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Abdalla Sidahmed Mohammed Babiker

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Robert A. Merkel
Major professor

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ENDOGENOUS PROTEINASES AND POSTMORTEM PROTEOLYSIS IN RABBIT LONGISSIMUS MUSCLE

BY

ABDALLA SIDAHMED MOHAMMED BABIKER

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ABSTRACT

Endogenous Proteinases and Postmortem Proteolysis in Rabbit Longissimus Muscle

BY

Abadalla Sidahmed Mohamed Babiker

Three experiments were conducted to study the role of some endogenous cysteine proteinases and postmortem proteolysis in the aging response of rabbit longissimus muscle. Leupeptin, an inhibitor of cysteine proteinases, was used to assess the role of these proteinases in postmortem proteolysis. Effects of electrical stimulation and conditioning temperature on subcellular distribution of some lysosomal enzymes and postmortem aging response also were assessed. In vivo injection of leupeptin decreased ($P<.001$) the activities of calcium-dependent proteinases and the soluble activities of cathepsins B and L. Leupeptin had no effect ($P>.05$) on the soluble activity of cathepsin H or the bound activities of cathepsins B, L and H. Leupeptin decreased ($P<.01$) myofibril fragmentation at 24, 72 and 168 h postmortem. Effects of electrical stimulation and conditioning temperature (2 or 22 C) on percent released activities of lysosomal enzymes depended on how total activity was calculated. Electrical stimulation increased (P

<.001) myofibril fragmentation at 24 h postmortem regardless of leupeptin and conditioning temperature treatments. The effect of high temperature conditioning (22 C for 4 h) on myofibril fragmentation was significantly reduced (P<.05) by leupeptin injection. Leupeptin, electrical stimulation and conditioning temperature had no consistent effect on electrophoretic patterns of myofibrillar proteins at 24, 72 or 168 h postmortem.

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INTRODUCTION

Because of the diet-health controversies and other economic considerations, there is a growing consumer demand for leaner, yet palatable meat (Dikeman, 1982; Kauffman, 1982). Hence, the meat industry has to produce leaner carcasses and rely on technology to ensure better meat quality (Tatum, 1981). Rapid chilling of lean carcasses increases their rate of heat dissipation, decreases the rate of proteolytic activity and increases the likelihood of cold-shortening resulting in tougher meat (Locker and Hagyard, 1963; Smith et al., 1976; Lochner et al., 1980).

Improvement in tenderness of lean carcasses could be achieved by high temperature conditioning for the first 3 to 4 h postmortem (Dutson et al., 1977; Lochner et al., 1980; Marsh et al., 1981; Marsh, 1983) or by carcass electrical stimulation (Savell et al., 1978b; Smith et al., 1977, 1979; Taylor and Cornell, 1985). It is generally agreed that high temperature conditioning produces its desirable effect on tenderness through enhancement of proteolytic enzymes activities, either lysosomal enzymes (Dutson et al., 1977; Moeller et al., 1976, 1977; Wu et al., 1981) or the calcium-dependent proteinases (CDPs)(Marsh, 1983). However, the mechanism(s) whereby electrical stimulation improves tenderness remain an enigma. While some investigators suggested that electrical stimulation

produces its effect through the release of lysosomal proteolytic enzymes (Dutson et al., 1980b; Wu et al., 1985), others maintain that electrical stimulation improves meat tenderness through fiber fracture (Marsh et al., 1981; Marsh, 1983).

Although CDPs and Cathepsins B, H and L have been demonstrated to be located inside skeletal muscle cells (Goll et al., 1983a; Koohmaraie, 1988), most of the evidence linking their activities to postmortem tenderization has been indirect. The observation are mainly through measurement of enzyme activities (Moeller et al., 1977; Dutson et al., 1980; Wu et al., 1985; Koohmaraie et al., 1987, 1988b; Calkins and Seideman, 1988) and attempts to correlate these activities to different measures of tenderness, or through the effect of purified enzymes on myofibrillar proteins and intact myofibrils (Schwartz and Bird, 1977; Okitani et al., 1980; Matsukura et al., 1981, 1984; Ouali et al., 1983, 1987; Koohmaraie et al., 1986). Both methods provide valuable insight regarding these proteolytic enzyme systems in muscle, but they do not provide direct evidence concerning the involvement of these enzymes in postmortem tenderization under varying conditions insitu.

Various enzyme inhibitors have been used to study protein turnover and to inhibit muscle wasting in dystrophic animals

(Bohley et al., 1977; Stracher et al., 1978a; Sugita et al., 1980; Sher et al., 1981). However, their use to assess the contribution of proteolysis to postmortem tenderization has not been explored to date. Leupeptin is a peptide-aldehyde, produced by actinomycetes, that is highly diffusible through cell membranes, and inhibits degradation of endogenous proteins in isolated muscle tissue (Libby and Goldberg, 1978) and in vivo (Stracher et al., 1978a, b; Sher et al., 1981). Leupeptin inhibits cathepsins B (Stracher et al., 1979; Kirschke et al., 1980; Sutherland and Greenbaum, 1983), cathepsin L (Kirschke et al., 1980) and CDPs (Toyo-Oka et al., 1978; Libby and Goldberg, 1980; Sher et al., 1981). Thus, leupeptin can be used as a probe to assess the contribution of CDPs, cathepsin B and L on the degradation of myofibrillar proteins and postmortem tenderization. It also can be used to determine whether proteolysis by these enzymes is the mechanism responsible for tenderization caused by high temperature conditioning and electrical stimulation.

This study has been undertaken to:

1) explore the in vivo use of enzyme inhibitors to assess the contribution of postmortem proteolysis of myofibrillar proteins to tenderness development.

2) determine the in vivo inhibitory activity of a cysteine proteinase inhibitor (leupeptin) against some lysosomal and sarcoplasmic cysteine protein-ases

3) determine whether proteolysis by these selected cysteine proteinases is responsible for the tenderizing effect of high temperature conditioning and electrical stimulation of lean rabbit carcasses.

2) determine the in vivo inhibitory activity of a cysteine
protease inhibitor (isopeptin) against some ixodoval and
macrophagic cysteine protein-ases

3) determine whether procaolysis by these selected
cysteine proteases is responsible for the pathogenic
deviation observed in [illegible]

Literature Review

Tenderness is probably the most important organoleptic characteristic of meat from the consumer standpoint (Deatherage, 1963; Jeremiah and Martin, 1982) and the predominant determinant of meat quality (Moeller et al., 1977). Over the past century, considerable research has been undertaken in search of the causes of the variation in tenderness of meat from different carcasses. A variety of antemortem practices and postmortem properties have been extensively studied (Asghar and Pearson, 1980).

It is generally agreed that intensive feeding produces carcasses with greater fat cover, higher marbling scores and higher quality meat (Utley et al., 1975; Young and Kauffman, 1978; Aberle et al., 1981). Indeed, studies on carcasses with different degrees of finish suggest that greater fat cover and marbling slow down the rate of heat dissipation during carcass chilling which lessens the extent of cold shortening, enhances the activity of autolytic enzymes or increases the duration of active proteolysis and thereby increases the ultimate tenderness of cooked meat (Merkel and Pearson, 1975; Smith et al., 1976; Lochner et al., 1980). Since research studies have failed to establish significant correlations between carcass fatness and meat tenderness (Cover et al., 1958; Batcher et

al., 1962; Crouse et al., 1978; Jost et al., 1983), it seems that the effect of carcass fatness on meat tenderness is indirect, probably by reducing the deleterious effects of rapid postmortem chilling.

In the past few years diet-health controversies involving meat and animal fat have received tremendous publicity, and consumers are concerned about the relation between life-threatening diseases and the consumption of meat fats (Dikeman, 1982). Because of this and other economic considerations, there is a growing consumer demand for meat that has less fat, yet is palatable, healthful and inexpensive; and these concerns are continuing to grow (McGill, 1981; Kauffman, 1982). Because of these changes in consumer desires and the escalating costs of production, it is impractical to adhere to the practice of producing expensive fat insulation. Thus, the necessity to produce leaner carcasses would appear inevitable and then rely on technology to ensure the production of desirable meat cuts.

Cold-shortening and Cold-induced Toughening

Since major meat markets are located some distance from production sites, the meat industry has to rely on refrigeration to prevent meat spoilage enroute. Rapid chilling of the carcasses has been shown to cause severe muscle contraction, a

phenomenon known as cold-shortening (Locker, 1960a). This was shown to be a major factor in the variation of lamb and beef tenderness (Locker and Hagyard, 1963; Marsh and Leet, 1966) of rapidly chilled carcasses.

In view of the observations on the relation between shortening and tenderness, Locker (1960a) postulated that it should be possible to improve the quality of the longissimus muscle by hanging the carcass in such a way that this muscle is prevented from shortening. These remarks served as a stimulus for investigators and many carcass suspension methods were developed to ensure muscle restraint during carcass chilling (Herring et al., 1965a, b; 1967b; Hostetler et al., 1970, 1972; Davey and Gilbert, 1973; Buege and Stouffer, 1974; Abban et al., 1975; Stouffer, 1977). These carcass suspension treatments were not well accepted by the industry. Smith et al. (1971) reported that these treatments had the disadvantage of resulting in irregularly shaped carcasses, which presented problems in fabrication. Since cold shortening occurs only when lean carcasses are chilled very rapidly, it was obvious that it could be avoided by slow chilling or by a longer period of holding at 16 C before chilling to 0 to 2 C or freezing. Many investigators compared carcass suspension methods and delayed chilling and concluded that delayed chilling appeared

to be the most practical method for industrial use (Smith et al., 1971; McCrae et al., 1971; Bouton et al., 1973). However, delayed chilling (holding carcasses at 16 to 18 C for 10 to 16 h) is not without problems. Bouton et al. (1973) reported that it is necessary to keep carcasses conditioned at this temperature at a low relative humidity. The combination of higher temperature and low humidity results in a large increase in evaporative weight loss.

High Temperature Conditioning

Early research on the phenomenon of rigor mortis and the changes occurring in muscle during rigor shortening (Bate-Smith and Bendall, 1947, 1949; Bendall, 1951; Marsh, 1954) expanded our knowledge and understanding of the factors involved in the onset of rigor mortis. These investigators have shown that postmortem muscle undergoes shortening during rigor mortis development and that this shortening is highly dependent on rigor temperature as greater shortening occurs at higher temperatures. Later, it was discovered that muscle shortening increased as temperature was lowered toward 0 C (Locker and Hagyard, 1963). These authors observed that minimum shortening occurred in the temperature range of 14 to 19 C and that shortening increased as the temperature was raised or lowered beyond that range.

Based on this knowledge and the relationship between shortening and toughness (Locker, 1960a; Marsh and Leet, 1966; Marsh et al., 1968), considerable research has been undertaken to determine the relationship of postmortem temperature to muscle shortening and tenderness. Since cold shortening occurs only in prerigor muscle, it is evident that it can be prevented by ensuring that rigor mortis is achieved before chilling or freezing the meat (Chrystall and Devine, 1985). Delayed chilling was one of the methods employed to ensure completion of rigor development and hence to avoid the deleterious effect of cold shortening. Smith et al. (1971) compared several mechanical and physical methods for increasing the tenderness of the longissimus muscle of beef. They reported that chilling beef carcasses in a 16 C cooler for the first 16 h postmortem resulted in a 40.2% increase in ratings of longissimus tenderness and a 47.5% decrease in shear force values. They concluded that chilling the carcasses for the first 16 to 20 h postmortem at 16 C appears to be the most practical method for industrial use since it involved no additional labor expense or any irregularity in carcass form when compared to other methods, i.e., carcass suspension, severance between vertebrae in five locations or severance of ligamentum nuchae or attachment of weights (Smith et al., 1971). Other studies

substantiated the observation that delayed chilling improves tenderness by reducing the amount of shortening at least in some muscles (McCrae et al., 1971; Bouton et al., 1973). Although holding carcass at 16 C ensures minimum shortening during rigor development and prevents cold-induced toughening, it requires longer periods to cool at that temperature.

Conditioning at even higher temperatures (37 C) has been employed earlier by Roschen et al. (1950) to achieve rigor development in 3 to 5 h postmortem and shorten the period required thereafter to achieve the beneficial effects of postmortem aging. Busch et al. (1967) investigated the effect of three storage temperatures (2 C, 16 C and 37 C) on the changes and relationships of certain chemical and physical properties of beef semitendinosus and psoas muscles. They observed that isometric tension values and shear force were somewhat related at 2 C, but no relationship existed at 16 or 37 C. They concluded that although considerable tension developed in the two muscles at 37 C, shear values decreased continuously indicating that factors other than shortening are more important at high temperatures and that these factors are temperature-dependent (Busch et al., 1967). These results shed some doubt on the relation between shortening and toughness

suggested earlier by Locker (1960a). Later Locker and Daines (1975) reported that in sternomandibularis muscle, the 25% shortening that occurred during rigor development at 34 C did not increase shear force over the 15 C controls; while at 37 C, a 32% shortening was accompanied with a significant decline in shear force values. Culler et al. (1978) found no significant differences in sarcomere length among tough, intermediate and tender groups of beef chilled at 2 C. Lochner et al. (1980) concluded that the generally recognized superior tenderness of well-finished beef is largely a consequence of slower cooling during the first 2 to 4 h postmortem and that except in very rapidly chilled lean carcasses, cold shortening is not a significant determinant of tenderness.

Regarding the mechanism by which high temperature conditioning improves tenderness, Dutson et al. (1975) showed that even when muscles are restrained, delayed chilling resulted in improved shear force, connective tissue tenderness and overall tenderness ratings. Their data indicated that carcass temperature in the first 12 h postmortem is important in determining meat tenderness. Moreover, tenderness improvement is probably caused by a combination of a reduction in cold shortening and an increase in autolytic enzymes activity (Dutson et al., 1975). Dutson et al. (1977) indicated

that although cold shortening is prevented or minimized by high-temperature prerigor conditioning, other factors which add to the increased tenderness must be operative since elevated temperature conditioning improved tenderness even when muscles are restrained to produce identical sarcomere lengths with muscles conditioned at low temperature. They suggested that increased activity of the lysosomal cathepsins (due to low pH and high muscle temperature), which hydrolyze myosin and alter troponin-T with the concomitant production of a 28,000 to 32,000 molecular weight subunit, is probably one factor responsible for the improved tenderness (Dutson et al., 1977).

The above observations and earlier findings that the release of lysosomal enzymes is magnified 4-fold when temperature is raising from 23 to 37 C (Weissmann, 1964), has led researchers to investigate the effects of high temperature conditioning on subcellular distribution of lysosomal enzymes and their possible effects on myofibrillar and connective tissue proteins. Moeller et al. (1976) reported that high temperature (22 C for 4 h followed by 12 C for 8 h) enhanced the disruption of lysosomal membranes as evidenced by a significant increase in percent of free enzyme activity at 12 h postmortem for both cathepsin C and β-glucuronidase. They concluded that some of the differences in tenderness produced

by high temperature (HT) treatments are possibly associated with the increased free lysosomal enzymes during the first 12 h postmortem. Moeller et al. (1977) observed that the fragmentation index was significantly different between HT (37 C) and low temperature (LT) (2 C) conditioned muscles (12 h), indicating that HT samples had probably undergone limited proteolysis resulting in a reduction of muscle fragment size after homogenization. Wu et al. (1981) also found a greater release of lysosomal ß-galactosidase and ß-glucuronidase, regardless of the time postmortem, when high temperature conditioning occurred. They also observed that inclusion of hyaluronidase or ß-galactosidase in the incubation medium resulted in an increase in the dissolution of collagen fibers by collagenase. Based on these observations, Wu et al. (1981) speculated that lysosomal glycosidases may also participate in the dissolution of collagen fibers during postmortem aging. Yates et al. (1983), using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reported that incubation of bovine muscle at 37 C promoted a more drastic proteolytic change in myofibrillar proteins than incubation at 4 C. They also reported that degradation of myosin and troponin-T were the most noticeable changes at 37 C. Those findings were substantiated by the work of Bechtel and Parrish (1983).

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Others also have reported that the enhanced tenderness of slowly chilled carcasses is not due solely to avoidance of temperatures which induce shortening but decreasing cooling rate during the first 2 to 4 h postmortem, enhances proteolytic enzymes activities (Lochner et al., 1980; Marsh et al., 1981). However, the point of controversy is the muscle pH associated with the elevated temperature treatment. The former group maintained that the most effective combination for improving tenderness is high temperature and low pH (Dutson et al., 1977; Moeller et al., 1976; 1977; Wu et al., 1981; Dutson, 1981; 1983). Evidence for this belief was derived in part from research on the release of lysosomal enzymes and their possible effects on myofibrillar proteins (Moeller et al., 1976; 1977; Wu et al., 1981; Yates et al., 1983). The other line of evidence comes from studies on electrical stimulation. Both high temperature and electrical stimulation decrease muscle pH while muscle temperature is still high, which results in tender meat. Thus, it was speculated that high temperature coupled with low pH is the most effective combination for inducing tenderness (Dutson et al., 1977; Dutson, 1981, 1983). Moreover, electrical stimulation of antemortem stressed (temperature and handling) beef (Dutson et al., 1982) did not affect palatability traits and did not lower ultimate pH

suggesting that the pH decline normally associated with electrical stimulation is necessary to produce the desired effects on palatability (Dutson et al., 1982). Marsh (1981, 1983), Lochner et al. (1980) and Marsh et al. (1981) maintained that it is high temperature coupled with high pH that produce the desired effects on meat tenderness. Again, many lines of evidence have been drawn to support this view. It was observed that tenderness was highly dependent on, and almost linearly related to, muscle temperature attained at 2 h postmortem (27 to 40 C) and that this relationship deteriorated rapidly as longer time intervals and lower temperature ranges were considered (Lochner et al., 1980). Another line of evidence came from their study of low frequency (2 Hz) electrical stimulation (Marsh et al., 1981). They substantiated their earlier contention (Lochner et al., 1980) that tenderness of beef is strongly influenced by muscle temperature in the first 3 h postmortem. Moreover, low frequency (2 Hz) electrical stimulation, which accelerates glycolysis but causes negligible tissue disruption, significantly toughens beef loin steaks (Marsh et al., 1981). Marsh (1983) reviewed the literature on high pH aging and made new interpretations from results of numerous workers to support the contention that high temperatures and high pH is the most effective combination to

produce the desired tenderization.

The effects of high temperature conditioning on myofibrillar proteins have been studied extensively. Myosin degradation in postmortem muscle has been shown to occur at 35 to 37 C with the concomitant production of polypeptides of 150 and 80 k daltons (Arakawa et al., 1976). Many other studies, using myofibrils or muscle homogenates incubated under various conditions of pH and temperature, have shown that degradation of myofibrillar proteins, as monitored by SDS-PAGE, was more extensive at higher temperatures (Samejima and Wolfe, 1976; Olson et al., 1977; Dutson et al., 1977; Cheng and Parrish, 1978; Yamamoto et al., 1979). Ikeuchi et al. (1980b) reported that a band at 30 kd appeared in the electrophoretograms and densitograms of muscle stored at 37 C for 12 h despite a great decrease in the extractability of myofibrillar proteins. They (Ikeuchi et al., 1980b) also reported the appearance of 30 and 27 kd bands and several unidentified bands between myosin heavy chain and actin with the concomitant degradation of troponin-T and myosin. Moreover, the intensity of these bands increased between 2 and 12 h at 37 C. Bechtel and Parrish (1983) examined the proteolytic breakdown of contractile proteins of bovine longissimus muscle strips using SDS-PAGE. They observed that samples stored at 4 C exhibited little proteolysis of the

major contractile proteins even after 14 d of storage. However, samples stored at 37 C showed significant degradation of myosin heavy chains after 1 d, and almost complete proteolysis of this protein after 14 d. Major breakdown products were observed in the 145 to 125 kd region. They (Bechtel and Parrish, 1983) concluded that substantial degradation of myosin and other muscle proteins can occur during the storage of meat and that this phenomenon is highly dependent on storage temperature. Yates et al.(1983) characterized the effects of different combinations of temperature and pH on proteolysis of myofibrillar proteins. They observed more extensive proteolysis of myofibrillar proteins in bovine muscle stored at 37 C when compared to that stored at 4 C and that degradation of myosin and troponin-T were the most noticeable changes at 37 C. They also reported that alterations in myosin and troponin-T were the most noticeable changes in ground beef incubated at pH 5.4 whereas troponin-T and α-actinin were altered at pH 7.0. They also noticed more troponin-T degradation at pH 5.4 and 37 C than at pH 7.0 and 4 C. Also, myosin degradation occurred to a much greater extent at pH 5.4 and 37 C than at pH 7.0 and 4 C and the cleavage site was frequently near the papain sensitive site of myosin (Yates et al., 1983). Other proteins also have

been reported to exhibit more proteolysis at higher storage temperatures than at lower temperatures, including desmin (Young et al., 1980) and connectin (Dutson, 1983).

Electrical Stimulation

Electrical stimulation (ES) has long been used to study the glycolytic pathway in muscle (Cori, 1945). Patents were issued to Harsham and Deatherage (1951) and Rentschler (1951) for its use as a method to improve meat tenderness in a much shorter time than required for conventional aging. The research undertaken by these investigators was not published and hence was not available to the scientific community.

The use of ES to hasten postmortem glycolysis, ATP dissipation and enhancing rigor development and the effect on tenderness was considered by deFremery and Pool (1960). They concluded that the rapid rigor development induced by ES was accompanied by a toughening effect in poultry muscles. In a later study (deFremery and Pool, 1963), it became evident that a rapid onset of rigor does not necessarily result in toughness. Chrystall and Devine (1985) suggested that the toughening that had been observed by deFremery and Pool (1960) would likely have developed in muscle which entered into rigor while ES was being applied.

ES studies on pig muscles and carcasses (Hallund and Bendall, 1965; Forrest and Briskey, 1967; McLaughlin, 1970) clearly demonstrated that ES caused an acceleration of glycolysis in prerigor muscles with a concomitant acceleration in the rate of pH fall. Chrystall and Devine (1985) noted that because of the risk of inducing the pale, soft exudative (PSE) condition in beef, these studies and those of Harsham and Deatherage (1951) went unregarded until a specific need arose in New Zealand to overcome the toughening induced by prerigor chilling and freezing of lamb.

Reapplication of ES as a practical approach to increase subsequent rates of postmortem glycolysis and hasten rigor development came from the study of Carse (1973). He indicated that stimulated carcasses put into a blast freezer 5 h postmortem were as tender as untreated carcasses held for 16 h before freezing. These findings indicate that the benefits of ES are not only restricted to its ability to hasten rigor development and allow meat to be chilled soon after slaughter without the damaging effects of cold temperatures, it also ensures that the aging process and associated improved tenderness will be achieved in a shorter time (Chrystall and Devine, 1985).

Many others have substantiated the beneficial effect of ES on meat quality (Chrystall and Hagyard, 1976; Grusby et al., 1976; Savell et al., 1976, 1978c; Smith et al., 1977, 1979; George et al., 1980). The improvement in tenderness by ES is less for carcasses that are likely to produce inherently tender meat (Smith et al., 1977; Savell et al., 1981), and for muscles that are restrained from shortening due to conventional carcass suspension (Smith et al., 1979; George et al., 1980; Elgasim et al., 1981; Moller et al., 1983). ES also was reported to decrease the variability in tenderness between carcasses of different animals (Smith et al., 1977; George et al., 1980). The possibility that ES increases the rate of tenderization during aging has received a lot of consideration. Savell et al. (1978b) reported a significant reduction in shear values for samples stimulated and aged for 7 d as compared to nonstimulated samples aged for 21 d. They concluded that ES could substantially reduce the time of cooler aging needed to assure optimum tenderness (Savell et al., 1978b). Later, Savell et al. (1981) reported that ES has the greatest impact on beef palatability if the aging period was 8 d or less; with longer aging the ES effects were negated. They also indicated that as aging time is reduced, the extent of ultimate tenderization appears to be influenced by the inherent

tenderness of the beef (Savell et al., 1981). Elgasim et al. (1981) confirmed these conclusions that regardless of postmortem chilling procedure, ES increases tenderness significantly. Taylor and Cornell (1985) reported that samples taken from ES carcasses 48 h postmortem and samples aged for 28 d at 1 C resulted in a similar improvement in tenderness compared to controls (samples nonstimulated taken at 48 h). These results have been substantiated by Babiker and Lawrie (1983) who reported that hot, deboned samples of ES carcasses held at 2 C were significantly more tender than either stimulated or nonstimulated samples held at 40 C. Contrary to these beliefs, George et al. (1980) suggested that the accelerated tenderization due to ES can be accounted for by the higher temperatures in stimulated muscles at the onset of rigor and that rapid cooling soon after death reduces the effect almost to zero. Some further evidence (Elgasim et al., 1981) for chilling rates affecting the aging process was that, at 24 h postmortem, there was little or no sarcomere degradation in stimulated beef held at 2 C as compared to stimulated beef held at 16 C for the first 12 h postmortem. But they (Elgasim et al., 1981) could not detect differences in tenderness (at 7 d) or sarcomere length due to these treatments. Moller et al (1983) investigated the effect of ES on tenderization of mutton

by aging. They concluded that ES was effective in producing substantial reduction in shear force values but only for muscles able to shorten and not for muscles restrained from myofibrillar shortening due to carcass suspension.

The mechanisms whereby ES improves tenderness have been the subject of controversy over the last decade. Uncertainty still exists on the mechanisms of tenderness improvement (Moller et al., 1983; Taylor and Cornell, 1985). Earlier work (Locker, 1976; Chrystall and Hagyard, 1976; Davey et al., 1976a; Hagyard et al., 1980) has indicated that the effectiveness of ES in improving tenderness is largely due to prevention of cold shortening. The severity of freezing, practiced in New Zealand, undoubtedly increases the extent of cold shortening and warrants the aforementioned reasoning. Since chilling to O C or lower is the usual practice in the U.S., comparison of sarcomere lengths in chilled muscle fibers from control and stimulated sides or carcasses revealed no differences and casts some doubt on the earlier conclusion that the effect of ES is by preventing cold shortening (Smith et al., 1977; Bouton et al., 1978; Savell et al., 1978a, 1979; Elgasim et al., 1981). These observations led to the belief that increased tenderness of stimulated carcasses is not simply due to prevention of cold shortening. However, muscles from

goat carcass sides that received ES have longer sarcomeres than control sides (McKeith et al., 1979). This discrepancy was explained by assuming that inadequate fat cover on goat carcasses allowed more rapid cooling and induced cold shortening in the unstimulated sides. Many other researchers have observed longer sarcomeres in muscles from ES sides (Bouton et al., 1980) or in muscles excised and frozen soon after ES (Nichols and Cross, 1980; Whiting et al., 1981) when compared to similarly treated muscles from nonstimulated sides. McKeith et al. (1980) observed that longissimus muscles from intact carcasses receiving ES had longer sarcomeres than muscles from stimulated sides of carcasses from steers. The latter, however, did not differ in sarcomere length from control sides. The disparity of these results have been reviewed by Asghar and Henrickson (1982).

Physical disruption of muscle fibers resulting from massive contractions during ES has been implicated as a cause of improved tenderness (Savell et al., 1978a). Other researchers also concluded from ultrastructural studies that ES causes disruption of sarcomere integrity, tearing and fragmentation of myofibrils at the Z-disks, appearance of contraction bands in some regions with superstretching of myofibrils in adjacent regions, intracellular edema and loss of

definition in the I-, A- bands and Z-disks (Will et al., 1980; Sorinmade et al., 1982). On the basis of these observations, the above mentioned workers proposed that ES improves tenderness through disruption of the muscle fibers. This proposition has been supported by Marsh and co-workers (Marsh et al., 1981; Marsh, 1983) and by Sonaiya et al. (1982) who reported that ES resulted in greater myofibril fragmentation index (MFI) but did not affect the time of appearance of the 30 kd peptide (SDS-PAGE) which is a measure of proteolysis. Many views that do not lend credence to this hypothesis have been reported. Histological studies by George et al. (1980) showed no indication of structural damage due to ES. McKeith et al. (1980) found no evidence for an increase in structural damage when intact carcass are stimulated and compared to nonstimulated sides. Savell et al. (1979) failed to find differences in MFI between stimulated and nonstimulated beef sides. Fabiansson and Libelius (1985) observed that contracture bands occurred in both nonstimulated and stimulated samples in the early postmortem period and concluded that contracture bands are a common artifact in improperly prepared tissue preparations, therefore, they are not a specific effect of ES.

The third explanation of the mechanism by which ES improves tenderness was that the enhanced activity of lysosomal enzymes in stimulated samples which results from lowered pH and concurrent high temperature, increases proteolysis of muscle proteins (Dutson et al., 1977). Further studies from their research laboratory revealed a significant increase in percent free activity and a significant decrease in specific sedimentable and total activities of ß-glucuronidase and cathepsin C in electrically stimulated sides as compared to nonstimulated control sides (Dutson et al., 1980a). Wu et al. (1985) reported that ES did not affect soluble activities but decreased microsomal activities of ß-glucuronidase, cathepsin B and cathepsin H. Therefore, due largely to the decrease in microsomal activities of these enzymes, percentage free activity was greater in ES samples relative to controls. They (Wu et al., 1985) concluded that because greater percentages of the activities of ß-glucuronidase and cathepsin B were significantly correlated with aging index that free lysosomal enzyme activities influenced the integrity of the Z-disks or thick filaments in postmortem muscle. These studies and others showing the lack of differences in sarcomere lengths between ES and control samples have led these workers to postulate that the rapid decrease in muscle pH may hasten rupture of lysosomal

membranes, releasing proteolytic enzymes when muscle temperature is still high. These conditions enhance the rate or duration of autolytic proteolysis which may partially explain the tenderization benefit derived from ES (Smith et al., 1977; Dutson et al., 1980b). Other workers substantiated this hypothesis of enhanced autolytic proteolysis due to ES treatment (Will et al., 1980; Elgasim et al., 1981; Fabianson and Libelius, 1985). To gain further evidence for their hypothesis, Dutson et al. (1982) investigated the effect of ES on antemortem stressed beef in which the ultimate pH remained high even after ES treatment. They failed to observe any effect for ES on meat palatability relative to the control and concluded that rapid pH decline is necessary for ES to produce its desired effects. Chrystall et al. (1982) obtained similar results for exercise-stressed beef in that ES slightly toughened loin roasts and significantly toughened leg roasts. They concluded that this toughening is likely a result of rigor occurring during or soon after ES so that no relaxation of muscle can occur when the current is switched off. Fjelkner-Modig and Ruderus (1983) reported that dark, firm and dry (DFD, i.e., high pH) meat resulting from exhaustion, whether stimulated or not, had a higher initial tenderness but showed less increase in tenderness than normal meat during storage.

It is, however, obvious in these studies that meat from stressed-stimulated animals has high tenderness ratings which are equal to, if not greater than, those of meat from rested-stimulated animals (Chrystall et al., 1982; Fjelkner-Modig and Ruderus, 1983). Based on these latter studies and other studies (Marsh et al., 1981), Marsh (1981, 1983) refuted the claim that ES tenderized meat through decreased pH and release of the lysosomal enzymes which degrade muscle proteins. They (Marsh et al., 1981) reported that slow glycolysis promotes tenderness provided that early and exceptionally fast chilling does not induce cold shortening and that low frequency (2 Hz) ES, which accelerates glycolysis but causes negligible tissue disruption, significantly toughens beef loin steaks. They (Marsh et al., 1981; Marsh, 1983) maintained that ES in its normal mode (50 to 60 Hz) produces its tenderizing effect mainly, and perhaps solely, by fiber fracture.

Studies relating the tenderizing effect of ES to degradation of myofibrillar proteins are limited. While Elgasim et al. (1981) reported considerable Z-disk degradation in stimulated and delayed-chilled samples as early as 24 h postmortem, Sonaiya et al. (1982) observed that the time of appearance of the 30 kd band (SDS-PAGE) was not influenced by ES despite the beneficial effects of ES on myofibrillar

ES despite the beneficial effects of ES on myofibrillar fragmentation index and shear force. Salm et al. (1983) using SDS-PAGE concluded that at normal chilling rate, ES enhanced degradation of the myofibrillar proteins, α-actinin and troponin-T, and increased the amount of the 30 kd peptide. Babiker (1985) also reported that ES when accompanied by high temperature incubation increased the breakdown of the myofibrillar proteins, myosin and troponin, which were degraded earlier during aging in stimulated high temperature incubation than in stimulated conventionally chilled samples. Recently, Ducastaing et al. (1986) confirmed these findings by indicating that the 30 and 32 kd peptides appeared in stimulated muscles as early as 4 h postmortem and only later in nonstimulated beef muscles as determined by SDS-PAGE. None of these studies, however, compared the effects of low vs high voltage stimulation or stimulation of stressed and rested animals, on the degradation of myofibrillar proteins to shed some light on the relation of ultimate pH to these postmortem properties.

Postmortem Proteolysis

As early as 1917, it was observed that meat tenderness increases substantially upon postmortem storage. Hoagland et al. (1917) reported that the principle effect of storage upon the organoleptic properties of beef was a marked increase in

tenderness. This finding has been substantiated by later researchers (Deatherage and Harsham, 1947; Ramsbottom and Strandine, 1949; Goll et al., 1964; Whitaker, 1964; Dutson and Lawrie, 1974; Dransfield et al., 1981; Etherington, 1981). In an attempt to explain the increase in tenderness, Hoagland et al. (1917) studied the chemical changes in postmortem muscle and observed that the changes that took place in 2 to 3 d were similar to, but less extensive than, those caused by enzymatic action when lean beef was autolyzed under aseptic conditions for periods of up to 100 d. They (Hoagland et al., 1917) concluded that the chemical changes which took place in beef muscular tissues during storage may be regarded as largely due to enzyme action.

In the ensuing years, numerous research approaches have been undertaken to test the hypothesis that proteolysis is important in postmortem tenderization. One of the first approaches attempted has been to follow protein solubility and some chemical constituents of meat (nonprotein nitrogen, NPN, free N-terminal groups, free amino acids, etc). Thus, Wierbicki et al. (1956) reported a decrease in water and K-citrate soluble nitrogen and concluded that postmortem tenderization may be due to certain ion-protein or protein-protein interactions (increasing the degree of protein

hydration) rather than classical proteolysis. While Locker (1960b) reported that small increases in free amino acids do occur postmortem, results of N-terminal analysis, nonprotein nitrogen, extractability and electrophoresis of structural protein gave essentially no evidence of proteolysis. Locker (1960b) concluded that proteolysis is not a significant factor in the tenderizing of beef by aging. Other researchers failed to correlate the increase in nonprotein nitrogen or free amino groups to postmortem tenderization (Davey and Gilbert, 1966; Parrish et al., 1969). Although Valin (1968) and Valin and Pinkas (1971) reported an increase in protein solubility with aging, Goll et al. (1964) reported that protein solubility changed during the first 6 h postmortem but not during a 6 to 312 h aging period. They concluded that protein solubility did not appear to be related to tenderness.

Changes in Sarcoplasmic Proteins. The lack of evidence for proteolysis affecting tenderness together with the observation of Sharp (1963) that no changes were detectable in the fine structure of myofibrils during 6 mo of aseptic storage led many investigators to believe that proteolysis is restricted to sarcoplasmic proteins (Sharp, 1963; Bodwell and Pearson, 1964; Scopes, 1964; Thompson et al., 1968). Indeed, an additional cationic band on electrophoretograms of aged meat was observed

by Fujimaki and Deatherage (1964) who inferred that this band originated from sarcoplasmic proteins. Neelin and Rose (1964) suggested that this band most likely originates from degradation of myoglobin. Aberle and Merkel (1966) identified some 15 anionic bands in electrophoretograms of sarcoplasmic proteins which exhibited more discrete boundaries in latter stages of aging. They also observed that certain new bands appeared or increased in intensity as aging progressed. However, the relation of these changes to tenderness were thought to be secondary (Neelin and Rose, 1964) because of the delay in their development. Thompson et al. (1968) also suggested that profile alterations in sarcoplasmic proteins during aging are not related to subsequent increases in tenderness. Hay et al. (1973b) suggested that the minor changes in chicken breast and leg sarcoplasmic proteins may reflect some changes in solubility and (or) denaturation of those proteins. Goll et al. (1970) reviewed the work on the changes in sarcoplasmic proteins during postmortem aging and concluded that sarcoplasmic proteins do not undergo large changes in composition during postmortem storage at temperatures of 5 C or lower and that these proteins do not experience extensive postmortem proteolysis.

Changes in Stroma Proteins. The major components of connective tissue in skeletal muscle are probably collagen and the ground substance (Asghar and Bhatti, 1987). Connective tissue is believed to be responsible for the background toughness of muscle (Herring et al., 1967a). Divergent views have been expressed about the changes in connective tissue. Most of the research on the effect of aging on connective tissue has been summarized in excellent reviews (Whitaker, 1959; Goll et al., 1970; Asghar and Yeates, 1978; Asghar and Bhatti, 1987). Accordingly, earlier workers, using alkali-insoluble nitrogen as an indicator of collagen content, failed to find changes in stroma proteins in muscle during postmortem storage for up to 15 to 30 d at 0 to 4 C (Wierbicki et al., 1954, 1955; Khan and van den Berg, 1964; Sayre, 1968; de Fremery and Streeter, 1969). Although those studies failed to show changes in the amount of stroma proteins during postmortem aging, the technique used is not sensitive enough to eliminate the possibility of occurrence of subtle changes in structure of connective tissue proteins, which render them more susceptible to solubilization by heating (Goll et al., 1970). Attempts to characterize subtle changes in collagen during postmortem aging were made by Sharp (1963) who reported that hydroxyproline extractability did not change during aseptic storage of beef

for 172 d at 37 C. Other investigators, however, reported that the percentage oftotal muscle hydroxyprolinesolubilizedby heating bovine muscles at 77 C for 10 min increased significantly after 10 d of storage at 4 C (Herring et al., 1967a; McClain et al., 1965, 1969). Earlier, Clayson et al. (1966) suggested that postmortem changes in collagen cross-linkages are probably not due to proteolysis but rather may be caused by traces of oxidized nitrogen in a highly reactive form such as nitroxyl radicals. This conclusion does not appear plausible because of the previous contention about the presence of ester-type cross-linkages in collagen which has been disproved (Asghar and Bhatti, 1987). Asghar and Yeates (1978) suggested that postmortem alterations in connective tissue which are reflected in weakening of collagen structure may be merely the consequence of low pH in postmortem muscle, thus increasing the swelling factor of collagen. Other investigators, however, believe that proteolysis, mainly by catheptic enzymes, plays a significant role in the alterations in connective tissue postmortem (Kopp and Valin, 1981; Wu et al., 1981, 1982).

Changes in Myofibrillar Proteins. Earlier, some investigators (Goll and Robson, 1967; Robson et al., 1967) interpreted their data on nucleosidetriphosphatase activity to mean that myosin, actin and tropomyosin had not undergone significant proteolysis

even after 312 h postmortem at 2 and 16 C. As has been reviewed earlier, degradation of myosin occurs faster at elevated conditioning temperatures but only slowly at 4 C (Arakawa et al., 1976; Dutson et al., 1977; Yamamoto et al., 1979; Yates et al., 1983; Bechtel and Parrish, 1983). However, the relation of myosin degradation to tenderness development is doubtful under conventional postmortem handling conditions (Goll et al., 1983a).

Depolymerization of F-actin in postmortem muscle has been suggested by Weinberg and Rose (1960) to be due to loss of forces that bind G-actin subunits. They suggested that such a release of actin would render the muscle more tender by weakening the muscle fiber ultrastructure. This suggestion was favored by King (1966) who reported the presence of an inextractable G-actin-myosin complex (G-actomyosin) during aging of fish muscle. Chaudhry et al. (1969) substantiated this hypothesis and suggested that fragmentation of F-actin may be caused either by pH-induced alterations in conformation of G-actin or by catheptic proteolysis of a protein which structurally supports the F-actin filaments. This proposition has been disproved by Ashgar and Yeates (1978) and Asghar and Bhatti (1987) who pointed out that the conditions in postmortem muscle become more conducive for the formation of F-actin

rather than its depolymerization which requires the presence of ATP, alkaline pH, low ionic strength and absence of the divalent cations Ca^{2+} and Mg^{2+}. This and other evidence including the observation that Mg^{2+}-induced ATPase activity, which depends on F-actomyosin, did not change in aged meat (Jones, 1972; Hay et al., 1973a) provides a strong argument against any changes in F-actin postmortem.

Although, both A-bands and I-bands remain nearly intact during storage (Goll and Robson, 1967; Stromer et al., 1967; Henderson et al., 1970; Goll et al., 1983a), progressive disintegration of Z-disks has been observed as one of the major consequences of postmortem aging (Stromer and Goll, 1967; Davey and Gilbert, 1969; Henderson et al., 1970; Goll et al., 1971). Many investigators associated this degradation of Z-disks with the loss of tensile strength of the myofibrils and the increased tenderness of aged meat (Busch et al., 1972; Penny et al., 1974; Davey et al., 1976b; Penny, 1980). Some earlier workers could not detect changes in the Z-disks (Sharp, 1963; Cassens et al., 1963). Others observed, that bovine muscle stored at 2 or 16 C for 13 d, had myofibrils with almost normal contractile properties as measured by their ability to superprecipate in response to ATP (Goll and Robson, 1967) and had clearly discernible A- and I-bands when examined by

electron microscopy (Stromer et al., 1967). Davey et al. (1976b) reported that the Z-disks of meat aged 3 d at 15 C remained organized and intact even after cooking. To reconcile this conflicting finding with their earlier observations that aging caused disruption of the Z-disks (Davey and Gilbert, 1964; Davey and Dickson, 1970), they suggested that Z-disks weakening is not identified unless aging is carried beyond the time of maximum tenderness development. Again, in a study of the effect of aging on meat using stretched muscle strips, Davey and Graafhuis (1976) observed that aging leads to opening of gaps between the A- and I-bands and that splitting occurs at the A-I junction.

Regarding these seemingly conflicting results about the disintegration of Z-disks upon aging, many investigators have observed that the degradation of Z-disks in red and white muscles was not identical. Hay et al. (1973a) reported that Z-disks degradation was apparent only in chicken breast muscle, whereas Z-disks in red leg muscle were refractory to the factors which result in the disruption of breast muscle Z-disks. This contention was largely supported by studies on pig muscles (Dutson et al., 1974; Abbott et al., 1977). Similarly Gann and Merkel (1978) reported that the Z-disks of type I (red) fibers from the longissimus muscle of young bulls

remained essentially unaltered 9 d postmortem, whereas those of type II (white) fibers showed limited degradation at 1 h postmortem with increases in degradation up to 48 h postmortem. They also observed that both fiber types underwent myofibrillar fragmentation at the level of the Z-disk and I-band which was independent of Z-disk degradation. These results might explain the disparity of earlier reports about Z-disk degradation and splitting of myofibrils in aged meat. The limitations of the microscopic studies in sample size (Asghar and Henrickson, 1982) and the myofibrils preparation buffer (Stromer and Goll, 1967) may cause these discrepancies regarding Z-disks degradation.

In view of the potential relation of Z-disk degradation to myofibril fragmentation and improved tenderness of aged meat, attempts were made to identify the protein(s) affected in the Z-disk. The most likely candidate is α-actinin, but it has been shown (Penny, 1972; Dayton et al., 1975, 1976b) that the α-actinin content does not change during aging nor does its property of binding to F-actin. Later, Nagainis and Wolfe (1982) and Nagainis et al. (1983) suggested the presence of a potassium iodide insoluble form of actin in the Z-disk which is possibly degraded during aging and may account for the loss of Z-disks.

Changes in the tropomyosin-troponin complex were reported earlier (Arakawa et al., 1970). Later, numerous investigators have consistently noticed the appearance of protein bands with molecular masses of about 30,000 daltons in aged meat (MacBride and Parrish, 1977; Olson and Parrish, 1977; Olson et al., 1977; Yamamoto et al., 1979; Penny,1980). Several in vitro studies suggested that this polypeptide originates from the proteolysis of troponin-T (Dabrowska et al., 1973; Samejima and Wolfe, 1976; Olson et al., 1977; Penny, 1980). Also, the intensity of the troponin-T band has been shown to diminish during aging with the concomitant appearance of the 30 kd band (Samejima and Wolfe, 1976; Olson et al., 1977; Penny, 1980). Others have suggested that the origin of the 30 kd band is a proteolytic fragment of myosin (Hay et al., 1973b; Okitani et al., 1981). Matsumato et al. (1983) demonstrated that tropomyosin was degraded by cathepsin D to a 30 kd fragment, whereas troponin-T was degraded to 33, 20 and 11 kd fragments. Furthermore, Koohmaraie et al. (1984a) observed the appearance of the 30 kd band concomitantly with the disappearance of desmin and troponin-T. This indicates that desmin might be the source of the 30 kd band. Regardless of the source, a parallel increase in tenderness with the increase in intensity of the 30 kd peptide has been reported by many researchers (Olson and

Parrish, 1977; MacBride and Parrish, 1977; Parrish et al., 1981; Salm et al., 1983). Penny and Dransfield (1979) suggested that measurement of the loss of troponin-T provides a good indicator of the rate and extent of tenderness changes during aging. However, George et al. (1980) failed to find any correlation between the loss of troponin-T and the decrease in shear force during aging. Penny (1980) noted that although troponin-T was degraded in cold-shortened muscles, there was no reduction in toughness. Parrish et al. (1981) failed to find differences in the 30 kd band between tender and tough E-maturity (old) beef. The relevance of troponin-T degradation to Z-disk disintegration and increased tenderness of aged meat has been questioned (Penny, 1980; Asghar and Bhatti, 1987). The loss of troponin-T from thin filaments would be expected to affect the binding of troponins I and C to tropomyosin and hence to thin filaments. The presence of troponins I and C in electrophoretograms of myofibrils indicates that they remained intact in postmortem muscle (Penny, 1980).

Besides the proteins of the thick and thin filaments, other proteins are now known to exist in the myofibrillar structure. These are collectively known as cytoskeletal proteins (Ashgar and Bhatti, 1987). Limited information is available on the location of some of these proteins in the

myofibril and on their fate during aging of meat. Connectin(titin) degradation has been explored by many investigators during the last decade. The first were Takahashi and Saito (1979) who reported that the amount of connectin decreased with increasing time of postmortem storage and that the loss in elasticity of muscle coincided well with the loss in connectin. They concluded that the continuous net structure of connectin is responsible for about 30 percent of the total elasticity of living skeletal muscle, and its degradation is responsible for postmortem tenderization of meat. Other investigators explored the effect of heating on connectin (King et al., 1981; King and Harris, 1982; King, 1984) and observed that connectin was extensively degraded when muscle samples were heated to 50 to 70 C. They implicated a carboxyl proteinase in its degradation because they noticed that connectin degradation was inhibited by pepstatin (King et al., 1981; King and Harris, 1982). They also reported that connectin, when intact, may contribute to tensile strength and toughness of cooked meat. However, King (1984) reported that the breakdown of connectin during heating at 60 to 80 C for 40 min was more extensive than during aging for 21 d at 2 C. He concluded that the partial proteolysis of connectin during storage at 2 C is unlikely to be responsible for the tenderization induced by aging. Locker and Wild (1984) reported that connectin survived aging at 15 C and that even

cooking at 80 C causes only random splitting of connectin to a smear with a molecular weight above 500 kd which should still be capable of contributing to structural strength.

Nebulin, another cytoskeletal protein, has been shown to be degraded during aging concomitantly with an increase in a protein band appearing between nebulin and connectin (Locker and Wild, 1984). They (Locker and Wild, 1984) claimed that these were the only changes on the same time scale as tenderization and questioned the location of nebulin in the N-line (Wang and Williamson, 1980) on the basis of these observations.

Desmin, yet another cytoskeletal protein also known as skeletin, is also found to be labile to proteolytic breakdown during aging. Young et al. (1980) concluded that the loss of desmin during postmortem storage probably accounts for the ease with which stored muscle disintegrates into individual myofibrils upon homogenization. They also stated that the disintegration of the cytoskeletal network can account for the increased tenderness after cooking of stored meat. This view has been largely supported by Koohmaraie et al. (1984a, b) who observed the disappearance of desmin in normal and cold-shortened beef muscle during postmortem aging.

Proteinases Involved in Postmortem Proteolysis

It is generally accepted that at least one or both of two proteolytic systems is responsible for muscle proteins

breakdown and the tenderizing effects associated with post-mortem storage. These are the lysosomal catheptic enzymes and the Ca^{2+}-dependent proteinases or CDP's (Penny, 1980; Dutson, 1983; Goll et al., 1983a; Etherington, 1984; Asghar and Bhatti, 1987).

Excellent reviews have appeared in the literature recently on cellular proteinases, their distribution, their possible physiological roles and classification (Khairallah et al., 1985; Asghar and Bhatti, 1987; Bond and Butler, 1987). These reviews also present the possible relation of different proteinase systems to postmortem tenderization (Dutson, 1983; Goll et al., 1983a; Etherington, 1984; Asghar and Bhatti, 1987; Koohmaraie, 1988). This literature review will be restricted to the CDP's and the lysosomal cysteine proteinases cathepsins B, H and L.

Calcium-Dependent Proteinases (CDP's). The first report on the existence of a Ca^{2+} activated cysteine proteinase, active at neutral pH, was that of Guroff (1964). Another group of investigators, simultaneously, demonstrated the existence of a

similar proteinase in skeletal muscle during their studies on phosphorylase kinase activation and named it kinase activating factor (KAF) (Meyer et al., 1964; Huston and Krebs, 1968). This Ca^{2+} dependent modification of proteins was found to be a phenomenon that is not limited to the case of phosphorylase kinase activation; but rather has much wider distribution. The report of Davey and Gilbert (1969) linking Ca^{2+} ions to postmortem tenderization and their observation that weakening and disappearance of Z-disks during postmortem aging was inhibited by EDTA, probably motivated the research on Ca^{2+}-dependent proteolysis in postmortem muscle. Hence, Busch et al. (1972) clearly demonstrated the Ca^{2+} dependence of myofibril fragmentation and Z-disks disappearance which led to their successful isolation of this proteinase (named calcium-activated sarcoplasmic factor [CASF]) from skeletal muscle. This proteinase was later purified and characterized by Dayton et al. (1976a, b). Besides KAF and CASF, this proteinase received a variety of different names from different groups of investigators. Among these names were calcium-activated neutral protease - CANP (Ishiura et al., 1978), calcium dependent neutral proteinase (Ducasting et al., 1985), calcium activated protease (Wheelock, 1982) and calpain (Murachi, 1983). CDP was found to be optimally active in the pH range of

6.5 to 8.0 in the presence of 1 to 2 mM Ca^{2+}, it had little activity below .1 mM Ca^{2+} and required a reduced sulfhydryl group for activity (Dayton et al., 1976b; Waxman, 1978). Soon after its purification, CDP was found to be localized inside skeletal muscle cells at the level of Z-disks and at the sarcolemma (Dayton and Schollmeyer, 1980, 1981; Ishiura et al., 1980). Goll et al. (1985) have indicated that CDP is present throughout the cytoplasm with no preferential location.

Disruption of the Z-disks structure by CDP (Busch et al., 1972) has been shown to result in meat tenderization (Penny et al., 1974; Slinde and Kryvi, 1986) and in myofibril fragmentation with concomitant tenderization (Slinde and Kryvi, 1986; Koohmaraie et al., 1987). Other studies have shown that treatment of purified myofibrils with CDP produced effects which closely resemble the effects of postmortem storage on the electrophoretograms of myofibrillar proteins (Olson et al., 1977; Cheng and Parrish, 1978; Yamamoto et al., 1979). Although some investigators reported low CDP activity in the psoas major (PM) muscle concomitantly with a low aging response as compared to a greater aging response in longissimus dorsi (LD), semitendinosus (SM) and biceps femoris (BF) muscle which have higher CDP activity (Olson et al., 1977; Koohmaraie et al., 1988b). Parrish et al. (1981) failed to find differences

in CDP activity in the LD of tough and tender beef. Further support for these criteria of involvement of CDP in the tenderization process came from the studies on the myofibrillar proteins that are degraded by CDP. Penny (1974) suggested that the observed destruction of the Z-disks by CDP is due to its digestion of α-actinin. It was later found that CDP does not degrade α-actinin but releases it from Z-disks (Suzuki et al., 1975; Dayton et al., 1975, 1976b). Ishiura et al. (1979) also have shown that CDP did not degrade myosin or α-actinin if the ratio of CDP to these proteins was (1:100); when this ratio was increased, CDP degraded myosin heavy chain and α-actinin. The actual substrates for CDP were found to be troponin-T, troponin-I, C-protein, tropomyosin (Dayton et al., 1975, 1976b) and the cystoskeleton proteins, desmin, titin and nebulin (Robson and Huiatt, 1983). However, degradation of these proteins by CDP does not explain the removal of Z-disks in postmortem muscle. Reinvestigation of the proteins found in Z-disks has raised the possibility that Z-disks contain an insoluble form of actin differing from thin-filament actin in its isoelectric pH, solubility in KI and antigenicity in mice (Nagainis and Wolfe, 1982; Nagainis et al., 1983). These workers suggested that digestion of this Z-disk actin by CDP might explain the disintegration of Z-disks in postmortem

muscle, however, this remains to be proven.

The questions raised about the possible involvement of the originally discovered CDP in postmortem tenderization include its requirement for mM concentration of Ca^{2+} in the sarcoplasm which is never achieved in muscle cells (Goll et al., 1983; Koohmaraie, 1988). The isolation of another form of CDP that requires uM concentrations of Ca^{2+} has been reported (Mellgren, 1980; Dayton et al., 1981; Szpacenko et al., 1981). This form of CDP, termed uM-CDP as opposed to mM-CDP, is thought to be similar to the originally discovered CDP (mM-CDP) but modified in a way such that its negative charge at pH 7.5 and its Ca^{2+} requirement for maximal activity are reduced. Soon after its discovery, involvement of the uM-CDP in postmortem tenderization has been determined using two lines of evidence. Ouali et al. (1983) compared the effects of postmortem storage on myofibrillar proteins and on myofibrils treated with uM-CDP and concluded that in beef and rabbit muscles incubation of myofibrils with uM-CDP mimicked the postmortem changes in the Mg-Ca-enhanced myofibrillar ATPase and in the banding pattern of myofibrillar protein on electrophoretograms. Koohmaraie et al. (1987) studied the effect of postmortem storage on both CDP's, their inhibitor and myofibril fragmentation and reported that, while the activity of mM-CDP remained constant up to 14 d

postmortem, there was a progressive decrease in the activities of uM-CDP and their inhibitor which paralleled the changes in MFI through d 14. They (Koohmaraie et al., 1987) suggested that uM-CDP, not mM-CDP plays an important role in myofibril fragmentation and tenderization during postmortem storage.

Another argument raised against the involvement of CDP's in the tenderization process was that they are optimally active at neutral pH and 25 C, conditions which exist in postmortem muscle for only a brief time postmortem. This has been refuted by the results showing that crude CDP (Suzuki et al., 1982) and purified uM-CDP (Koohmaraie et al., 1986) retain some activity under conventional postmortem conditions. Koohmaraie et al. (1986) reported that uM-CDP had 24 to 28% of its optimal activity at pH 5.5 and 5 C and that this level of activity was sufficient to cause most of the known changes associated with postmortem tenderization. Further evidence for the involvement of CDP's in the postmortem tenderization process came from the study of Koohmaraie et al. (1988a) in which beef muscle slices were incubated in the presence of Ca^{2+} or the Ca^{2+} chelators EDTA or EGTA; and postmortem changes in MFI and in electrophoretic banding pattern of myofibrillar proteins were monitored. They concluded that postmortem changes in MFI and the appearance of the 30 kd band was accompanied by the loss

of desmin and troponin-T. These effects were completed after 24 h of Ca^{2+} treatment while EGTA and EDTA completely inhibited these changes even after 7 d postmortem, suggesting that postmortem tenderization may be associated with CDP's activity rather than catheptic enzymes.

Lysosomal Cysteine Proteinases Cathepsins B, H and L.

Cathepsin B was discovered over 30 years ago by Greenbaum and Fruton (1957). Its molecular mass varies between 24 to 28 kd in various tissues, and it was found to exist in multiple forms with the isoelectric pH ranging from 4.7 to 5.6 (Barrett, 1973; Locnikar et al., 1981; Turk et al., 1984). This may possibly explain why the optimum pH for its activity varies widely between 3.5 to 6.0 (Asghar and Bhatti, 1987). The presence of cathepsin B in skeletal muscle has been documented by direct cytochemical localization (Bird et al., 1978, 1980; Bird and Carter, 1980).

Cathepsins H and L were discovered by Kirschke and coworkers (Kirschke et al., 1977a, b). Cathepsin H was characterized as an aminopeptidase as well as an endopeptidase. It is a thiol enzyme with an isoelectric pH of 7.1. It splits proteins, amino acid derivatives and selected N-protected amino acid derivatives optimally at pH 6.0. It is strongly inhibited by leucyl-chloromethan and SH-blocking substrates. However,

leupeptin showed only a weak inhibitory effect compared to its action on cathepsins L and B (Kirschke et al., 1977b). Cathepsin L was characterized as a thiolproteinase with a molecular mass of 23 to 24 kd and an isoelectric pH of 5.8 to 6.1. Its optimum pH for digestion of proteins is close to 5.0. It does not hydrolyze esters, and it splits synthetic low molecular weight substrates only to a low degree. Leupeptin is a strong inhibitor to cathepsin L. Cathepsin L exists in multiple forms and was shown to be the most active endopeptidase in rat liver lysosomes (Kirschke et al., 1977a). Locnikar et al. (1981) reported that cathepsin H exists in two forms with isoelectric points (PI) of 7.1 and 7.3. The presence of cathepsin H in skeletal muscle has been demonstrated by Stauber and Ong (1982) using immunofluorescence. With the same technique, Stauber and Ong (1981) have also demonstrated the presence of cathepsin B in skeletal muscle. Cathepsin L has only recently been localized in rabbit skeletal muscle by immunohistochemical techniques (Taylor et al., 1987). Other evidence supporting the existence of these three proteinases inside skeletal muscle fibers comes from the findings of many investigators that the activities of cathepsins B, H and L were increased several fold in cultured myotubes as compared to pre-fusion myoblasts (Bird et al.,

1981; Kirschke et al., 1983) and in cultured leg muscle myoblasts derived from embryos of dystrophic chicken as compared to those from normal chicken (Sohar et al., 1985).

Evidence for the involvement of these lysosomal cysteine proteinases came from in vitro studies with purified enzymes and myofibrils or myofibrillar proteins. Cathepsin B has been shown to degrade myosin and actin at pH optima of 5.2 and 5.0, respectively (Schwartz and Bird, 1977). They also noted that soluble denatured myosin was degraded more extensively than insoluble native myosin. Bird et al. (1978) extended these studies and demonstrated the ability of cathepsin B to degrade myosin and actin contained in the structural configuration of myofilaments and myofibrils. Hirao et al. (1984) confirmed these results and suggested that skeletal muscle cathepsin B may participate in the degradation of muscle proteins in vivo. Of great significance to postmortem aging response and the changes that actually occur in myofibrillar proteins during aging are the findings of Noda et al. (1981a), who reported that cathepsin B degraded myosin heavy chain into fragments of 170, 160 and 145 kd and that troponin-T and troponin-I were degraded to fragments of 30, 18 and 14.8 kd. They (Noda et al., 1981a) also reported that cathepsin B degraded actin and tropomyosin very slowly, and it did not affect myosin light

chains or α-actinin. When glycerinated myofibrils were incubated with cathepsin B, Noda et al. (1981a) observed the disappearance of Z-disks in the early stages of incubation followed by disappearance of M-lines and a decrease in the intensity of A-bands after swelling of the myofibrils. Hirao et al. (1984) also reported that cathepsin B did not degrade α-actinin. These finding are consistent with the well documented changes occurring in myofibrils and in the banding pattern of myofibrillar proteins during postmortem aging. Recently, Ouali et al. (1987) reported that myofibrils incubated with cathepsin B showed ultrastructural modifications at the level of Z-disk, M-line and A-band and that on electrophoretograms cathepsin B affected the proteins with molecular weights below actin (43 kd). Cathepsin B was also shown to degrade collagen with a pH optimum of 4.5 to 5.0 (Burleigh et al., 1974). The pH optimum for degradation of native insoluble collagen by cathepsin B was found to be about pH 3.5 (Burleigh et al., 1974; Evans and Etherington, 1978).

Cathepsin H also has been reported to degrade myosin and to possess a 5-fold greater specific activity for myosin compared to cathepsin B (Bird and Carter, 1980). While cathepsin H did not degrade actin (Kirschke et al., 1981), Katunuma and Kominami (1983) have shown that it did degrade

troponin-T. However, comparison of the effects of cathepsin H with those of other lysosomal proteinases on myofibrils revealed that cathepsin H caused little myofibrillar protein degradation and that it did not affect the ultrastructure of myofibrils after 6 h of incubation (Ouali et al., 1987).

Cathepsin L is the most active lysosomal cysteine proteinase as it has greater than 10-fold higher specific activity for myofibrillar proteins than other mammalian cysteine proteinases (Kirschke et al., 1980). Bando et al. (1986) estimated the amount of cathepsin L in skeletal muscle to be about 1.94 $\pm$ .60 ug/mg tissue (39 $\pm$ 12 ng/mg protein). Cathepsin L was reported to degrade myosin heavy chain optimally at pH 4.1 with some degradation products visible at pH 3.6 and 5.0 (Okitani et al., 1981). Its activity against myosin has been shown to be ten times greater than that of cathepsin B and two times greater than cathepsin H (Bird and Carter, 1980). Cathepsin L also degrades actin (Kirschke et al., 1981); elastin (Mason et al.,1986) and collagen (Kirschke et al, 1981, 1982; Mason et al., 1984) extensively at pH 3.5, but more slowly at pH 6.0. Matsukura et al. (1981) studied the action of cathepsin L on myofibrillar proteins degradation in isolated form or assembled in myofibrils. They observed that cathepsin L degraded myosin heavy chain producing fragments of 160, 92, 83 and 60 kd. It also degraded myosin light chains. The degradation of myosin was found to be most severe at pH 4.2. They also reported that cathepsin L degraded actin into fragments of 40, 37 and 30 kd at pH 4.7. Cathepsin L also degraded troponin-T and troponin-I

into fragments of 30 and 13 kd at pH 3.7 to 6.7. The degradation of α-actinin was reported to occur at pH 3.0 to 3.5 producing a major fragment of 80 kd. Cathepsin L did not degrade tropomyosin or troponin-C (Matsukura et al., 1981). Moreover, cathepsin L degraded myosin heavy chain, α-actinin, actin, troponin-T and troponin-I assembled in myofibrils with the concomitant production of fragments primarily at 160 and 30 kd regions, at pH 5.0 (Matsukura et al., 1981). Later, these results were confirmed by the observation that cathepsin L caused fragmentation of myofibrils at pH 5.5 and 6.0, and at pH 5.5 and 37 C caused the disruption of the lateral arrangement of myofibrils, discontinuity of the N_2 -lines and loss of M-lines and Z-disks in glycerinated muscle fibers (Matsukura et al., 1984). Because these changes are similar to the changes observed in postmortem aged muscle, it was concluded that cathepsin L might be more responsible for these changes than CDPs since it has an optimum pH nearer to the ultimate muscle pH than the CDPs (Matsukura et al., 1984).

Substantial evidence exists for the role of each of these proteinase systems in the postmortem tenderization process. Early investigators have identified postmortem proteolysis with both enzyme systems (Yamamoto et al., 1979; Penny and Ferguson-Pryce, 1979; Ouali and Valin, 1981). Calkins and Seideman (1988) reported that while the initial (d 1) shear force was correlated to uM-CDP activity, the overall change in shear force (d 1 to d 14) was correlated to the activity of cathpsins B and H. They also stated that cathepsins B and H

accounted for 35 and 58%, respectively, of the variation in shear force between d 1 to d 14 and d 3 to d 6. Since 41.6% of the aging response occurred between d 3 and d 6, they suggested that uM-CDP helps to establish initial (d 1) tenderness; but that cathepsins B and H are responsible for the tenderization that occurs later during aging (Calkins and Seideman, 1988). However, Koohmaraie et al. (1988b) reported that the activities of cathepsins B, H and L are the same in muscles with different aging response while uM-CDP activity closely paralleled the aging response, being high in longissimus dorsi, intermediate in biceps femoris and low in psoas major muscles. Moreover, Koohmaraie et al. (1988a) observed that when muscle slices were incubated in the presence of the Ca^{2+}-chelators, EDTA or EGTA, the activities of cathepsins B, H and L remained unaffected while the activity of CDP's was inhibited concomitantly with lack of changes in MFI or SDS-PAGE patterns of myofibrillar proteins. From these data, Koohmaraie et al. (1988a, b) concluded that the changes observed during postmortem storage appeared to be associated with CDP's activity rather than catheptic enzymes.

In a study of protein degradation in isolated rat skeletal muscles, Rodemann et al. (1982) observed that the sulfhydryl inhibitor, mersalyl, completely inactivated CDP's without altering overall protein breakdown or the stimulation (45 to 140%) of protein breakdown induced by the Ca^{2+}-ionophore A-23187. They concluded that Ca^{2+} appeared to promote protein breakdown by stimulating synthesis of prostaglandin E_2 which

in turn activates the lysosomal apparatus (Rodemann et al., 1982). Conversely, Lowell et al. (1986) observed that agents which inhibit lysosomal proteinase activity (NH_4 Cl, chloroquine or leupeptin) failed to diminish the release of N^{τ} methylhistidine by perfused muscles of starved or fed rats despite a 25 to 35% inhibition of total protein breakdown. They suggested that the complete breakdown of myofibrillar proteins occurs via a nonlysosomal pathway. These reports add to the controversy as to which proteolytic system is involved in the degradation of myofibrillar proteins observed during postmortem aging.

Despite all the existing evidence for the role of proteolysis in the postmortem tenderization process, factors other than proteolysis have been implicated in isolated reports. Hattori and Takahashi (1979) reported that Ca^{2+} ions alone at a concentration of 10^{-4} M bind to some Z-disk constituents and induce weakening of the Z-disk structure which is enhanced by the continuous tension in muscle and associated rigor bonds. Using the release of α-actinin from Z-disks as a criterion, these investigators reported that the Ca^{2+}-induced weakening of Z-disks occurred without concomitant release of α-actinin and that this process was therefore, not due to the proteolytic action of CDP's. They inferred that the Ca^{2+}-induced weakening of Z-disks predominates over CDP proteolysis and that it is the major factor in the characteristic weakening of Z-disks (Hattori and Takahashi, 1982). Recently, this group concluded that the postmortem weakening of Z-disks is nonenzymatically

induced by the raised sarcoplasmic Ca^{2+} ion concentration of 10^{-4} M and that Ca^{2+} ions probably solublize the amorphous material of Z-disks, leaving unchanged the Z-filaments composed of α-actinin (Takahashi et al., 1987a).

Takahashi et al. (1982, 1985) isolated a new protein, paratropomyosin, extracted from myofibrils upon treatment with 10^{-4}M Ca^{2+}. This protein was found to facilitate the dissociation of rigor bonds. These investigators inferred that paratropomyosin released by Ca^{2+} ions played a major role in the characteristic weakening of rigor linkages. Very recently, Takahashi et al. (1987b) studied the effects of paratropomyosin and tropomyosin on the myofibrillar ATPase activities. They concluded that paratropomyosin is able to bind to thin filaments and its site of binding to F-actin is different from that for tropomyosin. Due to its greater affinity for the myosin binding site on actin, paratropomyosin competes for the binding site and helps weaken rigor linkages (Takahashi et al., 1987b). These two inferences of Takahashi and his group clearly suggest that the raised Ca^{2+} concentration in postmortem muscle (to 10^{-4}M) produces tenderization by two mechanisms unrelated to proteolysis, namely, the Ca^{2+} weakening of Z-disks and the release of paratropomyosin which weakens the rigor bonds.

Endogenous and Exogenous Inhibitors

The proteolytic activities discussed here seem to be under rigorous control in living cells. Present evidence suggests that specific endogenous inhibitors are synthesized in cells

and used as one of many ways to control their proteolytic enzymes. The most characterized inhibitor thus far is the CDP's-inhibitor, calpastatin, which was discovered soon after the purification of CDP's (Okitani et al., 1976; Waxman and Krebs, 1978; Otsuka and Goll, 1980). Divergent views have been expressed about its molecular weight, its inhibitory mechanism and concentration of Ca^{2+} needed for its binding to the enzymes (Goll et al., 1983b; Suzuki et al., 1987). However, it is known to co-exist with CDP's in skeletal muscle and to inhibit both CDP's, i.e., the uM and mM forms, respectively, CDP-I and CDP-II (Cottin et al., 1981; Szpacenko et al., 1981).

Endogenous inhibitors for cathepsins B, H and L are less well characterized. Chromatography of a concentrated muscle extract on Sephadex G-75 resolved two peaks that had inhibitory activity to cathepsin B corresponding to molecular masses of 12,500 and 62,000 daltons (Schwartz and Bird, 1977). These inhibitors were found in skeletal muscle of the rat, cow, rabbit, pig and human (Bird et al., 1978). They were later purified from muscle of many sources and found to be active against cathepsins B and H (Lenney et al., 1979; Brooks and Bird, 1981; Roisen et al., 1983). Despite the presence of many cysteine proteinase inhibitors, including α_2 -macroglobulin, cystatins, α-cysteine proteinase inhibitor (α-CPI) and liver cysteine proteinase inhibitors (CPI-A and CPI-B) in liver cells and plasma (for review see Barrett et al., 1984), there is a carcity of information on endogenous inhibitors for cathepsin L in skeletal muscle. Wood et al. (1985) isolated a low

molecular weight inhibitor (12400 to 14000 daltons) from chicken skeletal muscle and reported that 1 ng of this inhibitor caused 50% inhibition of cathepsin L activity and 13% inhibition of cathepsin H activity; however, 10 ng inhibited only 45% of cathepsin B activity. This inhibitor was similar and crossreacted with egg-white cystatins indicating that they are closely related (Wood et al., 1985).

Many exogenous inhibitors of cysteine proteinases have been reported. These include iodoacetate, iodoacetamide, N-ethylmaleimide and other alkylating agents (Bird and Carter, 1980) which are used to differentiate these enzymes from other classes of enzymes. Diazomethylketones also are found to be potent inhibitors of cysteine proteinases. These were first introduced by Leary et al. (1977) and Leary and Shaw (1977) and were found to be specific in their inhibitory activity towards cathepsin B and L (Kirschke and Shaw, 1981). Some tripeptidylchloromethyl ketones have been synthesized and reported to effectively inhibit the CDP's (Sasaki et al., 1986).

Many inhibitors have been isolated from cultures of some fungi genera (Streptomyces and Aspergillus) and found to be potent inhibitors of cysteine proteinases. L-trans-epoxysuccinyl-leucylamido (4-guanidino)butane commonly known as E-64 was isolated by Handa et al. (1978) and found to inhibit cathepsins B, H and L (Hasida et al., 1980; Noda et al., 1981; Barrett et al., 1982) and the CDP's (Sugita et al., 1980).

Leupeptins are potent proteinase inhibitors which have been

isolated from many species of Actinomycetes (Aoyagi et al., 1969a, b), characterized by Kondo et al. (1969) and their structure determined, and chemically synthesized by Kawamura et al. (1969). The potential of leupeptin as a therapeutic agent in muscular dystrophies has been investigated. Because of its inhibitory activity against papain, the most characterized cysteine proteinase, its low molecular weight, nontoxicity and nonimmunogenicity. Libby and Goldberg (1978) measured leupeptin's effect on protein degradation in rat skeletal and cardiac muscle in vitro. They reported that leupeptin decreased protein degradation in these tissues, and in muscle from denervated and dystrophic rats. They also reported that homogenates of leupeptin-treated muscles had decreased cathepsin B activity. Libby et al. (1979) reported that leupeptin (30 uM) diminished net proteolysis in cultured fetal mouse hearts by 50% and retarded cardiac atrophy. Besides, they observed that leupeptin did not alter microscopic appearance, pattern of contraction, rates of protein synthesis, protein leakage or concentrations of ATP, lactate dehydrogenase and creatine kinase. Hence, they concluded that leupeptin appears to be useful for studies of protein turnover, in maintaining tissues in culture and possibly in the therapy of certain diseases. Many reports showed that in vivo injection of leupeptin in dystrophic chickens and mice prevented or delayed the degeneration of muscle tissue which is characteristic of this disorder (Stracher et al., 1978a, b; Sher et al., 1981). This inhibition of muscle degeneration has been

attributed to leupeptin inhibition of cathepsin B and the CDP's (Stracher et al., 1979; Sher et al., 1981). In vitro studies by many investigators have demonstrated the inhibitory effect of leupeptin on cysteine proteinases. Toyo-Oka et al. (1978) reported that leupeptin was effective in inhibition of purified CDP at equimolar concentration, and that the mode of inhibition was noncompetitive. Libby and Goldberg (1980) demonstrated that leupeptin (2 uM) inhibited most of the activity of CDP's prepared by isoelectric precipitation at pH 4.9. Leupeptin also was found to inhibit cathepsin B and L effectively at micromolar concentrations (Kirschke et al., 1980; Schwartz and Barrett, 1980; Libby and Goldberg, 1980). Cathepsin H, however, was not affected by 1 uM leupeptin and even 21 uM gave only 29% inhibition of purified cathepsin H (Schwartz and Barrett, 1980). Kirschke et al. (1980) reported that leupeptin (30 uM) gave only 50% inhibition of cathepsin H activity. Injection of leupeptin also caused inhibition of cathepsin B in muscle with the activity returning to normal after 4 h (Sutherland and Greenbaum, 1983). Information on the effect of in vivo administration of leupeptin on the activities of cathepsins L, H and the CDP's is scarce. In one report (Sher et al., 1981), it was claimed that intraperitoneal injection of leupeptin into dystrophic mice led to abolishment (approximately 90%) of CDP activity within 24 to 48 h and a return to original activity after 5 to 6 d. Leupeptin and possibly other proteinase inhibitors reviewed in this section, therefore, offer potential for studying the relationship of

proteolysis to postmortem tenderization in meat because of their selective inhibition of enzymes.

MATERIALS AND METHODS

Experiment I Effects of Electrical Stimulation and Storage Temperature on Muscle Temperature and pH.

Twenty male New Zealand white rabbits weighing about 3 kg were slaughtered in groups of four animals. After exsanguination, they were assigned to the following treatments: 1) Non-stimulated, high temperature (22 C) conditioned (NS,HT); 2) Non-stimulated, low temperature (2 C) conditioned (NS,LT); 3) Electrically stimulated, high temperature (22 C) conditioned (ES,HT) and 4) Electrically stimulated, low temperature (2 C) conditioned (ES,LT). Five animals received each treatment. Electrical stimulation was performed soon after bleeding according to procedures described by Chrystall and Devine (1983) for rats. Stainless steel needles were inserted into the right shoulder and left hind foot to avoid skin resistance (Devine and Chrystall, 1984). Stimulation was provided by a Grass S5 stimulator (Grass Medical Instruments, Quincy, MA) delivering square waves with 14.3 pulses/s and durations of 5 ms at 80 v for 3 min. Low voltage electrical stimulation was chosen to avoid muscle cell disruption which is believed to be the primary mechanism of tenderizing caused by high voltage electrical stimulation. Immediately after stimulation, each rabbit was skinned, the head removed and then eviscerated. The carcass was hung from its hind legs after tying them together

and assigned to its respective temperature treatment of either 2 or 22 C.

Longissimus muscle temperature and pH were monitored every 15 min until the temperature of the muscle held at 2 C reached 2 C. Temperature was determined by inserting a thermometer directly into the caudal end of the longissimus muscle (about 1 cm depth). Muscle pH was determined on samples of the longissimus muscle by homogenizing 500 mg of the samples in 2.5 ml of 5 mM sodium iodoacetate, .15 M KCl (Chrystall and Devine, 1983) and reading the pH of the slurry with a Radiometer PHM82 standard pH-meter. The samples were taken alternately from the left and right longissimus muscles, about 1 cm apart, starting from the caudal end of the muscles and progressing cranially. After the muscle temperature of the carcasses at 2 C reached 2 C, carcasses in the HT treatment were moved to the cold room (2 C). Samples for pH measurement were then taken every 4 h up to 16 h postmortem and then at 24 h and 48 h postmortem.

Experiment II Effect of Leupeptin Injection on the Activity of Ca^{2+}-Dependent Proteinases (CDP's) and Cathepsins B, H and L.

Sixteen rabbits averaging about 3 kg body weight were injected intraperitoneally with either 0, 20, 100, or 200 mg of leupeptin (Peptides International, Louisville, KY), in .9%

saline per kg of body weight to determine the most effective dose for inhibiting CDP's, cathepsin B and L. Intraperitoneal injection was chosen because intravenous injection of leupeptin at 100 mg/kg body weight was found to kill the animals possibly due to respiratory paralysis (Tanaka, 1983). One h after injection (Sutherland and Greenbaum, 1983), the animals were sacrificed and dressed as described earlier except that they did not receive ES or conditioning temperature treatments. Immediately after dressing the longissimus muscle was removed, put on ice and taken to a cold room 4 C. The muscles were trimmed of fat and connective tissue, ground with a Kitchen Aid food grinder equipped with a 3 mm plate, and samples were taken to determine the activities of CDP's, cathepsins B, H and L. Crude muscle homogenates were used for the assay of catheptic enzymes and a crude preparation of CDPs was used rather than purified enzymes. This approach was chosen in order to mimic insitu carcass conditions as closely as possible.

Calcium-Dependent Proteinases (CDP's) (EC 3.4.22.17).

Crude CDP's were prepared as described by Koohmaraie et al. (1984c). One hundred g of muscle were suspended in 2.5 volumes (v/w) of .17 M Tris-HCL, 4 mM ethylenediamine-tetraacetic acid (EDTA), 5 mM 2-mercaptoethanol (MCE), pH 7.9.The suspension was then homogenized in a Waring Blendor using three bursts of 30 s duration with a 90 s cooling period between bursts. The

homogenate was centrifuged at 14,000 x g for 2 to 3 h in a Sorvall superspeed RC2-B automatic refrigerated centrifuge at 4 C. The pH of the resulting supernatant was then adjusted to 4.50, isoelectric point of rabbit CDP (Imahori, 1982), by dropwise addition of 1 N acetic acid. After 20 min on ice, samples were centrifuged at 14,000 x g for 20 min and the supernatant discarded. The resulting pellet was suspended in 20 ml of 50 mM Tris-HCL, 1 mM EDTA, 5 mM MCE, pH 7.6 using a glass homogenizer. The pH was then adjusted to 7.0 to 7.30 and the samples clarified at 148,000 x g and 2 C for 60 min in Beckman model L5-75 ultracentrifuge. The resulting supernatant was designated crude CDP. Protein of the crude CDP preparation was quantitated by the Biuret method (Gornall et al., 1949).

Crude CDP activity was assayed according to the method of Dayton et al. (1976a). Aliquots of crude CDP were mixed with 1.5 ml of 5 mg/ml Casein-Hammersten (U.S. Biochemical Corporation, Cleveland, OH) in 100 mM Tris-acetate, 100 mM KCl, 10 mM MCE, . 5mM $CaCl_2$, 1 mM NaN_3 , pH 7.6 (Tsuji and Imahori, 1981), at CDP to casein ratio of 1:20. Total reaction volume was brought to 2 ml with glass-distilled deionized water. Control tubes for enzyme contained the above assay medium except that $CaCl_2$ was replaced with 10 mM EDTA and control tubes for the substrate contained no enzyme. After 60 min at 25 C, the reaction was stopped by addition of 2 ml of

cold 5% trichloroacetic acid (TCA). The assay tubes were then centrifuged at 1,000 x g in a Sorval general purpose RC-3 automatic refrigerated centrifuge for 60 min. Absorbance of the clear supernatant was measured at 278 nm, and CDP activity expressed as total absorbance units /100 g muscle.

Catheptic Enzymes Activity. Crude muscle homogenates were prepared by the procedure of Moeller et al. (1976) as modified by Moeller et al. (1977). Five g of ground muscle were suspended in 50 ml of .25 M sucrose containing .02 M KCl and homogenized using a Brinkman polytron at a setting of 7, with 3 bursts of 20 s each and 30 s cooling time between bursts. The homogenate was filtered through two layers of cheesecloth and the pH adjusted to 7.30 with .1 M KOH. The adjusted filtrate was centrifuged at 105,000 x g in a Beckman L5-75 ultracentrifuge at 2 C for 2 h, to obtain an unsedimentable fraction (supernatant) and a sedimentable fraction (pellet). The sedimentable fraction was resuspended and homogenized in .25 M sucrose, .02 M KCl and .1% Triton X-100. Protein concentration of the two fractions was determined by Biuret method, and aliquots were diluted with a .1% solution of Brij-35 to contain 1 mg protein/ml. These aliquots were used for the assay of the activities of cathepsins B, H and L.

Cathepsins B, H and L activities were determined fluorometrically utilizing methylcoumarylamide substrates (Barrett,

1980) as described by Kirschke et al. (1983).

Cathepsin B (EC 3.4.22.1). A .25 ml aliquot of the diluted fractions was mixed with .25 ml of .1% Brij-35 and .25 ml of 352 mM KH_2PO_4 /48 mM Na_2HPO_4 , 4 mM EDTA and 10 mM MCE, at pH 6.0. Control tubes contained .1% Brij-35 instead of the enzyme. The tubes were prewarmed in a 40 C waterbath for 5 min. The reaction was started by adding .25 ml of 20 uM N-α-Benzyloxy-carbonyl-L-Arg-L-Arg-7-Amino-4- Methylcoumarin.2HCL (Z-Arg-Arg-NMec, Bachem Fine Chemicals, Torrance, CA).

Cathepsin H (E 3.4.22.16). Again .25 ml of diluted fractions were mixed with .25 ml of .1% Brij-35 and .25 ml of 200 mM KH_2PO_4 /200 mM Na_2 HPO_4 , 4 mM EDTA and 10 mM MCE, at pH 6.8. After 5 min at 40 C, the reaction was started by adding .25 ml of 20 uM Arg-7-Amino-4-Methylcoumarin (Arg-NMec, Enzyme Systems Products, Livermore, CA). Control tubes containing no enzyme were included in each assay.

Cathepsin L (EC 3.4.22.15). A .25 ml aliquot was mixed with .25 ml of .1% Brij-35 and .25 ml of 340 mM Na acetate/60 mM acetic acid, 4 mM EDTA, 10 mM MCE at pH 5.5. Since the substrate is not specific for cathepsin L, control tubes were prepared by mixing .25 ml aliquot with .25 ml of .1% Brij-35 containing 4 uM N-Benzyloxycarbonyl-L-phe-Diazomethane (Z-phe-phe-CHN_2 , Enzyme Systems Products, Livermore, CA) and .25 ml assay buffer. Z-phe-phe-CHN_2 is a potent inhibitor of cathepsin

L (Kirschke et al., 1983). Tubes were incubated at room temperature for 30 min. After a 5 min preincubation at 40 C, the reaction was started by adding .25 ml of 20 uM N-α-Benzyloxycarbonyl-L-phenylalanyl- L-Arginine-7-Amido-4-Methylcoumarin.HCL (Z-phe-Arg-NMec, Bachem Fine Chemicals, Torrance, CA).

For all three enzymes, after addition of substrate, the contents were mixed using a Vortex and the tubes were incubated for 10 min at 40 C. Reactions were stopped by the addition of 1 ml of cold 100 mM Na chloroacetate (Eastman Kodak Company, Rochester, NY) in a buffer containing 30 mM Na acetate, 70 mM acetic acid, at pH 4.3. Fluorescence was then measured in a Varian-330 spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The instrument was zeroed against the reaction stop buffer and set to read 1000 arbitrary units with .5 uM 7-amino-4-methylcoumarin (Sigma Chemical Company, St. Louis, MO) in the buffered Na chloroacetate solution (Barrett, 1980). Thus, for the 10 min reaction time, a reading of 1000 corresponds to 100 u Units of activity in the tube (1 unit is the release of 1 mmol of product/min). All the methylcoumarin substrates and Z-phe-pheCHN$_2$ were stored as 10 mM solutions in dimethylsulfoxide (Sigma) at 4 C.

Experiment III Effects of ES and High Temperature Conditioning on Proteolysis and Aging Response of Rabbit Longissimus Muscle.

From experiment I, the length of time for the rabbit carcasses held at 2 C to reach 2 C in the longissimus muscle was found to be 4 h. The most effective concentration of leupeptin to inhibit cathepsins B and L was found to be 100 mg/kg body weight (experiment II). These data were utilized in experiment III. Thirty-two rabbits (averaging about 3 kg live weight) were allotted to a symmetrical two-level factorial design in incomplete blocks. The factorial design includes: 1) electrically stimulated vs nonstimulated carcasses (ES vs NS); 2) carcass storage temperature of 2 (LT) or 22 C (HT); and 3) with (L) or without (NL) leupeptin injection. The incomplete blocks were constructed according to the procedures outlined by Gill (1978) as shown in Table 1. Each of the four interactions was used for confounding in one replicate and hence, full information on the main effects and 3/4 information on the interactions was obtained.

TABLE 1. CONSTRUCTION OF INCOMPLETE BLOCKS

Replicate	1	2	3	4
Defining contrast	AB	AC	BC	ABC
Block 0	(1),c,ab,abc;	(1),b,ac,abc;	(1),a,bc,abc;	(1),ab,ac,bc
Block 1	a,b,ac,bc;	a,c,ab,bc;	b,c,ab,ac;	a,b,c,abc

(1) = NS, LT, NL (control)
a = ES, LT, NL (main effect of ES)
b = NS, HT, NL (main effect of temperature)
c = NS, LT, L (main effect of leupeptin)

ab = ES, HT, NS (interaction between ES and temperature)
ac = ES, LT, L (interaction between ES and leupeptin)
bc = NS, HT, L (interaction between temperature and leupeptin)
abc = ES, HT, L (3 factor interaction)

Leupeptin injection, slaughtering and the assignment of carcasses to their respective ES and temperature treatments were done as described for experiments I and II. After application of ES, samples were taken from the longissimus muscle for measuring myofibrillar fragmentation index (MFI), cathepsins B, H and L and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Thus, about 15 g of muscle were removed from the caudal end of the longissimus muscle soon after ES, taken to a 4 C cold room and trimmed of fat and connective tissue. Duplicate samples of 4 g were used for the assessment of MFI and cathepsins B, H and L activity. The rest of the muscle was frozen in liquid nitrogen and stored at -90 C until used to isolate myofibrils for SDS-PAGE. At 4 h postmortem, another 4 g of muscle was removed for the measurement of lysosomal ß-glucuronidase and of cathepsins B, H and L to assess the effect of ES on the subcellular distribution of these enzymes. Immediately following this sample, the rabbit carcasses of the high temperature conditioning treatment (22 C) were moved to the cold room (2 C). Samples for assessment of MFI and SDS-PAGE also were taken at 24, 72 and 168 h postmortem. At each sampling time approximately 12 to 15 g

were removed alternately from the right and left longissimus muscle progressing cranially about 1 cm apart. Samples for MFI were processed immediately, and samples for SDS-PAGE were frozen in liquid nitrogen and stored at -90 C until used.

Myofibrillar Fragmentation Index (MFI). Proteolytic effects on myofibrils due to treatment were assessed by determination of MFI according to the procedure of Olson et al. (1976) as modified by Culler et al. (1978), except that ethylene glycol-bis-(ß-aminoethyl ether) N, N, N', N'-tetra acetic acid (EGTA, Sigma Chemical Company, St. Louis, MO) was used in the isolating medium instead of EDTA. Also for the 0 h samples, sedimentation was performed at 2,500 x g for 10 min (Sonaiya et al., 1982) instead of 1,000 x g for 15 min. Longissimus muscle samples for MFI determination were taken at 0 h, 24 h, 72 h and 168 h postmortem. At each time, duplicate samples of 4 g of muscle were scissor-minced and suspended in 10 vol (v/w) of a 2 C isolating medium containing 100 mM KCl, 1 mM EGTA, 1 mM $MgCL_2$, 1 mM NaN_3 and 20 mM K phosphate, at pH 7.0. Samples were then homogenized for 30 s in an Eberbach blender, the homogenate sedimented at 1,000 x g for 10 min and the supernatant decanted. The sediment was resuspended in 10 vol of isolating medium using a glass rod and sedimented again at 1,000 x g for 15 min. The supernatant was decanted, mixed with the first supernatant and saved for assessment of

lysosomal enzymes activities. The sediment was resuspended in 2.5 vol of isolating medium and passed through a polyethylene strainer (Tupperware) to remove connective tissue and debris. Additional 2.5 vol of isolating medium were used to facilitate passage of myofibrils through the strainer. Protein concentration of the suspension of myofibrils was determined by the Biuret procedure of Gornall et al. (1949). An aliquot of the myofibril suspension was diluted with isolating medium to a protein concentration of .5 mg/ml and a total volume of 8 ml in 13 x 100 mm borosilicate disposable culture tubes. The tubes were stirred vigorously (vortexed) and absorbance of the diluted myofibril suspension read immediately at 540 nm in a Spectronic 20 (Milton Roy Company, Rochester, NY). Absorbance of duplicate tubes was averaged and multiplied by 200 and recorded as MFI value.

Lysosomal Enzymes Activities. Preparation of the crude muscle homogenates was slightly different from that described for experiment II. The two supernatants resulting from the centrifugation steps in the MFI procedure were saved. The combined supernatant was centrifuged at 105,000 x g for 2 h at 2 C to yield an unsedimentable fraction (supernatant) and a microsomal fraction (pellet). The microsomal fraction was homogenized in 10 ml of MFI isolating medium containing .1% Triton X-100 to disrupt lysosomal membranes. The myofibril

suspension, after MFI determination, was used as the nuclear fraction. At 4 h postmortem, 4 g of muscle were removed, minced with a scissors and homogenized as for the MFI procedure. The resulting supernatants were pooled and centrifuged as above. The myofibrillar suspension was used as the nuclear fraction. Protein concentration in all fractions was determined by Biuret procedure (Gornall et al., 1949), and aliquots diluted with .1% Brij-35 to a protein concentration of 1 mg/ml as described earlier. These diluted fractions were used for the assessment of the activity of ß-glucuronidase (4 h postmortem sample only), cathepsins B, H and L (at 0 and 4 h).

Cathepsins B, H and L Activity. The activity of cathepsins B, H and L was assayed as described in the methods of experiment II.

ß-glucuronidase Activity (EC 3.2.1.31). Activity of ß-glucuronidase was assessed by the fluorometric procedure described by Moeller et al. (1976) as modified by Wu et al. (1985). Assay tubes contained: .25 ml of diluted fractions, .25 ml of .1% Brij-35 and .25 ml of buffer containing 300 mM sodium citrate, 4 mM EDTA, at pH 5.0. Control tubes containing no enzyme were run in each assay. Tubes were preincubated at 40 C for 6 min. The assay was started by adding .25 ml of 200 uM 4-methylumbelliferyl-ß-D-glucuronide

(Sigma Chemical Company, St. Louis, MO). After 30 min at 40 C, the reaction was stopped by adding 1 ml of 500 mM $Na_2 CO_3$, at pH 10.4 and placing the tubes on ice. Fluorescence was measured in a Varian SF-330 spectrofluorometer at excitation and emission wavelengths of 360 and 448 nm, respectively. Percent released activity was calculated from the fluorescence units of the unsedimentable fraction and the total fluorescence units (sum of unsedimentable, microsomal and nuclear fractions).

SDS-PAGE. Frozen muscle was thawed at 4 C for 2 h, and 4g were minced with a scissors and used for isolation of myofibrils according to the procedure of Goll et al. (1974). Samples were suspended in 10 vol of a standard salt solution (100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3 and 20 mM K phosphate, pH 6.8), homogenized for 10 s with a Brinkman polytron and centrifuged at 1,000 x g for 10 min (2,500 x g for 0 h samples). The sediment was washed again with 6 vol of standard salt solution and centrifuged as above. The sediment was washed twice in 8 vol of standard salt solution, homogenized and passed through a household polystyrene strainer and centrifuged as above. The sediment was again washed twice in 6 vol of standard salt solution containing 1% (v/w) Triton X-100, homogenized as described above and centrifugated at 1,500 x g for 10 min. The resulting sediment

was washed once in 8 vol of standard salt solution and 4 times in 8 vol of 100 mM KCl, 1 mM NaN_3 by stirring vigorously with a glass rod, and centrifuged each time at 1,500 x g for 10 min. The resulting sediment was homogenized for 5 s twice in 8 vol of the 100 mM KCl/1 mM NaN_3 solution, and centrifuging each time at 1,500 x g for 10 min. This final sediment was homogenized in about 2 vol of the 100 mM KCl/ 1 mM NaN_3 solution and the protein concentration of the myofibril suspension determined by the Biuret procedure of Gornall et al. (1949). One-half ml of each sample was dissolved in 2 ml of sample buffer (62.5% Tris-HCL, pH 6.8, 2% SDS, 5% MCE, 10% glycerol and .02% bromophenol blue). The samples were then heated in a boiling waterbath for 5 min and frozen and stored at -30 C until used for electrophoresis. About 30 to 40 ug of protein were added to each lane of the gel.

Electrophoresis was conducted on slab gels according to the procedure of Laemmli (1970) with some modifications. Gels containing 4% (stacking gel) and 12.5% (separating gel) acrylamide were prepared from stock solution of 30% acrylamide having a 37.5 to 1.0 ratio of acrylamide: N, N'-bis-methylene acrylamide. Final concentrations in the separating gel were: .375 M Tris-HCL (pH 8.8) and .1% SDS. The gels were polymerized chemically by addition of .05% by volume of tetramethylethylenediamine (TEMED) and ammonium persulfate.

Eleven to 12 cm gels were prepared in glass rectangular plates (15 cm total length) with an inside diameter of 1.5 mm. The stacking gels of 4% acrylamide and a length of 3 to 4 cm contained .125 M Tris-HCL (pH 6.8) and .1% SDS and were polymerized chemically as described above. The electrode buffer (pH 8.3) contained .025 M Tris, .192 glycine and .1% SDS. Electrophoresis was performed with a current of 16mA per gel until samples had focused, then at 24 mA per gel until bromophenol blue reached to within .5 cm of the bottom of the gel. Proteins were then fixed with 25% TCA for 20 to 30 min and stained with a .1% Coomassie brilliant blue R250 in 50% methanol, 7% acetic acid, overnight. The gels were then destained by repeated washing in 20% methanol, 7% acetic acid to a clear background.

Reagents used for electrophoresis were from Bio-rad (Rockville Center, NY), except for Tris/glycine used in electrode buffer and MCE used in sample buffer which were from Sigma Chemical Company (St. Louis, MO). TCA, glycerol, acetic acid and methanol were obtained from Mallinkrodt, Inc. (Paris, KY).

Assessment of CDP's Activity. Due to the problems encountered in the isoelectric precipitation procedure used in experiment II and due to longissimus muscle sample limitation from the rabbits used in experiment III, CDP's activity and

the effect of leupeptin injection on it were assessed on 12 additional rabbits (6 control and 6 injected with 100 mg/kg body weight of leupeptin). Rabbits were injected, euthanized and dressed as previously described. The longissimus muscle was removed, trimmed of visible fat and connective tissue, frozen in liquid nitrogen and stored at -90 C until used for extraction of CDP's. Chromatographic separation of CDP's and their endogenous inhibitor on DEAE-Sephacel (Pharmacia, Upsala, Sweden) was performed as described by Dayton et al. (1976a) with some modifications. Muscle samples were thawed at 4 C for 90 min and 50 g samples were taken, chopped into small pieces and suspended in 2.5 volumes (v/w) of 100 mM Tris-HCL, 4 mM EGTA, 10 mM MCE at pH 8.30. Homogenization was done in a Waring Blendor (3 bursts of 30 s each and 90 s between bursts). Homogenates were then centrifuged at 25,000 x g for 4 to 5 h. The resulting supernatant was passed through two-layers of cheesecloth, pH adjusted to 7.50 and left at 4 C overnight. This was necessary to avoid a dialysis step and still have clear supernates which would not clog the resin. The adjusted supernatants were again centrifuged at 25,000 x g for 2 to 3 h, passed through wetted glass wool and loaded onto columns (2.5 x 40 cm, Pharmacia) that had been equilibrated with elution buffer (20 mM Tris-HCL, .1 mM EDTA, 10 mM MCE at pH 7.35). When the sample front reached the

bottom of the column (judged by the red myoglobin color), all the material that did not adsorb to the resin was collected. After all supernatant was loaded, columns were washed with elution buffer and the eluent was collected until the last red drops of the samples. This collection of unadsorbed material from the samples was undertaken because preliminary work showed that leupeptin passed through the resin and eluted with the unadsorbed material. After all the unadsorbed material was collected, collection was stopped and the columns washed with elution buffer until the absorbance (278 nm) of the overflow was less than .4. The CDP endogenous inhibitor was eluted with .145 M NaCl in elution buffer. CDP's were eluted collectively with .5 M NaCl in elution buffer. Thirty fractions were collected for each step using a fraction collector (LKB, Bromma, Sweden) and the flow rate was 30 ml/h. The CDP's inhibitor was discarded. Fractions collected with .5 M NaCl in elution buffer were screened for activity of the CDP's using a $CaCl_2$ assay medium and an EDTA assay medium for control tubes (same as mentioned for experiment II, except that 100 mM KCl was deleted). The reaction mixture contained .25 ml fraction, 1.5 ml $CaCl_2$ or EDTA medium and .25 ml H_2O. All reaction conditions and reading of absorbance were as described for experiment II. The fractions containing the CDP's activity were pooled and the volume measured. The

pooled CDP's were mixed with the unadsorbed material collected off columns, and the activity of CDP's was measured on .5 ml of this mixture as described. The calcium-dependent caseinolytic activity was calculated and expressed as total OD units /50 g muscle. The percentage inhibition of CDP's activity due to leupeptin injection was calculated.

Statistical Procedures. Data for experiments I and II were analyzed using a randomized complete block design with four blocks (blocking factor was time as it was possible to handle only four animals at a time) and four treatments per block.

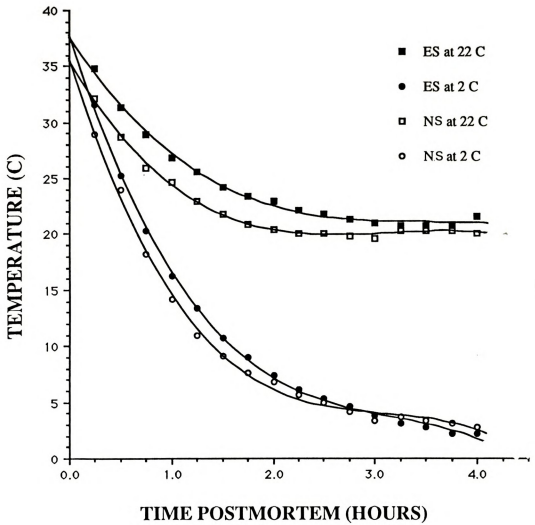
Data for experiment III were analyzed using a symmetrical 2-level factorial design in incomplete blocks. Four replicates were used, and each interaction term was confounded in one replicate. Thus, the eight treatment combinations of the 2^3 factorial were done in two blocks for each replicate (Gill, 1978).

RESULTS AND DISCUSSION

Experiment I

The decline in muscle temperature postmortem for non-stimulated (NS) and electrically stimulated (ES) rabbit carcasses, aged at 2 C or 22 C is shown in Figure 1. It is evident that up to at least 3 h postmortem the curves for ES animals are higher (higher temperature) compared to those of their NS counterparts. For the low temperature (LT) aged carcasses from both NS and ES treatments the rate of temperature fall per 15 min (dT/dt) is 1.74 C and 1.96 C, respectively. Most of the change in (dT/dt) occurred in the first 2 h postmortem being 3.16 C for NS carcasses and 3.46 C for ES carcasses. After 2 h the rate of temperature fall was greatly diminished being .5 C for NS and .65 C for ES carcasses per 15 min. For the high temperature (HT) aged carcasses the overall rate of temperature fall per 15 min (dT/dt) was .81 C and .89 C for NS and ES carcasses, respectively. Again, most of the change in dT/dt occurred in the first 2 h postmortem being 1.7 C for both treatments and decreased to .04 C and .18 C for NS and ES carcasses, respectively. These results are in agreement with results of many investigators who have shown similar trends. Wu et al. (1985) reported that, for both bull and steer carcass sides, muscle temperature measured at 2 h and 6 h postmortem was

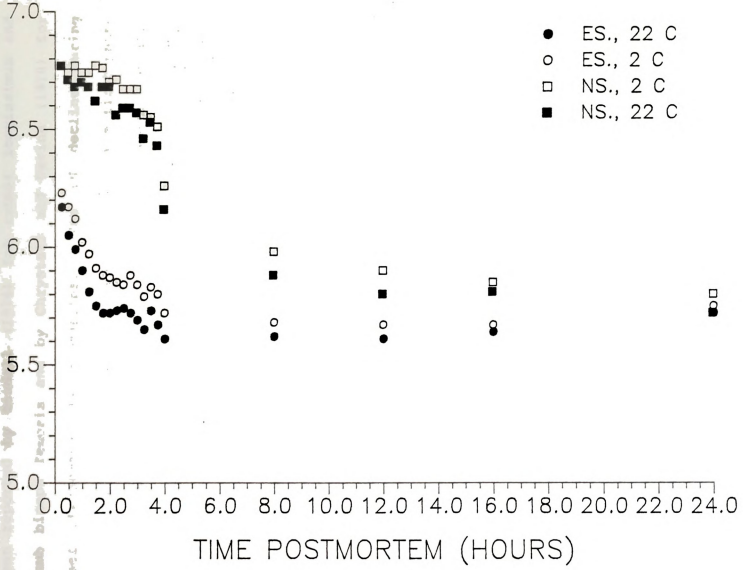
FIGURE 1. POSTMORTEM TEMPERATURE DECLINE FOR ELECTRICALLY STIMULATED (ES) AND NONSTIMULTED (NS) LONGISSIMUS MUSCLE OF RABBIT CARCASSES HELD AT 2 C OR 22 C


ES at 22 C
ES at 2 C
NS at 22 C
NS at 2 C
TEMPERATURE (C)
40
35
30
25
20
15
10
5
0
0.0
1.0
2.0
3.0
4.0
TIME POSTMORTEM (HOURS)

about 4 C higher in ES sides as compared to NS sides. Elgasim et al. (1981) reported similar curves for ES and NS beef sides aged at 2 C or 16 C. It is clear from the temperature curves reported by these investigators that the curves for ES sides were higher than those for NS sides. Elgasim et al. (1981) also reported that at 2 C the ES sides cooled faster than NS sides in the first h postmortem. It is obvious from Figure 1 that in the first 15 min postmortem the temperature of the ES/2 C carcasses dropped by 6.3 C, whereas that of NS/2 C carcasses dropped by 4.9 C. These results agree well with those of Elgasim et al. (1981). A Student t-test (Gill, 1978) on dT/dt for paired samples (NS vs ES) showed no significant difference ($P < .05$) in the mean decline of muscle temperature at 2 C and at 22 C.

Postmortem decline in pH for NS and ES carcasses aged at 2 C or 22 C is presented in Figure 2. The ES carcasses have a significantly lower initial (15 min) pH than NS carcasses ($P < .001$). This result is consistent with results of many other investigators (deFremery and Pool, 1960; McLoughlin, 1970; Bendall et al., 1976; Elgasim et al., 1981; Taylor and Cornell, 1985). The initial pH decline during stimulation calculated as the difference in pH between NS and ES carcasses is found to be .52 pH units (range .41 to .70) for the carcasses aged at 2 C, and .60 pH units (range .40 to .77)

FIGURE 2. EFFECT OF ELECTRICAL STIMULATION AND CARCASS CONDITIONING TEMPERATURE ON THE RATE OF pH FALL OF RABBIT LONGISSIMUS MUSCLE


7.0
6.5
6.0
5.5
5.0
PH
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0 22.0 24.0
TIME POSTMORTEM (HOURS)
ES., 22 C
ES., 2 C
NS., 2 C
NS., 22 C

for the carcasses conditioned at 22C. Similar results have been reported by Bendall (1976) for rabbit longissimus and lamb biceps femoris and by Chrystall and Devine (1978) for beef sternomandibularis muscles. The pH decline during stimulation is termed delta pH and represents the first phase in hastening the process of rigor development (Bendall et al., 1976; Chrystll and Devine, 1978). The second phase, the subsequent rate of pH fall per unit time (dpH/dt = 15 min) was -.034 for both NS and ES at 2 C and -.041, -.037 for NS and ES at 22 C, respectively, and was not significantly different among treatments ($P < .05$). These values are lower than those reported for beef muscles (Bendall et al., 1976; Chrystall and Devine, 1978) and rabbit longissimus muscle (Horgan and Kuypers, 1985). This may be due to the variability between the rabbits in this study. However, Bendall et al. (1976) and Horgan and Kuypers (1985) reported that (dpH/dt) was not significantly different between NS and ES animals. Such results are in agreement with the results of this experiment. Again, at 4 h postmortem of longissimus muscles, ES animals had lower ($P < .01$) mean pH values than those of NS animals. The significantly lower pH for ES animals (summed over temperature) as compared to NS animals at .25 and 4 h postmortem agrees with results of other investigators (Fabiannson and Reutersward, 1985; Tayler and Cornell, 1985)

who reported significant differences in pH for ES and NS beef animals up to 4 to 6 h postmortem. However, the effect of the temperature alone on pH was not significant at .25 or4 h postmortem. For the NS carcasses the time course forthe achievement of a pH value of 5.80 was 12 h for the HT carcasses and 24 h for the LT carcasses. This is in close agreement with the results of other researchers (Marsh, 1954; Cassens and Newbold, 1967). It is also quite apparent from Figure 2 that for most of the time periods pH of the LT carcasses was nearly .1 pH units higher than that of the HT carcasses for the NS treatment and exceeded .1 pH units for the ES treatment.

It is evident that muscle pH dropped only slightly below pH 5.80. Hence, pH 5.80 was taken as the ultimate pH in this study. Thus, for the ES treatment, pH of the HT carcasses dropped to 5.81 in 1.25 h, whereas that of the LT carcasses dropped to a similar value in 2.5 to 3.0 h. This, when compared to 12 h and 24 h needed for the NS/HT and NS/LT carcasses, respectively, to attain a similar pH value, reconfirms the importance of electrical stimulation in hastening rigor and allowing carcasses to be chilled sooner without the complicacy of cold-shortening.

Experiment II

The effect of in vivo injection of leupeptin on the activities of cathepsins B, L, H and CDP is summarized in Table 2. Mean activities of these proteinases from 4 replicates are shown, and the standard deviation of each mean is shown in brackets. It is apparent from the standard deviations that considerable variation exists in the procedures used in this study. These results differ from the results of other investigators due either to differences in muscle extraction procedures or in expression of results. Kirschke et al. (1983) extracted rat muscle in a 100 mM acetate buffer at pH 5.5 containing .2% Triton X-100. They reported that the specific activities of cathepsins B, L and H were 46, 6 and 99 u Units/mg protein, respectively. Wu et al. (1985) expressed the activities of cathepsins B and H in units /g of beef muscle. Percent inhibition of the activities of cathepsins B, H, L and CDPs is presented in Table 3. It is obvious that percent inhibition of the activities of cathepsins B, L and CDPs increased with increase in the amount of leupeptin injected. However,the difference in percent inhibition between the 100 mg/kg and the 200 mg/kg levels is small. This fact, together with the high cost of leupeptin warranted the use of the 100 mg/kg level. As

TABLE 2 - EFFECT OF LEUPEPTIN INJECITON ON THE ACTIVITIES OF CDP AND CATHEPSINS B, L AND H IN RABBIT LONGISSIMUS MUSCLE[a]

Enzyme	Fraction[b]	Leupeptin Level (mg/kg) 0	20	100	200
Cathepsin B[c]	US	25.9(6.1)	18.9(5.8)	6.4(2.2)	4.0(.8)
	S	20.5(5.5)	13.4(4.1)	10.0(1.8)	8.5(1.6)
Cathepsin L[c]	US	19.5(2.5)	11.0(3.0)	4.6(1.4)	2.1(.9)
	S	17.3(4.1)	11.4(1.3)	8.8(1.7)	5.3(1.9)
Cathepsin H[c]	US	219.1(45.3)	212.3(49.0)	211.0(44.7)	212.9(43.0)
	S	31.3(5.7)	30.7(5.5)	25.0(6.8)	19.6(8.9)
CDP[d]		23.5(6.8)	19.2(6.0)	11.0(11.8)	8.9(5.9)

[a] Means and standard deviations.
[b] US = Unsedimentable fraction (105,000 x g supernatant)
S = Sedimentable fraction (105,000 x g pellet)
[c] - Specific activity in u Units mg protein^{-1}.min^{-1}.
[d] - Total activity in absorbance units/100g muscle.

expected, leupeptin inhibited cathepsin H only slightly. This finding agrees well with results of many investigators who observed that cathepsin H is only slightly inhibited by leupeptin (Kirschke et al., 1980; Schwartz and Barrett, 1980). The small means and large standard deviations of the percent inhibition of cathepsin H in Table 3 are due to the fact that some leupeptin injected animals showed a slightly higher cathepsin H activity than control animals.

For cathepsins B and L, the percent inhibition is greater in the soluble fraction (unsedimentable fraction) as opposed to the lysosomal (sedimentable) fraction. This suggests that although leupeptin crosses the sarcolemma, it may not easily cross lysosomal membranes. However, the free activities Of cathepsins B and L, which are inhibited the most, are of the greatest interest in postmortem proteolysis. The large standard deviations reflect the large variability between animals in this study. Other investigators have studied the inhibitory effect of leupeptin on some of these proteinases. Libby and Goldberg (1978) incubated rat muscle strips in a buffer containing 25 uM leupeptin and reported a 26 and 34% inhibition of cathepsin B activity in soleus and extensor digitorum longus muscles. Injection of leupeptin

TABLE 3 - PERCENT INHIBITION BY LEUPEPTIN OF THE ACTIVITIES OF CATHEPINS B, L, H AND CDP IN RABBIT LONGISSIMUS MUSCLE[a]

Enzyme	Fraction[b]	Leupeptin Levels (mg/kg) 20	100	200
Cathepsin B	US	27.5(12.8)	75.5(5.5)	83.9(4.7)
	S	34.0(13.5)	47.4(22.3)	57.7(6.0)
Cathepsin L	US	43.8(14.6)	75.6(9.4)	89.3(4.9)
	S	31.0(20.3)	46.6(17.5)	69.4(6.8)
Cathepsin H	US	9.2(11.2)	9.5(15.1)	8.5(17.0)
	S	7.6(13.2)	21.6(26.3)	33.4(37.0)
CDP		17.7(14.3)	57.6(35.2)	64.1(13.7)

[a] Means and standard deviations.
[b] US = Unsedimentable fraction (105,000 x g supernatant)
S = Sedimentable fraction (105,000 x g pellet)

into rats at 20 and 200 mg/kg decreased cathepsin B activity in muscle 42 and 70%, respectively (Sutherland and Greenbaum, 1983). Their values differ from the values reported in Table 3, being higher for the 20 mg level and lower for the 200 mg level. This discrepancy may be due to the extraction procedure used by Sutherland and Greenbaum (1983). They extracted the muscle in a 1% Triton X-100 solution which is different from the buffer used for fractionation of muscle extracts in this experiment. Calculation of an average percent inhibition from both fractions (US and S) for the 20 and 200 mg levels gives 30.8 and 70.8% inhibition which are closer to the values reported by the above mentioned investigators. No data were found in the literature describing the effect of injection of leupeptin on cathepsin L activity.

For CDP, data on the effect of in vivo injection of leupeptin upon the activity of this proteinase are scarce. Stracher et al. (1979) using hemoglobin as a substrate at 37 C in Tris-HCl buffer at pH 8.0 reported that leupeptin inhibited 48% of the neutral proteinase activity in cultured muscle cells. However, the conditions used in their assay are not suitable for CDP activity. CDP is known to be optimally active at pH 7.5, not pH 8.0. Also CDP activity is linear at 25 C, but not at 37 C (Dayton et al, 1976a; b). Moreover,

hemoglobin has been reported as a poor substrate for CDP unless it was denatured (Ishiura et al., 1979). In addition, Stracher et al. (1979) did not use Ca^{2+} or a sulfhydryl reducing agent which are essential for CDP activity. Thus, it is not clear which enzyme these investigators were measuring, although their text is highly suggestive that they were referring to CDP. Later, the same group (Sher et al., 1981) reported that intraperitoneal injection of leupeptin at 12 mg/kg body weight in dystrophic mice led to abolition (90%) of CDP activity within 24 to 48 h. However, they did not present any data to support that claim. In experiment II, difficulty was encountered in the preparation of crude CDP by the isoelectric precipitation method at pH 4.5. It has been observed that most of leupeptin was lost in the supernate and did not precipitate with the enzyme. Thus, large variability exists between animals which is reflected in the high standard deviations. Hence, the percent inhibition of CDP activity as reported in Table 3 may not reflect the true inhibition of CDP activity by the levels of leupeptin studied.

It is clear from Table 3 that no matter what level of leupeptin was used, inhibition was not complete and activity of all these proteinases was still measurable. At the 100 mg level of leupeptin injection activities of the CDPs, cathepsin B and L present at 0 h were 42.4, 24.5 and 24.4%,

respectively, in the soluble fraction. Whether these retained activities are enough to envoke the changes seen in muscle proteins during postmortem storage remains to be established.

The results obtained from Experiments I and II were utilized in Experiment III. Four h were needed for the temperature of the carcasses allotted to the low temperature aging treatment to drop to 2 C. Also, at that time the carcasses alloted to ES treatment had already reached their ultimate pH (5.80). Hence, 4 h was taken as the sampling time to assess the effects of ES and aging temperature on subcellular distribution of lysosomal enzymes. From Experiment II, the 100 mg leupeptin/kg body weight level was selected as an effective and the most economical dose of leupeptin for the inhibition of CDPs, cathepsins B and L. Thus, all rabbits in experiment III were injected with either .9% saline (NL) or 100 mg leupeptin/kg body weight dissolved in .9% saline (L).

Experiment III

Effects of ES, Conditioning Temperature and Leupeptin on Cathepsins B, L and H Activities. In order to mimic insitu carcass conditions, enzyme assays were performed using crude homogenates rather than purified enzymes. Fpr the CDPs, after purification by ion-exchange chromatography to separate them

from their inhibitor, the purified enzymes were added back to unadsorbed material to create the crudest system possible for measuring CDPs activity. Table 4 shows the means and standard errors of the activities of cathepsins B, L and H at 0 h postmortem. Analysis of variance revealed that ES decreased ($P < .05$) the free (US) activity of cathepsin L but had no effect ($P > .05$) on the free activities of cathepsins B and H or the bound (S) activities of any of these enzymes. Since aging temperature treatment commenced after taking the 0 h samples, the effect of temperature on the activities of these enzymes is not significant ($P > .05$) and has no practical importance. Leupeptin, on the other hand, decreased ($P < .001$) the free activities of cathepsins B and L but had no effect on the free activity of cathepsin H. This is consistent with the observation that leupeptin, at low levels, does not inhibit cathepsin H (Kirschke et al., 1980; Schwartz and Barrett, 1980). While leupeptin had no effect on the bound (S) activities of cathepsins B and L, it increased ($P < 0.05$) the bound activity of cathepsin H.

TABLE 4 - MAIN EFFECTS OF ELECTRICAL STIMULATION, AGING TEMPERATURE AND LEUPEPTIN ON THE ACTIVITIES OF CATHEPSINS B, L AND H AT ZERO HOUR POSTMORTEM[a]

		TREATMENTS						
Enzyme	Fraction	NS	ES	LT	HT	NL	L	SE[b]
B	US	3.10	3.32	3.20	3.22	4.42***	1.99***	.34
	S[c]	3.20	3.40	3.24	3.36	3.07	3.52	.23
L	US	3.59*	2.98*	3.36	3.21	5.31***	1.26***	.29
	S[c]	3.33	3.24	3.29	3.29	3.50	3.07	.21
H	US	19.79	21.29	20.05	21.04	20.76	20.33	.98
	S[c]	6.62	6.24	6.08	6.78	5.92*	6.94*	.36

[a] Activities expressed as umoles of product released.min^{-1}.4g muscle.$^{-1}$

[b] SE = Standard error of mean.

[c] Activities represent the sum of the activities in the sedimentable and nuclear fractions.

* - Significantly different (P <.05).

*** - Significantly different (P <.001)

Since no interaction term is significant, the free activities of cathepsins B, L and H were pooled over ES and aging temperature treatments to show the effect of leupeptininjection on these activities. Means of the activities of the CDPs, cathepsins B, L and H for noninjected vs leupeptin injected animals are presented in Table 5, along with percent inhibition of these activities. Comparison of the percent inhibition data to those in Table 3 (Experiment II) revealed that the percent inhibition of cathepsin B activity is lower in experiment III (75 vs 55). This discrepancy could be due to the change in the extraction procedure in experiment III compared to experiment II. The percent inhibition of cathepsin B activity reported in Table 3 may have been overestimated since Sutherland and Greenbaum (1983) reported a 70% inhibition of cathepsin B activity in rats with 200 mg of leupeptin/kg body weight. Thus, the percent inhibition of cathepsin B activity reported in Table 5 is probably more realistic than the value presented in Table 3. The percent inhibition of cathepsin L activity is the same for both experiments and higher than that for cathepsin B activity. Percent inhibition of CDP activity is also slightly lower than has been reported (Table 3). This may also be due to the change in procedure. However, the procedure used for this experiment reduced the variability from that of the isoelectric precipitation procedure used in Experiment II.

TABLE 5 - PERCENT INHIBITION OF THE ACTIVITIES OF CDP, CATHEPSINS B, L AND H BY LEUPEPTIN

Enzyme	No Leupeptin	Leupeptin	SE	% Inhibition
CDP[a]	37.31	19.58	2.62	47.5
Cathepsin B[b,c]	4.42	1.99	.34	55.0
Cathepsin L[b,c]	5.31	1.26	.29	76.2
Cathepsin H[b,c]	20.76	20.33	.98	2.1

[a] CDP total activity (OD units/50 g muscle)
[b] Catheptic enzymes total activity in the US fraction expressed as umol of product released $min^{-1}.4$ g $muscle^{-1}$.
[c] Activities pooled over ES and conditioning temperature treatments.

The inability of leupeptin to completely inhibit CDP, cathepsin B and L may be due to low levels of leupeptin reaching the muscular tissue. Indeed studies with rats (Tanaka, 1983) have demonstrated that leupeptin is rapidly metabolized in the intact animals. Furthermore, others have demonstrated the ubiquitous distribution of a leupeptininactivating enzyme in mouse tissue (Place et al., 1985). Place et al. (1985) reported that two leupeptin inactivating enzymes were resolved by ion exchange chromatography of rat liver homogenates. Their preliminary studies indicated that the first enzyme referred to as leupeptinase 1 may be a metalloenzyme and probably inactivates leupeptin by cleaving its peptide bonds. Thus, it is possible that leupeptin used in this study may have been degraded at least in part; and the actual bioactive amount of leupeptin that reached the muscle was too low to completely inhibit these enzymes.

Effect of ES and Conditioning Temperature on Subcellular Distribution of Catheptic Enzymes. High temperature conditioning and electrical stimulation have been shown to enhance the release of lysosomal enzymes during postmortem storage. This effect was thought to be due to lowered pH and high carcass temperature which would lead to rupturing of lysosomal membranes (Moeller et al., 1976; 1977; Dutson et al., 1980a; Wu et al., 1985). ß-glucuronidase has been used as a

lysosomal marker enzyme in different subcellular fractions (unsedimentable, microsomal and nuclear fractions) because it has no known inhibitor. Thus, an increase in the percent of free (unsedimentable) activity of ß-glucuronidase is taken as an indication of increased disruption of lysosomal membranes and release of other lysosomal enzymes (Moeller et al., 1976; 1977; Dutson et al., 1980a; Wu et al., 1985).

To assess the effects of electrical stimulation and high temperature conditioning on the release of lysosomal enzymes, all sample was taken at 4 h postmortem and processed as described in the Materials and Methods. Activities of ß-glucuronidase, cathepsins B, L and H were determined in the unsedimentable (free), microsomal and nuclear fractions (bound). The total activities of these enzymes were calculated by summing the activities of all three fractions. Percent-free activities were calculated by dividing the activity in the unsedimentable fraction by the total activity in all three fractions or by a total activity calculated by summing the activities in the unsedimentable and microsomal fractions (Wu et al., 1985).

Table 6 shows the subcellular distribution of the activities of ß-glucuronidase, cathepsins B, L and H as affected by ES and conditioning temperature treatments. For ß-glucuronidase HT conditioning increased the activity in the US fraction ($P < .001$), the S fraction ($P < .05$) and the total

activity ($P < .001$). ES increased the activity in the US fraction ($P < .05$) but had no effect on the activity in the S fraction or total activity ($P > .05$). Neither ES nor temperature treatment had a significant effect on the subcellular distribution of the activities of cathepsins B, L or H. It is important to emphasize that high temperature conditioning was applied for 4 h only in this study (experiment III). Others have used 12 to 60 h (Moeller et al., 1976, 1977; Wu et al., 1981). Regarding the effect of HT on ß-glucuronidase distribution, these results do not support the findings of earlier investigators (Moeller et al., 1976) who reported that HT decreased total activity of ß-glucuronidase 12 h postmortem but did not affect the free (US) activity. These investigators in a later study (Moeller et al., 1977), however, reported that HT did not affect the total activity of this enzyme but increased its free activity. Others reported an increase in free (US) activity of this enzyme and a decrease in its bound (S) activity between 12 and 60 h postmortem when HT conditioning was imposed (Wu et al., 1981). Results for the effect of ES on the distribution of ß-glucuronidase activity (Table 6) are not in agreement with results of other investigators. Dutson et al. (1980a) reported that ES did not affect the free specific activity of ß-glucuronidase but decreased the sedimentable (S) and total activities of this enzyme. Later, the same group (Wu et al., 1985) reported that

TABLE 6 - THE EFFECT OF ES AND CONDITIONING TEMPERATURE ON SUBCELLULAR DISTRIBUTION OF CATHEPTIC ENZYMES AT FOUR HOURS POSTMORTEM

Enzyme	LT	HT	NS	ES
ß-Glucuronidase[a]				
US	2.01	2.84***	2.29	2.66*
S	1.87	2.16*	1.93	2.11
Total[b]	5.06	6.30***	5.44	5.92
Cathepsin B[c]				
US	.93	1.05	.90	1.07
S	.30	.25	.27	.28
Total[b]	1.96	2.02	1.90	2.08
Cathepsin L[c]				
US	.73	.83	.69	.86
S	.18	.16	.16	.17
Total[b]	1.51	1.64	1.60	1.54
Cathepsin H[c]				
US	5.35	5.80	5.45	5.70
S	.62	.59	.63	.59
Total[b]	7.04	7.49	7.18	7.35

[a] Activity in fluorescence units x 10^{-3}.min^{-1}.g $muscle^{-1}$.

[b] Total activity calculated by summing the three fractions.

[c] Activity expressed as umoles of product released min^{-1} g $muscle^{-1}$.

* - Significantly different (P <.05).

*** - Significantly different (P <.001).

sES decreased the activity of ß-glucuronidase in the edimentable (S) fraction but had no effect on the free (US) activity. No reports were found in the literature describing the effects of HT on the distribution of the activities of cathepsins B, L and H between the S vs US fractions. However, Moeller et al. (1976; 1977) reported contradictory results regarding the effect of HT on the distribution of cathepsin C. Moeller et al. (1976) observed that HT did not affect the activity of cathepsin C in the US fraction but decreased total activity of the enzyme. Later, Moeller et al. (1977) demonstrated that HT decreased cathepsin C activity in all fractions. The results for the effects of ESupon the distribution of cathepsins B and H in the present study do not agree with those of Wu et al. (1985) who observed that ES decreased the microsomal (S) activity of these enzymes. The discrepancy among these results may be due to the sampling time which was 4 h postmortem in the present study as opposed to 12 h or later in the above mentioned studies. Moreover, it could be due to species differences (rabbits vs beef cattle or lambs). Thus, further study is evidently needed to confirm the effects of ES and HT on the distribution of cathepsins B, L and H between tissue fractions.

The effects of HT and ES on the release of these lysosomal enzymes is presented in Table 7. Earlier reports have shown that percent free activities of ß-glucuronidase and

TABLE 7 - THE EFFECT OF ES AND HIGH TEMPERATURE CONDITIONING ON THE RELEASE OF LYSOSOMAL ENZYMES AT FOUR HOUR POSTMORTEM[a]

Enzyme	LT	HT	NS	ES	SE
ß-Glucuronidase					
% Free[b]	52.9	56.1	53.2	55.8	1.6
% Free[c]	42.1	45.0	41.6	45.5*	1.5
Cathepsin B					
% Free[b]	71.9	78.2***	74.7	75.4	1.4
% Free[c]	47.1	50.0	46.8	50.2	2.3
Cathepsin L					
% Free[b]	74.8	79.3	76.7	77.5	2.6
% Free[c]	43.0	47.4	40.4	50.0*	3.4
Cathepsin H					
% Free[b]	88.8	90.5*	88.9	90.4*	.7
% Free[c]	73.0	77.1	73.1	77.1	2.3

[a] Percent free activity.
[b] Calculated using total activity as US + S.
[c] Calculated using total activity as US + S + NF.
* - Significantly different (P <.05).
*** - Significantly different (P <.001).

cathepsin C were increased by HT conditioning (Moeller et al., 1976; Wu et al., 1981). Furthermore, ES has been shown to increase the percent free activities of ß-glucuronidase, cathepsins C, B and H (Dutson et al., 1980a; Wu et al., 1985). The results reported here (Table 7) show that HT conditioning does not affect the percent free activities of ß-glucuronidase or cathepsin L but increased ($P < .05$) the percent free activities of cathepsins B and H only when the activity in the nuclear fraction (NF) was omitted from the total activity. Moreover, ES had no effect on percent free activities of ß-glucuronidase, cathepsins B or L, but increased ($P < .05$) that of cathepsin H. However, when the NF activity was included in the calculation of total enzyme activity, ES increased ($P < .05$) the percent free activities of ß-glucuronidase and cathepsin L but had no effect on cathepsins B and H. The results reported for the effects of HT upon the percent free cathepsin C activity are contradictory. In one paper Moeller et al. (1976) observed that HT increased percent free activity of this enzyme, and in a subsequent paper the same group (Moeller et al., 1977) reported no such effect. These discrepancies may be due to the sampling time or the species studied or both.

Effect of ES, Conditioning Temperature and Leupeptin on Aging Response of Rabbit Longissimus.

Myofibrillar Fragmentation Index. Table 8 shows the means of the main effects of conditioning temperature, ES and leupeptin injection treatments on MFI. Analysis of variance revealed that HT has no effect on MFI ($P < .05$). Moeller et al. (1977) reported that HT increased muscle fiber fragmentation when compared to LT conditioned bovine muscle. However, their procedure is different from the MFI procedure used in the present study, and the two measurements may not be comparable. ES increased MFI ($P < .001$) only at 24 h postmortem. Further aging reduced the effect of ES on MFI to the level of NS values. Reports on the effect of ES upon MFI are conflicting. While Savell et al. (1979) reported that ES did not affect MFI, Sonaiya et al. (1982) observed that ES increased MFI when compared to the NS control. However, the data reported here are consistent with the well documented acceleration of postmortem aging caused by ES (George et al., 1980; Elgasim et al, 1981; Savell et al., 1981). Most of the changes in MFI of the ES muscle samples occurred by 24 h postmortem with little increase thereafter.In the NS muscles, as is apparent from Table 8, MFI continued to increase in magnitude up to 168 h postmortem. These results are as expected and consistent with previously reported data.

TABLE 8. THE EFFECT OF HT CONDITIONING, ES AND LEUPEPTIN INJECTION ON MYOFIBRILLAR FRAGMENTATION INDEX[a]

Time	Treatments		
	LT	HT	SEM[b]
0	44.20	43.35	1.15
24	67.77	66.80	1.28
72	70.43	69.68	2.15
168	72.85	72.00	2.50
	NS	ES	SEM[b]
0	43.05	44.50	1.15
24	63.83	70.55***	1.28
72	68.15	71.95	2.15
168	72.30	72.55	2.50
	NL	L	SEM[b]
0	44.45	43.10	1.15
24	69.95	64.42***	1.28
72	73.20	66.90**	2.15
168	76.00	68.86**	2.50

[a] Values of MFI reported as (A_{540} X 200).
[b] Standard error of means.
** Significantly different (P <.01).
*** Significantly different (P <.001).

It is evident from Table 8 that leupeptin injection decreased ($P < .01$) MFI values at 24, 72 and 168 h postmortem. This observation clearly indicates that myofibril fragmentation is caused by some proteolytic enzyme(s) which is(are) inhibited by leupeptin. Since cathepsin H activity is not inhibited by leupeptin (Table 5), it may be concluded that this and possibly other catheptic enzymes play little role in myofibrillar fragmentation. This conclusion is consistent with the recent observation that cathepsin H showed little degradation of any of the myofibrillar proteins when incubated with purified rabbit myofibrils (Ouali et al., 1987). Koohmaraie et al. (1988a) have shown that myofibril fragmentation was completely inhibited when muscle slices were incubated in the presence of the Ca^{2+} chelators EDTA and EGTA. While cathepsins B, H and L remain fully active, CDPs were completely inactivated in the presence of Ca^{2+} chelators. Therefore, Koohmaraie et al. (1988a) concluded that myofibril fragmentation was caused by CDPs rather than catheptic enzymes. Additionally, cathepsin D, a carboxyl proteinase, which also is not affected by leupeptin, likewise does not appear to be involved in myofibrillar fragmentation. In agreement with this conclusion, Matsukura et al. (1984) concluded that in spite of the loss of the Z-disk, fragmentation of myofibrils was not caused by cathepsin D. Leupeptin inhibited the activity of CDPs and the soluble (US)

activities of cathepsins B and L (Table 5). Hence, it is difficult to draw conclusions about the specific role of each of these enzymes in myofibrillar fragmentation. Calculation of the percent change in MFI between 0 and 24 h postmortem revealed that for the leupeptin-treated animals a 49% change occurred by 24 h as opposed to 57% change in control animals. Since CDP's, were inhibited to a lesser extent than cathepsins B and L (Table 5), the CDP's, may be more involved in myofibrillar fragmentation than cathepsins B and L. This is consistent with earlier observations that myofibrillar fragmentation was inhibited by calcium chelators (EDTA, EGTA) which inhibit CDP activity (Koohmaraie et al., 1988a). The increase in MFI of leupeptin injected animals with time postmortem may be due to the inability of leupeptin to completely inhibit CDP, cathepsins B and L activities. The remaining activity of these enzymes may be enough to evoke the observed changes in MFI. In this regard, Koohmaraie et al. (1986) reported that under low temperature aging, activity of the uM-CDP was decreased to about 28% of that observed under optimal conditions of 25 C and pH 7.5; but this level of residual activity was enough to cause most of the postmortem changes in MFI and polyacrylamide gel electrophoresis profiles of myofibrillar proteins.

The results for MFI have been confirmed by phase contrast microscopy of myofibrils (data not shown). It was apparent

that myofibrils of the leupeptin treated animals were broken into larger fragments as opposed to those from noninjected animals. Also, the majority of the myofibrillar fragments from leupeptin treated animals had intact Z-disks even up to 168 h postmortem. Myofibrillar fragments from control animals lost most of their Z-disks by 72 h postmortem, but remnants of Z-disks could still be seen in some fragments even after 168 h postmortem. Since MFI is highly correlated with shear force values of cooked meat (Olson and Parrish, 1977; Culler et al., 1978; Koohmaraie, 1988), it may be concluded that leupeptin inhibited the postmortem tenderization process.

SDS-PAGE of Myofibrillar Proteins. The major changes in myofibrils occurring during the postmortem aging process, which are associated with tenderization, have been reviewed by many investigators (Parrish, 1977; Goll et al., 1983a; Koohmaraie et al., 1986; Koohmaraie, 1988). These changes include myofibril fragmentation and weakening of Z-disks, which have been discussed in the previous section. Besides, they include changes in the banding pattern of myofibrillar proteins on SDS-polyacrylamide gels. These changes include the well documented loss in the intensity of the desmin and troponin-T bands with the concomitant appearance of protein bands at the 30 kilodaltons (kd) region. The major contractile proteins, myosin and actin, are not affected during postmortem aging

(Olson et al., 1977; Penny, 1980). The banding pattern of myofibrillar proteins on SDS-polyacrylamide gels is shown in Figures 3 to 6. Since similar results were obtained for all replicates, gels of only two replicates are presented. For comparison, beef LD muscle sample taken at similar periods postmortem and purified in the same manner were included in all gels (lanes 2, 3, 4 and 5 corresponding to 0, 24, 72 and 168 h postmortem periods) in Figures 3 to 6. The desmin (Des) and troponin-T (TN-T) bands are lost by 168 h postmortem in beef muscle. Also, new protein bands appear at 32, 30 and 27 kd by 72 h and increased in intensity at 168 h postmortem (bands a, b, c, respectively). These results are consistent with data for beef reported by Goll et al. (1983a). In addition to the above-mentioned changes in beef muscle, there is a shift in the protein band corresponding to troponin-I (TN-I) to a slightly lower molecular weight (band d) at 168 h postmortem. This indicates that troponin-I is also digested by 168 h postmortem, and this change has not been reported previously. Olson et al. (1977) observed a loss in the intensity of TN-I band in their crude-CDP-treated myofibrils, but they did not observe this shift in the TN-I band in aged muscle of beef. The failure of earlier investigators to observe this change may be due to the use of rod gels which are difficult to align, and this small change may have been overlooked. Also, 100 ug of myofibrillar proteins are typically loaded on rod gels, and this may result

in broadening of protein bands and make it difficult to notice this small change. In rabbit longissimus muscle, the changes in banding pattern of myofibrillar proteins due to treatments (ES, aging, temperature and leupeptin) are not consistent with those reported for beef muscle. In addition to the beef muscle samples, Figure 3A shows the 0, 24, 72 and 168 h samples of NS, LT, NL (lanes 6 to 9) and the corresponding time periods for those of NS, LT, L (lanes 10 to 13), respectively of rabbit longissimus muscle. It is obvious that the desmin and troponin-T bands are still present even at 168 h postmortem in the rabbit muscles. No bands are apparent in the 27 to 32 kd region (bands a, b and c), and the troponin-I band is intact. Figure 3B shows the rabbit samples of ES, LT, NL (lanes 6 to 9) and those of ES, LT, L (lanes 10 to 13) corresponding to 0, 24, 72 and 168 h postmortem, respectively. A loss in the intensity of TN-T band and appearance of new protein bands in the 27 (b) and 32 (a) kd regions (lanes 8 and 9) are evident in the ES, LT, NL samples. Additionally, two bands appeared just below TN-I in the 24 and 72 h samples (lanes 7 and 8). These changes are completely inhibited by leupeptin (lanes 10 to 13). The appearance of the 32 and 27 kd bands in the rabbit muscle (Figure 3B, lanes 8 and 9) with no band at 30 kd (position b) is consistent with observations of other investigators whonoted that unlike beef muscle, aging or treatment with proteinases produced new protein bands at 32 and 27 to 28 kd position with

CODE FOR FIGURES SHOWING THE SDS-PAGE RESULTS

Abbreviations used:

KD = kilodalton
AC = α-Actinin
Des = Desmin
TNT = Troponin-T
TM = Tropomyosin
TNI = Troponin-I
TNC = Troponin-C
LC = Myosin Light Chain
a = 32000 dalton band
b = 30000 dalton band
c = 27 to 28000 dalton band
d = band appearing as a shift in TNI molecular weight

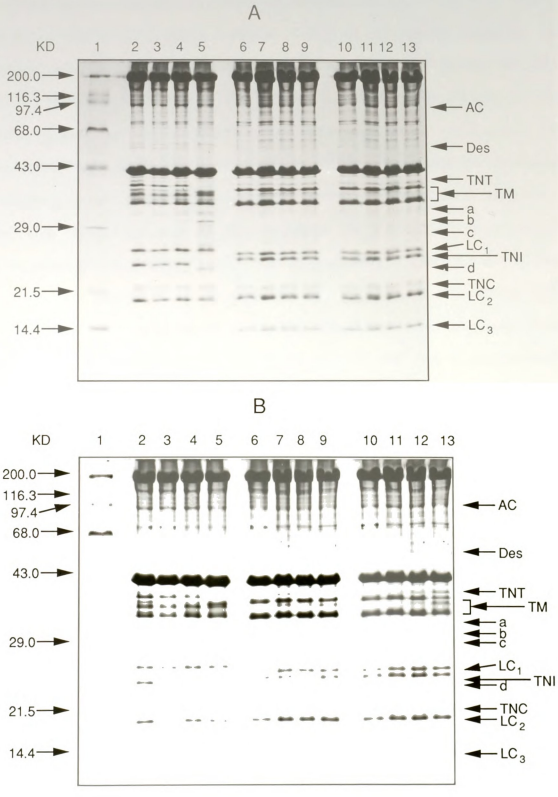
Molecular Weight Markers:

Protein	Molecular Weight (kd)	
Myosin Heavy Chain	200.0	
ß-Galactosidase	116.3	
Phosphorylase b	97.4	
Bovine Serum Albumin	68.0	
Ovalbumin	43.0	
Carbonic Anhydrase	29.0	
Soyabean Trypsin Inhibitor	21.5	
ß-Lactoglobulin	18.4	(Figure 6A)
Lysozyme	14.4	

FIGURE 3. ELECTROPHORETIC PATTERN OF MYOFIBRILLAR PROTEINS FROM NONSTIMULATED AND ELECTRICALLY STIMULATED RABBIT CARCASSES (REPLICATE 4) AGED AT 2 C.

A. Molecular weight markers (lane 1); beef sample (lanes 2 to 5); rabbit samples from NS, NL (lanes 6 to 9) and NS, L (lanes 10 to 13).

B. Molecular weight markers (lane 1); beef sample (lanes 2 to 5); rabbit samples from ES, NL (lanes 6 to 9) and ES, L (lanes 10 to 13).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TNT
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TNI
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TNI
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TNC
LC2
LC3

no band at the 30 kd position (Takahashi et al., 1987 a; Ouali et al., 1987) in rabbit muscle. Figure 4A shows samples of rabbit muscle for NS, HT, NL (lanes 6 to 9) and those of NS, HT, L (lanes 10 to 13). In the NL samples, the 32 and 27 kd bands appeared after 24 h (lane 7); but they decreased in intensity by 168 h postmortem (lane 9) possibly due to further proteolysis as reported by Penny (1980). For the L samples, the two bands (a and c) are evident at 24 h but completely disappeared from the 72 and 168 h samples. Samples from the ES, HT, NL (lanes 6 to 9) and the ES, HT , L (lanes 10 to 13) are presented in Figure 4B. Again, the NL samples clearly show the 32 and 27 kd bands (a and c) at 24, 72 and 168 h postmortem (lanes 7, 8 and 9, respectively). While the L samples show many faint bands in the 27 to 32 kd region, Figures 5 and 6 show, beside the beef muscle (lanes 2 to 5) in each, the samples from 8 rabbits (corresponding to the same treatment combinations mentioned above) for a second replicate. These gels include the following treatments: NS, LT, NL (lanes 6 to 9), NS, LT, L (lanes 10 to 13) Figure 5A; ES, LT NL (lanes 6 to 9), ES, LT, L (lanes 10 to 13) Figure 5B; NS, HT, NL (lanes 6 to 9), NS, HT, L (lanes 10 to 13) Figure 6A; ES, HT, NL (lanes 6 to 9) and ES, HT, L (lanes 10 to 13) Figure 6B. In this replicate, the results are mixed and inconsistent compared to those in Figures 3 and 4. While some samples do not show the bands a and c, others show very faint bands regardless of

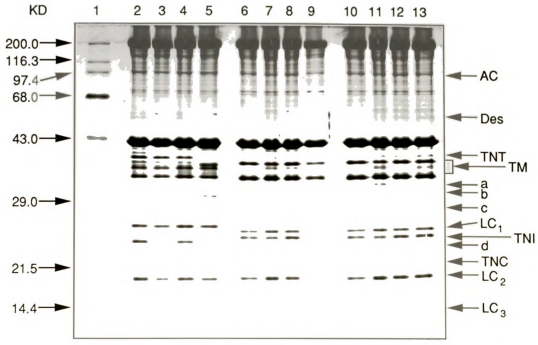
leupeptin treatment. These data reflect the variability among the rabbits used in this study. For the electrophoretograms in Figures 3 to 6 the procedure used for myofibril purification was that of Goll et al. (1974). The variability between rabbits in the appearance of the breakdown products at 32 and 27 kd regions also has been observed in preliminary experiments utilizing two other procedures of myofibrillar purification. Namely, the procedure outlined by Bendall (1961) and that of Etlinger et al. (1976) as modified by Takahashi et al. (1987a). In their study, Takahashi et al. (1987a) presented an electrophoretogram of aged rabbit samples showing the appearance of 27 and 32 kd bands by d 2. Their electrophoretogram may contain samples from one rabbit or aged samples from different rabbit carcasses which show these bands. Since only one electrophoretogram is presented (Takahashi et al., 1987a), it is difficult to know whether all the rabbit carcasses used in their study behaved in the same way or whether they also encounter variability in the appearance of proteolytic products at the 27 to 32 kd region. Others have observed these bands when purified rabbit myofibrils were incubated with purified enzymes (Ouali et al., 1987), and hence their results may not be comparable to the results in Figures 3 to 6. Thus, it is difficult to draw conclusions about the effect of the treatments used here (electrical

FIGURE 4. ELECTROPHORETIC PATTERN OF MYOFIBRILLAR PROTEINS FROM NONSTIMULATED AND ELECTRICALLY STIMULTED RABBIT CARCASSES (REPLICATE 4) AGED AT 22 CFOR 4 H AND THEN AT 2 C.

A. Molecular weight markers (lane 1); beef (lanes 2 to 5); rabbit samples from NS, NL (lanes 6 to 9) and NS, L (lanes 10 to 13).

B. Molecular weight markers (lane 1); beef (lanes 2 to 5); rabbit samples from ES, NL (lanes 6 to 9) and ES, L (lanes 10 to 13).

A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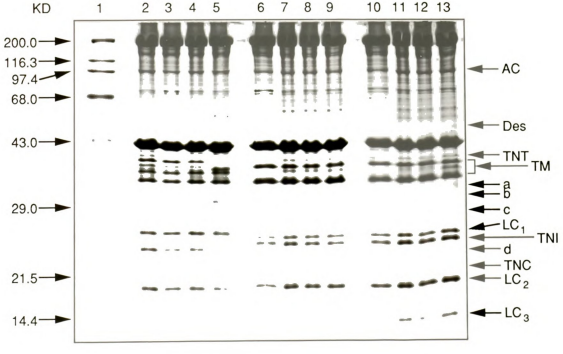
TNC
LC2
LC3

B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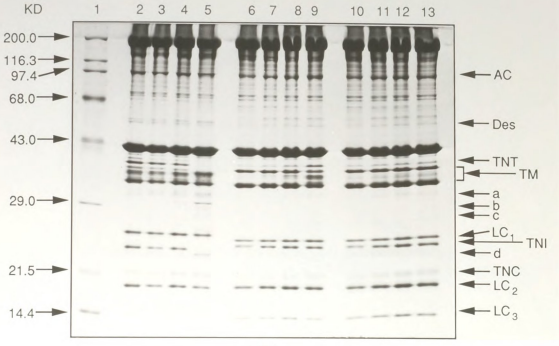
b
c
LC1
TNI
d
TNC
LC2
LC3

FIGURE 5. ELECTROPHORETOGRAMS OF MYOFIBRILLAR PROTEINS FROM NONSTIMULATED AND ELECTRICALLY STIMULATED RABBIT CARCASSES (REPLICATE 1) AGED AT 2 C.

A. Molecular weight markers (lane 1); beef samples (lanes 2 to 5); rabbit samples from NS, NL (lanes 6 to 9) and NS, L (lanes 10 to 13).

B. Molecular weight markers (lane 1); beef samples (lanes 2 to 5); rabbit samples from ES, NL (lanes 6 to 9) and ES, L (lanes 10 to 13).

A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LC1
TNI
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TNC
LC2
LC3

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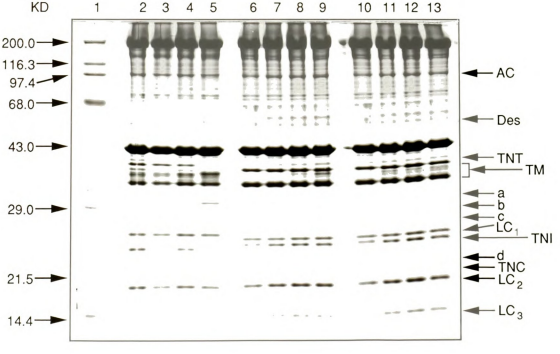
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AC
Des
TNT
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a
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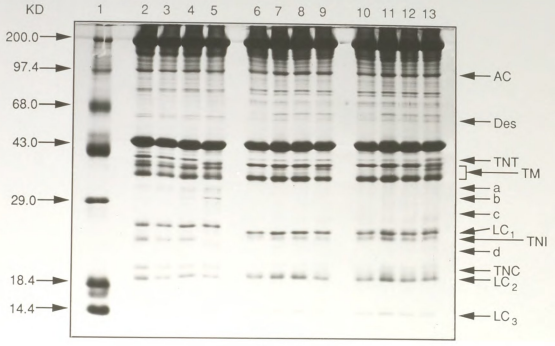
LC1
TNI
d
TNC
LC2
LC3

FIGURE 6. ELECTROPHORETOGRAMS OF MYOFIBRILLAR PROTEINSFROM NONSTIMULATED AND ELECTRICALLY STIMULATED RABBIT CARCASSES (REPLICATE 1) AGED AT 22 C (4 H) THEN AT 2 C.

A. Molecular weight markers (lane 1); beef samples (lanes 2 to 5); rabbit samples from NS, NL (lanes 6 to 9) and NS, L (lanes 10 to 13).

B. Molecular weight markers (lane 1); beef samples (lanes 2 to 5); rabbit samples from ES, NL (lanes 6 to 9) and ES, L (lanes 10 to 13).

A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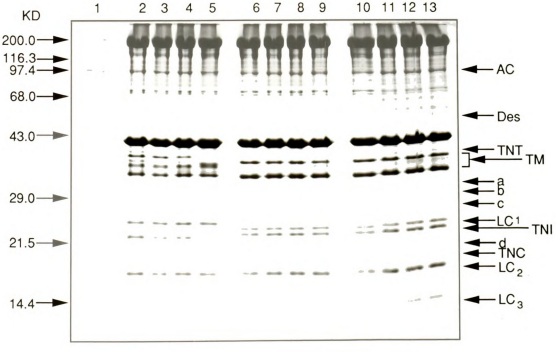
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stimulation, aging temperature and leupeptin injection) on the banding pattern of myofibrillar proteins postmortem storage.

SUMMARY

The effects of electrical stimulation (ES) and high temperature (HT) conditioning on the activities of cysteine proteinases, the subcellular distribution of lysosomal enzymes and the aging response in the longissimus muscle, have been studied with 32 New Zealand white rabbits. Leupeptin, a cysteine proteinase inhibitor, has been used to assess the role of cysteine proteinases (CDP, cathepsins B, L and H) in the aging response of rabbit longissimus muscle. High temperature (HT) conditioning for 4 h postmortem increased ($P < .05$) the percent free activities of cathepsins B and H but did not affect ($P > 0.05$) those of ß-glucuronidase and cathepsin L. ES increased ($P < .05$) the percent free activities of ß-glucuronidase, cathepsins L and H when the activity in the nuclear fraction (NF) was omitted from the total activity. ES had no effect ($P > .05$) on percent free activity of cathepsin B. While HT had no effect ($P > .05$) on myofibrillar fragmentation index (MFI) measured at 0, 24, 72 or 168 h postmortem, ES increased ($P < .001$) MFI value at 24 h with no further effect up to 168 h postmortem. Neither HT for 4 h nor ES had a consistent effect on the banding patterns of myofibrillar proteins as assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Leupeptin inhibited 47.5%, 55% and 76.2% of the activities of CDPs, cathepsin B and cathepsin L, respectively. It

had minimal effect on the activity of cathepsin H. Furthermore, leupeptin decreased ($P < .01$) MFI values measured at 24, 72 and 168 h postmortem compared to no leupeptin treatment. Leupeptin had no consistent effect on the banding patterns of myofibrillar proteins. The appearance of degradation products in the 27 to 32 kd region of SDS-polyacrylamide gels was variable for the rabbit muscles used in this study.

It is concluded from these studies that the aging response in the rabbit longissimus muscle as measured by MFI is, at least in part, due to the activities of either CDPs, cathepsin B, cathepsin L or all of them. Myofibril fragmentation may not be due to the activity of cathepsin H and possibly the other catheptic enzymes which are not inhibited by leupeptin. Moreover, the effects of HT and ES on the release of lysosomal enzymes is not consistent and may depend on the inclusion or omission of the activity in the NF fraction when total activity is calculated. The tenderizing effect of ES is due to increased myofibril fragmentation in the ES carcasses and may not be due to release of lysosomal enzymes or increased proteolysis of myofibrillar proteins. It is also concluded that while the injection of enzyme inhibitors offers a great potential to study postmortem aging, leupeptin may not be the most suitable candidate for this purpose because of methodological problems as described below. It has been observed in Experiment II and other preliminary work during

preparation of the CDPs by the isoelectric precipitation procedure that most of leupeptin remained in the supernate and did not precipitate with the enzyme. Also purification of CDPs by ion-exchange chromatography resulted in the loss of leupeptin in the void volume. Furthermore, leupeptin did not completely inhibit CDPs or cathepsins B and L. In addition, the rabbits did not prove to be a suitable model for postmortem aging studies since the appearance of proteolysis products is variable among rabbits of the same breeding. Further studies using other inhibitors and other species are needed to study the role of individual enzymes (CDPs, cathepsins B or L) in postmortem proteolysis.

APPENDIX

Appendix

Conditions for enzyme assays.

1. Calcium-Dependent Proteinases (CDPs).

   Substrate - Casein (for qualitative assays).

   Optimal pH - 6.5 - 8.0 (7.6 for rabbit CDPs).

   Optimum temperature - 25 C gives linear activity up to 60 min, at 37.5 C CDPs rapidly autolyzes in the presence of Ca^{2+} (Complete autolysis after 5 min).

   Other conditions - Presence of 1 mM Ca^{2+} and reduced -SH groups are essential for activity.

   Activity - Absorbance (278 nm) of peptides soluble in 2.5% TCA. [A_{278} of assay tubes (Ca^{++}) - A_{278} of control tubes (EDTA)]/Volume of CDP used for assay X Total CDP volume = Total Activity in OD units/grams muscle used.

2. Lysosomal Enzymes:

   i. Cathepsin B:

   Substrate - Z-Arg-Arg-NMec (km = 2.5 X 10^{-5}).

   - Z-phe-Arg-NMec (km = 7.3 X 10^{-5}).

   Optimum pH - 6.0 (synthetic substrates)

   - 4.0-6.5 (muscle proteins)

i. Cathepsin B (Continued)

Optimum temperature - 30-40 C giving linear activity at 40 C up to 10 min.

Other conditions - Disodium-EDTA (2 mM) and reduced -SH groups are essential for activity.

Sensitivity to Z-phe-phe-CHN_2 - 1.0 X 10^{-4} M gives 50% inhibition.

ii. Cathepsin L:

Substrate - Z-phe-Arg-NMec (km = 7.0 X 10^{-7} M).

Optimum pH - 5.5 (synthetic substrates)

- 3.0-6.5 (muscle proteins)

Optimum temperature - 30-40 C giving linear activity at 40 C up to 10 min.

Other conditions - Disodium-EDTA (2 mM) and reduced -SH groups are essential for activity.

Sensitivity to Z-phe-phe-CHN_2 - 5.0 X 10^{-8} M gives 50% inhibition Z-phe-phe-CHN_2 was used at 1.0 X 10^{-6} M (100% inhibition) for calculation of cathepsin L activity.

iii. Cathepsin H:

Substrate - Arg-NMec (km = 1.5 X 10^{-4} M)

Optimum pH - 6.8 (synthetic substrate)

- 5.5-6.5 (muscle proteins) has weak activity against myofibrillar proteins.

iii. Cathepsin H (continued)

Optimum temperature - 30-40 C giving linear activity at 40C (up to 10 min)

Other conditions - Disodium-EDTA (2 mM) and reduced -SH groups are essential for activity.

Lysosomal enzymes activity calculation

Assay tubes - contain enzyme and substrate

Control tubes - contain no enzyme. For cathepsin L these contain enzyme and substrate and Z-phe-phe-CHN_2.

Fluorometer - 360 nm excitation
460 nm emission

set up such that 1000 arbitrary units correspond to the release of 1 nmol of product.

Calculation - Assay tubes - control tubes = delta F. A delta F of 1000 corresponds to 0.1 m units in a 10 min assay (1 m unit is the release of 1 nmol of product/min).

Specific activity - (delta F X 0.1)/(1000 X mg of protein) gives activity in m units.min^{-1}.mg $protein^{-1}$, i.e., nmol of product released .min^{-1}.mg^{-1}.

Total activity - (Activity/mg protein) X total protein.

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