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Review

Role of muscle endopeptidases and their inhibitors in meat tenderness

M.A. Sentandreu, G. Coulis and A. Ouali*

Muscle Biochemistry Group, SRV, INRA de Theix, 63122 Saint Genès Champanelle, France (Tel.: +33-4-73-62-41-63; fax: +33-4-73-62-42-68; e-mail: ouali@clermont.inra.fr)

Postmortem meat tenderness improvement is generally assumed to result from the softening of the myofibrillar structure by endogenous proteolytic enzymes. In this context, the present paper is a broad overview of the different peptidases so far identified in skeletal muscle, their specific inhibitors and their respective potential role in postmortem muscle. A series of petidase families have been thus considered including calpains, cathepsins, proteasomes, caspases, matrix metallopeptidases (MMP) and serine peptidases.

Introduction

For decades, consumers considered tenderness as the most important quality attribute of meat. In a review of factors influencing consumption, selection and acceptability of meat purchases, Jeremiah (1982) concluded that the most common cause of unacceptability in beef was toughness and that this was a common problem in pork and lamb. In contrast to mammals, fish flesh often undergoes very rapid softening rendering it unappealing to consumers (Ashie & Simpson, 1997; Crapo, Himelbloom, Pfutzenreuter, & Chong, 1999; Jiang, 2000).

In mammals and poultry, inconsistency and variability in meat texture have been identified as two of the major problems that face the meat industry. The main origin of this variability is the large biological diversity of skeletal muscle (Pette & Staron, 1990). No one muscle within an animal is identical with a second, an heterogeneity which can be even observed between fibers within a given muscle. This structural, metabolic and contractile heterogeneity of muscle tissue reflects its high degree of functional specialization and is the basis of its functional plasticity and adaptability. Therefore, it is not surprising to observe, in standardized processing conditions, a large animal variability in the rate and extent of postmortem meat texture development.

A prerequisite for an accurate control of this biological variability and for a better standardization of meat tenderness is to improve our understanding of the underlying mechanisms. For years, this objective has been the focus of meat scientists but a lot of questions still remain unanswered. There is however a consensus supporting the concept that the meat tenderizing process is primarily enzymatic in nature, physico-chemical conditions (pH, osmotic pressure) modulating positively or negatively the proteolytic action of endogenous peptidases (Ouali, 1992).

The purpose of the present paper is therefore to overview the general properties of the major peptidases so far identidied in skeletal muscle and their possible contribution to the meat tenderising process. Different families of peptidases will thus be examined in this review including calpains, cathepsins, proteasomes, caspases, matrix metallopeptidases (MMP) and serine peptidases. As it will be subsequently addressed, endogenous peptidase inhibitors, which play a major role in controlling the activity of mature enzymes, often appear to be better predictors of meat tenderness than target peptidases themselves. Hence, peptidase inhibitors also have to be considered in this context.

Underlying mechanisms of meat tenderisation

Conversion of muscle into meat is a three-step process. The pre-rigor phase (few min to 30 min postmortem in beef), during which muscle remains excitable, might correspond to the duration of survival of the nervous system (Chrystall & Devine, 1985). The rigor phase, during which energy rich compounds (ATP, PC,

* Corresponding author.

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glycogen, ...) are exhausted, can be highly variable in length depending on muscle types, animal species and chilling conditions. Upon rigor onset, muscle elasticity decreases and at its completion, the tissue reaches its maximum toughness. The last step is the tenderizing phase, which differs in length with the chilling conditions as well as between muscles, individual animals and animal species. Most changes in post-rigor meat texture result primarily from a weakening of the myofibrillar structure. Collagen concentration and its extent of cross-linking define the background toughness which discriminates meat cuts consumed as steaks or roast from those ascribed for cooking in highly humid atmosphere; cooking in such conditions enabling the conversion of collagen to gelatin.

The rate and extent of meat tenderization are known to be highly variable. Optimum tenderness can thus be reached within few hours in chicken and turkey breast muscles but the same process would need up to 4–6 days in pork and lamb muscles and 10–15 days in bovine muscles (Monin and Ouali, 1991; Ouali, 1991, 1999). In addition, within a given species, large animal variability in the tenderizing profile of bovine *Longissimus* muscle was reported (Ouali, 1991; Valin, 1995; Zamora, 1997; Zamora *et al*., 1996). In the same way a similar variability was observed between muscles from the same animal (Dransfield & Jones, 1981; Ouali, 1981; Valin, 1995; Zamora, 1997). One possible cause of this variability could be the difference in the enzyme content and more likely in the enzyme/inhibitor ratio, a parameter reflecting the efficiency of the proteolytic systems. The rate of muscle growth in living animals and the rate of postmortem tenderization are highly dependent on the endogenous peptidase efficiency. As pointed out earlier, decrease of protein degradation by reducing protease activity could be a more efficient way to promote muscle growth (Goll, Kleese, & Szpacenko, 1989). This statement strongly suggests that peptidase inhibitors have an essential role, a feature supported by several studies emphasizing that the best predictors of meat tenderness are the concentrations of inhibitors. This is the case for calpastatin, a calpain inhibitor, cystatins, a family of cysteine peptidase inhibitors (Ouali & Talmant, 1990; Shackelford *et al*., 1991) and serine peptidase inhibitors (Ouali, 1999; Zamora, 1997; Zamora *et al*., 1996). However, apart from calpastatin, the specific calpain inhibitor, little is known about the potential endogenous inhibitors of the other peptidases present in skeletal muscle cells and susceptible to be involved in the postmortem conversion of muscle into meat (Ouali *et al*., 1995; Zeece, Woods, Keen & Reville, 1992).

The calpain system

General features

Amongst the cysteine peptidases, calpains represent nowadays one of the most important groups, and include a great number of intracellular structurally related peptidases. The most investigated ubiquitous calpains including μ-calpain or calpain 1 (active at μM calcium concentration), m-calpain or calpain 2 (active at mM calcium concentration) and μ/m-calpains (active at intermediate calcium concentration) are heterodimers composed of two subunits. The 80 kDa catalytic subunit is responsible for the peptidase activity and is unique to each enzyme while the 30 kDa regulatory subunit is common to all of them. As depicted in Fig. 1A, the typical four domain structure of the catalytic subunit is comprised of Domain I (autolytic activation), Domain II (cysteine catalytic site), Domain III (switch domain) and Domain IV (calmodulin-like calcium binding Domain). Besides these well-known calpains and with the development of molecular cloning, a large number of new calpains have been identified in mammals and other organisms. All members of this superfamily contain homologous catalytic domains (Domain II) but some of them lack one or more of the three other domains or have specific inserts in their structure (Fig. 1b and c). Based on their domain composition and their tissue distribution, members of the calpain superfamily are now classified within three different groups: ubiquitous calpains (μ, m and μ/m calpains), tissue specific calpains (calpain 3 or p94, calpains nCl-2, nCl-4, Lp85 and Rt88, ..) and atypical calpains (calpains 5, 6, 10, ...) (Ma *et al*., 2001; Ono *et al*., 1998, 1999; Sorimachi, Ishiura, & Suzuki, 1997; Suzuki, Sorimachi, Ishiura, & Ohno, 1995).

The activity of ubiquitous calpains is mainly controlled by calcium ions, phospholipids and calpastatin, their specific inhibitor (Saido *et al*., 1994). Calcium ions are known to bind to Domains III and IV of the catalytic subunit and to Domain VI of the regulatory subunit. More recently, the recombinant Domain II fragment corresponding to the catalytic domain was shown to exhibit a calcium dependent proteolytic activity suggesting an additional calcium binding site in Domain II (Hata *et al*., 2001). Another important feature concerns the activation of calpains by phospholipids originally suggested by Coolican and Hathaway (1984). In this respect, the possible function of calpain Domain III emerged from the 3D structure of m-calpain (Strobl *et al*., 2000). The folding of this domain was very similar to C2 domains originally identified in protein kinase C and found later in many different proteins (Rizo & Sudhof, 1998). The C2 domain can bind phospholipids in a calcium-dependent manner and is thought to be responsible for orchestrating calcium and membrane regulation of enzyme activity. In m-calpain, Domain III was shown to be a calcium-regulated phospholipid-binding Domain. Binding of phospholipds to Domain III also enhances by 1–10-fold the calcium binding capacity of that Domain suggesting that the two processes take place in a cooperative manner

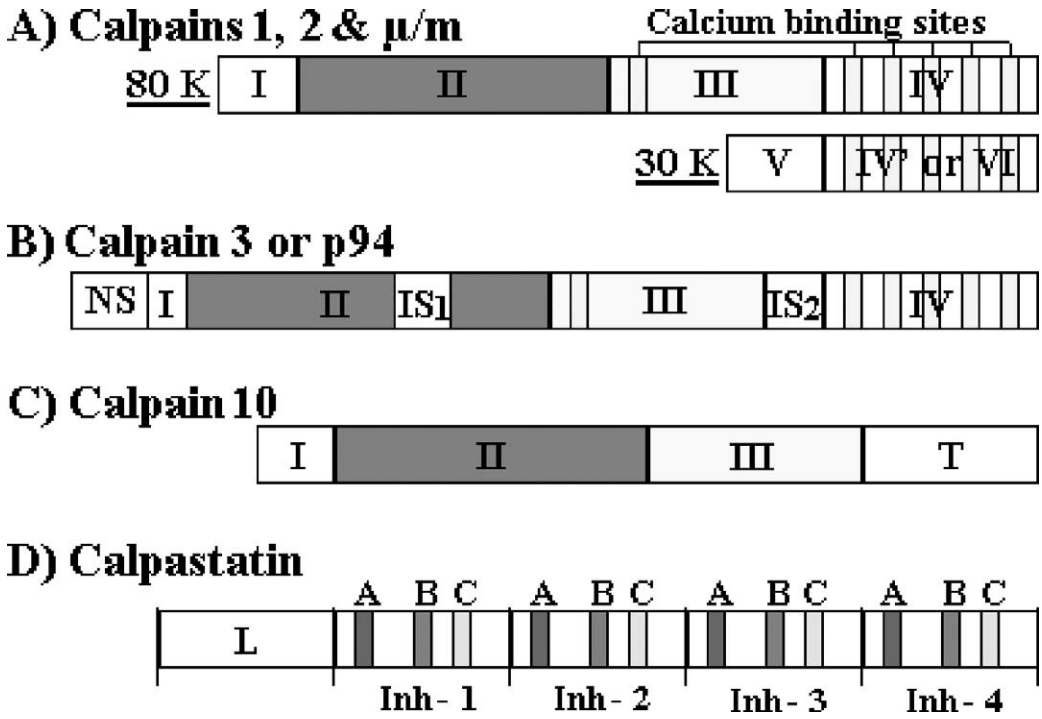

Fig. 1. Schematic structure of the different calpains expressed in skelatal muscles and their specific inhibitor designated calpastatin. (A) Ubiquitous calpain 1 (μM-), calpain 2 (mM-) and chicken μ/m calpain; (B) Muscle specific calpain 3 or p94; (C) Ubiquitous structurally different calpain designated calpain 10; (D) Schematic structure of calpastatin, the specific calpain inhibitor. Abbreviations used: ***I***, N-terminal Domain; ***II***, cysteine catalytic Domain; ***III***, Domain III or switch Domain; ***IV***, calcium binding or calmodulin-like Domain of the 80 kDa catalytic subunit; ***V***, N-terminal Domain of the small 30 kDa subunit; ***VI*** or ***IV'***, calcium binding or calmodulin-like Domain of the 30 kDa subunit; ***NS***, N-terminal inserted sequence; ***IS1***, first insert of calpain p94; ***IS2***, second insert of calpain p94; ***T***, Domain T first identified in an atypical calpain from C. elegans (Wilson *et al.*, 1994); ***L***, N-terminal Domain of calpastatin; ***Inh-1*** to ***4***, repetitive inhibitory Domains of calpastatin; ***A***, ***B***, ***C***, particular regions necessary for the inhibitory activity of the isolated sub-Domains. and for the inhibitory activity of calpastatin itself (adapted from Sorimachi *et al.*, 1997, Ma *et al.*, 2001; Takano & Maki, 1999).

(Tompa, Emori, Sorimachi, Suzuki, & Friedrich, 2001). Taken together, these findings stressed that regulation of calpain activity by calcium is very likely more complex than expected.

The calpain inhibitor or calpastatin

Calpastatin, a specific inhibitor of the ubiquitous calpains, is a polymorphic protein (Mr between 50 and 172 kDa) comprising generally four similar domains each of which exhibits a calpain inhibitory activity and a N-terminal domain (Domain L or XL) of variable size (Goll, Thompson, Taylor, Ouali, & Chou, 1999) (Fig. 1d).

Several isoforms of calpastatin have been reported and these are believed to result from various cellular events including:

- alternative splicing (De Tullio, Sparatore, Salamino, Melloni, & Pontremoli, 1998; Geesink, Nonneman, & Koohmaraie, 1998; Maki *et al.*, 1991; Lee *et al.*, 1992),
- differing start sites of translation/transcription (Imajoh, Kawasaki, Emori, & Suzuki, 1987; Wang, Wei, Miao, Liu, & Koide, 1994; Cong, Thompson, Goll, & Antin, 1998),
- differing states of phosphorylation (Averna *et al.*, 2001; Pontremoli *et al.*, 1991, 1992).

Interestingly, the presence of two calpastatin isoforms expressed mainly in fast-twitch (first peak eluted from anionic exchange column) and slow-twitch muscles (coeluted with calpain 1 or later in the salt gradient) were reported in bovine, ovine and pork muscle (Ouali & Talmant, 1990). As a result, no calpain 1 activity can be detected in the last muscle type. According to previous findings, the major calpastatin isoform present in slow-twitch muscles might correspond to the phosphorylated form of calpastatin and reciprocally this will be the minor form in fast-twitch muscles (Pontremoli *et al.*, 1991, 1992). Calpastatin behaves as a reversible competitive inhibitor of calpains and its interaction is calcium dependent. It binds to the calmodulin-like domains of the large (Domain IV) and small subunits (Domain IV' or VI) of calpains (Takano & Maki, 1999).

Physiological functions of calpains

The precise *in vivo* functions of calpains have not been clearly identified. The ubiquitous and constitutive expression of calpains 1 and 2 strongly suggest that they are involved in basic and essential cellular functions. This may be one of the reasons why we cannot specify precisely the physiological functions of calpains. Nevertheless, most muscle fiber proteins, especially cytoskeletal proteins, are known to be potential biological substrates for calpains (Goll *et al.*, 1999; Huff-Lonergan & Lonergan, 1999). Moreover, mutation within the calpain 3 or p94 gene was found to be responsible of the LGMD type 2A (Limb Girdle Muscular Dystrophy type 2A) (Richard *et al.*, 1995). More recently, the gene encoding for calpain-10, a newly identified ubiquitous calpain, has been associated with type 2 diabetes (Evans *et al.*, 2001; Sreenan *et al.*, 2001; Yang *et al.*, 2001). Similarly, the increase in calpain Lp82 activity, a calpain mainly expressed in the lens, was correlated both spatially and temporally with cataract formation in rats (Baruch *et al.*, 2001). Taken together, these findings strongly suggest a contribution of calpains to different types of pathologies and support a central role of these enzymes in cell biology and physiology. Although calpains are assumed to contribute to a series of intracellular events including signal transduction, cell cycle, cell differentiation and apoptosis (Carafoli & Molinari, 1998; Goll *et al.*, 1999; Ono *et al.*, 1998; Sorimachi *et al.*, 1997; Wang, 2000), much remains to be learned about them and their physiological functions.

Muscle calpains

In muscle tissue, μ- and m-calpains have been found in all animal species whereas μ/m calpain seemed to exist only in chicken muscle. Besides the extensively studied ubiquitous calpains, a muscle specific calpain designated p94 with reference to its molecular mass, has been identified at the gene level (Sorimachi *et al.*, 1989) but because of its high instability, the protein has never been purified. In addition to the four typical domains found in ubiquitous calpains, p94 contain three specific inserts NS, IS1and IS2 located in Domain I, Domain II and Domain IV of the large subunit, respectively (Fig. 1b). Interest in this muscle specific calpain has grown tremendously since its implication in LGMD2A was demonstrated (Richard *et al.*, 1995). More recently, a new calpain designated calpain 10 and expressed in all rat, mouse and human tissues investigated including skeletal muscle has been described. In skeletal muscle, calpain 10 was predominantly located at the level of the cellular membrane. Calpain 10 differs from the other ubiquitous calpains by the lack of Domain IV (calcium binding domain) and the presence of a non calcium binding domain or T Domain suggesting that its activity may not be dependent on calcium (Ma *et al.*, 2001) (Fig. 1c).

Calpains and meat texture

As calpains were believed to play a major role in postmortem tenderization of meat, they have been and are still extensively investigated by meat scientists. The first evidence supporting this assumption was the rapid loss of Z-disks in calpain-treated skeletal muscle myofibrils, a change often associated with meat tenderness (Busch, Stromer, Goll, & Suzuki, 1972). Since then, a large set of structural and biochemical evidences reported in the literature strengthened the primary role of calpains in postmortem proteolysis and some authors even stressed forward that calpains are responsible for up to 95% of all proteolytically induced postmortem tenderization (Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001a, 2001b). However, using a mathematical model based on calpain activity, Dransfield (1992) pointed out that this model predicted accurately the ultimate tenderness of normal pH meat but failed to provide a good prediction of the texture of high pH meat which had always been found abnormally tough. This finding strongly suggested that some other systems are implicated in that case. We have recently shown that the specific structural changes observed in high pH meat (Guignot, 1992) are wholly comparable to those obtained upon treatment of glycerinated fibers with purified 20S proteasome. The proteasome might be therefore a good candidate for postmortem tenderization of high pH meat (Dutaud, 1998; Ouali, 1999). On the other hand, different sets of evidence supporting the role of calpains in postmortem muscle came from studies dealing with the identification of biological markers of meat tenderness. Most of them demonstrated that the calpain/calpastatin concentration ratio and, though to a lesser extent, the muscle concentration of calpains, are good predictors of the ultimate tenderness of beef (Ouali & Talmant, 1990; Shackleford *et al.*, 1991; Thomson, Dobbie, Cox, & Simmons, 1999). However, it is still not known whether postmortem proteolysis involves primarily μ- or m-calpain or both (Boehm, Kendall, Thompson, & Goll, 1998; Delgado *et al.*, 2001a, 2001b) and what calpain substrates are most important to tenderization (Huff-Lonergan & Lonergan, 1999).

Besides the implication of ubiquitous calpains in the meat tenderizing process, some recent investigations provide contradictory findings about a possible contribution of calpain p94 (calpain 3) in that process. Using a specific antibody directed against calpain 3 to quantify the protein by western blot analysis, Parr *et al.* (1999) failed to find any relationship between p94 level and meat tenderness. By contrast, Ilian *et al.* (2000) reported a good correlation between calpain 3 mRNA level and the ultimate tenderness of different bovine and ovine muscles. However, as pointed out by Sorimachi *et al.* (1989, 1990), the mRNA concentration is not representative at all of the amount of calpain 3 really expressed in the muscle tissue.

The lysosomal cathepsins

General features

The term "cathepsins" generally designates peptidases located in the lysosomes and mostly active at acidic pH. This is indeed a complex group of enzymes that includes exo- and endo-peptidases belonging to cysteine (cathepsins B, H, L, X, ...), aspartic (cathepsins D and E) and serine (cathepsin G) peptidase families. The cathepsins known to be expressed in muscle tissue and relevant to our purpose include six cysteine peptidases (cathepsins B, L, H, S, F and K) and one aspartic peptidase, i.e. cathepsin D. Cathepsin E, the other lysosomal aspartic peptidase has never been demonstrated to be expressed in muscle cells. In muscle tissue, cathepsin E was located within the macrophages. A brief description of the major cathepsins exhibiting endopeptidase activity and supposed to have or might have some relevance in skeletal muscle is shown in Table 1.

As for many other peptidases, cathepsins are synthesized as proenzymes that are further transformed into the mature active enzymes by cleavage of the N-terminal propeptide. This process can be done either by autolytic cleavage or by the action of other peptidases (B. Turk *et al.*, 2000). Cysteine lysosomal peptidases belong to the papain family, having similar amino acid sequence and folds. Although their properties are similar, lysosomal cysteine peptidases are immunologically distinct (Barrett, Rawlings, & Woessner, 1998).

Active cathepsins represent a high hydrolytic potential since their total concentration in cells can be higher than 1 mM (Lloyd & Mason, 1996). Their activity is controlled by several factors including pH, redox potential, extent of precursor activation and specific endogenous inhibitors. Cathepsin L is very unstable at neutral and alkaline pH (Dufour, Dive, & Toma, 1988) but most of the other cathepsins are stable over a wide range of pH values. Nevertheless, they are generally believed to work only in acidic environment like the lysosomal compartment (B. Turk *et al.*, 1999). By contrast, cathepsin S has been shown to be stable and active above pH 7.0, indicating a possible role outside lysosomes (Kirschke, Wiederanders, Brömme, & Rinne, 1989). Hydrolytic efficiency of cathepsins *in vivo* is mainly driven by the ratio of the precursor/mature enzyme concentration and by the inhibitor/cathepsin balance (Lloyd *et al.*,1996; B. Turk, Dolenc, Turk, & Bieth, 1993). Endogenous inhibitors also seems to play a major role in the regulation of the enzymes implicated in postmortem muscle tenderisation (Ouali, 1999; Shackleford *et al.*, 1991).

Physiological function

Cathepsins have been traditionally thought to accomplish intralysosomal protein degradation in a non-selective way (Chapman, Riese, & Shi, 1997). However, there is growing evidence supporting their implication in a large number of biological events. During the last decade, cathepsins have been extensively investigated and this has led to the discovery of new cathepsins and has improved our understanding of their

Table 1. Characteristics of the well known cathepsins with endopeptidase activity existing in skeletal muscle[a]

Name	EC Number	Classification and type	Size (kDa)	Optimum pH range	pI	Observations
Cathepsin B	3.4.22.1	Cysteine FamilyCl (papain family)	30 kDa (25 + 5)	5.5–6.5	4.5–5.5	Additional peptidyl dipeptidase activity
Cathepsin L	3.4.22.15	Cysteine Family C1	28 kDa (24 + 4)	5.5–6.5	5.0–6.3	Strong activity against proteins, less againstsynthetic substrate
Cathepsin H	3.4.22.16	CysteineFamily C1	28 kDa (23 + 5)	6.5–6.8	6.0–7.1	Strong aminopeptidase activity
Cathepsin S	3.4.22.27	Cysteine Family C1	24 kDa	6.0–6.5	6.3–7.0	Stable and active at basic pH (7.5)
Cathepsin K	3.4.22.38	Cysteine Family C1	29 kDa	6.0–6.5	—	High collagenolytic activity
Cathepsin F	3.4.22.41	Cysteine Family C1	—	5.2–6.8	—	High expression in skeletal muscle
Cathepsin D	3.4.23.5	Aspartyl Family A1 (pepsin family)	45 kDa (30 + 15)	3.0–5.0	6.8	One of the most abundant cathepsin in lysosomes
Cathepsin E	3.4.23.34	Aspartyl Family A1	42 kDa	3.0–3.5	4.1	Can form dimers of 84 kDa. Stabilized in the presence of ATP. Present in muscle but only in macrophages.

[a] Data adapted from Barrett *et al.* (1998) and from a series of web-sites including NC-IUMB (http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html); MEROPS database (http:/www.merops.co.uk) and BRENDA database (http://www.brenda.uni-koeln.de).

biological role by providing evidence supporting specific and individual functions of these enzymes. Although the specific functions are sometimes associated with the restricted localisation of some of the newly discovered enzymes, recent studies suggested that ubiquitous cathepsins (B, H, L, D, F, S and K) can also be implicated in more specialized processes (V. Turk, Turk, & Turk, 2001). Cathepsins B, H and L are thus involved in the development of cancers either directly through the degradation of extracellular matrix or indirectly by activating other peptidases (Sloane, 1990; Kos & Lah, 1998). Caspases are the primary peptidases responsible for programmed cell death or apoptosis (Adrain & Martin, 2001; Earnshaw *et al.*, 1999; Hengartner, 2000). However, recent findings suggest that cathepsins may contribute to that process either directly (Pennacchio *et al.*, 1998) or indirectly through the activation of caspases (Ishisaka *et al.*, 1998). Cathepsins also play an important role in the antigen presentation mediated by the major histocompatibility complex (MHC) class II, a process which is essential for the normal processing of antigen peptides by the immune system. In this regard, cathepsins S and L and probably also cathepsins B, D or F would be implicated (Chapman, 1997; Turk *et al.*, 2001).

On the other hand, the action of cathepsin K is decisive for bone remodeling in which bone formation and resorption is continuously being carried out (Barrett *et al.*, 1998). The high hydrolytic potential of cathepsins, together with their ability to act outside the lysosomes, makes them essential for a series of biological cellular events including the activation or processing of other enzymes and peptide hormones (B. Turk, Turk, & Turk, 2000).

If cathepsins are involved in numerous and important biological processes, it is not surprising that an inadequate regulation of their activities, under abnormal conditions, can led to the apparition of serious diseases such as cancer, atherosclerosis, Alzheimer's disease, multiple sclerosis or muscular disorders (Kirschke, Barrett, & Rawlings, 1995). In inflammatory myopathies, there is an important increase of endogenous muscle cathepsin activity suggesting that cathepsins, together with the action of other peptidases, constitute an important mechanism of muscle fiber degeneration (Kumamoto *et al.*, 1997).

Cathepsins and meat texture

The role of endogenous muscle cathepsins in postmortem meat tenderisation has been a controversial question during the last decades. Lysosomal cathepsins and calpains are the two most widely studied endogenous enzymatic systems in relation to meat texture development (Jiang, 2000). The lack of consensus among meat scientists during this time comes undoubtedly from the complexity of the muscle structure and of the tenderising mechanisms. Ideas discarding the contribution of cathepsins in the development of meat tenderness are mainly based on two types of observations. First, cathepsin activities failed to explain differences in tenderness of meat samples (Koohmaraie, Seidman, Schollmeyer, Dutson, & Babiker, 1988; Whipple *et al.*, K1990). Second, inhibition studies showed that some cathepsin inhibitors were not able to suppress postmortem proteolysis, but on the contrary a general cysteine peptidase inhibitor like E-64, which inhibits both calpains and cathepsins, effectively prevented postmortem proteolysis. As a result, major role was assigned to calpains (Hopkins, 2000; Mestre-Prates, Ribeiro, & Dias Correia, 2001). In addition, cathepsin D has been suggested to be not decisive in meat tenderisation for the following reasons:

- it loses much of its activity at low temperature;
- its optimum pH is far from postmortem pH meat (see Table 1);
- pepstatin, a cathepsin D inhibitor, did not influence the ageing process (Uytterhaegen, Claeys, & Demeyer, 1994).

Another reason to reject cathepsin action on myofibrillar proteins has been that there is little or no actin and myosin degradation during ageing, two proteins highly sensitive to the action of cathepsins (Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991; Ouali *et al.*, 1987, 1988). Another major argument to support that cathepsins could not participate in postmortem proteolysis has been the fact that they are contained into lysosomes, and so they might have no access to the myofibrillar structure without a prior breakdown of the lysosomal membrane (Koohmaraie, 1996). Despite all these negative arguments, research efforts in meat science carried out over the last years have partly clarified this controversy.

Histochemical studies have demonstrated that, as meat ageing proceeds, there is a progressive disruption of lysosomes so that after 14 days of storage, lysosomes breakdown is almost complete (Zeece *et al.*, 1992). This was supported by the considerable shift in cathepsin activity from the lysosomal/ microsomal fraction to the soluble fraction (O'Halloran *et al.*, 1997). Lysosomal breakdown may be explained by the decrease in pH while carcass temperature is still high (Moeller, Field, Dutson, Landmann, & Carpenter, 1977) or by the failure of ionic pumps in lysosomal membranes during rigor development consecutively to the depletion of ATP stores (Hopkins, 2000). On the other hand, free cathepsin activity, especially that of cathepsins B, H and L, is correlated with meat tenderness from one day postmortem till the end of the ageing period (Calkins & Seidman, 1988; Johnson, Calkins, Huffman, Johnson, & Hargrove, 1990; O'Halloran *et al.*, 1997).

Different industrial applications have been developed in order to improve meat tenderness, giving rise to variable effects. Electrical stimulation induces an accelerated pH drop, a change that may enhance muscle

proteolysis through an increase in the release of lysosomal enzymes and, thereby accelerating the tenderising process in the early storage period (Geesink, Van Laack, Barnier, & Smulders, 1994). However, some other collateral negative effects in the final quality of meat have been reported, depending on the way in which this technique is applied. This can explain why electrical stimulation not always succeeds in improving final meat tenderness (Barnier, 1995; Maribo, Ertbjerg, Andersson, Barton-Gade, & Moeller, 1999).

Incubation of myofibrils or glycerinated fibers with a lysosomal extract results in some of the ultrastructural changes observed during meat ageing including the degradation of myofibrils near the N_2 lines and at the A–I junction area. Moreover, some fragments with molecular masses close to 155 and 90 kDa, probably originating from myosin heavy chain degradation, have been identified in stored meat (Yates, Dutson, Caldwell, & Carpenter, 1983). Like myosin, actin seems to be also partially degraded but this degradation occurs probably after a minimum of 7–10 days postmortem (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). Cathepsins degrade many other structural and contractile proteins, so that it is possible that they show more affinity for these other proteins than for actin and myosin filaments in muscle. Even if many of the ultrastructural postmortem changes in muscle can be mimicked by treatment of myofibrils with lysosomal extracts, some others such as the decrease in Z-line density are only explained by treatment of myofibrils with calpains or proteasome (see below). Because all these structural changes identified in postmortem muscle cannot be explained by the action of one proteolytic system, a synergistic action of calpains and cathepsins must be considered (Ouali, 1992).

Therefore, while calpains may be responsible of the changes in the early postmortem period (24 h), a relevant contribution of cathepsin could be expected thereafter (Calkins & Seidman, 1988; Zeece *et al.*, 1992). There are some studies that have not succeeded in explaining differences in meat tenderness by the determination of calpain and/or cathepsin activity levels (Ouali, 1992). However, both calpastatin and cystatins, the endogenous specific inhibitors of calpains and cathepsins respectively, have been proven to be better predictors of meat quality than activity of the calpains or cathepsins themselves (Johnson *et al.*, 1990; Shackleford *et al.*, 1991). It seems clear that endogenous inhibitors continue to exert an important regulation of peptidase activities in postmortem muscle. Hence, it is not surprising that differences in meat quality are more closely related to the enzyme/inhibitor ratio than to the enzyme activity levels by themselves (Ouali, 1992). In this context, cystatins are much less studied inhibitors than calpastatin. The major reason is very likely the difficulty to measure cystatin levels. This determination has been done by indirect methods based on the following activity assessment:

- measurement of the total inhibitory activity of a muscle extract against a standardized solution of papain (Ouali *et al.*, 1995),
- measurement of cathepsin B and L activity before and after cystatin removal using an affinity column (Koohmaraie & Kretchmar, 1990).

Further research is needed in order to develop better and direct methods to quantify cystatins with the aim to better understand how cystatins regulate cathepsins in postmortem muscle. *In vivo*, cystatins are located in the cytoplasm, separated from cathepsins, which remain inside lysosomes. In this situation, unwanted cathepsin activity outside lysosomes is prevented by cystatins. In postmortem muscle, on the contrary, the physiological situation is different because lysosomal breakdown induces a release of cathepsins to the cystosol where cystatins are. In this case, the cathepsin/cystatin balance would drive the effective activity level of cathepsins. However, very little is known about muscle cystatins and additional work will be required to improve our understanding of the *in vivo* and postmortem biological roles of these inhibitors often considered to be target proteins for prognosis, diagnosis and therapy in cancer (Kos & Lah, 1998). On the other hand, meat science has been traditionally focused on the study of cathepsins B, H, L and D in muscle, but these investigations should be extended to other recently identified cathepsins. All these findings would suggest that the role of cathepsins and their inhibitors in relation with meat quality development must be reconsidered.

Cystatins: a family of cysteine peptidase inhibitors

The term 'cystatin' refers to a group of homologous and evolutionary related cysteine peptidase inhibitors (Barrett, 1986, 1987; Kominami & Ishidoh, 1995; Muller-Esterl *et al.*, 1985; Rawlings & Barrett, 1990; V. Turk *et al.*, 1986) which are inactive against other classes of peptidases (serine-, aspartyl- and metallopeptidases). On the basis of their primary structure, there are four distinct families of cyteine peptidase inhibitors, which are recognized as belonging to the cystatin superfamily (Rawlings & Barrett, 1990). Family 1 cystatins, also designated stefins, are low molecular weigth proteins (10–14 kDa) containing one copy of the basic inhibitory structure but no intramolecular disulfide bridges. The most well characterized members of this family are stefin A and stefin B. As stefins lack the signal sequence present in secreted proteins, they are generally found intracellularly. Family 2 cystatins, also designated cystatins, are low molecular weight proteins (10–14 kDa) containing one copy of the basic inhibitory structure and at least one intramolecular disulfide

bridge. Cystatins are predominantly extracellular. Cystatin C and chicken cystatin are the most representative of this cystatin family. Family 3 cystatins, also designated kininogens, are high Mr cystatins (> 50 kDa) containing generally three copies of the basic inhibitory structure and several disulfide bonds. The kininogen family comprises three subclasses referred to as low Mr (LMW), high Mr (HMW) and T- kininogens differing by their molecular masses and their specificity towards cysteine peptidases. They are generally present in body fluids. Family 4 cystatins are glycosylated protein inhibitors of intermediate Mr containing two copies of the basic inhibitory structure and several intramolecular disulfide bridges.

All members of the cystatin superfamily inhibit cysteine peptidases such as papain and the major lysosomal peptidases including cathepsins B, H and L. In addition, kininogens have been shown to inhibit the ubiquitous calpains, which are calcium dependent cysteine peptidases (Ishiguro *et al.*, 1987).

In muscle tissue, several low Mr cystatin-like proteins have been described (Bige *et al.*, 1985; Schwartz & Bird, 1997) but as no sequence was available, it was and is still impossible to certify that these are really members of the cystatin superfamily. Indeed, their identification as cystatin was only based on their closely similar molecular mass. This statement is true for most of the other cysteine peptidase inhibitors isolated from skeletal muscles of various animal species (Ouali *et al.*, 1995; Zeece *et al.*, 1992). Importance of cystatins in post-mortem and living muscles is supported by their identification as potent markers of meat tenderness (Barnier, 1995; Shackleford *et al.*, 1991) and their possible use as biological markers of different pathologies in humans (Strojan *et al.*, 2000; Kos & Lah, 1998).

The proteasome complexes

General features

The 20S proteasome is a 700,000 dalton, cylinder-shaped structure arranged as four axially stacked heptameric rings composed exclusively of either alpha-subunits (two outer rings) or beta-subunits (two inner rings), respectively. The multiple catalytic sites of this proteolytic complex, classified within the Ntn-hydrolase family (Lowe *et al.*, 1995; Seemuller *et al.*, 1995), are exclusively associated with the beta-subunits and, because of the particular structural topology of the particle, they were assumed to be all sequestered within the hollow cavity of the cylinder. The eukaryotic proteasome is characterized by three major activities of differing specificity against short synthetic peptides: a chymotrypsin-like activity, a trypsin-like activity and a peptidyl-glutamyl hydrolyzing activity (Coux, Tanaka, & Goldberg, 1996). Within the cell, the 20S proteasome exists either in a free state or associated with large regulatory complexes. It can thus bind one or two 19S complexes (PA700) responsible for the ATP-ubiquitin dependent hydrolytic activity of the resulting 26S complex (Hendil, Hartmann-Petersen, & Tanaka, 2002). It can also bind one or two 11S activator complexes (PA28) (Baumeister, Walz, Zuhl, & Seemuller, 1998; Bochtler, Ditzel, Groll, Hartmann & Huber, 1999; Coux *et al.*, 1996; DeMartino & Slaughter, 1999). Recent findings further suggest a possible simultaneous binding of PA700 and PA28 activators to the 20S proteasome. Such an hybrid proteasome complex is probably more efficient for the generation of peptide antigens (Hendil, Khan, & Tanaka, 1998; Tanahashi *et al.*, 2000). The proteasome population appears to be highly polymorphic and this polymorphism may also exist within each sub-group of proteasome complexes (Dahlmann, Ruppert, Kloetzel, & Kuehn, 2001; Dahlmann, Ruppert, Kuehn, Merforth, & Kloetzel, 2000). Furthermore, the different regulatory complexes and sub-populations of proteasomes were shown to have different distributions between tissues (Noda, Tanahashi, Shimbara, Hendil, & Tanaka, 2000) and within mammalian cells (Brooks *et al.*, 2000), a finding which probably reflects the importance of this proteolytic system in multiple crucial cellular processes. Proteasomes are widely distributed in mammals since they were found in all tissues and cells investigated so far.

In eukaryotic cells, proteasomes play a major role in non-lysosomal proteolysis (Attaix, Combaret, Pouch, & Taillandier, 2001; Coux *et al.*, 1996; DeMartino & Slaughter, 1999; Hilt & Wolf, 1996; Stoltze, Nussbaum, Sijts, Emmerich, Kloetzel Stricklin, & Schild, 2000). Proteasomes have a central role in multiple cellular events including:

- antigen processing (Gaczynska, Rock, Spies, & Goldberg, 1994; Rock *et al.*, 1994; Stoltze *et al.*, 2000),
- cell differentation (Baz, Henry, Caravano, Scherrer, & Bureau, 1997a; Baz, Henry, Château, Scherrer, & Bureau, 1997b; Henry, Baz, Château, Scherrer, & Bureau, 1996; Henry *et al.*, 1997),
- apoptosis (Andersson, Sjostrand, Petersen, Honarvar, & Karlsson, 2000; Masdehors, Merle-Beral, Magdelenat, & Delic, 2000; Pasquini *et al.*, 2000)

They are also involved in accelerated degradation of muscle tissue associated with certain diseases and sepsis (Attaix & Taillandier, 1998; Hobler *et al.*, 1999; Lecker, Solomon, Price, Kwon, Mitch, & Goldberg, 1999; Mitch & Goldberg, 1996).

The activity of the 20S proteasome is under the control of specific activators (PA700 and PA28) and inhibitors. Whereas activators have been well characterized (see above), little is known about the potent endogenous protein inhibitors of the proteasome (DeMartino & Slaughter, 1999). Nevertheless, several protein inhibi-

tors of the proteasome have been identified (Guo, Gu, & Etlinger, 1994; Li, Gu, & Etlinger, 1991; Ma, Slaughter, & DeMartino, 1992; Murakami & Etlinger, 1986). We have recently purified and partially characterized a 70 kDa proteasome inhibitor from bovine skeletal muscle (Dutaud, 1998). The peptidyl-glutamyl hydrolysing activity of the 20S proteasome decreased linearly as the concentration the muscle inhibitor was raised. The inhibition was further found to be non-competitive suggesting that the inhibitor did not bind to the same site than the substrate and that it might act through a structural modification of the complex (Dutaud, 1998).

Protein inhibitors were generally discovered by assaying fractionated tissue extracts for their ability to inhibit the 20S proteasome, but in most cases they have not been tested for their ability to affect the proteasome activity in the presence of activators. Thus, the physiological role of these proteins may be to inhibit the 20S proteasome and/or to regulate proteasome–activators interaction or their hydrolytic activity. Additional work will be required to advance our understanding of the mechanisms by which these proteins regulate proteasomes together with their physiological significance.

Protein degradation by the 20S proteasome

Because of the properties of the 26S proteasome which comprised a protein unfolding system, i.e. the 19S complex, the 20S proteasome is somtimes considered to be unable to hydrolyse native proteins especially muscle proteins. However and although still unexplained, there is a large set of evidence suggesting that myofibrillar proteins and myofibrils themselves can be hydrolysed upon treatment with purified 20S proteasome (Koohmaraie, 1992; Mykles, 1989a, 1989b; Mykles & Haire, 1991, 1995; Taylor *et al*., 1995). In addition, whether no structural changes was observed by Koohmaraie (1992) in myofibrils treated with partially purified 20S proteasome, he noted a degradation of troponin C and myosin light chains by this complex, a finding supporting a possible action of the 20S proteasome on myofibrils. Hydrolysis of native non muscle proteins, i.e. casein and glucose-6-phosphate dehydrogenase, by the 20S proteasome has been also reported (Dahlmann, Kuehn, Grziwa, Zwickl, & Baumeister, 1992; Friguet, Szweda, & Stadtman, 1994).

Proteasomes in muscle tissue

All subtypes of proteasome complexes exist in skeletal muscle (Dahlmann *et al*., 2000, 2001). As assessed by ELISA, the 20S proteasome concentration was found to be muscle type dependent being highest in oxidative and oxidative–glycolytic bovine muscles and lowest in glycolytic muscles. In bovine muscles, the proteasome concentration ranged from 200 to 350 μg/g of wet tissue (Dutaud, 1998).

The 20S Poteasome in meat texture development

Effect of temperature and pH on proteasome activity

As assessed in vitro, using fluorescent synthetic substrates, the temperature effect on the trypsin and chymotrypsin like activities of the 20S proteasome were not very different from that reported for calpains. About 15–20% of the maximum activity indeed remains at 5°C. Fuhermore the enthalpy of activation (13.6 Kcal M^{-1}) is wholly comparable to the value obtained for calpains (11 Kcal M^{-1}) (Dutaud, 1998; Ouali, 1976).

As the pH decreases below 7.0, both activities are similarly affected and reach a minimum value at pH 5.0–5.5 corresponding to 25% of the maximum activity. This result is not far different from the values reported for calpains (Koohmaraie, Schollmeyer, & Dutson, 1986).

Contribution of the 20S proteasome to postmortem meat texture development

The 20S proteasome has been shown to degrade myofibrils and to cause significant damage of the M- and Z-lines as does calpains (Dutaud *et al*., 1996; Taylor *et al*., 1995). Based on some specific ultrastructural changes observed in high pH meat (Guignot, 1992) and in postmortem slow-twitch oxidative or type I muscles, recent investigations suggested that the 20S proteasome might be the main proteolytic system of concern in postmortem tenderization of these types of meat (Dutaud, 1998; Dutaud *et al*., 1996; Ouali, 1999). In slow twitch bovine muscles, i.e. *Diaphragm pedialis* muscle, the main ultrastructural change was indeed an enlargement of the Z-line with more or less density loss (Ouali, 1999). In high pH veal meat, the sequential postmortem ultrastructural changes started by an enlargement of the Z-line followed by the appearance of an amorphous protein material recovering more or less the I-band. Increasing the storage time to 8–10 days led to a total or partial disappearance of this amorphous structure and of the Z-band density (Guignot, 1992). As neither calpains nor cathepsins are able to mimick these structural changes, one possible candidate could be the 20S proteasome. This assumption was verified by incubating glycerinated fibers with purified proteasome, a treatment leading to a wholly comparable sequential myofibrillar ultrastructural changes (Dutaud, 1998; Dutaud *et al*., 1996; Ouali, 1999). In the presence of proteasome inhibitors, i.e. lactacystin (Fenteany *et al*., 1995) or MG132 (Shinohara *et al*., 1996) at a final concentration of 1 mM, the 20S proteasome was completely inhibited and no change was observed after incubation suggesting that the observed effects can be ascribed to the action of this complex. The 20S proteasome appeared therefore to be the only one proteolytic system able to reproduce *in vitro* the particular postmortem changes occurring in slow-twitch muscles and in high

pH meat, a finding supporting a possible implication of the 20S proteasome in the meat tenderising process.

Caspases: apoptosis peptidases

General features

Caspases constitute a new family of cysteine peptidases (Alnemri *et al*., 1996; Barrett *et al*., 1998) characterized by their ability to cleave proteins after aspartic acid residues. Their primary function is their contribution to programmed cell death or apoptosis (Earnshaw, Martins, & Kaufmann, 1999; Hengartner, 2000; Kos & Lah, 1998). However, these peptidases could also have many other key roles in various cellular events (Beere, 2001). Based on their substrate specificity (nature of the four N-terminal residues preceding the cleavage site), their extent of sequence identity and their structural similarities, about fourteen different caspases have been so far identifed in different tissues and cells from humans all the way down to insects, nematodes and hydra. Several sets of evidence mainly originating from caspase knockouts experiments have suggested some functional redundancy in caspases which could be explained, in part, by functional compensation (Beere, 2001). In other words, the absence of one caspase may lead to the over expression of another caspase allowing continuity in the apoptotic process. A number of complex signalling pathways leading to apoptosis have been recently defined (Adams, Gielen, Hambrecht, & Schuler, 2001; Earnshaw *et al*., 1999; Hengartner, 2000; Kos & Lah, 1998). The common feature of all pathways is the ultimate activation of a series of caspases (Hengartner, 2000).

Caspases in muscle tissue

Programmed cell death has been extensively studied in mononucleated cells. But what happens in multinucleated cells alike mature muscle fibers? The occurrence and significance of apoptosis in multinucleated, differentiated cells like skeletal muscle cells, is controversial in the current literature. However, there is growing evidence suggesting that apoptosis is a feature in skeletal muscle (Adams *et al*., 2001; Liu & Ahearn, 2001; Sandri & Carraro, 1999). In agreement with this statement, the expression of different caspases (caspases 1, 3, 6, 8 and 9 ….) and of various apoptosis regulators (activators or inhibitors of apoptosis) has been noticed in skeletal muscle cells (Belizario, Lorite, & Tisdale, 2001; Biral *et al*., 2000; Jin, Wu, Tian, & Gu, 2001; Sandri *et al*., 2001).

Caspases and apoptosis in meat science

Death within a living tissue may occur by either necrosis or apoptosis. After animal exsanguination, muscle cells will be engaged in a cell death program but which program is of concern in postmortem muscle remains unknown. As necrosis has never been reported in postmortem muscle, the initiation of the cell death through an apoptotic-like process would be the most probable issue. But what could be the signalling pathway leading to apoptosis and cell death after animal exanguination? One possibility would be an irreversible alteration of myofibres at the neuromuscular junction where activators of the apoptotic cascade could be produced. Such assumption is supported by the rapid decrease in the efficiency of low voltage electrical stimulation (Valin, 1982) which results from a rapid postmortem alteration of the nervous system, the major conductor of low voltage current (Chrystall & Devine, 1985). Similarly, denervation was shown to induce apoptosis in adult skeletal muscle, a process often associated with a fragmentation of myofibrils (Adams *et al*., 2001; Liu & Ahearn, 2001; Sandri & Carraro, 1999). Another initiating site for the development of the apoptotic cell death process in postmortem muscle could be the dysfunction of mitochondria (deterioration of the oxidative chain and increase in intracellular free radical concentration) occurring soon after death (Halliwell, 1991; Machlin & Bendich, 1987; Renerre, 1999). Indeed, mitochondria alteration might be another initiator of apoptosis through the release of caspase activating factors such as cytochrome c (Adrain & Martin, 2001). These are two possible pathways leading to muscle cell death but other pathways may exist (Hengartner, 2000; Torriglia *et al*., 2000). With respect to meat science, nothing is known about the molecular mechanisms of cell death, but this preliminary step might be of crucial importance in postmortem muscle since all subsequent changes contributing to the conversion of muscle into meat will be highly dependent on this early event.

Inhibitors of caspases and apoptosis

A large number of proteins are involved in the regulation of the apoptotic program leading to cell death (Adrain & Martin, 2001; Earnshaw *et al*., 1999; Koseki *et al*., 1998; Hengartner, 2000; Sandri & Carraro, 1999). They act either as inhibitors or activators of that process. With regards to inhibitors, the terms of apoptosis repressor (AR) or inhibitors of apoptosis protein (IAP) are generally preferred because the mode of action of these proteins is still not well known. Indeed, it is often not clearly established whether these inhibitors really inactivate the enzymes or inhibit some protein-protein interactions necessary for activation of the enzymes or for the formation of essential intermediate complexes. Nevertheless, as reviewed by Ekert, Silke, and Vaux (1999), there is clear evidence suggesting that some of them behave as tight binding inhibitors of various caspases. These authors also emphasized that some caspase protein inhibitors were unexpectedly classified as serpin-like proteins, a large family of serine peptidase inhibitors (see below).

Matrix metallopeptidases

General features

Matrix metallopeptidases (MMPs), also designated matrixins, represent a large family of structurally related zinc metalloendopeptidases responsible for connective tissue catabolism (Birkedal-Hansen *et al*., 1993; Parsons *et al*., 1997). At least 18 different MMPs with Mr ranging from 25,000 to 75,000 have been identified so far (Barrett *et al*., 1998). These enzymes play a central role in normal embryogenesis and tissue remodeling as well as in many diseases (Bode *et al*., 1999; Skiles *et al*., 2001). In addition, MMPs and their inhibitors are suspected to be implicated in programmed cell death or apoptosis (Mannello & Gazzanelli, 2001). MMPs are synthesized and secreted from a number of cell types including fibroblasts and muscle cells (Balcerzak, Querengesser, Dixon, & Baracos, 2001; Guerin & Holland, 1995; Nagase, 1996). Each one of these enzymes has a specific function, together with a particular specificity towards collagen substrates (Bode *et al*., 1999; Skiles *et al*., 2001). In all tissues and cells examined, they are secreted in a latent form and further activated sequentially in a cascade initiated by plasmin, tissue kallikrein, membrane-type MMP (MT-MMP) or other proteolytic enzymes (Parsons *et al*., 1997). The importance of matrixins in both physiological and pathological catabolism of connective tissue has been emphasized because little MMP activity can be detected in normal steady-state tissues, but the synthesis of many MMPs increased drastically upon muscle injury, denervation, anoxia and a series of other pathological conditions. The biological activities of MMPs are further controlled during the steps of their activation from inactive precursors as well as through interaction with specific inhibitors. In this respect, three types of protein inhibitors of MMPs have been described: the Tissue Inhibitors of Metallo-Peptidases (TIMP), the alpha-macroglobulin and the precursor of amyeloid beta-protein (Bode *et al*., 1999; Nagase, 1996; Parsons *et al*., 1997; Skiles *et al*., 2001).

MMPs in muscle tissue

In skeletal muscle, the extracellular matrix is structurally and functionally complex. This matrix, which contains collagen (types I, III, IV, V and VI), glycoproteins (laminin and fibronectin) and proteoglycans, contributes to the mechanical characteristics of muscle and to the transmission of force from muscle to tendon. MMPs and their specific inhibitors (TIMPs) are synthesized and secreted by fibroblasts and muscle cells, and their expression seems to be fiber type dependent (Balcerzak *et al*., 2001; Guerin & Holland, 1995; Singh *et al*., 2000). Their secretion is concomitant with the synthesis of the different proteins necessary for the sequential processing required for pro-MMPs to mature active MMPs through limited proteolysis (plasminogen activator, tissue kallikrein, plasminigen activator inhibitor, MT-MMPs, ….) (Balcerzak *et al*., 2001; Guerin & Holland, 1995; Singh *et al*., 2000). Most MMPs and their inhibitors are present in skeletal muscle, and although the exact function of each of these peptidases in the degradation of the connective tissue is still not well known, this machinery is able to denature the fibrillar collagen (collagenases MMP-1, MMP-13, …) which then can be degraded to small peptides by gelatinases (MMP-2, MMP-9, …) (Balcerzak *et al*., 2001). Amongst the connective tissue components and related structures, MMPs were shown to degrade the different types of collagen together with the cytoskeletal proteins connecting the sarcolemma to the extracellular matrix, the membrane-associated dystroglycans, and the neuromuscular endplates (Birkedal-Hansen *et al*., 1993; Frisdal *et al*., 2000; Kherif *et al*., 1999; Nagase, 1996; Schoser & Blottner, 1999; Yamada *et al*., 2001).

Specific protein inhibitors of MMPs or TIMPs

Four different TIMPs have been identified in mammals. They were designated TIMP-1, TIMP-2, TIMP-3 and TIMP-4, all having a molecular mass ranging from about 20 to 25 kDa as assessed from their amino acid sequence. However, depending on their degree of glycosylation, the mature forms of TIMPs might exhibit higher apparent Mr values when analyzed by SDS-PAGE. For example, the Mr of TIMP-1, a mannose-rich sialoglycoprotein was estimated to be 28,000 instead of 20,000 (Carmichael *et al*., 1986; Stricklin & Welgus, 1983), whereas the unglycosylated protein TIMP-2 showed a similar Mr of about 22,000 with both techniques (De Clerck, Yean, Ratzkin, Lu, & Langley, 1989; Herron, Banda, Clark, Gavrilovic, & Werb, 1986). All seemed to bind tighly to their target MMPs, leading to very stable complexes (K_d values in the nanomolar range or lower) but they are not hydrolysed by their respective target enzymes as are serpins, a large family of serine peptidase inhibitors. TIMP -1 to -3 are present in bovine tissues and expressed by cultured myogenic cells, whereas no analysis has performed so far for the presence of TIMP-4 (Balcerzak *et al*., 2001). However, because these MMPs inhibitors are ubiquitous and found in all tissues and cells investigated, TIMP-4 is probably expressed in muscle tissue as it is in bovine Adrenal Cortex (partial genomic sequence of bovine TIMP-4 submitted to EMBL/genbank by Hosseini and Pepper in 1997).

MMPs in the meat science field

Because collagen does not undergo drastic changes in meat stored at low temperatures (0–4°C), meat scientists have poorly studied MMPs. However, the absence of changes in connective tissue is based on routinely measured factors (solubility, macroscopic structural changes, degree of crosslinking,….) and other molecular aspects could be involved. These include:

- the disorganization of the neuromuscular junction,
- the alteration of the dystrophin-associated complex which spans the sarcolemmal membrane linking the intracellular cytoskeleton to the basement membrane, and then to the endomysium and the whole extracellular matrix,
- degradation of the proteins in charge of the spatial organization of the collagen fibrils such as colligin (Ball, Jain & Sanwal, 1997) and decorin (Weber, Harrison, & Iozzo, 1996).

Almost all the proteins involved in these connecting structures are susceptible to proteolysis by MMPs (see above for references) and, for some of them, by plasmin, an abundant peptidase located in the vicinity of the sarcolemma (personal observations). Skeletal muscle basement membrane can thus be degraded by locally generated plasmin (Hantai & Festoff, 1987). Detailed investigations on these proteins (agrin, laminin, perlican, dystrobrevin, fibronectin, colligin, decorin, collagen IV, dystroglycan, synaptic proteins,....) in postmortem muscle would be necessary to highlight changes affecting, at a molecular level, the connective tissue associated structures and their possible relation to meat texture and muscle cell death. On the other hand, MMPs and associated peptidases such as plasmin together with MMPs inhibitors also would have to be better characterized in postmortem muscle.

Muscle serine peptidases

General features

Serine peptidases are the largest group of proteolytic enzymes (Barrett *et al.*, 1998). Amongst them, the most widely known are probably the digestive peptidases (trypsin, chymotrypsin) and the pool of peptidases involved in the blood cloating process (thrombin, plasmin,....). Although the biological function is not clearly understood for all serine peptidases identified so far, they are found in most tissues, cells and body fluids. As they are involved in a large set of cellular events, aspects dealing with their biological and physiological functions which might be encoded in their C-terminal sequence (Krem, Rose, & Di Cera, 1999), will not be developed in this review, which will focus on their role in muscle tissue. The reader should refer to other reviews on this topic (Gettins, Patston, & Olson, 1996; Potempa *et al.*, 1994).

Serine peptidases in muscle tissue

Based on the loss of proteolytic activity following injection with '48/80', a compound that disrupts mast cells, it was suggested that most, if not all, serine peptidases having a neutral or alkaline optimum pH in crude muscle homogenates, originated from mast cells and not from striated muscle cells (Bird & Carter, 1980; Kuo, Giacomelli, Kithier, & Malbora, 1981; Libby & Goldberg, 1980; McKee, Clark, Benilich, Lins, & Morgan, 1979; Woodbury, Gruzenski, & Lagunoff, 1978).

However, immunolocalization studies indicated that muscle cells may contain neutral serine peptidases (Kay, Heath, Dahlmann, Kuehn, & Stauber, 1985; Stauber, Fritz, Dahlmann, & Reinauer, 1983). More recently, Margolius (1996) reported the presence, in skeletal muscle cells, of Kallikrein, a serine peptidase assumed to play an important role in the regulation of muscle cell metabolism and homeostasis (Mayfield, Shimojo, & Jaffa, 1996). Based on in vitro studies, muscle cells were shown to express and secrete the urokinase-type of plasminogen activator (Festoff, Rao, Maben, & Hantai 1990a, 1990b; Vult Von Steyern & Josefsson, 1995). In addition, embryonic and neonatal muscle express prothrombin mRNA which may be activated to act locally (Citron, Smirnova, Zoubine, & Festoff, 1997; Kim, Buonanno, & Nelson, 1998). In muscle, thrombin is believed to play a major role in the degradation and remodeling of the neuromuscular junction and the synapse as well as in inflammatory pathologies affecting skeletal muscles (Akaaboune *et al.*, 1998; Mbebi, Hantai, Jandrot-Perrus, Doyennette, & Verdiere-Sahuque, 1999). Morover, the expression of thrombin, thrombin receptor and protease nexin 1, a thrombin inhibitor, are strictly co-regulated in developing and denervated skeletal muscle (Kim *et al.*, 1998).

As reviewed by Birkedal-Hansen *et al.* (1993) several serine and metallopeptidases are present in the extracellular matrix. Amongst the serine peptidases, plasmin and its precusssor plasminogen are highly concentrated in muscle tissue (10–100-fold higher than the matrix-metallopeptidases) and seem to be located outside the cell in close association with the plasma membrane (personal observation). Taken together, these findings support the existence in muscle cells of different intracellular serine peptidases including thrombin, as well as a high concentration of plasmin in the extracellular compartment.

Serine peptidase inhibitors

Serine peptidase inhibitors constitute a complex family of functionally related proteins which can be classified within about 10 different families based on:

- their amino-acid sequences,
- the number and localization of the active sites,
- the mechanism of peptidase inhibition,
- the number of disulfide bridges (Laskowski & Kato, 1980).

Amongst them, the most important family is undoubtly the serpins, an acronym for serine proteinase inibitors (Carrell & Travis, 1985), a family first identified by Hunt and Dayhoff (1980). According to their primary sequence homology, the serpin family has been

first divided into two sub-groups designated ov-serpins (intracellular serpins homologous to ovalbumin and encoded by genes lacking the petide signal) and serpins (encoded by genes containing a peptide signal at their N-terminal end) (Korpula-Mastalerz & Dubin, 1996; Worrall, Blacque, & Barnes, 1999). More recently, efforts to elucidate the phylogeny of the vertebrate serpin superfamily led to the proposition of a new classification mainly based on the structure of their repective genes (number of introns and exons and their relative position at DNA level). This approach led to the identification of at least six subfamilies (Ragg *et al.*, 2001).

Although most serpins exhibit inhibitory activity against serine peptidases by acting as suicide inhibitors, some of them, like ovalbumin, colligin, tyroxine-binding protein, angiotensinogen and others, were classified as non-inhibitor serpins and were supposed to perform other functions (Ball, Jain, & Sanwal, 1997; Doolittle, 1983; Gettins *et al.*, 1996; Potempa *et al.*, 1994; Renold-O'Donnell, 1993; Worrall *et al.*, 1999). In most cases, the target enzymes of inhibitory serpins are serine peptidases. However, several examples have suggested that serpins might have a broader specificity than originally expected and, that their function is not restricted to serine peptidases. Interleukin-1-Beta-converting peptidase, which is currently characterized as a cysteine peptidase (Cerretti *et al.*, 1992), was shown to be inhibited by proxvirus serpins (Ray *et al.*, 1992). More recently, another serpin designated endopin and isolated from adrenal medulla chromaffin cells was found to inhibit the Prohormone Thiol Protease (PTP), a cysteine peptidase converting specifically inactive proenkephalin to active enkephalin by limited proteolytic cleavage (Hwang *et al.*, 1999). On the other hand, uterin serpins from cattle, sheep and pigs are potent inhibitors of pepsin, an aspartyl peptidase (Mathialagan & Hansen, 1996). Serpins were thus the first example of cross-class protein peptidase inhibitors and, as pointed out by Salvesen (1993), it is the geometry of the substrate-binding cleft and not the catalytic mechanism of peptidases that dictates selectivity for inhibitors.

Serpins regulate diverse physiological processes such as coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation, neoplasia and viral pathogenesis (Potempa *et al.*, 1994). The molecular structure plasticity and physical properties of serpins permit these proteins to adopt a number of different conformations under various physiological conditions (Janciauskiene, 2001; Whisstock, Skinner, & Lesk, 1998). Alterations of the serpin structure are often responsible for diverse pathologies, sometimes referred to as conformational diseases (Carrell & Gooptu, 1998; Janciauskiene, 2001).

In muscle tissue, several serpins have been described including Protease Nexin 1, a thrombin inhibitor (Festoff, Rao, & Hantai, 1991; Verdiere-Sahuque *et al.*, 1996), plasminogen activator inhibitor or PAI (Fibbi *et al.*, 2001) and kallistatin, a kallikrein binding protein (Richards, Chao, & Chao, 1997). Muscle cells also express alpha-1- antichymotrypsin and beta-amyloid protein precursor (Akaaboune, Verdiere-Sahuque, Lachkar, Festoff, & Hantai, 1995). Amongst a series of serine peptidase inhibitors fractionated from bovine skeletal muscle (Ouali *et al.*, 1995; Rouchon, 1995; Tassy, 1998), two serpins have been purified and further identified from their N-terminal sequence as antithrombin III and endopin respectively (Tassy, 1998).

Serine peptidases and meat tenderisation

Since the beginning of the 1980s and for the above mentioned reason (peptidases suspected to be only located in mast cells), very few studies on the serine peptidases have been conducted by meat scientists, and nothing is known about to their potential role in meat tenderization. However, whereas some authors found no change in the ultimate toughness of beef cuts injected with PMSF (*P*henyl-*M*ethyl-*S*ulfonyl-*F*luoride), an irreversible inhibitor of serine peptidases, a higher ultimate toughness in PMSF injected beef was reported, suggesting a possible contribution of unidentified serine peptidases to muscle proteolysis (Uytterhaegen *et al.*, 1994; Alarcon-Rojo & Dransfield, 1995). This assumption was supported by recent findings showing that amongst 15 different quantitative biological and physico-chemical measured variables, including levels of calpain 1, calpain 2, calpastatin and cysteine peptidase specific inhibitors, the *in vivo* concentration of endogenous serine peptidase inhibitors was found to be the best predictor of ultimate beef tenderness (Ouali, 1999; Zamora, 1997; Zamora *et al.*, 1996) (Table 2). Therefore, although our knowledge of these enzymes in muscle tissue is still extremely limited, we might keep in mind that the proteolytic potential represented by serine peptidases is probably very important and these would merit more consideration in the meat science field.

Muscle cysteine and serine peptidase inhibitors

Although skeletal muscle has been poorly investigated, several papers reported the presence, in that tissue, of protein inhibitors directed towards cysteine or serine peptidases (Bige, 1985; Bige *et al.*, 1985; Gopalan, Dufresne, & Warner, 1986; Kim, Chung, Woo, Ha, & Chung, 1992; Kuehn, Rutschmann, Dahlmann, & Reinauer, 1984; Matsuishi, Okitani, Hayakama, & Kato, 1988; Matsumoto, Okitani, Kitamura, & Kato, 1983; Noguchi & Kandatsu, 1969; Ouali *et al.*, 1986, 1995; Schwartz & Bird, 1977; Zabari, Berri, Rouchon, & Ouali, 1991; Zeece *et al.*, 1992). However, the classification of these inhibitors remains difficult or even impossible since no sequences are available so far. Attempting

Table 2. Biological predictors of bovine Longissimus muscle toughness after 8 days of storage: list of the most significant variables with their partial correlation values and the sign of this correlation (adapted from Zamora, 1998)

Quantitative variables	Partial r^2	Toughness correl. sign
Serine peptidase inhibitors	0.40	Positive
Rate of pH fall	0.25	Positive
LDH activity	0.24	Negative
Extent of osmotic pressure increase	0.13	Negative
Rate of calpain activity drop	0.09	Negative
Extent of pH fall	0.03	Positive

to fill in this gap, a complete overview of muscle peptidase inhibitors was initiated a decade ago by analysing several fractions obtained from a crude muscle extract after size exclusion chromatography on sephadex G100. As shown in Fig. 2, four fractions designated F1–F4 according to their decreasing molecular mass were separated. Whereas Fractions F1, F3 and F4 have been partially analysed and the inhibitors purified from each of them further characterized, nothing has been done on fraction F2.

Fraction F1 was shown to contain at least three cysteine peptidase inhibitors with Mr of about 43,000, 60,000 and 69,000, and three serine peptidases inhibitors with Mr close to 60,000, 63,000 and 70,000, respectively (Ouali *et al.*, 1995; Rouchon, 1995). Partial sequences were obtained for two of the serine peptidase inhibitors (Mr of 60 and 70 kDa) which were identifed as Antithrombin III and endopin (Tassy, 1998), two serpins originally identified in bovine blood and bovine chromaffin cells, respectively (Hwang *et al.*, 1999; Mejdoub *et al.*, 1991).

From fraction F3, a 34 kDa cysteine peptidase inhibitor was purified and characterized, but no partial sequence of this protein is presently available (Berri, Rouchon, Zabari, & Ouali, 1998; Berri, Venien, Levieux, & Ouali, 1996; Zabari, 1993). Nevertheless, this inhibitor showed a different Mr than either stefin, cystatins or kininogens, the three major groups of the cystatin superfamily, suggesting that this inhibitor is not a cystatin and that its target enzymes could be cysteine peptidases other than cathepsins, i.e. caspases.

From fraction F4, a 14 kDa cysteine peptidase inhibitor was purified (Bige, Ouali, & Valin, 1985) together with two serine peptidase inhibitors with Mr of about 11,000 and 14,000, respectively (Zabari, 1993). Unfortunately, as no sequence is available for these low Mr cysteine and serine peptidase inhibitors, they can not be classified yet.

This rapid overview clearly demonstrates the complex pattern of peptidase inhibitors in muscle crude extracts, a fact that is reflected by the abundant literature in the meat science field (Bige, 1985; Bige *et al.*, 1985; Gopalan *et al.*, 1986; Kim *et al.*, 1992; Kuehn *et al.*, 1984; Mat-

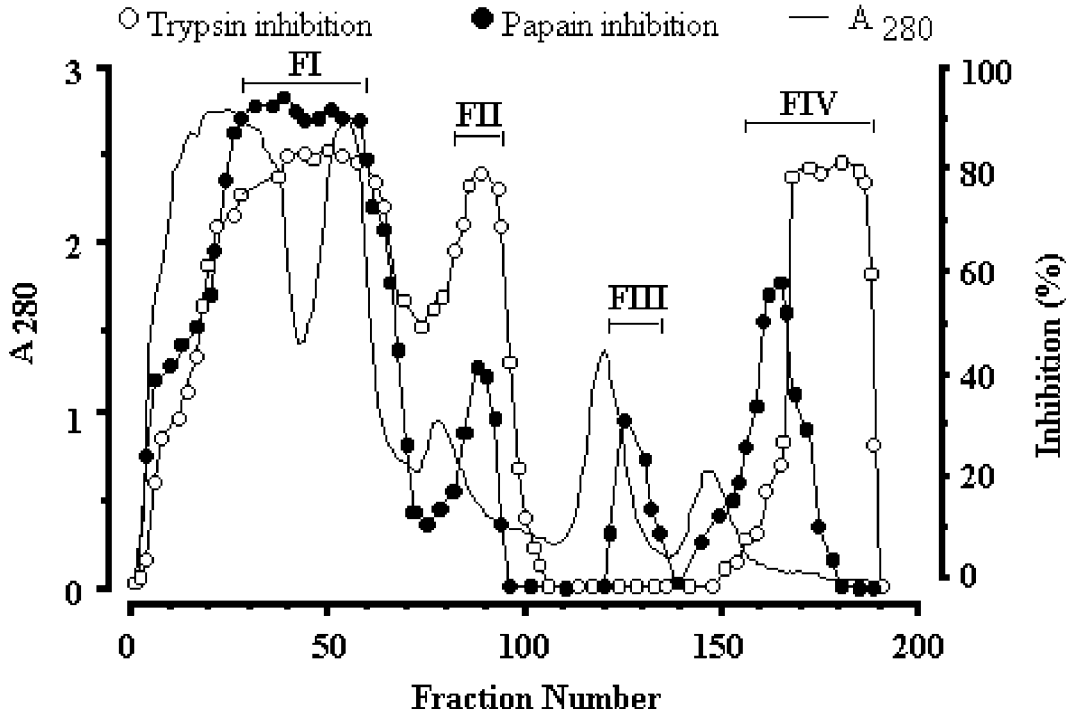

Fig. 2. Elution profile of inhibitory activities from a bovine muscle crude extract run on a Sephadex G100 column (5 × 100 cm). Fractions were tested for their inhibitory activity against cysteine (papain as a model) and serine (trypsin as a model) peptidases (Ouali *et al.*, 1995).

suishi *et al*., 1988; Matsumoto *et al*., 1983; Ouali *et al*., 1986, 1995; Noguchi & Kandatsu, 1969; Schwartz & Bird, 1977; Zabari *et al*., 1991; Zeece *et al*., 1992). It further emphasises the absolute need for a better characterisation of these proteins in muscle. It seems that peptidase inhibitors are often better markers of meat quality than their target enzymes (Ouali, 1999; Ouali & Talmant, 1990; Shackleford *et al*., 1991; Zamora, 1997; Zamora *et al*., 1996). Hence, this will be very likely a prerequisite for a better understanding of the enzymatic tenderisation of meat and its management by industry and animal producers. Moreover, such approach might constitute another way to identify the peptidases involved in that process.

Conclusions

Besides calpains and cathepsins, which have been the most extensively investigated so far in this context, the present review emphasizes that muscle tissue contains numerous other peptidases able to degrade a large set of muscle protein components. Some of these peptidases have been recently discovered and not yet considered in the meat science field. This is the case for members of the caspase family and for serine peptidases, which have been first considered to be located outside the muscle cell. Updating our knowledge on the different proteolytic systems identified in muscle tissue and further characterizing their potential role in postmortem muscle would invite us to reconsider the proteolytic mechanisms of the meat tenderising process and to consider peptidases other than the extensively studied calpains and cathepsins.

The present review further points out that, with regard to meat texture, we must consider not only the proteolytic enzymes but also their natural endogenous inhibitors, which are often better markers of this major meat quality attribute than the target peptidases themselves. Little is known about peptidase inhibitors in muscle tissue. Data available about their identity and their classification are, in most cases, very scarce. Consequently, meat scientists will have to clarify the complex pattern of peptidase inhibitors in muscle tissue and to characterize them including their identification and relationship compared to similar inhibitor families extensively investigated in other mammalian tissues and cells. The rapid development of Proteomic tools will probably be very helpful to achieve this goal.

The main challenge in meat science will be to identify biological markers of meat tenderness enabling a classification of carcasses in quality groups and an adjustment of the prices to the expected quality. It is worthy to note that a similar objective is already planned for the identification of biological quality markers of fresh meat cuts and the resulting processed meat products including dry-cured ham, sausages, … (Toldra & Flores, 1998, 2000). It is well recognized that peptidase inhibitors and their target enzymes are good predictors of meat tenderness at least in beef. In this context most research programmes are restricted to calpains and, though to a much lesser extent, to lysosomal cathepsins. In the light of the data recently available in the literature about a series of new proteolytic systems and their specific protein inhibitors, these studies must be extended to systems other than calpains and cathepsins. Amongst them, caspases, a newly identified group of cysteine peptidases never considered with regard to meat science, serine peptidases and serpins, the major family of serine peptidase inhibitors, and the proteasome appear to be a priority. Capases represent a good example since, after animal exanguination, muscle cells are engaged in a cell death programme in which these peptidases might very likely play a central role. Moreover, the cell death programme takes place in the first few minutes postmortem, a period known to be essential for the following time course development of meat tenderness.

Quantification of peptidases in muscle tissue has been done so far through measurement of their activity after partial fractionation of muscle extracts. However, this is only possible when the fractionation procedure is simple and short. Otherwise, proteolytic enzymes are not analysed. This was and still is the major limiting factor for the quantification of a series of peptidases including proteasomes, serine peptidases and many others. This also explains why lysosomal cathepsins have been less extensively studied than calpains. In addition, as no highly specific substrates are available for these enzymes and because only coarse fractionation procedures are generally applied, we can not certify that the measured activity is due to one enzyme or to a pool of co-fractionated enzymes. A good example of that situation is the coelution of the 20S proteasome with calpain 1 and, though to a lesser extent, with calpain 2 upon fractionation of the calcium peptidases by cation exchange chromatography (Fernandez, 2000). As the proteasome complex is able to hydrolyse calpain themselves and all calpain fluorescent or protein substrates, what exactly is the activity measured with this type of substrates? For protein quantification including proteolytic enzymes, it would be suitable to switch towards more specific techniques like ELISA (Enzyme Linked ImmunoSorbent Assay) or other immunoassay tools allowing us to work on crude muscle extracts and to be sure of the nature of the quantified antigen. Such techniques will further enable to work on large sets of samples (more than 200–300 samples can be analysed in one day) instead of 3–4 which is the maximum we can actually analyse in one day for calpain quantification. Immunochemical methods will therefore be very promising in our search for biological markers of meat quality. As a result, most research presently carried out in our laboratory involves development of ELISA tests for quantification of different proteolytic enzymes in muscle crude extracts.

Such tests are already available for proteasome, for some serpins and will be available soon for the different calpains and their inhibitor as well as for the major cathepsins.

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