Proteases and Meat Tenderization

James David Morton[a], **Zuhaib Fayaz Bhat**[a], **and Alaa El-Din Ahmed Bekhit**[b], [a] Lincoln University, Canterbury, New Zealand; and [b] University of Otago, Dunedin, New Zealand

Glossary

Actin A small globular protein important in the cytoskeleton of all eukaryotic cells and forms the major component of the thin filaments of myofibrils.
Aging The process where meat held at refrigerated temperatures becomes more tender as a result of proteolysis.
Apoptosis A controlled process of cell death mediated by caspases.
Calpains A family of calcium-dependent, cytoplasmic cysteine proteases found in most animal cells.
Calpastatin A protein which is a specific, reversible inhibitor of calpain
Caspases A family of proteases important in apoptosis
Cathepsins The proteases located in the lysosomes which are active under acid conditions.
Collagen Most abundant family of structural proteins in the connective tissue of animals.
Glycogen Storage form of glucose found in the muscle and liver of animals.
Myofibrils The elongated structural elements of muscle cells which are responsible for contraction.
Myosin An ATP-dependent enzyme which makes up the thick filaments in myofibrils and acts as a molecular motor to cause contraction following binding of actin.
Proteases The enzymes which break down other proteins by hydrolysis of peptide bonds
Proteasome A large structure in the cytoplasm with a set of proteases which are responsible for ATP-dependent proteolysis of ubiquitin-labeled proteins.
Rigor or rigor mortis Stiffening of muscles after death due to the irreversible binding of myosin to actin after ATP levels have declined in post-mortem muscle.
Sarcomere The repeating subunit of myofibrils in striated muscle.
Ubiquitin Small cytoplasmic proteins which binds to unwanted proteins and presents them to the proteasome.

Muscle to Meat

People have cooked and consumed muscles from mammals, birds and fish for thousands of years. Meat continues to be an important source of protein and other micronutrients in the human diet. The characteristics of the meat we eat are the result of the composition of the muscle it was and the processes that occur to it after the death of the animal. Meat is derived from skeletal muscle and meat quality varies with the composition of those muscles. All the muscles have long myofibrils consisting of repeating sarcomere which are responsible for the contraction function of muscles (Ertbjerg and Puolanne, 2017). Surrounding these myofibrils is a variable amount of connective tissue principally made up of collagen.

The process of muscle turning to meat begins as the animal is slaughtered and the circulation ceases (Bate-Smith and Bendall, 1947). In the absence of oxygen and external fuel, the muscle metabolizes its glycogen reserves to maintain homeostasis and preserve its ATP level. This anaerobic glycolysis leads to an increase in lactic acid and a decline in pH. Typically when the pH drops to around 5.5, glycolysis stops and the ATP levels fall. At this stage the sarcomere lengths are fixed as myosin binds to actin and the muscle enters rigor (Matarneh et al., 2017).

Tenderness

The texture or tenderness of meat is one of the most important quality attributes for consumers (Miller et al., 2001) and is largely dictated by the physical and biochemical status of proteins in the meat. Tenderness is a particular issue with the meat from mammals such as cattle, pigs and sheep. Customers are willing to pay a premium for meat that is more likely to be tender. Tenderness is measured on cooked meat either subjectively with consumer panels (Boleman et al., 1997) or objectively by measuring the shear force to cut through a defined cross-section of meat (Shackelford et al., 1995). These measurements have shown a high level of variability with high proportions of unacceptably tough beef but also lamb and pork (Bickerstaffe et al., 2001). The overall shear force consists of a background component, which is directly related to the proportion and type of collagen (Purslow, 2014), and another variable component resulting from the myofibrillar proteins. This second component increases to a maxima as the muscle goes into rigor and then decreases over time as the rigor is resolved

in a process known as aging or tenderization (Wheeler and Koohmaraie, 1994). The peak toughness is related to the sarcomere length which is determined by the conditions, temperature, pH and restraint, at the time the muscle goes into rigor (Marsh and Leet, 1966). The increase in tenderness with aging is the result of the breakdown of key structural proteins in the myofibrils by endogenous proteases and varies with species and muscles (Kemp et al., 2010). Although there is agreement on the importance of enzymes in aging there is still controversy over the contribution of individual proteases. A candidate enzyme must be found in the muscle, have access to the myofibrils, be active post-mortem and should be able to reproduce the *in vivo* pattern of proteolysis *in vitro* (Koohmaraie and Geesink, 2006).

Calpains

The family of proteases that best fits the above criteria is the calpains (Goll et al., 2003). These proteases were originally found in skeletal muscle (Dayton et al., 1976) but are now known to be ubiquitous in animal cells. Their defining characteristic is that they require calcium for activation. Fourteen genes for calpain have been identified in the human genome (Ono et al., 2016) and three of these, calpain 1, 2 and 3, have been thought to have a role in tenderization. Calpain 1 and 2 have similar structures with an 80 kDa large subunit which contains the active site and a 28 kDa small subunit. They vary in the amount of calcium required for half maximal activity of approximately 50 μM for calpain 1 and 500 μM for calpain 2 which gave them their earlier names of μ-calpain and *m*-calpain respectively (Goll et al., 2003). These values are considerably greater than the physiological levels of calcium which are of the order of 0.1 μM. An initial autolysis step lowers the calcium requirement of both calpains with autolyzed calpain 1 having a half maximal activity near 3 μM (Goll et al., 2003). There is also evidence that this autolysis makes the calpain 1 less stable and leads to further autolysis and denaturation (Geesink and Koohmaraie, 2000). Measurement of autolyzed calpain is used to indicate calpain activity (Raser et al., 1995). The calpains have a complex specificity (Shinkai-Ouchi et al., 2016) and often modify the activity of their substrates by making single cuts Many of the substrates of calpains are cytoskeletal proteins and they are involved in changes in cell structure such as the development of muscle fibers (Sorimachi and Ono, 2012).

The calpain hypothesis for aging of meat begins with the decrease in ATP levels post mortem (Koohmaraie and Geesink, 2006). The cell cannot maintain low sarcoplasmic calcium as the active pumps in the sarcoplasmic reticulum and mitochondria require ATP. With the rise in calcium calpain 1 is activated (Hopkins and Thompson, 2001) and this operates on specific cytoskeletal proteins which are involved in maintaining the structure of the myofibrils. The most important of these are thought to be titin, the giant protein which connects the m-line to the z-disk, desmin, which connects bundles of myofibrils to each other, and vinculin in the costameres, the structures which attach myofibrils to the sarcolemma (Taylor et al., 1995). Nebulin and troponin T, key components of the thin filaments, are also calpain substrates. This proteolysis destabilizes the myofibrils and leads to tenderness. Importantly actin and myosin, the most abundant proteins in the myofibrils are neither good calpain substrates nor extensively proteolysed in post mortem meat.

The pattern of proteolysis when myofibrils were incubated with purified calpain 1 or 2, is similar to what is seen in aged meat (Huff-Lonergan et al., 1996). It was also found that infusion with calcium tenderised meat (Koohmaraie, 1990) while zinc and other more specific calpain inhibitors inhibited aging (Uytterhaegen et al., 1994). Calpain 1, rather than calpain 2, is considered the key enzyme, based on its lower requirement for calcium and its autolysis pattern (Koohmaraie et al., 1987). Calpain 1 is autolyzed in the first few days post-mortem where more proteolysis occurs while most calpain 2 can be intact weeks after death (Morton et al., 1999). This was confirmed by experiments in mice where knocking out the calpain 1 gene prevented post-mortem proteolysis of the muscle (Geesink et al., 2006). There is however evidence that calpain 2 may be important in the later stages of aging in some muscles (Colle and Doumit, 2017).

An important part of the calpain system is calpastatin, a protein with the sole known function of reversible, calcium-dependent inhibition of calpain. The inhibitory region of calpastatin binds to either side of the active site of calpain and blocks access to substrates (Moldoveanu et al., 2008). The importance of calpastatin is evident from the strong correlation between calpastatin levels and the rate of aging both between (Ouali and Talmant, 1990) and within species (Shackelford et al., 1991). Increasing the expression of calpastatin either in transgenic animals (Kent et al., 2004) or with β-agonists (Koohmaraie et al., 1991) leads to reduced post-mortem proteolysis and tougher meat. Increased calpastatin can be the mechanism when changes to increase animal growth sometimes lead to tougher meat as in the callipyge sheep (Koohmaraie et al., 1993). Genetic variation in calpastatin is linked to tenderness in cattle (Casas et al., 2006) and is the basis of commercial testing for tenderness potential.

A third isoform, calpain 3, is found in large amounts in muscle and is bound to titin, one of the key target proteins in aging (Sorimachi et al., 1995). Mutations in the calpain 3 gene are responsible for a muscle wasting disease in humans, limb girdle muscular dystrophy type 2A. Calpain 3 levels were correlated with aging in lamb (Ilian et al., 2004) but not in pork (Parr et al., 1999). However it is unlikely that calpain 3 plays a dominant role in aging as knocking out the calpain 3 gene did not affect post-mortem proteolysis in mice (Delgado et al., 2001) and it is not inhibited by calpastatin (Ono et al., 2004).

Other Endogenous Proteases

Calpains are not the only proteases in muscle and there is evidence that other proteases have either a direct role in aging or interact with calpain. The cathepsins are the proteases which digest cellular components in the lysosome and are active at the acid pH of

meat (Sentandreu et al., 2002). However they would only have access to the myofibrils under conditions where the lysosomal membranes have been ruptured. They also proteolyse actin and myosin and there is only very limited breakdown of these proteins during aging (Mikami et al., 1987). The proteasome is another candidate protease. This is found in large amounts in the muscle and normally works by an ATP-dependent process involving recognition and proteolysis of ubiquitin-labeled proteins (Robert et al., 1999). In the absence of ATP, as in post-rigor muscle, the proteasome is no longer ubiquitin dependent and has been shown to maintain activity for at least one week post-mortem (Lamare et al., 2002). It can reproduce some of the characteristics of aging and proteolysis in chilled meat is slowed by proteasome inhibitors (Houbak et al., 2008) but the overall pattern of proteolysis differs from normal aging.

Recent theories of early aging have considered it as a process of apoptosis or controlled cell death (Becila et al., 2010). The caspases are the key enzymes activated in apoptosis and there is evidence that caspase 3 can reproduce many of the characteristics of post-mortem proteolysis in myofibrils (Kemp and Parr, 2008). Caspases have also been suggested to be the target of the serine peptidase inhibitors or SERPINs which have correlated with toughness in beef (Herrera-Mendez et al., 2009). There is considerable evidence that the caspases interact with calpains and are able to proteolyse calpastatin (Wang et al., 1998). Thus the current view of post-mortem aging is centered on calpains but involves interactions with several other groups of proteases.

Proteomic approaches have identified several groups of proteins which affect aging. These suggest that the extent of proteolysis is determined by the action of other proteins which protect either the proteases or their substrates (Lana and Zolla, 2016). These include heat shock or chaperone proteins and enzymes involved in metabolism (Gagaoua et al., 2015). There is also a strong correlation of tenderness with proteins which protect against oxidation (Lana and Zolla, 2015) and it is known that calpain is very susceptible to oxidation (Lametsch et al., 2008). The activity of proteases can also be affected by post-translational modification. Calpain 1 activity is changed by phosphorylation either of the protease or its substrates (Li et al., 2016).

The rate and extent of aging is greatly impacted by the changes in temperature, pH and ionic strength within the muscle cells during the hours immediately in following slaughter (Marsh et al., 1987). There is also a relationship with ultimate pH with muscles of intermediate pH 5.8–6.1 being toughest (Purchas, 1990). Many of these changes can be explained by the effect of the conditions on the balance of calpain 1 activity, autolysis and denaturation (Geesink and Koohmaraie, 2000; Mohrhauser et al., 2014).

Exogenous Proteases and Tenderisation

The highest value muscles on a carcass are those that can be used for grilling but only a small proportion of the muscles become that tender even with optimal aging (Sullivan and Calkins, 2011). The remaining muscles typically have higher levels of connective tissue which cannot be cleaved by the endogenous enzymes. These tough cuts of meat have been successfully tenderised by the use of plant proteases principally papain from papaya, bromelain from pineapple, ficin from figs and actinidin from kiwifruit (Bekhit et al., 2014). The proteases can be infused or injected into the meat or used as a marinade. These plant preparations are often crude extracts with a variety of proteases with low specificity and digest all meat proteins both from the myofibrils and connective tissue. They are very effective at decreasing the shear force of meat but some of them, for example papain, tend to over-digest the meat and leave it with a mushy texture unlike normally aged meat (McKeith et al., 1994). Others, such as actinidin (Lewis and Luh, 1988) and some of the microbial enzymes (Ryder et al., 2015), are more specific and lead to a more controlled tenderness development. The proteolysis following application of infusion of kiwifruit juice to lamb was partly due to activation of calpain 2 (Han et al., 2009).

Conclusions

Aging or tenderization of meat occurs in refrigerated conditions and is the result of interactions between endogenous proteases and muscle proteins. Calpain 1 is believed to be the main protease responsible for meat tenderization during post-mortem aging, however, there is evidence for the involvement of other proteolytic system either direct or indirectly in the aging process. Calpastatin, the specific calpain inhibitor, modulates the activity of calpain 1. The extent of aging is also affected by other proteins and the temperature and pH of the meat Aging alone cannot tenderize certain meat cuts and interventions, such as the use of exogenous proteases, could improve the tenderness level.

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