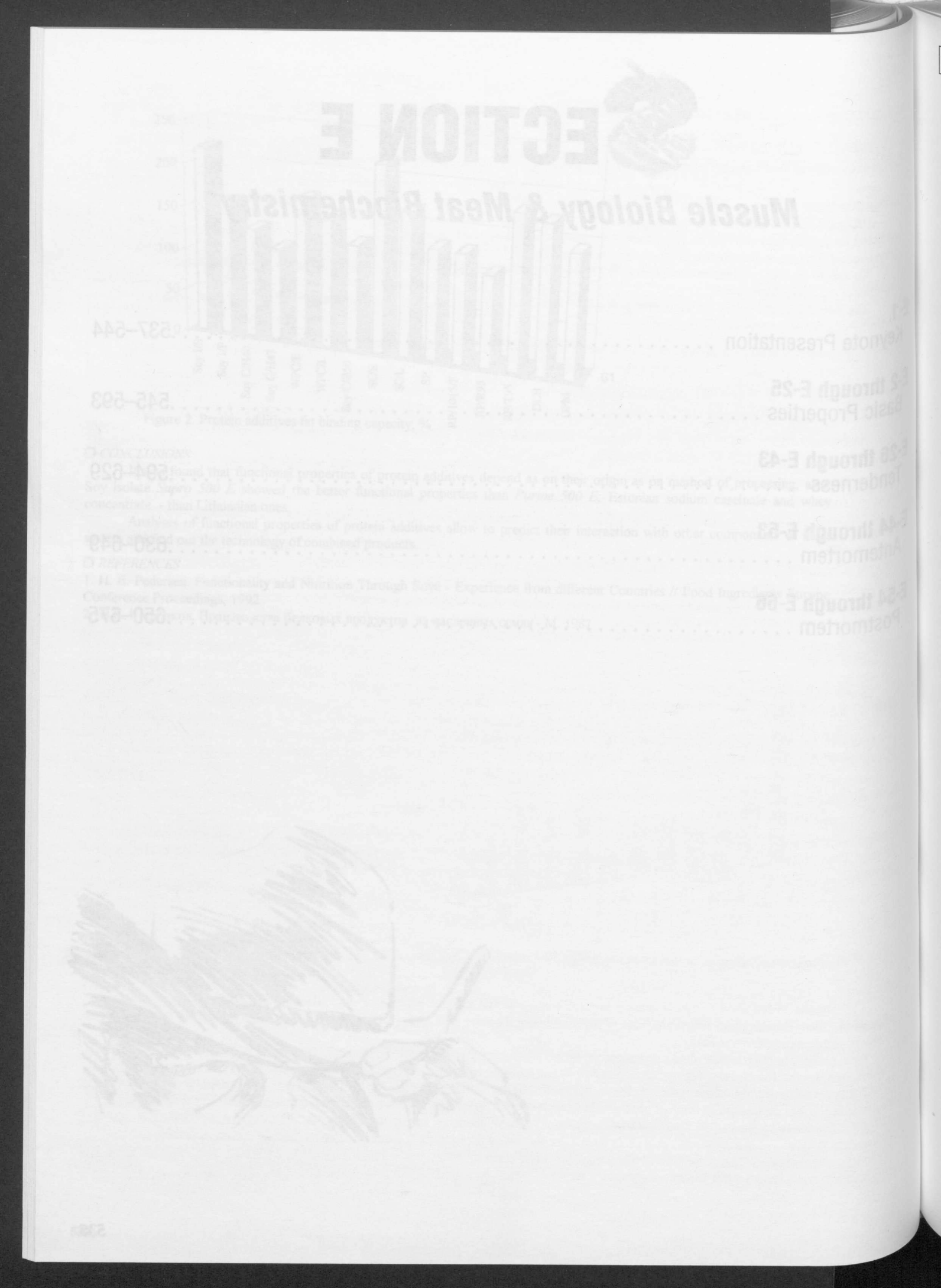
SECTION E

Muscle Biology & Meat Biochemistry



E1

DOES PROTEOLYSIS CAUSE ALL POSTMORTEM TENDERIZATION, OR ARE CHANGES IN THE ACTIN/MYOSIN INTERACTION INVOLVED?

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ABSTRACT

This paper proposes two new hypotheses for the mechanisms that cause changes in toughness during postmortem aging: 1) the increase in toughness observed during the first 24-36 hr postmortem (the time depending on species, temperature, etc.) is caused by a change in the actin/myosin interaction from a weak-binding state to a strong-binding state; this increase in toughness may be accompanied by and exacerbated by shortening; and 2) the decrease in toughness that occurs after 24-36 hr postmortem (the time depending on species, etc.) is caused by two factors; a) a weakening of the actin/myosin interaction; and b) calpain-induced proteolytic degradation of costameres, of intermediate filaments that constitute the intermyofibrillar linkages in muscle fibers, and of the attachments of titin and nebulin to the Z-disk. Contrary to the widely accepted conclusion that Z-disk degradation has an important role in postmortem tenderization, Z-disks seem to remain robust during postmortem storage. Furthermore, it is proposed that, in most situations, especially those involving postmortem storage at 2-4° C, changes in the actin/myosin interaction have far larger effects on toughness than proteolysis. Recent x-ray crystallographic structures of actin and the myosin cross-bridge have shown that myosin can make at least four contacts with actin and that changes in the number of these contacts is associated with a weak (resting muscle) to strong (contracting muscle) transition. Changes in the number and nature of the actin/myosin contacts during postmortem storage are likely to have marked effect on muscle toughness. Rigor-shortened myofibrils lengthen after 36-72 hr postmortem; the Mg^{2+} -modified ATPase activity first increases by 30-80% and then decreases back to its at-death level, and the rate of superprecipitation of reconstituted actomyosin suspensions is more rapid in preparations made from postmortem muscle than in preparations from at-death muscle. Those observations suggest that the actin/myosin interaction changes during postmortem storage. Future research on the nature of the nucleotide bound to myosin in postmortem muscle and on the effect of ATP on the interaction of actin and myosin prepared from postmortem muscle may provide interesting new insights into postmortem tenderization.

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[b]Abbreviations used are: AM, actomyosin; M, myosin; AM·ATP, AM·ADP·P_i, etc., ATP bound to actomyosin, ADP and inorganic phosphate resulting from hydrolysis bound to actomyosin, etc.; TM, tropomyosin; TN, troponin.

INTRODUCTION

Rather than attempting a comprehensive review of the vast literature on postmortem tenderization, this article will present two new hypotheses concerning the mechanisms that cause changes in toughness during postmortem aging: 1) that the increase in toughness observed during the first 24 -36 hr postmortem is caused by a change in the nature of the actin/myosin interaction from a weak to a strong binding state similar to that now known to occur during muscle contraction; that this increase may be accompanied by and be significantly exacerbated by shortening; and that the actin/myosin interaction changes or weakens after 24 - 36 hr postmortem so that much of the increase in toughness caused by the strengthening of this interaction is now alleviated; and 2) that most of the proteolytically induced decrease in toughness that occurs after 24 - 36 hr postmortem results from calpain degradation of titin and nebulin at sites in the I band near the Z-disk and from calpain degradation of costameres and intermediate filaments linking adjacent sarcomeres in the muscle fiber rather than from Z-disk degradation *per se*. These two hypotheses are based in the assumption that the data published by Wheeler and Koohmaraie (1994) accurately reflect the changes that occur in tenderness during postmortem storage at 2 - 4 ° C (Fig. 1), although the magnitude of these changes and time postmortem at which they occur will vary among species and among animals within species. For example, in some animals or in some muscles, the magnitude of the large increase in toughness, shown in Fig. 1 as occurring after 24 hr postmortem may be greater or less than that observed by Wheeler and Koohmaraie. Similarly, in some animals or in some muscles, the degree of postmortem tenderization may be greater than that in Fig. 1. Despite these variations, it seems likely that the general pattern of postmortem changes in toughness follows that shown in Fig. 1; 1) an initial increase in toughness followed by a rapid decrease that matches or usually exceeds the initial toughness increase, all of which occurs during the first 1-4 days postmortem, depending on species, muscle, and

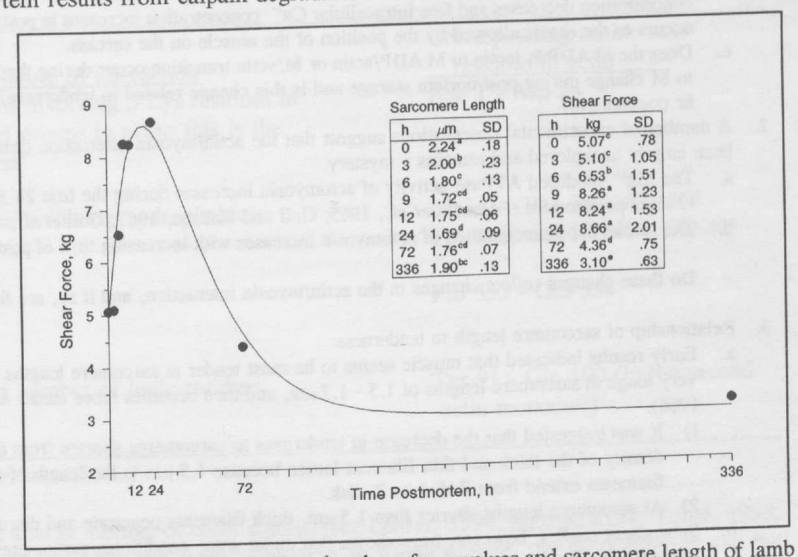

Fig. 1. Changes in Warner-Bratzler shear force values and sarcomere length of lamb longissimus thoracic et lumborum muscle during postmortem storage. This figure has been adapted from Wheeler and Koohmaraie shown (1994).

other conditions; and then 2) a gradual decrease in toughness that extends for a period of 7 - 14 days postmortem.

Accepting this as the general pattern of postmortem changes in toughness the two central questions are: 1) what causes the increase in toughness during the first 24 - 36 hr postmortem? and 2) what causes the decrease in toughness after the first 24 - 36 hr postmortem? The answers to these questions, based on the two hypotheses listed at the beginning of this review, are summarized in Fig. 2 and 3. Although it is generally believed that postmortem shortening causes increased toughness and that proteolysis is responsible for postmortem tenderization, the hypotheses summarized in Fig. 2 and 3 propose that as yet uncharacterized changes in the nature of the actin/myosin interaction have a significant role both in postmortem toughening and in the large decrease in toughness that occurs before 72 hr postmortem (Fig. 1). Also, although it is widely believed that proteolytic degradation of the Z-disk is responsible for postmortem tenderization, recent evidence indicates that proteolytic degradation of costameres, intermediate filaments, and titin and nebulin contribute substantially to postmortem tenderization (Robson *et al.*, 1991; Taylor *et al.*, 1995a). Some of the reasons for believing that factors other than sarcomere shortening or Z-degradation are important in postmortem toughening or tenderization, respectively, are summarized in the following two sections.

WHAT CAUSES THE INCREASE IN TOUGHNESS DURING THE FIRST 24 - 36 HOURS POSTMORTEM?

Following the initial seminal observation that shortening has profound effects on toughness of excised muscle strips stored at different temperatures (Marsh and Leet, 1966; Fig. 2), a number of groups reported that shortened sarcomere lengths were highly related to increased muscle toughness (see Davis *et al.*, 1979; Herring *et al.*, 1965, as examples). Other groups, however, found little relationship between sarcomere length and toughness (see Culler *et al.*, 1978; Parrish *et al.*, 1979, as examples). In a reexamination of this question, Smulders *et al.* (1990) found instances where muscles having sarcomere lengths of 1.6 - 1.7 μm were among the most tender of the muscles examined in 67 animals. In light of this data it is difficult to conclude that there is a direct causative relationship between sarcomere length and tenderness, and Smulders *et al.*, (1990) suggested that the rate of postmortem glycolysis (low pH at 3 hr postmortem) was a significant factor affecting the relationship between sarcomere length and muscle toughness. If the muscle pH was 6.3 or less at 3 hr postmortem (rapid postmortem glycolysis), the correlation between sarcomere length and panel tenderness was 0.16 for the 48 animals tested. This observation clearly shows that the increased toughness associated with shortened sarcomeres in some animals is not simply due to an increased mass of myofibrillar protein per unit of cross sectional area, as is commonly supposed, but that some other factor(s) must be involved. What is this factor(s)?

The x-ray crystallographic structures of actin (Holmes *et al.*, 1990; Kabsch *et al.*, 1990) and of the S1 head of myosin (Rayment *et al.*, 1993b) have now been determined, and the nature of the interaction between actin and myosin has been ascertained at the molecular level by combining the crystallographic structures with "low resolution" (30Å) electron denisty maps of the actin/myosin complex

WHAT CAUSES THE INCREASE IN TOUGHNESS DURING THE FIRST 24-36 HOURS POSTMORTEM?

A. Changes in the Actin/Myosin Interaction.
   1. The x-ray crystallographic structure of the myosin "head", and reconstruction of this structure with the crystallographic structure of actin and low resolution electron density maps of the actin/myosin complex (Rayment *et al.*, 1993a; 1993b) together with a variety of other evidence have shown that there are at least two states of the actin/myosin complex: 1) a weak-binding state; and 2) a strong-binding state.
      a. The weak-binding state corresponds to the M·ADP·P_i/actin state, and the strong-binding state, which is the force-producing state, corresponds to the M·ADP/actin or M/actin state (Lehrer, 1994).
      b. The high (1-5 mM) ATP and low (< 1μM) free Ca^{2+} concentrations in living or at-death muscle disfavor the actin/myosin interaction. As ATP concentration decreases and free intracellular Ca^{2+} concentration increases in postmortem muscle, the actin/myosin complex is formed and shortening occurs to the extent allowed by the position of the muscle on the carcass.
      c. Does the M·ADP·P_i/actin to M·ADP/actin or M/actin transition occur during the first 24-36 hr postmortem? Does the nature of the nucleotide bound to M change during postmortem storage and is this change related to tenderness? Is formation of the strong-binding complex reversed after 24 - 36 hr postmortem?
   2. A number of experimental observations suggest that the actin/myosin interaction changes during postmortem storage, but the nature of this change has been largely unexplored and remains a mystery.
      a. The Mg^{2+}-modified ATPase activity of actomyosin increases during the first 24-36 hr postmortem and then decreases back to its at-death level after 13 days postmortem (Fujimaki *et al.*, 1965; Goll and Robson, 1967; Goll *et al.*, 1970; Robson *et al.*, 1967).
      b. The rate of superprecipitation of actomyosin increases with increasing time of postmortem storage (Arakawa *et al.*, 1970a; 1970b; Goll *et al.*, 1970).

      Do these changes reflect changes in the actin/myosin interaction, and if so, are they related to changes in tenderness?
   3. Relationship of sarcomere length to tenderness:
      a. Early results indicated that muscle seems to be most tender at sarcomere lengths of 2.0 - 2.5 μm, less tender at sarcomere lengths of 1-7 - 2.0 μm, very tough at sarcomere lengths of 1.5 - 1.7 μm, and then becomes more tender at very short sarcomere lengths of 1.5 μm or less (Marsh and Leet, 1966).
         1) It was suggested that the decrease in tenderness as sarcomeres shorten from their resting length (2.3 - 2.5 μm) to 1.5 μm is due to an increased density of the thick and thin filament lattice because 1.5 μm is the length of thick filaments and is the sarcomere length at which myosin thick filaments extend from Z-disk to Z-disk.
         2) At sarcomere lengths shorter than 1.5 μm, thick filaments penetrate and disrupt the Z-disk structure, causing an increase in tenderness.
      b. Subsequent studies, however, have shown that under some conditions, sarcomere length is not related to tenderness (Smulders *et al.*, 1990); hence it is unlikely that there is a causal relationship between sarcomere length and tenderness.

Fig. 1. Proposed causes for the increase in toughness that occurs during the first 24-36 hours postmortem, and the evidence supporting this suggestion.

A. Weakening of the Actin/Myosin Interaction.
1. Several kinds of indirect evidence suggest that the actin/myosin interaction changes after 24 - 36 hr postmortem; this change may involve a partial and gradual shift from a strong-binding to a weak-binding state. Could such a change cause the decrease in toughness between 24 and 72 hr postmortem?
   a. Rigor-shortened sarcomeres seem to lengthen slightly after 24 - 36 hr postmortem (Gothard *et al.*, 1966; Stromer *et al.*, 1967; Takahashi *et al.*, 1967; Wheeler and Koohmaraie, 1994).
   b. Increasingly lower ATP concentrations are needed to dissociate the actin/myosin complex after 2 or 7 days of postmortem storage than is needed to dissociate the actin/myosin complex from at-death muscle (Fujimaki *et al.*, 1965).
   c. The Mg^{2+}-modified ATPase activity of actomyosin decreases back to that of actomyosin from at-death myosin after 24 - 36 hr postmortem (Goll and Robson 1967; Ouali and Valin, 1980; Robson *et al.*, 1967).

Brief treatment with m-calpain causes a 20 - 30% increase in the Mg^{2+}-modified ATPase activity of myofibrils; longer incubation with m-calpain causes the Mg^{2+}-modified ATPase activity to decrease back to control levels (Suzuki and Goll, 1974).
Brief tryptic treatment causes lengthening of contracted myofibrils (Goll *et al.*, 1971).
Could some of the postmortem changes in the actin/myosin interaction be the result of very limited proteolysis, perhaps involving the N-terminus of myosin LC1?

B. Proteolytic Cleavage of Certain Cytoskeletal Proteins.
1. Severing the connection of titin and nebulin to the Z-disk.
   a. Conversion of titin from the T1 to T2 form (Huff-Lonergan *et al.*, 1995; Taylor *et al.*, 1995a) is caused by cleavage of the long titin molecule near its N-terminus (the Z-disk end); much of this degradation occurs during the first 72 hr postmortem (Bandman and Zdanis, 1988; Huff-Lonergan *et al.*, 1995; Taylor *et al.*, 1995a).
   b. Nebulin is rapidly degraded in postmortem muscle; most nebulin is cleaved to small fragments within 72 hr postmortem (Huff-Lonergan *et al.*, 1995; Taylor *et al.*, 1995a).
2. Degradation of costameres and of intermediate filaments that link myofibrils to the sarcolemma and to each other within the muscle fiber.
   a. Costamere proteins such as vinculin, dystrophin, and desmin are rapidly degraded in postmortem muscle and degradation of vinculin is related to tenderness (Taylor *et al.*, 1995a).
   b. Desmin is a principal component of the intermediate filaments linking adjacent myofibrils to each other and is largely degraded within 3 days postmortem (Robson *et al.*, 1991).

The calpains quickly convert titin from the T1 to the T2 form and rapidly degrade nebulin in myofibrils (Thompson *et al.*, 1993).
The calpains rapidly degrade dystrophin (Cottin *et al.*, 1992), vinculin (Goll *et al.*, 1983b), and desmin (O'Shea *et al.*, 1979).
Therefore, it seems likely that the calpains are responsible for the proteolytic degradation of these cytoskeletal proteins.

Fig. 2. Proposed causes for the decrease in toughness that occurs after the first 24-36 hours postmortem and some of the evidence supporting these suggestions.

(Milligan *et al.*, 1990). This information has shown that, in the rigor (no ATP) state, which was the only state at which electron density maps could be obtained, at least four sites on the myosin head contact actin and that two adjacent actin monomers in the same strand of the double stranded actin filament are involved in the actin/myosin rigor interaction (Rayment *et al.*, 1993a; Table 1). In addition to the interaction sites listed in Table 1, recent studies have suggested that the N-terminus of the myosin light chain 1 (the large essential light chain) may also be able to interact with actin after the initial myosin/actin contacts are made.

Table 1. Sites of interaction between myosin S1 and actin[a]

Amino acid residues in myosin	Nature of interaction	Corresponding amino acid residues in actin
Tyr 626 - Gln 647 (interface of 50 - (interface of 50 - and 20 kDa fragments)	Ionic interactions involving 5 Lys residues in S1 and 6 COOH groups in actin; this is the "weak interaction"	Asp 1 - Asp 24
Pro 529 - Lys 553 ("lower" portion of 50 kDa fragment)	Steric interaction involving hydrophobic residues	Ile 341 - Phe 352, Ala 144 - Thr 148, and His 40 - Gly 42
Arg 405 - Tyr 415 (loop in 50 kDa fragment)		Pro 332 - Glu 334
Lys 567 - His 578	Flexible loop, a number of ionic residues involved	Tyr 91 - Glu 100 (in the second actin monomer)

[a]Much of this information is based on data from Rayment *et al.* (1993a)

The x-ray crystallographic information together with a large number of other studies (see Lehrer, 1994; Squire, 1994; Yount, 1993, for recent reviews) have shown that at least two states of the actin/myosin interaction exist in living muscle, the weak-binding and the strong-binding state. The actin/myosin interaction undergoes a weak to strong binding transition during each contractile cycle with the strong-binding state being the force-producing state. Moreover, the weak-to strong-binding transition is a highly cooperative process that involves both conformational changes in the myosin head and changes in the thin filament. Briefly, these cooperative changes may be summarized somewhat simplistically as follows:

1. In resting muscle or in the non-force-producing state, myosin interacts weakly with actin; some of the myosin-binding sites on actin are sterically blocked by tropomyosin.

WHAT CAUSES THE DECREASE IN TOUGHNESS AFTER THE FIRST 24 - 36 HOURS POSTMORTEM?

The current evidence suggests that there are at least two causes for the decrease in muscle toughness following 24 - 36 hr postmortem (Fig. 3). The first of these, a weakening of the actin/myosin interaction, is related to the strengthening of the actin/myosin interaction that was proposed to cause the increase in toughness observed during the first 24 - 36 hr (Fig. 1, 2). As described in the discussion outlining the reasons for believing that the increase in toughness is due to changes in the actin/myosin interaction, there also has been very little attention given to possible changes in actin/myosin interaction that would cause a decrease in toughness after 24 - 36 hr postmortem. Hence, the evidence that weakening of the actin/myosin interaction causes a decrease in muscle toughness during postmortem storage is indirect.

It seems likely, however, that most of the decrease in toughness observed between 24 and 72 hr in Fig. 1 is the result of a weakening of the actin/myosin interaction. First, the extent of this decrease seems to be related to the extent of the increase in toughness during the first 24 hr, *i.e.*, a large increase in toughness is usually accompanied by a large decrease in toughness and *vice versa*. This suggests that there has been some kind of a reversal of the changes that caused the toughness. If the decrease in toughness between 24 and 72 hr (Fig. 1) were due to proteolysis, the extent of this decrease would be approximately the same, regardless of the initial increase in toughness. Second, a number of studies have shown that rigor-shortened myofibrils lengthen slightly after 24 - 36 hr postmortem (Fig. 1, 3). Furthermore, Herlihy *et al.* (1972) found that shortened myofibrils in mouse biceps brachii muscle could be stretched from 1.7 μm back to 2.0 μm *in situ* by extending the fore legs of the mouse. Electron microscopy showed that this stretching was accompanied by a sliding of filaments and reappearance of the H-zone in rigor-shortened muscle. Stretching was also accompanied by a variable amount of muscle damage suggesting that some fibers had a larger proportion of the strong-binding actin/myosin interaction than others, as discussed in the preceding section. Third, it was shown a number of years ago (Fujimaki *et al.*, 1965) that lower concentrations of ATP are required to dissociate the actin/myosin complex in myosin B (actomyosin) prepared from postmortem muscle than in myosin B prepared from myosin immediately after death (Fig. 3). This observation again suggests that the actin/myosin interaction is weakened during postmortem storage. Finally, the Mg^{2+}-modified ATPase activity of actomyosin prepared from muscle after 24 - 36 hr postmortem decreases from the elevated activity observed at 24 hr postmortem back to the activity of actomyosin prepared from at-death muscle (Fig. 3). Although the nature of the changes that cause these alterations in postmortem muscle is unknown, collectively they indicate that the actin/myosin interaction has been affected and probably weakened after 24 - 36 hr of postmortem storage. It is reasonable to believe that this weakening leads to a decrease in toughness. Postmortem weakening of the actin/myosin interaction could be caused by very limited proteolysis of the loop from Lys 567 to His 578 (Table 1), by cleavage of the N-terminus of LC1 so it cannot contact actin, by oxidation of certain SH groups, or by a combination of these and other possibilities. It was reported a number of years ago that brief trypsin treatment of myofibrils that had been "supercontracted" by addition of ATP *in vitro* resulted in a lengthening of the severely shortened sarcomeres from an average length of 1.2 μm to an average length of 1.7 μm (Goll *et al.*. 1971). Moreover, trypsin treatment for 1-2 min caused a 10-30% increase in the Mg^{2+}-modified ATPase activity of reconstituted actomyosin and greatly increased the rate of superprecipitation of these same preparations (Goll *et al.*, 1971). Longer periods of trypsin treatment resulted in a decrease in the Mg^{2+}-modified ATPase of reconstituted actomyosin. These changes are similar to the changes observed in postmortem muscle. After the discovery of calpain, it was shown that incubating myofibrils with partly purified calpain for 1-2 min causes a 25-35% increase in the Mg^{2+}-modified ATPase activity of these myofibrils, whereas longer periods of calpain treatment results in a decrease in Mg^{2+}-modified ATPase activity (Suzuki and Goll, 1974). Consequently, a proteolytic enzyme endogenous to muscle is also capable of inducing changes in the properties of actomyosin that resemble those changes that occur during postmortem storage (Suzuki and Goll, 1974). Because the nature of the actin/myosin interaction in postmortem muscle has not been studied in detail, it is possible at present only to speculate on the nature of these changes and the factors that cause them.

Proteolytic Cleavage of Certain Cytoskeletal Proteins.

It has long been believed that proteolysis has an important role in postmortem tenderization (Goll *et al.*, 1983a), and recent evidence has shown that the calpain system seems to be responsible for most of the proteolytic degradation of myofibrillar proteins that occurs during the first 7-10 days postmortem (Goll *et al.*, 1970; 1983a; 1992; Koohmaraie, 1988; 1992; Stromer *et al.*, 1974; Taylor *et al.*. 1995a), the period when most of changes in muscle toughness occur. Although the prevailing view suggests that postmortem tenderization is caused by calpain-induced degradation of the Z-disk, recent evidence has shown that the Z-disk remains quite robust during the first 7-10 days postmortem. Myofibrils prepared by homogenization and used in the myofibril fragmentation index assay are not sheared at the Z-disk but break in the I-band area next to the Z-disk, even after 7 days postmortem (Taylor *et al.*, 1995a). The most commonly observed ultrastructural changes in postmortem muscle are breaks or "tears" in the I-band area, often at the level of the N_2 band or near the Z-disk, and loss of the costamere structure, resulting in separation of the sarcolemmal membrane from the myofibril (Taylor *et al.*, 1995a, 1995b).

These recent ultrastructural observations together with Western blot analyses showing that costamere proteins such as desmin, vinculin, and dystrophin and the giant cytoskeletal proteins, nebulin and titin, are rapidly degraded during postmortem storage has led to the conclusion that proteolytically-induced tenderization in muscle is not the result of Z-disk degradation but rather is caused by calpain-cleavage of specific cytoskeletal proteins (Huff-Longergan *et al.*, 1995; Taylor *et al.*, 1995a). These recent findings can be summarized as follows.

1. During the first 3-4 days postmortem, both costameres and N_2 lines are almost completely degraded in postmortem muscle. Costamere degradation results in separation of the sarcolemma from the myofibrils and may increase tenderness.

2. All of the proteins known to constitute costameres are very susceptible to calpain degradation, and three of them, desmin, dystrophin, and vinculin, are rapidly degraded during postmortem storage. Degradation of these three proteins occurs during the same time that costamere structures are degraded (Taylor *et al.*, 1995a).

3. Nebulin and titin have been shown to anchor thin and thick filaments, respectively, to the Z-disk. Calpain rapidly degrades nebulin to smaller polypeptides and rapidly removes the N-terminal part of titin, which is the Z-disk end of the long titin molecule, thereby severing the connection of titin with the Z-disk (Thompson *et al.*, 1993). Severing the connection of nebulin and titin with the Z-disk would significantly weaken the myofibril lattice and would increase tenderness. The N_2 line evidently is an area where the titin and nebulin molecules may coalesce in the I-band; calpains rapidly destroys the N_2 line structure (Goll *et al.*, 1991), and the postmortem disappearance of the N_2 line occurs at the same time that nebulin and titin are degraded in postmortem muscle.

Consequently, this recent evidence indicates that the proteolytically associated tenderization that occurs during postmortem storage is caused by calpain cleavage of costamere proteins; calpain-induced selective cleavages of nebulin and titin, destroying the attachments of these two proteins to the Z-disk; and calpain cleavage of intermediate filament proteins such as desmin that form intermyofibrillar linkages connecting adjacent myofibrils within a muscle fiber rather than frank Z-disk degradation.

CONCLUSIONS

Postmortem tenderization is a complex process that almost certainly is affected by a large number of factors including rate of glycolysis, rate of pH decline, osmolarity of muscle cells, temperature, and genetic factors inherent to the animal, among others. It seems possible that these many factors exert their effects through a few basic processes: 1) the nature of the actin/myosin interaction, especially the proportion of myosin cross-bridges that are in a strong-binding state, and the subsequent weakening of this interaction; and 2) calpain-induced cleavage of certain cytoskeletal proteins that constitute the costameres, intermyofibrillar linkages, and Z-disk/sarcomere connections. Although postmortem proteolysis has been studied extensively, the nature of the actin/myosin interaction in postmortem muscle has not received much attention. Studies such as determining the amount and type of nucleotide bound to myosin in postmortem muscle, learning the effect of ATP on the interactions of actin and myosin from postmortem muscle, and determining whether very limited proteolytic cleavages of actin or myosin occur during postmortem storage should indicate whether changes in the actin-myosin interaction have an important role in postmortem tenderization.

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