Postmortem Changes in Muscle Extracellular Matrix Proteins

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Introduction

It has long been recognized that meat tenderness is related to both the components inside the muscle fibers (predominantly the myofibril) and the connective tissues surrounding the fibers (predominantly collagen). It has also been shown that the tenderness of meat increases with time of storage postmortem. Most work attempting to determine the mechanism of this postmortem tenderization has concentrated on the changes that occur in the myofibril that pack the muscle cell fiber. Although this part of muscle clearly has a major role in meat tenderness, the components of the extracellular matrix may play a previously unrecognized role as well. In this review I plan to summarize briefly the properties of the different components of the muscle extracellular matrix, their macroscopic, microscopic, and ultra structural locations; potential proteases and their inhibitors which might play a role in possible degradation processes, and current information about postmortem changes in these molecules and structures. The literature on the extracellular matrix is vast; most of it relates to matrix material in non-muscle tissues. I will not attempt to cover all this material, but will try to provide references to key reviews. The reader is referred to the excellent book by Bailey and Light (1989) and the reviews by Light (1987), Bailey (1989), and McCormick (1989) for more extensive background material which includes a meat perspective.

Components of the Extracellular Matrix in Muscle

Muscle cells are mechanically attached to bones via a complex mixture of macromolecules which is collectively referred to as connective tissue. There are three major layers called the epimysium, the perimysium, and the endomysium (Figure 1). The epimysium is a thick layer which surrounds a whole muscle. Within a muscle there are further divisions between groups of muscle fibers (called bundles) that are surrounded by the perimysium. Blood vessels, fat cells (this is the region where marbling occurs), and nerves are embedded in this layer. A still finer layer of material encapsulates each individual muscle cell and is referred to as the endomysium. The epimysium and perimysium are visible to the naked eye, and the number and diameter of muscle fiber in the muscle bundles is often referred to as the muscle texture. The endomysium can only be observed microscopically, and it too has substructure when viewed with the electron microscope. Surrounding each muscle fiber is a coating referred to as the basal lamina or basal membrane (see Inoue, 1989; Sanes, 1994; Adachi et al., 1997 for reviews and the paper by Nishimura et al., 1997 describing this region in bovine muscle). A diagram showing the arrangement of this structure is shown in Figure 2. Immediately adjacent to the muscle protein-phospholipid containing cell membrane (called the *plasmalemma*) is a zone referred to as the *lamina lucida* or *lamina rara*. The next layer above this is called the *lamina densa.* The *lamina lucida* and *lamina densa* are so named because the former has sparse filamentous components while the latter is more densely packed. The next distal layer is the *reticular lamina*, which although it is less densely packed, contains larger diameter collagen fibrils. All of these layers contain a complex mixture of proteins, glycoproteins, and proteoglycans. A list of the major biopolymers in the muscle extracellular matrix is shown in Figure 3.

Collagen is the major protein component in terms of amounts with values of approximately 10 to 30 mg/g of muscle wet weight. However, it only composes about 40% of the dry weight of the extracellular matrix (Bailey and Light, 1989). There have been 19 different collagen types identified, with each named by a Roman numeral in the order discovered (see Kadler, 1994; Prockop and Kivirikko, 1995 for review). Collagen type I is found in highest amounts in the body and make up the largest proportion in muscle (Bailey and Light, 1989). Type III constitutes about one third of the total bovine intramuscular collagen (Burson and Hunt, 1986; Bailey and Light, 1989). These two types, along with types II,

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Reciprocal Meat Conference Proceedings, Volume 50, 1997.

FIGURE 1.

Connective tissue layers in muscle

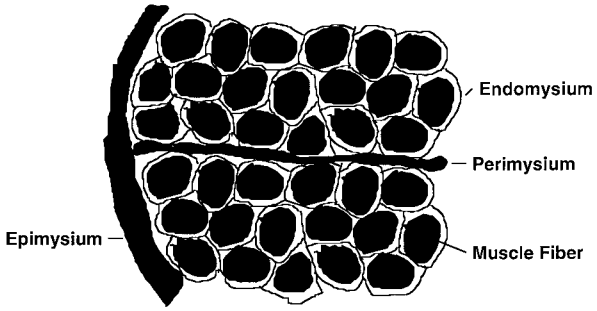
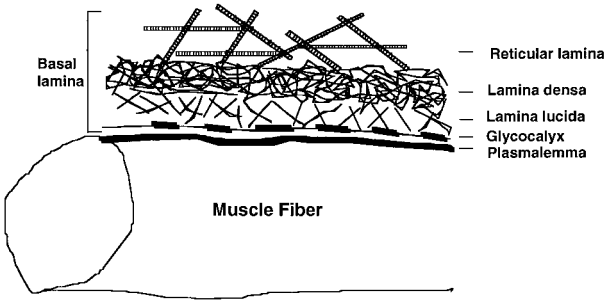


FIGURE 2.

Diagram of Basal lamina



V, and XI, are classified as fibril forming collagens since they will spontaneously assemble into the quarter staggered structures with 67 nm periodicity. Approximately 90% of the intramuscular collagen is found in the perimysium (McCormick, 1994). Type IV, in contrast to the fibril forming types, assembles into a "chicken-wire" network type arrangement. Type VI collagen is found in high concentrations near the muscle-tendon junctions (Senga et al., 1995). The different collagen types have different distributions in the connective tissue layers with type I dominating in the epimysium, type III being concentrated in the perimysium, and type IV being the major component in the endomysium/ basal lamina (Figure 3) (Bailey and Sims, 1977; Bailey et al., 1979). The collagen amount changes little during muscle growth (Goll et al., 1963); the levels are actually somewhat higher in muscle from very young animals (Bailey and Light, 1989). Muscle collagen is synthesized in both actual muscle cells (Beach et al., 1982; Beach et al., 1985; Rao et al., 1985) and in fibroblasts between the muscle cells. Collagen goes through a complex series of changes after synthesis (Figure 4). First, the polypeptide chains undergo hydroxylation of a large number of prolines and several lysines plus several sugar molecules are added (glycosylation). Second, a collagen molecule is formed by the assembly of three polypeptide chains into a triple helix for most of the length. This procollagen is then secreted into the extracellular space. Procollagen peptidases remove segments at both the amino and carboxyl ends. A few of the lysine and hydroxylysine side chain amino groups are converted to aldehydes by oxidative deamination by the enzyme lysyl oxidase. These aldehyde groups condense with a side chain amino group from an adjacent polypeptide (both intramolecular or intermolecular) to form a covalent bond (see Bailey, 1989 and Kadler, 1994 for review). If the aldehyde is formed from a lysine and a hydroxylysine aldehyde, the bond is called an aldimine (Figure 5). If the bond is between a hydroxylysine and a hydroxylysine aldehyde, then an oxoimine is formed. The presence of these compounds can be determined by reducing the bonds with sodium borohydride and measuring the resulting hydoxylysinonorleucine (HLNL) or dihydroxylysinonorleucine (DHLNL) respectively (Bailey and Light, 1989). In the tissue a third lysine side chain can combine with either of the aldimine or oxo-imine to form a ring structure called a pyridinoline; again if a the cross link is derived from 3 lysines the compound is called lysylpyridinoline (LP) and if from 2 lysines and a hydroxylysine is called hydroxylysylpyridinoline (HP). Bovine muscle collagen contains about 0.25 moles of HP and 0.03 moles of LP per mole of collagen(Field et al., 1996; Bosselmann et al., 1995). Additional cross-links can be formed involving a histidine side chain along with a pair of lysines. The formation of compounds derived from three side chains, which are referred to as mature cross-links, is believed to be the major factor responsible for stabilizing the insoluble macromolecular collagen complex. It is still not clear whether all the important collagen cross-linking compounds have been identified. However, the increased levels of crosslinks with age probably accounts for the decreased collagen solubility in older animals (Reagan et al., 1976).

The elastin content is very low–approximately 1/20th of the collagen (Bailey and Light, 1989; Karatzas and Zarkadas, 1989). Its primary location is the epimysium and perimysium (Rowe, 1986). Both coarse (5 to 10 mm) and fine (1 to 2 mm) fibers were found, with the fine fibers following the same direction as the coarse collagen fibrils (Rowe, 1986). The content of elastin in muscle is quite variable with the bovine semitendinosus having 37% of its connective tissue as elastin while in longissimus it make-up less than 2% of this fraction (Bendall, 1967). In muscles such as the *longissimus*, the protein is quite rare in the perimysium and may be restricted mainly to the walls of small blood vessels. Elastin is similar to collagen in that it has intermolecular cross-links formed from lysine side chains and the cross-links are formed subsequent to lysyl oxidase modification. However, the cross-linked amino acids are different with desmosine and isodesmosine being the major products; both are formed from 4 lysine side chains.

Several glycoproteins play an important role in extracellular matrix structure and assembly (Paulsson, 1992). These

FIGURE 3.

Components of the Muscle Extracellular Matrix

Name	Properites	Location		
		Epimysium	Perimysium	Endomysium
Collagens				
Type I	Fibrous	***	*	*
Type III	Fibrous	*	***	*
Type IV	Network	-	-	***
Type V	Fibrous	*	*	**
Type VI	Fibrous	*	**	*
Type VII	Anchoring	-	*	*
Elastin	Elastic	**	**	*
Laminin	Heterotrimer	-	-	***
Fibronectin	Homodimer	*	*	*
Perlecan	Large proteoglycan (PG)			**
Decorin	Smal Leu-rich PG	-	**	**
Tenascin-X	Large glycoprotein	-	*	*
Heparin sulfate PG		-	-	*

proteins are secreted by muscle cells as evidenced by their presence in the extracellular matrix of cultured muscle cell lines (Olwin and Hall, 1986; Rao et al., 1985; Brandan et al., 1991). Laminin is a large protein containing 3 non-identical polypeptides (370 kDa, 205 kDa, 220 kDa) with a total size of greater than 800,000 daltons (Timpl and Brown, 1994; Malinda and Kleinman, 1996). The protein is disulfide linked and possesses a cross-like shape when viewed by rotary shadowing. There are a large number of isoforms; the major muscle type, also called laminin-2 or merosin, has a subunit composition of $a_2 b_1 g_1$. Different isoforms are found associated with the surface of most of the muscle cells, at the neuromuscular junction and at the myotendon junctions (Wakayama et al., 1997). Laminin is found in high concentrations in the basal lamina (30% by weight–Bailey and Light, 1989) and is believed to function as a link between type IV collagen and dystroglycan embedded in the muscle cell membrane (Ervasti and Campbell, 1993; Hopf and Hoch, 1996; Cohen et al., 1997; Denzer et al., 1997).

Fibronectin is a homodimeric protein with the two chains disulfide linked (Kosmehl et al.,1996; Poets et al., 1996; Johansson et al., 1997). It is found in the endomysium and perimysium (Gulati et al., 1982; Bertolotto et al., 1983). The protein binds to laminin, collagen, and the transmembrane protein integrin (Schnapp et al., 1995).

Tenascin-X is a 500 kD protein expressed predominantly in heart and skeletal muscle (Matsumoto et al., 1994; Burch et al., 1995; Chiquet-Ehrismann, 1995; Schoser and Goebel, 1996). The protein binds heparin but not laminin, fibronectin or collagen. It is believed to play an important role in muscle development.

Proteoglycans are covalent complexes of proteins with several classes of polysaccharide. A list of the different glycosaminoglycans (GAG) is shown in Figure 6. The chain lengths vary considerably. These components are highly negatively charged and believed to confer water binding and viscosity to the extracellular matrix. Several proteoglycans, including perlecan (Couchman et al., 1995) and decorin (Andrade et al., 1991; Eggen et al., 1994; Iozzo, 1997) are found in muscle. Decorin is present in highest concentration and appears to constitute about 2/3 of the total proteoglycans in bovine skeletal muscle (Eggen et al., 1994). The protein core of muscle decorin appears to be identical with the fibroblast form but the attached GAG components are different (Andrade and Brandan, 1991). Decorin has a 40,000 dalton protein core and attached dermatin and chondroitin sulfate groups to give a total size of about 90,000 daltons. The protein has been shown to bind to both collagen and fibronectin. It has been proposed that decorin coating protects collagen from proteolytic attack, but if re-

FIGURE 4.

Synthesis and Assembly of Collagen

Intracellular

- Synthesis of procollagen chains
- Hydroxylation of prolines and lysines, glycosylation
- Formation of triple helix collagen molecules

Extracellular

- Proteolytic removal of N- and C- terminal peptides
- Assembly of collagen molecules into fibrils
- Oxidative deamination of Lys side chains by lysyl oxidase
- Condensation of Lys or OHLys aldehydes with Lys
- Formation of mature crosslinks

FIGURE 5.

Crosslinking Compounds in Collagen

Precursors	Class name	Reduced compound	Abbreviation
Lys & Lys aldehyde	Aldimine	Lysinonorleucine	LNL
Lys & OHLys aldehyde	Oxo-imine	Hydroxylysinonorleucine	HLNL
OHLys & OHLys aldehyde	Oxo-imine	Dihydroxylysinonorleucine	DHLNL
2 OHLys & 1 Lys	Pyridinoline	Lysylpyridinoline	LP
3 OHLys	Pyridinoline	Hydroxylysylpyridinoline	HP
His & OHLys & Lys		Histidinohydroxylysinorleucine	

moved itself by proteolysis, the collagen may become susceptible to proteolysis (Pedersen et al., 1996).

In addition to these components, a large group of enzymes referred to as matrix metalloproteinases (MMP) are found associated with the macromolecules outside cells. At least 20 different members of this class have been identified (Baramova and Foidart, 1995; Corcoran et al., 1995; Murphy and Knauper, 1997); a partial list is shown in Figure 7. All of these proteases are structurally related (Figure 8). All are glycoproteins, contain both a structural zinc atom and another near the active site, are calcium activated, and have maximal activity near neutral pH. All of the extracellular matrix macromolecules can be degraded by one or more members of this protein class. In spite of different substrate preferences, all have sequence similarities. These proteases are secreted as inactive precursor proteins; proteolytic activation may occur by a separate group of proteases, by other members of the MMP class, or autocatalytically. They bind tightly to several of the extracellular matrix proteins including collagen and laminin. These enzymes have been found in muscle (Chin and Werb, 1997) and some have been shown to be secreted by satellite cells (Guerin and Holland, 1995).

There is also a group of small molecular weight (~20,000 daltons) proteins which inhibit the action of the MMPs. These proteins are called tissue inhibitors of matrix metalloproteinases and abbreviated as TIMP (Denhardt et al., 1993; Leco et al., 1997). All are glycoproteins and each member of the 4 types identified to date (TIMP-1, -2, -3, -4) have 12 cysteine residues in identical positions. The TIMPs are also secreted with a signal peptide which is cleaved to release the inhibitory activity. Thus there are multiple levels for control of this extracellular matrix system: transcription of MMP genes, translation of MMP message, proteolysis of pro MMPs, transcription of TIMP genes, translation of TIMP message, proteolysis of proTIMPs.

Postmortem Changes in Extracellular Matrix Proteins

For many years it was believed that the collagen in meat remained unchanged during postmortem aging. This was based on the failure to see any significant increase in water soluble hydroxyproline containing compounds even after one year of aseptic storage at 37°C (Sharp, 1959 quoted in Lawrie, 1991). Evidence that at least a portion of collagen is highly resistant to postmortem degradation comes from electron microscopic observations on skeletal muscle from a human cadaver after 2100 years underground storage in China (Anon, 1976). These workers found collagen fibrils, with their distinctive banding pattern, were still visible in the tissue. Attempts to find increases in the proportion of soluble collagen during meat aging have been largely unsuccessful (Jeremiah and Martin, 1981; Gerrard et al., 1987; Seidemann et al., 1987). The neutral and salt soluble collagen proportions also remained the same through 21 days of storage of bovine longissimus (Pierson and Fox, 1976). The proportion of soluble collagen decreased from 5.36% to 4.23% during the first 21 days of postmortem storage in a different study (Kruggel and Field, 1971).

The content of free hydroxyproline, however, nearly doubles in bovine muscle within the first 14 days postmortem (Feidt et al., 1996). Since the only proteins containing this amino acid are collagen and elastin and since the collagen content is about 20 fold greater than elastin, it appears likely that collagen is the source of this increase. The failure to see increases in soluble collagen may reflect the fact that any soluble collagen formed is rapidly degraded to the amino acid level by endogenous exo- and endopeptidases. It should be noted that in spite of this increase, the proportion of collagen degraded must be very small since numerous studies have found no statistical decline in total collagen during aging.

The thermal stability of collagen also decreases approximately 5 to 8°C during the first 7 to 10 days postmortem (Judge and Aberle, 1982; Bernal and Stanley, 1986). This change may be related to changes in the glucosaminoglycans (GAG) (see below) associated with the collagen since it has been shown that the collagen shrinkage temperature is very sensitive to the GAG content (Bailey and Light, 1989).

The structure of the endomysium/basal lamina changes little from death to postrigor when viewed by scanning electron microscopy (Rowe, 1989). The only significant early postmortem change is the appearance of holes in the plasmalemma. However, scanning electron microscopy showed

FIGURE 6.

Properties of the Glycosaminoglycans (GAG)

Type	Repeating Unit Composition	Repeating Unit No.
Hyaluronic acid	Glucuronic acid- N-acetyl glucosamine	50-10000
Chondroitin sulfate	Glucuronic acid- N-acetyl galactosamine 6-SO4	20-60
Dermatin sulfate	Iduronic acid - N-acetyl galactosamine 4-SO4	30-80
Heparin sulfate	Copolymers of: Glucuronic acid - N-acetyl glucosamine & Iduronic acid - N-acetyl glucosamine	
Keratin sulfate	N-acetyl galactosamine -galactose	5-40

Adapted from Bailey and Light, 1989

FIGURE 7.

Key Matrix Metalloproteinases

MMP	Enzyme name	Molecular wt.	Known substrates
MMP-1	Interstitial collagenase	57/52 kD	Collagen I, II, III, VII, X, gelatin, PG core protein
MMP-2	Gelatinase A	72 kD	Gelatin, collagen IV, V, IX, X, elastin, fibronectin, PG core
MMP-3	Stromelysin-1	60/55 kD	PG core , fibronectin, laminin, elastin, collagen IV, V, IX, X, proMMP-1
MMP-7	Matrilysin	28 kD	Fibronectin, laminin, gelatin, collagen IV, PG core, proMMP-1, proMMP-2
MMP-8	Collagenase	75/65 kD	Same as MMP-1
MMP-9	Gelatinase B	92 kD	Gelatin, collagen IV, elastin, PG core
MMP-10	Stromelysin-2	60/55 kD	Same as MMP-3
MT-MMP	Membrane-type	63 kD	MMP-2 activation?

degradation of the endomysial regions of bovine muscle after 4 days postmortem (Varriano-Marston et al., 1976). Recent work has demonstrated extensive structural damage to the perimysium and endomysium with more extended aging (Nishimura et al., 1996). Changes were minor during the first 10 days postmortem, but progressive deterioration was observed between 14 and 28 days. The 100 to 200 µm sheets of collagen fibrils in the perimysium appears to break down into small collagen fibrils with diameters of 4 to 8 µm. The endomysium honeycomb structure developed holes and the fine collagen network became less dense. The authors proposed that degradation of the glycoproteins and proteoglcans which bind to the collagen fibrils might be responsible for the altered structural appearance of the extracellular matrix. Nishimura and coworkers also proposed that postmortem tenderization occurred primarily in the myofibrillar component during the first 10 days after death but changes were mainly in the extracellular compartment subsequently.

Although there is evidence that elastin does turn over in living muscle (approximately 13% of the precursor labeled lysine administered at 5 days of age in the rat appear in the excreted desmosine/isodesmosine at one year of age–Morris and Stone, 1995; Stone et al., 1997), it appears unlikely elastin degradation has much to do with postmortem tenderization since its content is so low in muscle and the protein is so resistant to numerous types of proteases. The most active protease against elastin (elastase) may be secreted by neutrophils in the extracellular matrix (Morris and Stone, 1995). Even autoclaving the protein does not change it's physical properties significantly (Lillie et al., 1994).

Postmortem changes in the proteoglycan compounds of muscle have been described recently. McIntosh (1967) showed that the relative viscosity of the mucoproteins declined and the soluble hexosamines increased during 28 days of aging. Nishimura and coworkers (1996) also found that the hexuronic acid containing proteoglycans decreased dramatically with time postmortem. The levels of high molecular weight PGs dropped from approximately 38 mg/100 g of muscle at death to 16 at 7 days postmortem. By 28 days after death, the PG content was approximately 2 mg/100 g. These workers also demonstrated the conversion of high molecular weight Alcian blue staining components on SDS gels to lower weight bands with decreasing staining intensity with postmortem storage. Transmission electron microscopic observation of the perimysium after Cuprolinic Blue staining demonstrated decreased staining intensity at later postmortem storage times. Scanning electron microscopy also showed a reduction of globular components which coat the collagen fibrils after postmortem storage. Eggen and Ekholdt (1995) have shown by gel filtration analysis that the high molecular weight GAG containing compounds in bovine muscle decline and smaller components increase during 21 days of postmortem storage. They also demonstrated that the decorin content declined postmortem. I could find no information on postmortem changes in laminin or fibronectin even though these glycoproteins make up a significant part of the extracellular matrix.

The proteases responsible for the postmortem changes in extracellular matrix components have not been identified. Early work suggested that catheptic enzymes may be responsible. Dutson and Lawrie (1974) showed that beta-glucuronidase activity increased three fold between 1 and 14 days postmortem in beef muscle. Wu and coworkers (1981) saw beta-galactosidase and beta-glucuronidase activity, both lysosomal enzymes, increased with postmortem storage time. These two enzymes increased the digestion of collagen by collagenase *in vitro*. It was suggested that breakdown of collagen postmortem may be accelerated by lysosomal glycosidases. Stanton and Light (1988) demonstrated that the changes in collagen peptides that occurred postmortem was similar to that obtained by *in vitro* incubation with crude spleen cathepsins. Stanton and Light (1987) also found that the proportion of perimysial collagen that could be extracted in denaturing solvents increased significantly after 14 days of aging.

A list of possible proteases and glucosidases which might break down the extracellular matrix postmortem is shown in Figure 9. These systems have only been briefly examined to date or have not been explored at all. Clearly more work needs to be done on postmortem changes in the extracellular matrix to better define its importance in relation to meat tenderness.

FIGURE 8.

Characteristics of the Matrix Metalloproteinases

Zinc ion at active site

Active at neutral pH

Secreted in latent form, proteolytically activiated

Glycoproteins

Amino acid sequence similarity

Inhibited by specific inhibitors (TIMPs)

Degrade at least one component of the extracellular matrix

FIGURE 9.

Possible Mechanisms of Postmortem Extracellular Matrix Degradation

1. **Cathepsins and glucuronidases from muscle fibers**
2. **Elastase from neutrophils**
3. **Matrix metalloproteinases**
4. **Plasmin from residual blood**

Acknowledgments

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison. Muscle Biology manuscript number 364.

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