Role of Proteinases and Inhibitors in Postmortem Muscle Protein Degradation

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Introduction

The lysosomal system contains a large number (15-20) of proteinases (cathepsins) within the membrane limited organelle and represents one of several pathways for intracellular degradation of muscle protein in vivo. The complement of proteinases within the lysosomal class is extensive in diversity of proteins hydrolyzed. The enzyme activities include exo- and endo-proteinases and, while they all share a common acidic pH optimum (3-4), many exhibit broad ranges of activity up to near neutrality.

The hypothesis that positive changes in meat texture result from proteolysis occurring during postmortem aging is one which has been widely ascribed to over time (Whitaker, 1959). Lysosomal proteinases were among the first enzymes to be investigated and initially thought to play a pivotal role in the process (Parrish and Bailey, 1966). However, with the discovery of other proteolytic systems such as the calpains (formerly CAF, Dayton et. al., 1975), relative importance of cathepsins has diminished in the recent past.

The relative contributions of calpain or cathepsin enzyme systems to the process putatively associated with tenderization has been debated extensively (Goll et. al., 1984; Kopp and Valin, 1980; Dutson, 1983; Etherington et. al., 1981; 1984; Ouali, 1990) and is beyond the scope of this presentation. There is considerable evidence based on in-vitro studies of calpain's action on myofibrils (Zeece et. al., 1986a), examination of myofibrillar changes induced postmortem (Olson et. al., 1977) and studies correlating enzyme activities with objective measurements of tenderness (Koohmaraie, 1988) that calpains play the predominant role. However, it should be kept in mind that cathepsins are also capable of rapidly degrading myofibrillar proteins and, unlike calpains, can also degrade connective tissue proteins (Etherington, 1984). It has also been suggested that calpains and cathepsins may function in some synergistic way to bring about the desired result (Ouali, 1990).

The attributes of cathepsins provide potential for significant contribution to postmortem proteolysis and meat texture and, thus, should not be overlooked. However, there are several obstacles to this involvement. The first includes the question of cathepsin containment within the lysosome during postmortem storage. It is obvious that enzymes confined within a vesicle would not exert any effect on muscle proteins. The extent to which the lysosomal membrane breaks down and releases catheptic enzymes during the aging process is not well established. Secondly, once lysosomal enzymes are released into the sarcoplasm, there are endogenous inhibitors present which could block their action.

Therefore, the purpose of this work is to summarize information concerning the properties of cathepsins, inhibitors of this class of proteinases, and implications for their role in postmortem protein degradation.

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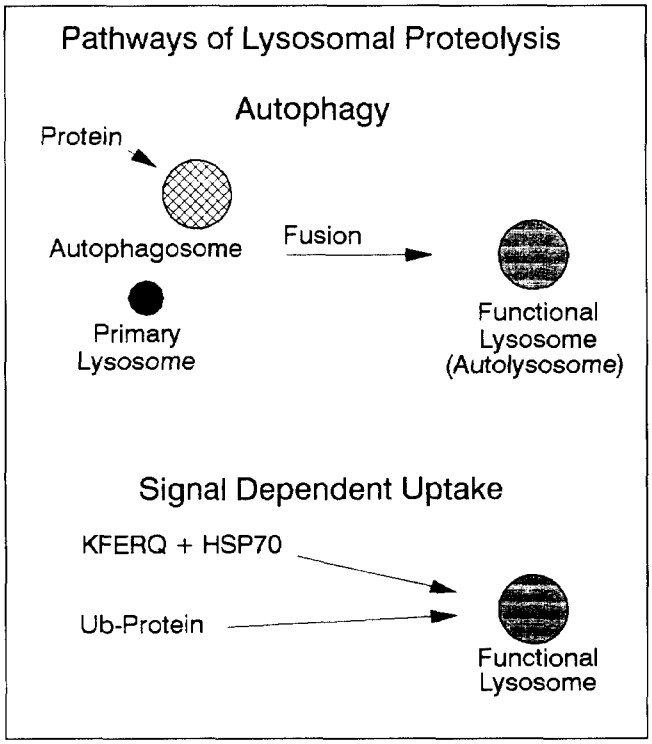
Lysosomal Enzymes in Muscle

Lysosomes have been identified in most cell types and specifically in muscle (Canonico and Bird, 1970). They are believed to function in vivo, in normal protein turnover. The system is greatly activated in response to unfavorable conditions, such as starvation and injury. Lysosomes are formed through an invagination of the golgi which pinches off a portion of the membrane containing bound hydrolytic enzymes (Holtzman, 1989). Lysosomal vesicles have a strongly acidic internal pH which is maintained by an ATP driven proton pump. There is also evidence of an active transport mechanism for cysteine (Pisoni et. al., 1990).

The mechanism by which proteins are taken into and degraded by the lysosomal system is not well characterized in muscle. One possible mechanism for in-vivo degradation is that proteins are digested by an autophagic process (Fig 1). In autophagy, cytoplasmic proteins are engulfed into a vacuole to become an autophagosome. The autophagosome fuses with a primary lysosome containing its complement of hydrolases and is then called an autolysosome (Pfeifer, 1987). The mechanism of protein degradation in the autophagic system is sometimes referred to as "bulk" protein degradation and logically should result in uniform protein halflives. Muscle proteins, however, exhibit widely divergent halflives and the role of the lysosomal system for basal protein turnover (especially myofibrillar) is thus thought to be a minor one. In addition, the long-used argument against lysosomal involvement in myofibrillar protein degradation is that myofibrils or their fragments have not been observed ultrastructurally within autophagic vacuoles (Goll et. al., 1989).

Alternatively, proteins may be selectively degraded by lysosomes through a signal-dependent mechanism. Recently, it has been reported that many short-lived proteins

Figure 1

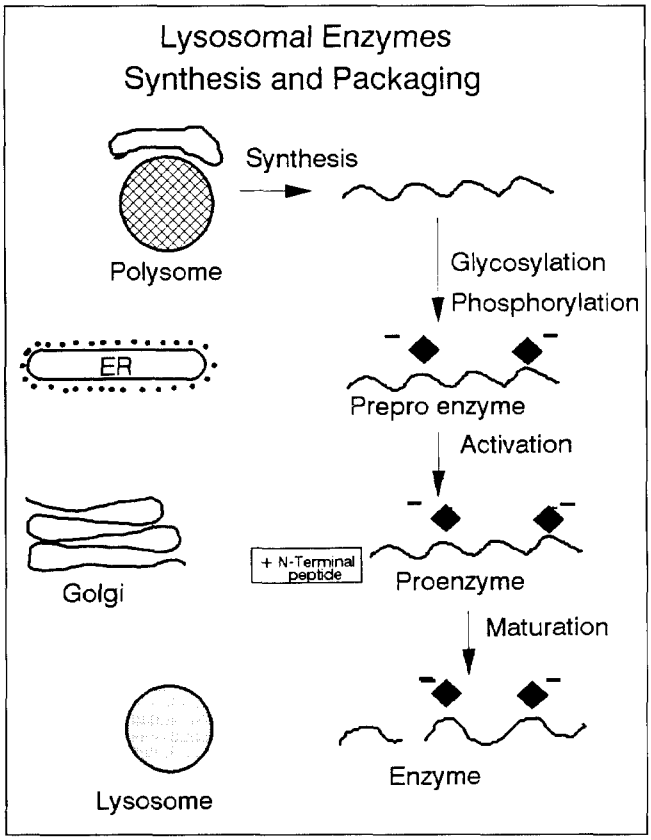


share a common 5 residue amino acid sequence, Lys-Phe-Glu-Arg-Gln (KFERQ) which provides a recognition point for lysosomal degradation (Dice, 1990). In this process, the KFERQ sequence is bound to a 70k-dalton protein produced by the heat shock mechanism (an HSP 70 protein) and the complex is rapidly degraded within the lysosome (Chiang et. al., 1989). A second signal-dependent process for targeting proteins for degradation by a lysosomal pathway involves ubiquitination of the protein. In this process, several molecules of the protein ubiquitin are covalently attached to the protein and the complex appears to be rapidly degraded in the lysosome (Doherty et. al., 1989). These newly-discovered processes for selective degradation show that protein degradation by lysosomes is more complex than the simple autophagic concept. This further suggests the possibility that disassembled myofibrillar proteins may also be degraded via a lysosomal pathway in vivo.

Synthesis and Trafficking of Cathepsins

Lysosomal enzymes appear to share a common process of synthesis and packaging into the organelle which can be illustrated using the sequence recently worked out for cathepsin D (Fig. 2) (Erickson, 1989). This enzyme is synthesized as a 36,779 M single polypeptide chain which contains a 20 residue signal peptide in its N-terminal region responsible for directing passage through the endoplasmic reticulum (ER). The enzyme is extensively glysopylated to produce a 53,000 M prepro form (Sameral et. al., 1986). The incorporated polysaccharides contain a large proportion of mannose residues which are subsequently phosphorylated. Limited proteolysis in the ER removes the signal sequence and other portions of the N-terminal. The enzyme

Figure 2



is then rapidly converted to the 51,000 and 48,000 dalton active forms. The function of phosphorylation of carbohydrate residues is putatively to enable recognition and binding of the enzyme to phosphomannosyl receptor proteins located in the golgi (Gieselmann et. al., 1983). The proteolytic modification steps which occur during translocation are thought to involve the participation of cathepsin B (Sameral et. al., 1989). Final maturation of the cathepsin occurs in an acidic compartment just prior to packaging into the lysosome. Exposure to an acidic environment is believed to promote autocatalytic activation, resulting in a two chain active molecule with M_r of approximately 14,000 and 32,000 for cathepsin D (Sameral et. al., 1986).

The attached carbohydrates and their sites of phosphorylation are an integral part of the process which ultimately delivers the cathepsin to the lysosome. An interesting consequence of alteration or deletions in phosphorylation sites of the mannose residues results in a secreted form of cathepsin. This is thought to be part of the process which produces a secreted form of cathepsin L from malignantly transformed mouse fibroblasts.

Properties of Catheptic Enzymes

There are 15 to 20 lysosomal cathepsins; however, only a few (principally endoproteinases) have been shown to be of significance to muscle protein degradation. This discussion will therefore focus on those with possible relevance to proteolysis in the postmortem muscle environment. The major cathepsins have been listed in Table 1 on the basis of

Table 1. Lysosomal Proteinases and Their Properties.

Cathepsin	Type	M_r	pH Optimum	Proteins Hydrolyzed	pI	Found in Muscle	E.C. Number
B[a]	Cysteine	27,411	3.5-6.0	M,A,TM,Tn-T,C	5.4	Yes	3.4.22.1
H	"	25,116	5.5-6.5	M (slowly)	7.1	Yes	3.4.22.16
J[b]	"	160 kDa	5.5-7.5[1]	*	5.8	?	—
L	"	23,720	3.0-6.0	T,M,N,α,A,D,TM, Tn-T,Tn-I,C,E	5.8-6.1	Yes	3.4.22.15
N	"	20,000	3.0-4.5	C	6.2	No	3.4.22.-
S[c]	"	24,000	6.0-7.5	C	7.0	No	3.4.22.-
T	"	35,000	6.0-7.5	*	—	No	3.4.21.16
D	Aspartyl	36,779	2.5-5.0	T,M,N,A,TM	5.5-6.5	Yes	3.4.23.5
E	"	90 kDa	<3.0	*	4.1-4.4	No	3.4.23.-
G[d]	Serine	27,500	6.5-8.0	C,E	10	No	3.4.21.20

Abbreviations as follows: Actin, A; α-Actinin, α; Collagen, C; Desmin, D; Elastin, E; Myosin, M; Nebulin, N; Titin, T; Tropomysin, TM; Troponins T & I, Tn-T, Tn-I.

[1]Cathepsin J may be identical with cathepsin C (dipeptidyl peptidase I) (Nikawa et al., 1992).

Most data regarding enzyme properties taken from Kirschke and Barrett (1987) with exceptions as noted:

[a]Deval et al. 1990; [b]Nikawa, et al. 1992; [c]Kirschke et al. 1989;

[d]Bohley and Seglen, 1992.

their catalytic mechanism: either cysteine, aspartyl or serine.

The cysteine-type cathepsins have a good potential for being active at postmortem muscle pH (5.3-5.5). While their optimas are low (3-4), they possess significant activity up to pH 7. This is particularly true for muscle forms of cathepsin L which, unlike its liver counterpart, does not completely inactivate until pH 7.5-8.0 (Zeece, unpublished results).

Cathepsin B is one of the more extensively researched cysteine-type proteinases and appears to be ubiquitously distributed in most mammalian tissues (Table 1). Precursor forms have not been well identified, but there are indications of a 41,000 dalton form in tumor cell cultures (Kirschke and Barrett, 1987). The mature form has a M_r of 30,000 (27,411 calculated from sequence data) and consists of two chains of 25,000 and 5,000 daltons (Deval et. al., 1990). There are multiple isoforms of the enzyme with pI's in the range of 5.4. Rat cathepsin B is composed of 252 amino acids, including 14 cysteine residues of which 12 are in disulfide linkages and one at the active site, Cys-25. This location for the active site places it in the light chain (Kirschke and Barrett, 1987).

Cathepsin B cleaves several synthetic substrates at pH 6.0. Among the best of these is carbobenzoxy-arginine-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-NMec) which is relatively specific for B (Table 2). In addition, it cleaves azocasein at pH 6.0 (Table 3). Cysteine-type cathepsins are very effectively inhibited by peptide chloromethyl ketones. Cathepsin B and L are rapidly and irreversibly inactivated by Pro-Phe-Arg-CH_2Cl. Similarly, diazomethane peptide derivatives such as Z-Phe-Phe(4-NO_2)-CHN_2 can readily inactivate cathepsins B and L. Virtually all cysteine-type cathepsins are inactivated by the epoxysuccinyl peptide E64 (L-3-carboxy-trans-2,3-epoxylpropyl-leucylamido[4-guanidino]butane).

Cathepsin H is also ubiquitous in mammalian tissues, has a M_r of 28,000 (25,116 from sequence data) daltons and contains 220 amino acids (Kirschke and Barrett, 1987). It contains 8 cysteine residues, 6 of which are in disulfide bonds and the active site cysteine is located at Cys-25. Cathepsin H is unique in that it possesses both endo- and exo-peptidase activities; with the exo activity limited to unblocked amino terminals. The optimum pH for both exo- and

Table 2. Comparison of Cathepsin Activities with Synthetic Substrates.

Substrate	Cathepsin* B			H			L			S		
	K_{cat}	K_m	K_{cat}/K_m	K_{cat}	K_m	K_{cat}/K_m	K_{cat}	K_m	K_{cat}/K_m	K_{cat}	K_m	K_{cat}/K_m
Arg-NMec	-	-	-	2.53	150	16.9[a]	-	-	-	-	-	-
Z Arg-Arg-NMec	1340	390	3420[b]	-	-	-	-	-	-	-	-	-
Z Phe-Arg-NMec	364	223	1632[b]	-	-	-	17.6	2.8	6286[b]	4.7	14.7	320[b]
Z Val-Val-Arg-NMec	14	31.9	450[b]	-	-	-	8.5	4.8	1771[b]	40.5	17.5	3214[b]

[a] Barrett and Kirschke (1981)

[b] Bromme et al. (1989)

*K_{cat} in sec^{-1}, K_m in μM and K_{cat}/K_m in mM^{-1} sec^{-1}

Table 3. Comparison of Cathepsin Activities with Protein Substrates.

	Cathepsin (Unit/μMole purified protein)[a]			
Protein Substrates	*B*	*H*	*L*	*S*
Collagen (insoluble) pH 3.5	65.2	0	558[c]	200[c]
Collagen (insoluble) pH 6.0	12.3	0	68.7	—
Azocasein pH 6.0	11.2[b]	1.85[b]	288[c]	382[c]
Azocasein pH 5.0 in 3.0 M urea	2.8	—	775[c]	697[c]

[a] from Barrett and Kirschke (1982)
[b] from Bromme et al. (1984)
[c] from Bromme et al. (1989)

endo-peptidase activity is 5.5 to 6.5 and its pI is 7.1 (Bohley and Seglen, 1992). Cathepsin H is also found in a two-chain form with M_r of approximately 23,000 and 5,000 daltons and the active site location is in the heavy chain.

Cathepsin H exhibits optimum activity with substrates such as Arg-NMec or azocasein at pH 6.5-6.8 (Tables 2 & 3). In general, however, its endoproteinase activity is weak and its true physiological function may come from its expoproteinase activity where it might aid in the further breakdown of peptides produced by the more potent endoproteinases such as cathepsin L (Bohley and Seglen, 1992).

Cathepsin J has been reported as a relatively high M_r (approximately 160 kdaltons) cysteine proteinase. It is not well characterized and recently it has been reported that cathepsin J and cathepsin C (dipeptidyl peptidase I) may be identical (Nikawa et. al., 1992).

Cathepsin L is found in muscle tissue and is probably the most potent protein hydrolase in the lysosome. The mature form has a M_r of 28,000 (23,720 from sequence data) and consists of 2 chains (22,000 and 6,000). The catalytic site (Cys-25) is located in the heavy chain (Bohley and Seglen, 1992). The enzyme is synthesized as an inactive precursor single polypeptide chain with a M_r of 38-40,000 daltons and is subsequently processed to an intermediate with M_r of about 34,000 daltons (Wiederanders and Kirschke, 1989).

Cathepsin L is most active with the synthetic substrate Z-Phe-Arg-NMec at pH 5.5 and degrades azocasein rapidly at pH 6.0 (Tables 2 & 3). The rate of azocasein degradation by cathepsin L is enhanced in the presence of 3 M urea. Thus, it is a useful way to discriminate between cathepsin B and L activities. Cathepsin L hydrolyzes proteins over a wide range of pH (3-7) in vitro. In addition to myofibrillar proteins, cathepsin L has very strong activity against various collagens and elastin (Kirschke and Barrett, 1987). Cathepsin L is effectively inhibited by various peptide chloromethyl ketone peptides and E64, as discussed above.

Cathepsin N is a cysteine proteinase with a M_r of approximately 34,000 and has been associated principally with spleen, placenta and leukocytes. It is a true collagenolytic enzyme and shows high activity at pH 3.5, but is not very active toward ZPhe-Arg-NMec. Cathepsin N also splits azocasein (Kirschke and Barrett, 1987; Bohley and Seglen, 1992).

Cathepsin S is a cysteine proteinase whose properties have only recently come to be known (Kirschke et. al., 1989; Bromme et. al., 1989). The tissue distribution was examined by immunoblotting and appears to be limited to kidney, spleen lymph nodes and lung. The enzyme has a M_r of about 24,000, a pI of 7.0 and is catalytically active at pH 6.0 to 7.5. It exhibits good activity with synthetic substrates such as Z-Val-Val-Arg-Nmec and much less activity with Z-Phe-Arg-NMec (Table 2). The relative activities of the cathepsin L and S can also be differentiated by means of pH. Cathepsin S hydrolyzes the Z-Phe-Arg-NMec substrate at pH 8.0, while cathepsin L does not (Bromme et. al., 1989). Cathepsin S also hydrolyzes azocasein (pH 5.0-8.0) in presence of 3 M urea, with rates (pH 5.0) similar to cathepsin L (Table 3).

Cathepsin T is a lysosomal cysteine proteinase occurring in the kidney, spleen, liver and intestine. It has a M_r of about 34,000 and digests azocasein. Even under optimal conditions, cathepsin T is a sluggish enzyme (Bohley and Seglen, 1992).

The two major aspartyl-type proteinases are cathepsins D and E. Cathepsin D is best known and appears to be ubiquitously distributed in mammalian cells and tissues. Cathepsin E, however, is primarily demonstrated in blood platelet and marrow. It has a relatively high M_r and is a dimer of 45,000 dalton subunits (Kirschke and Barrett, 1987). Cathepsin E has not been demonstrated to exist in muscle tissue.

Cathepsin D is an aspartyl proteinase with a M_r of approximately 42,000 for the mature (two-chain form). It is widely distributed in mammalian tissues and cells. The enzyme is synthesized as a higher M_r protein and subsequently processed as described above. The proteinase has very strong activity relative to other cathepsins (except perhaps L). Cathepsin D shows some substrate specificity and prefers to cleave between adjacent hydrophobic residues. It exhibits strong activity from pH 2.5 to 5.0 and there is little or no activity at pH 7.0 (Kirschke and Barrett, 1987). Cathepsin D activity is affected by temperature and exhibits a maximum activity with hemoglobin at temperatures of between 45 and 50C (pH 3.5); However, cold temperatures greatly inhibit activity. There is little or no activity at 5C (pH 5.5) (Zeece et. al., 1986b). Conversely, it shows considerable thermal stability and retains 50% of its activity after 30 min at 50C (pH 5.5) (Draper and Zeece, 1989).

Cathepsin D has been reported to cleave synthetic substrates such as pyroglutamyl-histidyl-pnitrophenylalanyl-phenylalanyl-alanyl-leucine amide (Pohl et. al., 1983). However, it is most routinely determined using the protein substrate hemoglobin at pH 2.8 to 4.0 (Barrett and Kirschke, 1981). Cathepsin D has been reported to cleave collagen (Scott and Pearson, 1978); however, recent investigations have not been able to repeat those results (Kirschke and Barrett, 1987). Cathepsin D is effectively inhibited by the microbial peptide pepstatin at low pH. The inhibition decreases with increasing pH and is essentially zero at pH 6.0 (Kirschke and Barrett, 1987).

Cathepsin G is the only serine-type proteinase presented in this review. It has a M_r of 30,000, very alkaline pI of 10 and exhibits optimum activity in the range of pH 6.5 to

8.0. Cathepsin G is not found in muscle. It is principally located in cell types associated with inflammatory response and is most noted for its ability to hydrolyze connective tissue proteins (Bohley and Seglen, 1992). Because cathepsin G's catalytic mechanism is of the serine type, its pH optimum is alkaline and thus has little potential for involvement postmortem.

Comparison of Cathepsin Action on Myofibrillar Proteins

The existence of lysosomes in muscle is now generally accepted and the effects of cathepsins on muscle proteins can therefore be considered valid possibilities. Myofibrillar protein degradation by cathepsins was first confirmed some time ago (Schwartz and Bird, 1977). A common characteristic of cathepsin action on myofibrils is degradation of myosin. This is in contrast to other nonlysosomal proteinases, such as the calpains, which do not degrade myosin (Goll et. al., 1984).

The hydrolysis of myosin heavy chain in vitro is slightly different depending on the cathepsin (B, D, or L) involved (Fig 3). Cathepsin D cleaves fast and slow myosin heavy chains at the rod-S1 junction to yield fragments of approximately 120,000 and 90,000 daltons (Dufour et. al., 1989; Zeece and Katoh, 1989). In contrast, cathepsin B was found to cleave fast and slow myosin heavy chains at the LMM-HMM junction. Cathepsin L cleaves myosin heavy chains first in the head-rod region as did cathepsin D. However, subsequent degradation was much more extensive and resulted in a number of soluble fragments in the 67-90,000 dalton region. In addition, it has been shown that the rates of degradation by these cathepsins is affected by the myosin isoform substrate. Fast muscle myosin is degraded at a substantially higher rate by either cathepsin B, D or L than slow myosin (Dufour et. al., 1989). However, cathepsin H only slightly degraded myosin heavy chain under the same conditions as used for the other cathepsins and is, therefore, considered much less effective.

Cathepsins also have substantial effects on other myofibrillar proteins (Table 1) . The large mass proteins of the myofibril, titin and nebulin are degraded by cathepsin D at pH 5.0 to 5.5 (Zeece and Katoh, 1989) and cathepsin L (Mikami et. al., 1987). Cathepsin L is the only proteinase of this group shown to degrade α-actinin at pH 5.0. This incubation resulted in a fragment of approximately 85,000 daltons (Matsukura et al 1981). Actin is susceptible to the action of both cathepsin B and D at pH 5.0 (D is the most active) and yields fragments of 35,000 and 12,000 daltons (Schwartz and Bird, 1977). Similarly, actin is hydrolyzed at pH 5.0 by cathepsin L to produce fragments of 40,000, 37,000 and 30,000 daltons after 24 hr of incubation (Matsukura et. al., 1981). Tropomyosin is degraded at pH 5.0 to 5.5 by cathepsins B, D or L (Noda et. al., 1981; Mikami et. al., 1987; Zeece and Katoh, .1989). Troponins, especially troponin T, are a site of action for cathepsin B, D or L (Matsukara et. al., 1981; Noda et. al., 1981; Zeece et. al., 1986b).

The action of these cathepsins on myofibrils results in fragments with M_r in the range of 30,000 daltons which are thought to be derived from troponin T. They are similar in size to those generated by the action of calpain on myofibrils and commonly observed in aged meat. Thus, these peptides are equated with proteolysis occurring as part of the

Figure 3

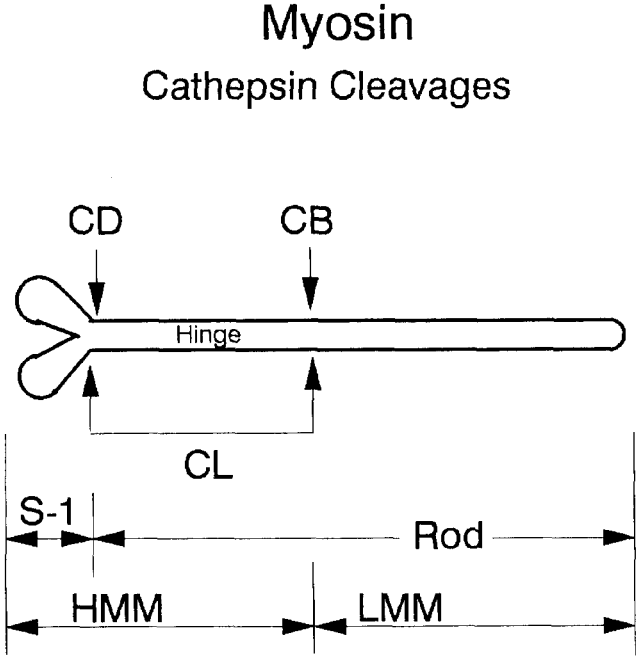


Table 4. Rates of Cathepsins B, L, N and S Activity on Connective Tissue.

Cathepsin	*Type of Connective Tissue Hydrolyzed*	K_{cat}/K_m[1] $(s^{-1}nM^{-1})$	*Cystatin*[2] *Inhibition* K_I *(nM)*
B	Collagen, Proteoglycans	1.5	10.6
L	Collagen, Proteoglycans, Elastin	44.0	0.021
N	Collagen	7.3	0.13
S	Collagen	0.21	0.11

[1]Type I collagen pH 3.4
[2]Chicken cystatin
From Maciewicz et al. (1987)

natural process of tenderization. It would be of interest to sequence these fragments so that positive identification can be made and provide clues as to the proteinase responsible for cleavage.

The incubation of myofibrils with cathepsins also has substantial morphological effects. When myofibrils are treated with cathepsin L (pH 5.0), the I band structure becomes disrupted, Z lines are lost, and fragmentation of the myofibril occurs at or near the Z line (Matsukara et. al., 1984; Mikami et. al., 1987). However, incubations of myofibrils with cathepsin D at pH 5.5 results primarily in A band alterations. There is a marked reduction in the length of the A band, but no increase in fragmentation is observed (Zeece et. al., 1986b; Zeece and Katoh, 1989).

Comparison of Cathepsin Action on Connective Tissue Proteins

Because connective tissue is a factor which contributes significantly to meat texture, it is appropriate to consider the collagenolytic activity intrinsic to several cathepsins (B,G,L,N and S) (Tables 3 & 4). First, cathepsin G can be omitted from further consideration as a potential participant in postmortem proteolysis for several reasons. Cathepsin G is unlikely to be involved in alteration of collagen in the postmortem environment because of its PH requirement. This serine proteinase may possess only limited activity at pH 5.5 (range is pH 6.5 to 8.0). Cathepsin G's principal target is cartilaginous collagen types and it is found primarily in neutrophils from which it is secreted in vivo as part of the inflammatory response mechanism (Bohley and Seglen, 1992). Until further information becomes available, its contribution to tenderization can be considered small.

The other cathepsins (B, L, N and S) have been shown to effectively hydrolyze a variety of connective tissue proteins. Cathepsin L acts on the nonhelical regions of type I collagen and results in depolymerization to individual alpha chains (Etherington et. al., 1980; 1981). Studies with spleen-derived enzymes have shown that cathepsins L and N have much higher activities toward type I collagen than cathepsins B and S (Table 4) (Maciewicz and Etherington, 1988). Cathepsins L and B also have substantial proteoglycan degrading abilities with L having the higher activity (Nguyen et. al., 1990). Similarly, cartilaginous collagens (types II, IX and XI) are extensively degraded by cathepsins B and L over a pH range of 3.5 to 7.0 and temperatures of 20C to 37C. Again, cathepsin L was found to have greater activity than B toward these collagen types (Maciewicz et. al., 1990).

Naturally occurring cysteine-type proteinase inhibitors (cystatins) for these enzymes have been identified. Preliminary experiments with the form found in chicken egg white have shown substantial inhibition of their activity. Effective inhibition of cathepsin L and N was achieved in the low nM range (Table 4) (Maciewicz et. al., 1987).

In summary, the strongest collagen degrading enzymes are cathepsins L and N, with cathepsin B being a close second. It is of interest to note that cathepsins B and L, but not N, are located in muscle. Assessment of their role in postmortem proteolysis will require location of these enzymes (and inhibitors) within muscle tissues.

Changes in Connective Tissue Postmortem

There has been a good deal of uncertainty and conflicting information concerning whether connective tissue is degraded during postmortem aging. The hypothesis has been that lysosomal enzymes, released in response to aging, are able to act on connective tissue proteins in muscle and thus contribute to increased tenderness (Moellar et. al., 1976; Dutson and Yeats, 1978; Wu et. al., 1981). In addition, since the action (in vitro) of catheptic enzymes on fibrous collagens results in soluble products, one should find an increase of these products in response to aging. However, this has never proved a very reliable indicator of tenderness.

Alternatively, it has been proposed by Etherington (1984) and Bailey and Light (1989) that proteolytic cleavages to perimysium and endomysium need not be extensive to bring about changes in structural integrity. For example, relatively few cleavages to the endomysial "net" which forms the major part of basement membrane could substantially reduce its structural integrity. Similarly, perimysial collagen cleaved at intermolecular cross-links could also be greatly altered. These investigators have strongly suggested that an increase in soluble collagen occurring as a result of postmortem degradation may be relatively small, difficult to measure and thus not a sensitive indicator. Alternatively, they propose that techniques such as isometric tension development, SDS-PAGE of isolated collagen and their CNBr fragments and immunological techniques be used.

Recently, evidence of proteolytic alteration of connective tissue during aging using electrophoretic methods has been reported (Stanton and Light, 1987; 1988; 1990). In addition, insoluble endomysial and perimysial collagen preparations treated with a spleen extract referred to as "crude cathepsins" resulted in similar proteolytic modifications. Incubations with crude cathepsins were performed at pH 5.5,(4C) for up to 24 hr. Approximately 10% to 15% of perimysial collagen was maximally solubilized and proteolytic damage was evident to collagen via SDS-PAGE and two-dimensional electrophoresis.

Cysteine Proteinase Inhibitors, the Cystatins

A class of potent cysteine-type proteinase inhibitor called cystatin has been discovered in muscle (Bige et. al., 1985). This discovery further complicates the question of whether cathepsins can participate in myofibrillar and/or connective tissue degradation postmortem.

The term "cystatin" has recently been adopted to refer to a group of homologous cysteine proteinase inhibitors which are evolutionarily related, but differ somewhat in molecular properties (Table 5) (Barrett, 1987). There are three distinct families of types of cysteine proteinase inhibitors which are recognized as belonging to the cystatin "super family." All of these proteins inhibit cysteine proteinases such as papain, and the lysosomal proteinases cathepsins B, H, and L. In addition, the kininogens have been shown to inhibit the calpains (Ishiguro et. al., 1987).

Family 1 cystatins (now referred to as cystatin A or B) are reported to be synonymous with stefins A and B. They contain approximately 100 amino acids and have a M_r of

Table 5. Properties of Cystatins.

Family 1	*Family 2*	*Family 3*
Cystatin A Stefin A	Cystatin 2 Bovine Colostrum	Kininogens
100 aa residue	115 aa residue	335 aa residue
10-11 kDaltons	12 kDaltons	60-140 kDaltons
No Trp, No disulphide bonds	2 disulphide loops	3 repeats of type 2 domain
Cystatin B	Extracellular	Extracellular
Stefin B		
Intracellular		

From Barrett (1987)

about 11,000. In general, cystatin A forms of inhibitor are slightly larger in M_r (12,000) and have lower pIs than cystatin B. Cystatins A and B do not contain trp and only cystatin B contains cys. Further comparison of the amino acid sequence of cystatins A and B also shows that both typically have N-terminal methionine (usually acetylated) and lack the signal peptide sequence required for secretion. Thus they are generally found intracellularly (Barrett, 1987).

Family 2 cystatins are slightly more complex and larger than those of family 1. They contain approximately 115 amino acid residues (approx. M_r 13,000). There are two intramolecular disulfide loops near the C-terminal end. Inhibitors representative of this class include human cystatin C, chicken eystatin (egg white), cystatin S and bovine colostrum cystatin (Barrett, 1987). Family 2 cystatins exist as multiple isoelectric variants with pIs in the range of 7 to 9. Thus they are somewhat more alkaline than Family 1 forms. They are also predominantly extracellular.

Family 3 cystatins are principally made up of the kininogens which are found in plasma. They are much larger than family 1 and 2 inhibitors (M_r 60,000 to 140,000) and essentially contain 3 replicas of the family 2 domain. In addition, family 3 cystatins are glycoproteins. The three domains of the kininogen molecule are not identical. The first segment has no inhibitory activity. The second segment inhibits papain, cathepsin L and calpain. The third inhibits some cysteine proteinases, but not calpain.

Family 1 and 2 cystatins are very effective inhibitors with Ki values in the low nanomolar range. They are generally believed to inhibit cathepsins B, H and L through a competitive type mechanism (Table 6) (Barrett, 1987). Family 3 cystatins also inhibit these cathepsins and calpain. However, the Ki is 1 to 2 orders of magnitude greater than that of type 1 and 2 inhibitors for the lysosomal enzymes. The interaction between enzyme and inhibitor (Family 1 & 2) involves the conserved region of the molecule (QVVAG, residues 53-57) and Gly-9. The glycine at position 9 is close to the inhibitory site in the molecule's native configuration and participates in binding to the enzyme by an electrostatic mechanism. This is known to be essential to inhibition because truncated cystatins (no Gly-9) have 1000-fold greater Ki.

Cystatins in Muscle

Schwartz and Bird (1977) first reported the presence of a cysteine proteinase inhibitor in rat skeletal muscle. They found that the amount of cathepsin B activity in a muscle extract increased with time up to about 24 hours. They further postulated the increase was due to the destruction of an endogenous inhibitor and isolated a crude inhibitor fraction on Sephadex G75, which contained two components of 12,500 and 62,000 daltons.

Subsequently, rat skeletal muscle was also reported to have several cysteine proteinase inhibitor forms. Size exclusion chromatography on Sephadex G100 resulted in forms with M_r of 52,000, 36,000, 28,000, 21,000 and 9,500 daltons which had varying degrees of inhibitory activity toward cathepsins B, L, and papain. The 9,500 and 52,000

Table 6. Inhibition of Cathepsins by Cystatins.[a]

	Cystatin				
Enzyme	*A*	*B*	*C*	*Chicken Cystatin*	*L-Kininogen*
Cathepsin					
B	8.2	73	0.25	1.7	600
H	0.31	0.58	0.28	0.064	1.2
L	1.3	0.23	0.005	0.019	0.017
Calpain	$>10^4$	$>10^4$	$>10^4$	$>10^4$	1.0

[a] K_1 values in nanomolar concentration, for inhibition of cysteine-type proteinases.
Data from A.J. Barrett (1987)

peaks inhibited all three enzymes. The 21,000 and 28,000 peaks inhibited papain, while the 36,000 peak inhibited cathepsin L. In addition, it was found that postmortem storage of rabbit skeletal muscle for 9 days resulted in little change in the amount of activity (Matsumoto et. al., 1983).

More recently, it has been reported that rabbit skeletal muscle contains predominantly two forms of cysteine proteinase inhibitor with M_r of 29,000 and 10,700. However, the 29,000 dalton form was converted to approximately 10,500 after reduction with 0.1% mercaptoethanol. Minor differences were reported in the inhibitory activity (inhibited cathepsin H most strongly) for the later form produced by reduction of the apparent trimer. The naturally isolated 10,700 dalton form inhibited cathepsin L most strongly. All forms were equally heat stable (Matshuishi et. al., 1988).

Cysteine proteinase inhibitors have also isolated from bovine skeletal muscle with M_r in the range of 62,000 and 14,000 (Bige et. al., 1985; Ouali et. al., 1986). Three variants of the 62,000 M_r form were reported and one of these may be a kininogen based on its extracellular location. Similarly, the 14,000 dalton form was found to exist as three charge variants with pIs of 6.2, 5.5, and a minor one of 5.1. The 14,000 dalton form inhibited cathepsin H more rapidly than cathepsin B. A competitive type inhibition mechanism was proposed for cathepsin H and noncompetitive type for cathepsin B. The authors further suggested that the inhibitor more closely resembled a family 1 cystatin (B) than cystatin C because of its acidic isoelectric points and lack of disulfide loops (no disulfide exchange could be demonstrated).

Cystatins function in vivo as regulators of cysteine proteinase activity. The precise location and mechanism by which cystatins function is still unclear but it is thought that they are distributed throughout the muscle cell and control unwanted proteolysis (Wood et. al., 1986; Barrett, 1987). The question of how cystatins in turn are down-regulated is an interesting one for meat science. It has been reported that cystatins can be inactivated by cathepsin D (Lenarcic et. al., 1991).

Cystatins Postmortem

Relatively little information is available concerning the effects of postmortem aging on cystatins. Matsumoto et. al., (1983) reported little change in inhibitor activity of rabbit skeletal muscle stored for up to 10 days. That general conclusion can also be made for beef muscle (Shackelford et. al., 1991). However, the level of cystatin inhibitor activity varies with species. Pork was found to have higher levels of cystatin than beef or lamb, but also became tender more quickly (Koohmaraie et. al., 1991).

In recent studies surveying the activity of several proteinases (cathepsins and calpains), inhibitors (calpastatin and cystatin) and correlations with objective measures of tenderness, it was found that the level of cystatin was significantly related (Shackelford et. al., 1991). The lack of change in cystatin activity during aging might be an explanation for the general lack of myofibrillar degradation by cathepsins postmortem. Cathepsin activity should be well favored (at least for L) in the postmortem environment and degradation of myosin heavy chain should be observable on SDS-PAGE. However, myosin degradation is not observed under normal aging conditions (Bandman and Zandis, 1988).

The question of cystatin's role postmortem may be a complex one. Cystatin exists in several forms, especially muscle (Schwartz and Bird, 1977; Bige et. al., 1985; Matsumoto et. al., 1983). Some forms in muscle extracts may originate from within the muscle cell, while others may have been derived from other tissues (e.g., blood and connective tissue). Therefore, assays of cystatin activity based on a simple homogenate may be misleading.

An experiment to monitor changes in cystatin inhibitor activity during postmortem storage of chicken muscle was conducted using two assay methods (Fig 4a & b). First, inhibitor activity was determined by measuring the inhibition of a standardized solution of the cysteine proteinase, papain. Second, determinations of inhibitor activity were made by measuring the relative activity of cathepsins B and L before and after its removal with an affinity column. This procedure is referred to as the ratio method (Koohmaraie and Kretchmar, 1990). The direct assay using inhibition of papain showed that inhibitor activity increased throughout the aging period with breast muscle having a peak of activity at 24 hours postmortem (Fig 4a). In contrast, the ratio method showed a general decrease in inhibitor activity after 6 days for both breast and thigh muscle. The breast samples again showed an increase at 24 hours using this method (Fig 4b).

The significance of the difference in these assay methods is unknown but it may mean that they are measuring different cystatin forms. Moreover, these results demon-

Figure 4a

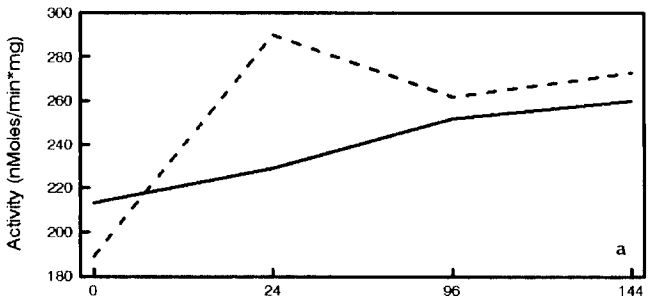
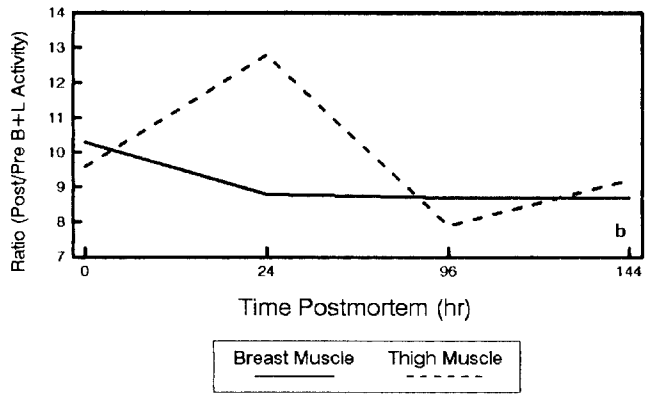


Figure 4b

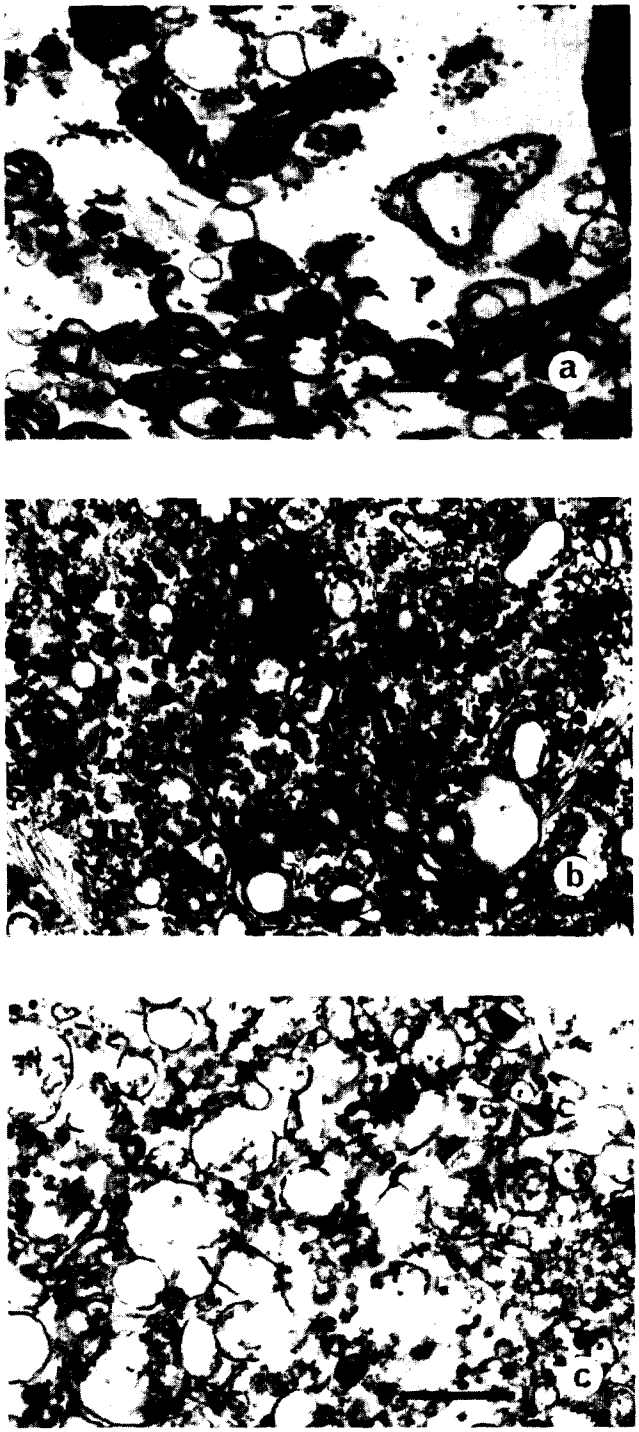


strate the variability in cystatin determinations and the need to develop more specific assays.

Lysosomal Integrity Postmortem

One of the most significant objections to the involvement of cathepsins in postmortem proteolysis is that they are contained within a membrane-limited organelle and thus unable to act. Early studies (summarized by Dutson, 1983) reported loss of lysosomal integrity and release of catheptic enzymes in response to aging. However, these studies only examined differences between sedimentable and nonsedimentable cathepsin activity which was found to increase with aging. The increase in nonsedimentable activity postmortem was further found to be enhanced by temperature treatment and electrical stimulation (Dutson, 1983).

Figure 5



An ultrastructural investigation was conducted of lysosomal membrane integrity as a result of normal aging (4C) in bovine sternomandibularis muscle for up to 14 days postmortem (Fig 5a, b & c). Examination of microsomal membranes prepared from at-death muscle showed many mitochondria and intact vesicles (Fig 5a). At the end of 7 days postmortem, intact mitochondria were difficult to find and many of the vesicles were disrupted (Fig 5b). Finally, at the end of 14 days postmortem there is almost complete vesicle disruption (Fig 5c).

Finally, lysosomal enzyme involvement as a part of the conditioning response is suggested by studies correlating cathepsin activities with objective measure of tenderness (Calkins and Seideman, 1988; Johnson et. al., 1990; Shackelford et. al., 1991). These studies provide circumstantial evidence that desirable meat texture is associated with proteolysis which is the result of the action of both lysosomal and nonlysosomal systems.

Conclusions

Lysosomal proteinases and their inhibitors logically have a role in postmortem muscle protein degradation. Cathepsin involvement may not be evident in the early phase (first 24 hrs) of postmortem aging unless the process is altered by use of electrical stimulation or higher temperatures. However, as aging proceeds there is likelihood of their importance to tenderness based on several points.

1. Lysosomes lose integrity under normal aging conditions and thus cathepsins are released into the cytoplasm. Evidence has been presented here showing loss of vesicle integrity.
2. Proteolytic alteration of muscle connective tissue (endomysium and perimysium) occurs during normal postmortem aging. Limited proteolysis of connective tissue during aging, like that occurring to myofibrillar protein, may not result in increased soluble products, but could have significant effects on structural integrity.
3. Evidence from several studies correlating cathepsin, calpain and respective inhibitor activities indicate a contribution of cathepsins to tenderness.

Future research to accurately assess cathepsin's and cystatin's role in postmortem proteolysis will require their localization at the electron microscope level. In addition, more selective assay methods for inhibitor activity determination will need to be developed.

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