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Muscle Proteinases and Meat Aging

Mohammad Koohmaraie

USRA-ARS, Roman L. Hruska US Meat Animal Research Center, P.O. Box 166, Clay Center, Nebraska 68933, USA

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ABSTRACT

The purpose of this manuscript is to review and summarize the results of experiments conducted in our laboratory regarding the mechanism of meat tenderization during post mortem storage of carcasses at refrigerated temperatures. Clearly, the conversion of muscle to meat and the subsequent tenderization process are complex phenomena and much remains to be learned. However, current experimental data suggest that proteolysis of key myofibrillar proteins is the principal reason for improvement in meat tenderness during post mortem storage. Speculatively, the weakening and/or degradation of Z-disks and degradation of desmin (and probably degradation of titin) are responsible for the increased fragility of myofibrils during post mortem storage. There is substantial experimental evidence suggesting that the calpain proteolytic system is responsible for post mortem proteolysis that results in meat tenderization. Calpain is the only proteolytic system that has all of the characteristics that are necessary for bringing about post mortem changes that result in meat tenderization. Undoubtedly, other factors (such as rate of pH and temperature decline during rigor development, ionic strength and others) influence the process. However, we believe that the rate and extent of post mortem proteolysis best explain the observed variation in tenderness at a constant age. Therefore, research efforts should be direct toward understanding the regulation of the calpain proteolytic system in post mortem muscle.

INTRODUCTION

The improvement in meat tenderness during post mortem storage of carcasses at refrigerated temperatures has been known since the turn

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of the century (Lehmann, 1907). However, the mechanism through which these changes are brought about has remained elusive and controversial.

Because consumers consider tenderness to be the most important organoleptic characteristic of meat, it is essential that we understand the mechanism of meat tenderization so that methodologies can be developed to manipulate the process advantageously. Undoubtedly, the mechanism of tenderization is complex and affected by a number of variables. Over the years, the following variables have been proposed to influence meat tenderness: animal age and gender, rate of glycolysis, amount and solubility of collagen, sarcomere length, ionic strengh, and degradation of myofibrillar proteins.

The purpose of this manuscript is to review and summarize the results of experiments conducted in this laboratory related to the role of endogenous proteinases in the post mortem tenderization process. The manuscript is not intended to be a comprehensive review of all factors affecting meat tenderness. Throughout this manuscript, post mortem storage is defined as holding of carcasses at refrigerated temperatures and should be distinguished from other methods such as high temperature conditioning. Also, our research efforts have been directed toward understanding the causes of variation in meat tenderness of animals slaughtered at similar ages and should not be extrapolated to other situations. For additional information, the reader is referred to a number of review papers written on this subject (Marsh, 1977; Penny, 1980; Robson *et al.*, 1981; Davey, 1983; Dutson, 1983; Goll *et al.*, 1983; Marsh, 1983; Robson & Huiatt, 1983; Robson, 1984; Dutson & Pearson, 1985; Greaser, 1986; Pearson, 1986; Asghar & Bhatti, 1987; Koomaraie, 1988; Koohmaraie (1992*a*,*b*; Marsh *et al.*, 1988; Ouali, 1990; Ouali, in press). Throughout this manuscript, due to space limitation, the original information source will be given only when the subject has not been addressed in these review articles.

Post mortem changes in skeletal muscle

Because of the number of recent reviews in this area (see above), only important changes as they relate to the objective of this manuscript will be discussed. During post mortem storage of carcasses, numerous changes occur in skeletal muscle, some of which result in the loss of tissue integrity which is translated into the improvement of meat tenderness. These changes include:

(1) Z-disk weakening and/or degradation which leads to fragmentation of myofibrils.

(2) Degradation of desmin which leads to fragmentation of myofibrils, probably through disruption of transverse crosslinking between myofibrils.
(3) Degradation of titin. Titin filaments, which connect myosin filaments, along their length, from the M-line to the Z-disk (Wang, 1985). Titin has been proposed to be involved in the regulation of the elasticity of the muscle (Wang *et al.*, 1991). When titin was preferentially destroyed by radiation (Horowits *et al.*, 1986) or controlled proteolysis (Yoshioka *et al.*, 1986), the tension of stretched muscle was reduced. Therefore, degradation of titin during post mortem storage would cause weakening of myofibril strength and, therefore, improvement in meat tenderness.
(4) Degradation of nebulin. Because of the location of nebulin in myofibrils (I-band), it is not clear how nebulin degradation will affect meat tenderness.
(5) Disappearance of troponin-T and simultaneous appearance of polypeptides with molecular weight 28–32 kDa. This is the most noticeable and reported change that occurs during post mortem storage. However, because of the location of troponin-T in myofibrils (i.e. I-band) it is doubtful that degradation of tropinin-T by itself will have a direct effect on meat tenderness. But, these changes (i.e. the disappearance of tropinin-T and appearance of 28–32 kDa polypeptides) seem to be good indicators of the extent of post mortem proteolysis. The origin of the 28–32 kDa polypeptides has not been determined and, therefore, these polypeptides could be from the degradation of any myofibrillar proteins with molecular mass greater than 32 kDa.
(6) Appearance of a polypeptide with a molecular weight of 95 kDa. Neither the origin nor its significance to meat tenderness is known.
(7) Perhaps the most important observation is that the major contractile proteins (myosin and actin) are not affected.

One of the important changes that occurs in the tissue is the ease of fragmentation of myofibrils under controlled homogenization, which does not occur in the unaged tissue. This phenomenon, first reported by Davey & Gilbert (1969), which is measured routinely by a number of laboratories, is called Myofibril Fragmentation Index (MFI) and is highly related to meat tenderness (for review see Parrish, 1977). Speculatively, the weakening and/or degradation of Z-disks and degradation of desmin (and probably degradation of titin) are responsible for the increased fragility of myofibrils during post mortem storage.

Mechanisms of post mortem changes in muscle tissue

Clearly, the changes discussed in the previous section are all produced by proteolytic action; and, therefore, the changes resulting in improvement in meat tenderness are produced by endogenous proteinases. This is not a new concept. As early at 1917, Hoagland *et al.* concluded that proteolysis was an important factor contributing to post mortem changes in skeletal muscle, including meat tenderness. Because the proteolytic changes that occur in skeletal muscle during post mortem storage are minimal, the classical methods failed to detect these changes, and, therefore, the pro-teolysis hypothesis was questionable until the advent of gel electrophoresis. Gel electrophoresis made it possible to demonstrate these minimal, yet significant, changes and, therefore, give credibility to the proteolysis theory. Based on the observation reported by numerous laboratories, Penny (1980) concluded that, 'there is no doubt that proteolytic enzymes are responsible for the changes during conditioning (post mortem storage).' Tenderness could also be improved by changes in the connective tissue; however, because proteolytic changes in collagen (the principle component of the connective tissue) during post mortem storage comparable to those of myofibrillar proteins have not been observed (Tarrant, 1987), the role of collagen is questionable at best. In addition, while collagen may affect meat tenderness of the same muscle obtained from young (e.g. 1 year old) and old animals (e.g. 7 years old), it is doubtful if any significant differences exist in collagen solubility of muscle (e.g. *longissimus dorsi*) from animals of similar age. We, therefore, have concluded that differences in the rate of myofibrillar protein degradation are the principal reason for the observed variation in tenderness of meat obtained from animals of similar age. Indeed, there is substantial experimental evidence in support of this theory. Some of these include: (1) infusion of carcasses with zinc chloride, which inhibits post mortem proteolysis, also inhibits the tenderization process (Koohmaraie, 1990); (2) muscle from β-adrenergic agonist-fed animals which undergo minimal or no post mortem proteolysis, also remains tough compared to muscle from untreated animals (Fiems *et al.*, 1990; Kretchmar *et al.*, 1990; Koohmaraie *et al.*, 1991*a*; Koohmaraie & Shackelford, 1991; Wheeler & Koohmaraie, in press); (3) differences in the extent of post mortem proteolysis are probably the reason for differences in meat tenderness between *Bos taurus* and *Bos indicus* breeds of cattle (Whipple *et al.*, 1990; Shackelford *et al.*, 1991); and (4) differences in rate of post mortem proteolysis are probably the reason for the observed differences in meat tenderness from pigs, sheep and cattle (Koohmaraie *et al.*, 1991*b*).

Proteinases involved in post mortem proteolysis

Currently, while the proteolysis theory is accepted by most, the question of proteinases involved has remained controversial. Proteinases should have the following characteristics to be considered as possible candidates for bringing about post mortem changes that result in meat tenderization: (1) be located within the skeletal muscle cell (for details see Goll *et al.*, 1983); (2) have access to the substrate (i.e. myofibrils); and (3) have the ability to degrade the same proteins that are degraded during post mortem storage. The proteolytic systems that have the potential to be involved in post mortem proteolysis include: (1) the lysosomal cathepsins; (2) the multicatalytic proteinase complex (MCP); and (3) the calpains. Current experimental evidence suggests that lysosomal cathepsins do not play a significant role in post mortem proteolysis. Some of this experimental evidence includes: (1) post mortem storage has no effect on actin and myosin, while among myofibrillar proteins these are the primary substrates for lysosomal cathepsins; (2) lysosomal cathepsins are normally located within lysosomes and must, therefore, be released to have access to myofibrils. While it has been assumed that during post mortem storage lysosomes are ruptured, thereby releasing cathepsins into the cytosol, there is no experimental evidence to support this hypothesis. On the contrary, the only experiment that has examined the accuracy of this hypothesis indicates that even after electrical stimulation and 28 days of storage at 4°C, lysosomal enzymes were still localized within lysosomes (LaCourt *et al.*, 1986). Because of these and other reasons (Koohmaraie, 1988; 1990; 1992*a*), we have concluded that lysomal cathepsins do not play a significant role in post mortem proteolysis.

The second candidate is the MCP. Until recently, no experimental data were available to determine the role of MCP in this process. We have recently purified and characterized MCP from ovine skeletal muscle (Koohmaraie, 1992*d*). Our results indicate that ovine skeletal muscle indeed contains MCP with similar biochemical properties of MCP isolated from other mammalian and non-mammalian tissues. Some of the characteristics of ovine skeletal muscle MCP include: (1) molecular mass of 600 kDa which dissociates into a series of low molecular polypeptides ranging from 21 to 31 kDa; (2) it has no proteolytic activity as isolated from tissue, but it can be reversibly activated by heating at 60°C and with pre-treatment with low concentration of sodium dodecyl sulfate (SDS); (3) maximum proteolytic activity is observed at pH 7·5–8·0 and 45°C, it retains about 2% of its maximum activity at 5°C and pH 7·5, and about 22% of its maximum activity at pH 5·5 and 45°C; and

(4) calcium chloride has no effect on its proteolytic activity. For more details of MCP characteristics, the reader is referred to excellent reviews (Rivett, 1989; Orlowski, 1990). Results of our experiments indicate that even after activation (by heating or incubation in the presence of SDS), myofibrils were a very poor substrate for MCP. We incubated myofibrils with MCP and analyzed the effects with SDS-PAGE, phase and electron microscopy. Morphologically, MCP had no effect on myofibrils and based on SDS-PAGE data, MCP only degraded troponin-C and myosin light chain-1 and -2. These results indicated that MCP does not play a major role in post mortem proteolysis that results in meat tenderization.

In contrast to lysosomal proteinases and MCP, substantial experimental evidence exists suggesting that calpains are the primary proteolytic system responsible for post mortem proteolysis that results in meat tenderization. There is considerable experimental evidence indicating that calcium causes weakening and(or) degradation of Z-disks. The first report that documented the role of calcium in Z-disk weakening was that of Davey & Gilbert (1969). They reported that EDTA inhibited the weakening and disappearance of Z-disks and speculated that EDTA probably acts by chelating calcium. Busch *et al.* (1972) provided further support by demonstrating that myofibril fragmentation was inhibited by EDTA and was induced by calcium. Koohmaraie *et al.* (1988*a*) also demonstrated that all post mortem changes were completed within 24 h when muscle slices were incubated with a buffer solution containing calcium chloride and none of the post mortem changes occurred if EDTA was included in the buffer instead of calcium chloride.

Acceleration of post mortem proteolysis and tenderisation processes

Based on the observation reported in the previous section, it became evident that the elevation of calcium concentration in post mortem muscle is the cause of post mortem tenderization. To determine whether these observation could be repeated *in situ*, lamb carcasses were infused with a solution of calcium chloride to increase the intracellular concentration of calcium (Koohmaraie *et al.*, 1988*b*). Results indicated that post mortem proteolysis and tenderization were accelerated such that ultimate tenderness values were obtained within 24 h of post mortem storage as opposed to 7–14 days in non-infused carcasses. Though these experiments were designed to activate calpains (Koohmaraie *et al.*, 1988*a*,*b*; for review see Koohmaraie, 1988; 1992*a*), we do not know the precise mechanism(s) through which calcium chloride infusion accelerates post mortem proteolysis and tenderness. However, we believe the primary mode of action of calcium is through activation of calpains (for review

see Koohmaraie, 1988; 1992*a*). There is no doubt that calcium will induce changes other than activation of calpains, however, these changes may not affect meat tenderness (Taylor & Etherington, 1991; Whipple & Koohmaraie, submitted). Regardless of the mechanism of action, calcium chloride infusion of whole carcasses or injection of cuts of meat is a very effective method of rapidly producing uniformly tender meat. The process has been very effective under all experimental conditions thus far examined, including: lamb carcasses (Koohmaraie *et al.*, 1988*b*, 1989; St. Angelo *et al.*, 1991); *Bos indicus* carcasses Koohmaraie *et al.*, 1990); beef round muscles (Wheeler *et al.*, 1991); and mature cow carcasses (Morgan *et al.*, 1991); post-rigor injection of beef *longissimus dorsi* muscle (Wheeler *et al.*, 1992) and post-rigor marination of beef steaks (Whipple & Koohmaraie, 1992).

Current experimental data suggest that of the three proteolytic systems discussed, the calpain proteolytic system is the best possible candidate for causing post mortem proteolysis and tenderization because: (1) calpains have an absolute requirement for calcium and, clearly, the evaluation of calcium is the reason for the observed changes in post mortem muscle that result in tenderization; (2) calcium has no effect on the activity of MCP (Koohmaraie, 1992*d*); (3) calcium not only does not stimulate cathepsins activity, but at 10 mM inhibits cathepsin B activity by 39% (Barrett, 1973); (4) of these three proteolytic systems, the calpains are the only system that precisely reproduces post mortem changes under in-vitro conditions; and (5) of these three proteolytic systems, calpain and MCP are localized in the cytosol, but cathepsins are located within lysosomes. While the precise location of MCP in relation to myofibrils is not known, calpains are localized primarily at the Z-disk (for μ-calpain: 66% on Z-disk, 20% in I-band, and 14% in A-band; Kumamoto *et al.*, 1992).

CONCLUSIONS

Clearly, the process of conversion of muscle to meat and the subsequent tenderization process is complex and much remains to be learned. Over the years, a number of factors have been proposed to influence the ultimate meat tenderness. These include rate of glycolysis, pH, sarcomere length, amount and solubility of collagen and post mortem proteolysis. Undoubtedly, all of these parameters and their interaction need to be considered to explain the observed variation in meat tenderness. Based on our current knowledge of the process, post mortem proteolysis is the most important of all and most other factors (such as rate of glycolysis, ultimate pH, rate of temperature decline) affect meat tenderness by their

influence on the proteolytic systems involved. Factors such as ionic strength and collagen solubility are probably involved in the process, but they cannot explain the differences observed in tenderness of meat obtained from animals of similar age. Rather, these factors set the so-called 'background toughness' (Marsh, 1977). For any theory to be valid, it must be able to explain the large variation observed in tenderness of meat from animals of identical backgrounds (e.g. variation observed in tenderness of meat from *Bos taurus* cattle slaughtered at 16–18 months of age). Clearly, collagen cannot explain these differences and neither can ionic strength. While ionic strength of post mortem muscle is double that of living tissue (equivalent to 165 and 280 mM NaCl; Winger & Pope, 1980–81) and that such a significant elevation in ionic strength would be expected to affect myofibrils which may lead to their instability, the question that needs to be addressed is: why would ionic strength be different in *longissimus dorsi* of animals of identical backgrounds. Let us examine two cases to see which of these three factors (collagen, ionic strength, and post mortem proteolysis) can explain the variation in meat tenderness.

Case 1: Tenderness of meat from β-adrenergic agonist-fed (BAA; L644,969 from Merck Sharp and Dohme) animals. When lambs (Kretchmar *et al.*, 1990; Koohmaraie & Shackelford, 1991; Koohmaraie *et al.*, 1991*a*) and steers (Wheeler & Koohmaraie, 1992) are fed BAA, the meat from their carcasses is tough and post mortem storage has no effect on it (i.e. it remains tough). Ultimate pH is proposed to cause elevation of ionic strength in post mortem muscle. The correlation between ionic strength and pH is reported to be about −0·90 (for review see Oulai, 1990). Since BAA feeding does not affected the ultimate pH of the muscle, one would expect that BAA should not effect ionic strength, yet meat from BAA-fed animals is not affect by post mortem storage (i.e. remains tough). Because the half-life of collagen is in excess of 200 days and BAA effects are evident after 2 weeks of feeding (Pringle *et al.*, 1993), toughness of meat from BAA-fed animals cannot be due to changes in collagen and, indeed, out data support this speculation (Koohmaraie & Shackelford, 1991). However, all data collected thus far indicate that lack of post mortem proteolysis is the reason for the toughness of meat from BAA-fed animals (Fiems *et al.*, 1990; Kretchmar *et al.*, 1990; Koohmaraie & Shackelford, 1991; Koohmaraie *et al.*, 1991*a*; Wheeler & Koohmaraie, submitted).

Case 2: Toughness of meat from *Bos indicus* as compared to meat from *Bos taurus*. It has clearly been documented that meat obtained from *Bos indicus* carcasses is significantly tougher than that obtained from *Bos taurus* carcasses (Ramsey *et al.*, 1961; Koch *et al.*, 1982; Peacock *et al.*,

1982; Crouse *et al.*, 1987; 1989). To identify the cause of these differences in tenderness, we (Whipple *et al.*, 1990; Shackelford *et al.*, 1991) determined a number of factors that are proposed to affect tenderness in meat obtained from *Bos taurus* and *Bos indicus* cattle raised under identical management practices (similar climate, diet, and slaughter age). Of all factors examined (pH and temperature decline, muscle composition, fiber-type composition and diameter, amount and solubility of collagen, sarcomere length, MFI and SDS-PAGE of myofibrillar proteins during post mortem storage), only post mortem proteolysis, determined by MFI and SDS-PAGE, was different. Because neither the pattern of pH decline nor ultimate pH was different, it was concluded that ionic strength (see above discussion on the relationship between pH and ionic strength) is not the cause of differences in tenderness of meat from these breeds of cattle. Data clearly suggest that the reduced rate of post mortem proteolysis in meat from *Bos indicus* carcasses was the only logical explanation for differences in tenderness of meat from these two breeds of cattle (Wheeler *et al.*, 1990; Whipple *et al.*, 1990).

Clearly, these two examples indicate that differences in the rate of post mortem proteolysis is the best explanation for the observed variation in meat tenderness.

Current experimental data suggest that the calpain proteolytic system is probably responsible for post mortem changes that result in improvement in meat tenderness. To manipulate the process, we must understand how calpains are regulated in post mortem muscle. Using a modelling approach, Dransfield (1992) demonstrated that 68% of the variation in toughness was accounted for by variation in μ-calpain activity. Identification of the regulatory mechanism for calpain in post mortem muscle could enable us to manipulate the process and, thereby, enhance the tenderization process. Recently, we have begun to determine the regulation of calpain in post mortem muscle (Koohmaraie, 1992*c*). Result indicate that pH and temperature, two key changes that occur in muscle during rigor development, have a dramatic effect on the inactivation of μ-calpain. We hope that such experimental approaches would lead to development of alternative carcass handling procedures during slaughter and early post mortem to maximize calpain potential and, therefore, improvement in the rate of tenderization.

Finally, we must develop the methodology to predict meat tenderness as early *post mortem* as possible and, ultimately, prior to slaughter. The development of such methodology would enable us to decide how a particular carcass should be marketed, depending upon its predicted eating quality. Variation in meat tenderness at the consumer level is one of the biggest problems that our industry is facing now. It is sobering to

realize that the only time that actual meat tenderness is known is when it is eaten. We must, therefore, collectively concentrate our efforts in developing the necessary methodology to predict meat tenderness prior to eating. We are placing special emphasis on knowledge acquisition to develop such technology.

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