presence of the substrate (Mellgren et al., 1982). Inomata et al. (1986) demonstrated that native CDP-I is an inactive form of the protease and that the protease must be autolyzed to be activated. Continued incubation in the presence of Ca^{2+} results in further autolysis of the large subunit with the eventual loss of enzymatic activity (Suzuki et al., 1981a,b; Mellgren et al., 1982; Parkes et al., 1985). This loss of activity is highly temperature-dependent (Koohmaraie et al., 1988d); a crude preparation of CDP-II incubated without Ca^{2+} was remarkably stable at 25°C for 7 h but when incubated with 6 mM Ca^{2+} at 25°C, catalytic activity decreased in 2 h to 10% of initial activity (Fig. 1).

Both forms of CDP affect myofibril similarly. The effects of CDP on myofibrils are summarized in Table 3.

Criteria for Involvement of a Protease in Postmortem Tenderization Process

In order for a protease to be involved in the postmortem tenderization process, it must have a number of characteristics. First, protease(s) must be located inside skeletal muscle cells (For detail, see Goll et al., 1983a). Secondly, the enzyme must have the capability to reproduce postmortem changes in myofibrils in an in-vitro system. Naturally, in an in-

Figure 1



Autolysis of CDP-II. CDP-II (in 20 mM Tris base, containing 0.1 mM EDTA, 10 mM β-mercaptoethanol with pH adjusted to 7.4 with 6N HCl) was incubated at 25°C in the presence of no Ca^{2+} (■) or 6 mM $CaCl_2$ (□). At various times, sample of this incubation was assayed for caseinolytic activity. Adapted from Koohmaraie et al. (1988d).

vitro system all conditions (pH, temperature, enzyme to substrate ratio . . .) are set to maximize enzyme activity. Finally, the enzyme must have access to the substrate, i.e., myofibrils. If a protease has these characteristics, it would be impossible to exclude its possible involvement in postmortem tenderization process.

Experimental Evidence

1. CDP-I is active under postmortem conditions.

The changes observed in myofibrils as a result of postmortem storage and incubation with CDP are summarized in Table 2. These results clearly indicate that CDP is capable of reproducing the majority of changes that are associated with aged muscle. In spite of the remarkable resemblance between the effect of postmortem aging and CDP on myofibrils, several legitimate questions have been raised concerning any involvement of CDP in the postmortem tenderization process. It must be clarified that these objections were raised mostly against CDP-II. These concerns included: 1) CDP-II is maximally active at pH 7.5 (Dayton et al., 1976b) and, therefore, would have very little activity, at pH 5.5 prevalent during postmortem storage; 2) CDP-II is maximally active at 25°C, whereas postmortem tenderization occurs at 2° to 4°C; and 3) the Ca^{2+} concentration requirement of CDP-II for activation exceeds the Ca^{2+} levels in postmortem muscle. On the basis of these arguments as well as others (Goll et al., 1983a), it would seem unlikely that CDP-II is involved in the postmortem tenderization process. In view of these concerns and because of the lack of any experimental data regarding a role of CDP-I in postmortem aging, we (Koohmaraie et al., 1986) conducted an experiment to examine the effect of CDP-I on myofibrils at conditions that exist during postmortem storage of meat (i.e., pH 5.5 to 5.8 and 5°C). CDP-I and myofibrils were purified independently and then incubated together at different temperatures (5°C and 25°C), and different pHs (5.5, 5.8, 6.2 and 7.5). Results based on SDS-PAGE, phase microscopy and electron microscopy indicated that CDP-I retained 24% to 28% of its activity at pH 5.5 to 5.8 and 5°C and, more importantly, this level of activity was sufficient to reproduce the changes in the myofibrils associated with postmortem storage. It was therefore concluded that, although the original questions which were raised concerning the involvement of CDP (i.e., CDP-II) in postmortem aging may have been valid because of pH and Ca^{2+} concentration required for activity, it seems reasonable to suggest that, with the identification of CDP-I, these questions can no longer be of legitimate concern.

Table 3. Shear Force Values, Ca^{2+}-Activated Proteases, Their Inhibitor and Catheptic Enzyme Acitvity in *Longissimus dorsi, Biceps femoris* and *Psoas major* Muscles.

	Longissimus	*Biceps femoris*	*Psoas major*
Shear Force, day 1 (kg)	8.25[a]	6.16[b]	3.99[c]
Shear Force, day 14 (kg)	4.97[a]	4.70[a]	3.83[a]
CDP-I[d]	91.35[a]	60.63[b]	49.70[c]
CDP-II[e]	108.02[a]	79.87[b]	50.40[c]
Inhibitor[f]	152.44[a]	148.57[a]	90.20[b]
Cathepsin B[g]	19.05	23.72	24.31
Cathepsin H[g]	59.06	54.77	52.66
Cathepsin L+B[g]	45.38	45.76	42.26

[a,b,c]Means within the same row with different superscripts differ ($P<0.05$).

From Koohmaraie et al. (1988a). Permission obtained from the Institute of Food Technologists and the authors.

2. CDP-I, not CDP-II, may be involved in postmortem aging.

Effect of postmortem storage on the activities of CDP-I, CDP-II and their inhibitor have been studied by three different groups of investigators (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987). Because the activity of CDP-II remained nearly constant throughout postmortem storage, and there was a progressive decrease in the activity of CDP-I, Koohmaraie et al. (1987) suggested that CDP-I, not CDP-II, may be involved in postmortem aging. The rationale for this suggestion derives from the observation that both CDPs undergo autolysis and presumably initial activation followed by catalytic activity upon continued autolysis in in-vitro assays where sufficient Ca^{2+} is available for CDP activation. Therefore, the rapid loss of CDP-I during postmortem storage (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987) may be a good indicator that CDP-I, unlike CDP-II, is activated under postmortem conditions. Indeed, Vidalenc et al. (1983) demonstrated that both CDPs were autolyzed in the presence of 3 mM Ca^{2+} whereas at 50 μM Ca^{2+} only CDP-I was inactivated, i.e., at a concentration which can be reached in muscle cells during postmortem storage (Nakamura, 1973a,b; Goll et al., 1983a). Therefore, it appears that under postmortem conditions only CDP-I is activated and that the reason for stability of CDP-II is insufficient Ca^{2+} concentration.

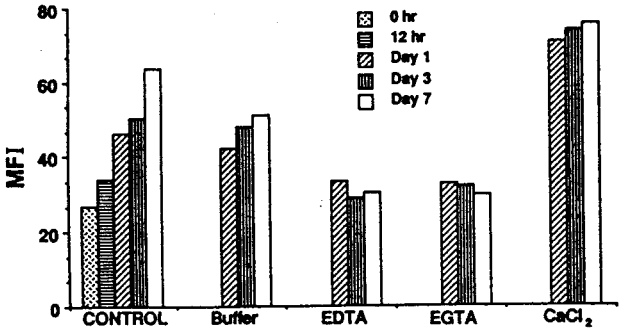
3. CDP and lysosomal enzyme activities in different muscles.

It has been postulated that either lysosomal enzymes or CDPs or the synergistic action of both classes of these proteases (CDPs and lysosomal enzymes) are responsible for postmortem changes in the skeletal muscle (Penny, 1980; Dutson, 1983; Goll et al., 1983a; Pearson et al., 1983; Dutson and Pearson, 1985; Greaser, 1986; Asghar and Bhatti, 1987). It is logical to assume that the class of protease responsible for postmortem aging should have higher activity in those carcasses with a high aging response. To avoid complications resulting from variability between animals, different muscles within the same carcass were used by Koohmaraie et al. (1988a) to relate aging responses (decline in shear force value) with protease activities, during postmortem aging. *Psoas major* (PM), *Biceps femoris* (BF) and *Longissimus dorsi* (LD) were selected based on previous reports (Olson et al., 1976; Dransfield et al., 1980-81). Our results (Table 3) indicated that between day 1 and day 14 postmortem, shear force values decreased greatly for LD and only slightly for PM. In an attempt to identify which protease class might be responsible for the observed differences in aging response, the activities of cathepsins B, H, and L, as well as the activities of CDP-I and CDP-II, were determined in LD, BF and PM muscles. Results (Table 3) indicated that regardless of the differences seen in the aging response, activities of cathepsins (B, H and L) were the same for all three muscles. However, in the case of CDP, activities followed the same pattern as the aging response; LD, which had the highest aging response, also had the highest CDP-I activity. In turn, PM, which displayed the least aging response, had the lowest CDP-I activity. Based on these results and others (Koohmaraie et al., 1986, 1987), it was concluded that the initial levels of CDP-I activity may determine the aging response of a given muscle.

4. Postmortem changes are Ca^{2+}-mediated.

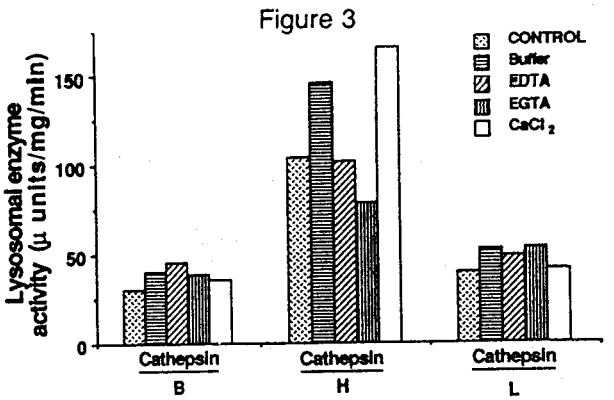
Davey and Gilbert (1969) were probably the first researchers to provide indirect evidence for involvement of Ca^{2+} in the postmortem aging process. Busch et al. (1972) demonstrated Ca^{2+} ions induced myofibril fragmentation and that

Figure 2



Effect of treatments on Myofibrillar Fragmentation Index (MFI). At 12 h postmortem longissimus dorsi muscle was sliced to 3-5 mm thick slices. Slices were then incubated for up to seven days in buffer (100 mM Tris base, containing 60 1 mM KCl, mM deoxycholate, 1 mM NaN_3 with pH adjusted to 7.2 with 1 N Acetic acid), buffer + 10 mM EDTA, buffer + 10 mM EDTA and buffer + 10 mM $CaCl_2$. Adapted from Koohmaraie et al. (1988b).

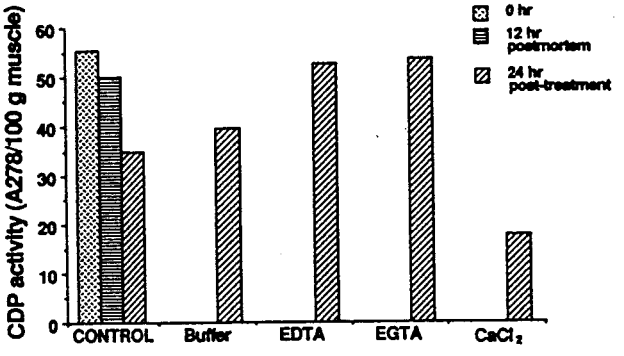
Figure 3



Effect of treatments on the activities of Cathepsin B, H and L activities. For detail see Figure Legend 2. Adapted from Koohmaraie et al. (1988b).

such fragmentation was inhibited by EDTA. Busch et al. (1972) suggested that Ca^{2+} exerts its effect by activating CDP. Using an approach similar to that of Busch et al. (1972), we (Koohmaraie et al., 1988b) attempted to sort out the contribution of lysosomal enzymes and CDPs to the postmortem aging process. We prepared slices from bovine *longissimus* muscle at 12 h postmortem and incubated these slices in different solutions to promote or inhibit activity of CDP. These solutions included: 1) buffer which consisted of 100 mM Tris containing 60 mM KCL, 1 mM deoxycholate, 1 mM NaN_3 and pH was adjusted to 7.2 with acetic acid, 2) buffer + 10 mM EGTA and 4) buffer + 10 mM $CaCl_2$. Deoxycholate was included to increase the permeability of the sarcolemma and to facilitate transfer of active compounds between the incubation buffer and the myofibrils. Sodium azide was included to prevent bacterial growth during the experiment. Results (Fig. 2) demonstrated that the myofibril fragmentation process was Ca^{2+}-mediated. This conclusion was based on the observation that MFI did not change when muscle slices were incubated in the buffer containing EDTA (chelator of divalent ions) or EGTA (chelator of Ca^{2+} ions) and that myofibril fragmentation was significantly accelerated when slices were incubated in the buffer containing $CaCl_2$. Results also indicated (see Koohmaraie et al., 1988b for details) that postmortem changes that occurred in nonincubated control muscle or in slices incubated with

Figure 4



Effect of treatments on the activity of Ca^{2+}-dependent proteases. For detail see Figure Legend 2. Adapted from Koohmaraie et al. (1988b).

buffer alone over a 7-day period had occurred rapidly, i.e., after 24 h of incubation in slices incubated in the presence of $CaCl_2$. However, in the presence of EDTA and EGTA none of these changes took place. These changes included myofibril fragmentation due to weakening of Z-disks, degradation of desmin and appearance of 30,000 dalton component.

To examine the effect of these treatments on CDPs and lysosomal enzymes, the activities of these proteases were determined at the beginning of the incubation period and 24 h after incubation. Catheptic enzyme activities were not affected by the treatments (Fig. 3). On the other hand, there was a marked effect on the activities of the CDPs (Fig. 4). CDPs are dependent on Ca^{2+} for activation. Upon activation, CDPs undergo autolysis with the eventual loss of activity (Fig. 1) and, therefore, if CDPs were activated by any of the incubation conditions, one would expect to have reduced activity of CDPs for that particular treatment. In the absence of Ca^{2+}, CDPs will not be activated and the activity of CDPs would be preserved due to lack of autolysis. Results presented in Fig. 4 show that in slices incubated with Ca^{2+} chelators (i.e., EDTA and EGTA) the activity of CDPs was preserved. On the other hand, slices incubated with $CaCl_2$ had significantly less CDP activity than any of the other treatments, indicating Ca^{2+}-dependent activation and autolysis of CDPs.

The results of this study (Koohmaraie et al., 1988b) indicated that postmortem changes in the myofibril were Ca^{2+}-dependent processes. This conclusion is based on the following observations: 1) The MFI changes were accelerated when slices were incubated with a solution containing $CaCl_2$, and these changes did not take place when slices were incubated with solutions containing Ca^{2+} chelators (Fig. 2); 2) The appearance of the 30,000 dalton component which is the most consistent change with postmortem storage (MacBride and Parrish, 1977; Olson et al., 1977, Penny, 1980; Samejima and Wolf, 1976; Yamamoto et al., 1979; Koohmaraie et al., 1984a,b,c; Koohmaraie et al., 1986) and disappearance of desmin (Robson and Huiatt, 1983; Young et al., 1981; Kohmaraie et al., 1984a,b,c) occurred within 24 h of incubation in the presence of $CaCl_2$ and did not take place in the presence of Ca^{2+} chelators. Because incubation of slices in EDTA and EGTA had no effect on the activities of cathepsins B, H, L and postmortem changes did not take place in the presence of EDTA and EGTA, it is concluded that lysosomal enzymes did not play any role in the changes in myofibril and myofibrillar proteins in this experiment. On the other hand, because incubation of slices in the presence of $CaCl_2$ accelerated postmortem changes and activated Ca^{2+}-dependent proteases, it is concluded that CDPs have a significant role in the postmortem tenderization of meat.

5. Acceleration of postmortem tenderization process.

To examine if the observations of Koohmaraie et al. (1988b) could be repeated in intact carcasses, thereby eliminating the need for extended postmortem storage to ensure meat tenderness; shortly after death, ovine carcasses were infused with $CaCl_2$ to increase the intracellular concentration of calcium (Koohmaraie et al., 1988c). An increase in free calcium concentration in prerigor muscle would theoretically result in muscle shortening similar to that of cold-shortening phenomenon (Locker and Hagyard, 1963). To avoid Ca^{2+}-induced shortening, low-frequency electrical stimulation (LFES) was used to diminish the ATP supply in the prerigor muscle. LFES was the method of choice since this method does not contribute significantly to tenderness as opposed to high-frequency electrical stimulation (Takahashi, et al., 1987a). To ensure that the observed effects, if any, were not due to infused water, a treatment which included LFES followed by infusion with H_2O was included. After LFES and prior to evisceration, the carcasses were infused through the carotid artery using a ham pumping device with water or water containing 0.3M $CaCl_2$. After infusion, calcium concentration in LD was 40 folds higher than that in control muscle and in those infused with H_2O alone (Table 5). Postmortem events (tenderization; proteolysis of myofibrillar proteins)

Table 4. Effect of Treatments on Calcium Content, Shear Forces and Enzyme Activities in Ovine *Longissimus* Muscle.

	Treatments			
Traits	*Control*	*Electrically stimulated*	*Electrically stimulated, then infused with H_2O*	*Electrically stimulated, then infused with C*
Ca (μg/g tissue)	53.0[a]	57.0[a]	56.9[a]	1577.0[b]
Shear force at day 1 (kg)	7.60[a]	6.32[b]	6.23[b]	3.56[c]
Shear force at day 6 (kg)	4.61[a]	3.60[ab]	3.03[b]	2.77[b]
Cathepsin B	42.9[a]	52.1[a]	68.3[ab]	68.7[b]
Cathepsin H	173.3	184.5	214.1	229.9
Cathepsin L	208.7	294.3	283.8	303.3
CDP-I	81.7[a]	77.3[a]	84.3[a]	0.0[b]
CDP-II	136.9[a]	124.8[a]	126.5[a]	57.6[b]
CDP-Inhibitor	78.3[a]	68.3[a]	69.2[a]	0.0[b]

[a,b,c]Means within the same row with different superscripts differ ($P<0.05$).

Adapted from Koohmaraie et al. (1988c).

Table 5. Effect of Infusion of Carcasses with NaCl and $CaCl_2$ on Shear Forces in Ovine *Longissimus* Muscle (N=6).

	Shear Force (kg/1.27 cm core)	
TREATMENT	*Day 1*	*Day 7*
Electrically stimulated	8.87[a]	6.29[a]
Electrically stimulated then infused with NaCl	8.07[a]	3.82[b]
Electrically stimulated then infused with $CaCl_2$	4.41[b]	4.06[b]

[a,b]means within the same column with different superscript differ ($P<0.05$).

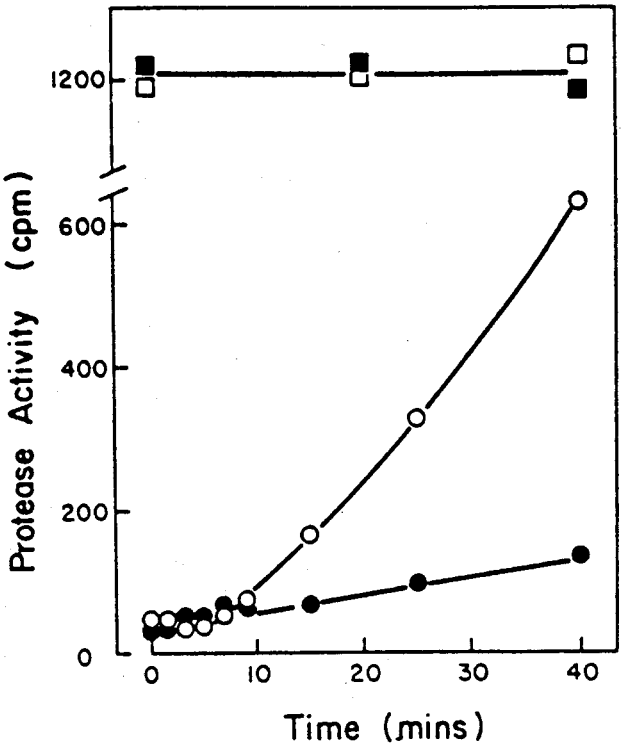
Adapted from Koohmaraie (1988b).

were completed by 24 h postmortem in carcasses infused with $CaCl_2$ (Table 4). To examine the effects of these treatments on the lysosomal enzymes and CDPs, the activities of cathepsin B, H, L, CDP-I, CDP-II, and CDP-inhibitor were determined at 24 h postmortem. All treatments had similar Cathepsin B, H and L activities. Infusion of carcasses with $CaCl_2$ caused a decrease in CDP-I, CDP-II and CDP-inhibitor activities. The CON, LFES and LFES + H_2O treatments had similar CDP-I, CDP-II and CDP-inhibitor activities. Since no treatment effect was observed on the activities of Cathepsin B, H and L, it was concluded that it seems unlikely that these Catheptic proteases were responsible for the observed tenderization due to infusion of carcasses with $CaCl_2$. However, results clearly indicated that postmortem tenderization is a Ca^{2+}-mediated process and because infusion of carcasses had a clear and definable effect (autolysis) on CDP-I, CDP-II and CDP-inhibitor, it appeared that Ca^{2+} exerts its effect through CDPs.

Wu and Smith (1987) presented evidence and concluded that elevated ionic strength in muscle during postmortem storage is one of the mechanisms responsible for the postmortem tenderization of meat. Therefore, it is possible that the observed effects in carcasses infused with calcium is due to elevation of ionic strength rather than to a proteolytic system. To ensure that the observed effects in carcasses infused with $CaCl_2$ were due to Ca^{2+} ions and not to an increase in ionic strength, carcasses were infused with NaCl (the same ionic strength as 0.3M $CaCl_2$ solution). Results (Table 5) indicated that infusion of carcasses with NaCl did not result in the acceleration of the postmortem tenderization process. Hence, it was concluded that the observed effects with $CaCl_2$ infusion of carcasses are due to Ca^{2+} ions and are not due to an elevation of ionic strength (Koohmaraie et al., 1988d).

Infusion of lamb carcasses with $CaCl_2$ results in loss of CDP-I activity and a significant decrease in the activities of CDP-II and CDP-inhibitor within the first 24 h. I have suggested that infusion of carcasses with $CaCl_2$ elevated the concentration of free Ca^{2+} which in turn activated CDPs to accelerate the postmortem tenderization process in these carcasses. Further detailed experiments must be conducted to test the validity of this hypothesis. However, several lines of evidence seem to support this hypothesis. It has been clearly demonstrated that CDPs autolyze in the presence of calcium, and continued incubation in the presence of calcium results in loss of enzymatic activity (Guroff, 1964; Suzuki et al., 1981a,b; Hathaway et al., 1982; Mellgren et al., 1982; Parkes et al., 1985; DeMartino et al., 1986; Imajoh et al., 1986; Inomato et al., 1986; Crawford et al., 1987). Therefore, the loss of CDP-I activity and a significant decrease in CDP-II activity in skeletal muscle of carcasses infused with $CaCl_2$ could be due to autolysis of CDPs. It is interesting to note that under normal postmortem conditions CDP-II is remarkably stable (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987). Koohmaraie et al. (1987) have attributed this observation to insufficient levels of Ca^{2+} to activate CDP-II. The accelerated postmortem changes in calcium-infused animals may result from not only more rapid activation of CDP-I but also activation of CDP-II. Since autolysis of CDPs in the presence of calcium is accompanied by loss of enzymatic activity, one has to question whether the process of autolysis and loss of CDP activity occurred before myofibrillar protein degradation and tenderization (Koohmaraie et al., 1988c). Crawford et al. (1987) reported that the loss of enzymatic activity due to autolysis in the presence of calcium is a slow process. Parkes et al. (1985) reported that for chicken CDP-II the autolysis process (i.e., degradation to smaller active-site-containing fragments) is complete in 2 h at 22°C. But the loss of enzymatic activity proceeds much more slowly such that even after 3 h of exposure to 5mM $CaCl_2$ at 22°C, CDP-II retained over 50% of its original activity. Results (Fig. 1) of recent studies on bovine skeletal muscle CDP-II (Koohmaraie et al., 1988d) indicated that after 1 h of exposure of 6mM $CaCl_2$ at 25°C, CDP-II loses 60% of its caseinolytic activity and after 4 h of exposure of $CaCl_2$ at 25°C, it had no caseinolytic activity. Since infusion of carcasses was done shortly after death (i.e. muscle temperature of about 37°C) one might conclude that CDPs are only active for a few h post-infusion and questions arise at this point whether CDPs could have such a major effect on muscle structure in such a short period of time. It is important to bear in mind that experiments designed to measure loss of activity due to autolysis (e.g., Parks et al., 1985; Koohmaraie et al., 1988d) were conducted in the absence of a substrate, i.e. CDP-II was exposed to $CaCl_2$ alone and at different times aliquots were withdrawn and assayed for enzymatic activity. DeMartino et al. (1986) demonstrated that the presence of substrate, casein, greatly reduced the rate of generation of active-site-containing fragments (Fig. 5). Because the formation of active-site-containing fragments is the initial step in the autolysis process and loss of enzymatic activity is the final step, it can be concluded that the presence of substrate greatly reduces the rate of loss of enzymatic activity. Therefore, in the presence of substrate, i.e. myofibrils, under in-vivo conditions even at high temperatures (i.e. conditions that exist during and after infusion of carcasses with $CaCl_2$) the loss of enzymatic activity due to exposure of CDP-I and CDP-II to $CaCl_2$ should be reduced to maintain the protease activity much longer than 4 h. Therefore, it is possible that the period during which CDP-I and CDP-II maintain catalytic activity could be long enough to produce the observed effects in ovine skeletal muscle after infusion of carcasses with $CaCl_2$. In spite of this indirect evidence supporting the hypothesis that Ca^{2+} exerts its effect through activation of CDPs, further key experiments will have to be conducted to ensure the accuracy of this hypothesis.

Figure 5



Effect of casein substrate on the autolysis-dependent formation of protease activity at 71 μM Ca^{2+} · CDP-II (220 μg/ml in 50mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol) was preincubated at 4°C in the presence of 2mM Ca^{2+} and in either the presence (●, ■) or absence (○, □) of 5 mg/ml casein. At various times, samples of this preincubation were diluted 500-fold in 50 mM Tris-HCl, pH 7.5, and assayed for protease activity at final Ca^{2+} concentrations of either 3571 (□, ■) or 71 μM (○, ●). Reproduced from DeMartino et al., (1986) with the permission of the American Society of Biological Chemists and the authors.

Which Proteolytic System is Involved?

Postmortem storage of carcasses causes numerous changes in skeletal muscle which lead to loss of structural integrity of this tissue. This loss of structural integrity is responsible for meat tenderization. It is generally accepted that proteolysis of myofibrillar proteins is responsible for this loss of structural integrity. Penny (1980) reported that "There is no doubt that proteolytic enzymes are responsible for the changes during conditioning (aging)." Hoagland et al. (1917) had already reported that proteolysis was an important factor contributing to postmortem changes in skeletal muscle, including increased tenderness. The question has been: Which proteolytic enzyme(s) is(are) responsible for the observed changes in the skeletal muscle?

There are many proteolytic enzymes in skeletal muscle; however, only Ca^{2+}-dependent proteases and certain cathepsins have been shown, to date, to degrade myofibrillar proteins. I believe that the experimental evidence supports the concept that CDPs are responsible for postmortem aging and that lysosomal proteases play a minor role, if any. In spite of overwhelming evidence in support of proteolysis as a causative process for postmortem changes, some still believe that proteolysis has no role in meat tenderization (Takahashi et al., 1987b).

Cathepsins are normally located inside lysosomes and must be released from this compartment to have access to the substrate, i.e. myofibrils. Evidence presented to indicate the release of cathepsins from skeletal muscle lysosomes is rather weak. An increase in the unsedimentable fraction (Moeller et al., 1976) has been interpreted as evidence for release of cathepsins from lysosomes. To clearly document the release of catheptic proteases from lysosomes, more selective and refined techniques must be used. Currently, antibodies generated against cathepsins B, D, H and L are available so that more selective techniques such as immunofluorescence methods could be used to document the release of lysosomal cathepsins from the lysosome to the cytosol of skeletal muscle cells during postmortem storage at 2° to 4°C. The release of cathepsin enzymes must be documented before we can discuss their role in postmortem skeletal muscle changes.

All catheptic proteases that degrade myofibrillar protein degrade the major contractile proteins, actin and myosin. It is crucial to note that SDS-PAGE studies by a large number of investigators have found no degradation of myosin heavy chain in muscle stored at 0° to 4°C (Arakawa et al., 1976; Olson and Parrish, 1977; Olson et al., 1977; Penny 1980; Koohmaraie et al., 1984a,b,c). Perhaps the best documentation of lack of myosin degradation during postmortem storage is reported by Bandman and Zdanis (1988). Using an antibody against myosin heavy chain, they did not detect myosin degradation products in extracts of postmortem muscle stored for four weeks at 4°C. In contrast, they were able to detect myosin degradation products after one day of postmortem storage at 37°C. Because they were able to detect fragments of myosin heavy chain from extract of muscle stored at 37°C, it can be concluded that the procedure (i.e. immunoblot) was sensitive enough to detect myosin heavy chain fragments if they had been generated when muscle was stored at 4°C. These results demonstrate that no myosin in degradation occurs at 0° to 4°C and yet at these temperatures, meat tenderization is clearly documented. Combination of these lines of evidence (i.e. cathepsins degrade myosin and no myosin degradation occurs during postmortem storage at 0° to 4°C) rules out the possible involvement of these enzymes in the meat tenderization process that occurs under refrigerated conditions. However, under some conditions, e.g. aging at temperatures above 16°C, catheptic proteases could contribute to postmortem tenderization by acting in concert with CDP, which is still involved in a major way (Goll et al., 1983a). Indeed, there are several reports indicating that degradation of myosin heavy chain occurs if muscle is stored at high temperatures (Arakawa et al., 1976; Samejima and Wolfe 1976; Yamamota et al., 1979; Ikeuchi et al., 1980; Bechtel and Parrish, 1983; Yates et al., 1983; Yu and Lee, 1986).

In contrast to cathepsins, Ca^{2+}-dependent proteases have all the characteristics that a proteolytic system must have to be involved in postmortem tenderization. These characteristics include: 1) endogenous to skeletal muscle cells; 2) cellular location; and 3) the ability to mimic postmortem effects on myofibrils. Results of numerous studies have left no doubt that the postmortem tenderization is a Ca^{2+}-mediated process (Davey and Gilbert, 1969; Busch et al.,

1972; Khan and Kim, 1975; Cheng and Parrish, 1977; Hattori and Takahashi, 1979, 1982; Takahashi et al., 1987b; Koohmaraie et al., 1988b,c,d). However, the question is: How does Ca^{2+} induce these changes? Calcium can exert its effects by activating CDPs (Busch et al., 1972; Cheng and Parrish, 1977; Goll et al., 1983a; Koohmaraie et al., 1988b,c,d) or independent of CDPs (Hattori and Takahashi 1979, 1982; Takahashi et al., 1987b). Hattori and Takahashi (1979, 1982) and Takahashi et al. (1987b) have reported that the postmortem weakening of Z-disks is non-enzymatically induced by the calcium ion itself. Since our data suggests that Ca^{2+} exerts its effect through activation of CDPs, it may be instructive, therefore, to review these findings and consider if they indeed provide a convincing argument against a major role for CDP in postmortem events leading to meat tenderization. The findings of Hattori and Takahashi (1979) have already been challenged by Goll et al. (1983a). Hattori and Takahashi (1979) found that myofibril fragmentation in the presence of Ca^{2+} was not prevented by iodoacetate. Since iodoacetate irreversibly inactivates CDP, they concluded that the myofibril fragmentation that occurs in the presence of Ca^{2+} and iodoacetate is therefore not due to the proteolytic action of CDP. As indicated by Goll et al. (1983a), iodoacetate reacts very slowly with CDP such that more than 40 min. (at 25°C and pH 7.5) are required for 5mM iodoacetate to cause a 60% inhibition of CDP activity. Hattori and Takahashi (1979) treated their myofibril preparation with 10mM iodoacetate for 30 min. at 5°C and pH 7.0 presumably to irreversibly inactivate CDP but they did not assay their myofibril preparation for CDP activity to indicate that it was inactivated. Therefore, it is possible that their observation of myofibril fragmentation in the presence of Ca^{2+} and iodoacetate could result from CDP before its complete inactivation by iodoacetate. Additionally, Hattori and Takahashi (1979) did not report the results of their fragmentation studies in the presence of iodoacetate. Later, Hattori and Takahashi (1982) reported that "When myofibrils were incubated with iodoacetate, leupeptin or E-64 to exclude the influence of contamination with muscle calpain (CDP) on the Z-disk weakening, they formed dense aggregates, which were not dispersed into myofibrils or myofibril fragments by homogenization. Such myofibril preparations were thus unsuitable for measurement of fragmentation indexes." Because of these observations, Hattori and Takahashi (1982) used the endogenous inhibitor of CDPs to separate CDPs contribution of CDP to the observed myofibril fragmentation in the presence of Ca^{2+}. Their results indicate that when exogenous CDPs are added to the myofibril, the fragmentation index increased by 293% and that this increase was completely inhibited by addition of a CDP-inhibitor. Since the addition of crude preparation of CDP-inhibitor to myofibrils did not prevent Ca^{2+}-induced fragmentation, they concluded Ca^{2+}-induced weakening of the Z-disk is not due to CDP. Their conclusion may not be correct, because purified myofibril are known to have CDP at the Z-disk level (Dayton and Schollmeyer, 1981) and Hattori and Takahashi (1982) did not demonstrate that the addition of CDP-inhibitor inhibits the activity of CDP that is associated with myofibrils.

Other studies (Penny and Ferguson-Pryce, 1979; Yates et al., 1983; Yu and Lee, 1986) have concluded that, depending upon the pH of muscle, different classes of proteases are responsible for meat tenderization. These studies imply that in low-pH meat, lysosomal enzymes cause meat tenderization whereas in high-pH meat, CDP is responsible for meat tenderization. Penny and Ferguson-Pryce (1979) reported that, above pH 6.0, disappearance of troponin-T in beef homogenate is accelerated by Ca^{2+}, but at pH values less than 6.0, the addition of EDTA increases the rate of loss of troponin-T. Their results should be interpreted with caution since the experimental conditions differ from conventional postmortem storage procedures (holding of carcasses at 4°C for 6 to 14 days). First, most of their experiments were conducted at 25°C and under these conditions up to 50% of the myosin heavy chains were degraded. As indicated earlier, neither CDP nor postmortem storage causes myosin heavy chain degradation. Secondly, since beef homogenates were adjusted immediately to pH 5.7, they do not mimic exactly the behavior of postmortem muscle. Under normal conditions, the pH of the muscle is initially about 7.0 and gradually decreases to 5.4 to 5.8 over a 24 h. period. Yu and Lee (1986) also concluded that the tenderization that occurs at pH 5.4 is caused by lysosomal proteases. As best as can be determined from their SDS-PAGE results and verified by personal communications, at pH 5.4 and 4°C, no myosin heavy chain degradation occurs. However, at pH 5.4 and 25° or 37°C, myosin heavy chain is extensively degraded. Because no myosin degradation occurred at pH 5.4 and 4°C and yet meat tenderization occurs (personal communication) under these conditions, again the contribution of lysosomal protease is questionable.

In summary, based on information currently available, it can be concluded that: 1) Postmortem tenderization of meat that occurs under refrigerated conditions is a Ca^{2+}-mediated process; 2) Lysosomal enzymes play a minor role, if any, in meat tenderization at refrigerated temperatures; 3) Because CDPs are the only proteases known, thus far, that are activated by Ca^{2+} and can reproduce postmortem changes in myofibrils associated with meat tenderization, they are most likely to be the causative agent for this process; 4) Protease(s) other than CDPs conceivably could be involved; however, their activity must be regulated by Ca^{2+} and be endogenous to skeletal muscle cells; and 5) Although it seems very unlikely, it may be judicious to consider the possibility that at least some of the postmortem changes responsible for meat tenderization may not be related to the proteolysis of myofibrillar proteins.

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