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Enzymes in Tenderization of Meat – The System of Calpains and Other Systems – a Review

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Tenderness of meat is considered as the most important feature of meat quality. Three proteolytic systems present in a muscle were examined as those which can play a role in the *postmortem* proteolysis and tenderization: the system of calpains, lysosomal cathepsins and MCP (multicatalytic proteinase complex). There are several theories (enzymatic or non-enzymatic) explaining the tenderization process. The calpain theory of tenderization was recognized as the most probable. During tenderization the main structures of a cytoskeleton are degraded as well as myofibril and cytoskeletal proteins. Meat becomes soft and the process of tenderization is accompanied by changes in the ultrastructure (degradation of the Z-line and the I-band). Many studies show that the system of calpains (especially calpain I and calpastatin) plays a major role in *postmortem* proteolysis and meat tenderization. However, recent studies show that proteasomes and caspases may be responsible for this process as well. This paper includes the characterization of calpains as well as describes the construction and functioning of the system of calpains. Additionally, this article presents factors influencing the activity of calpains. It was also mentioned that other systems, such as proteasomes and caspases, may be involved in *postmortem* tenderization of meat.

INTRODUCTION

Tenderness of meat is the most important quality distinguishing feature of meat in consumer evaluation [Koohmaraie, 1994, 1996; Boleman *et al*., 1995; Miller *et al*., 2001; Koohmaraie & Geesink, 2006; Destefanis *et al*., 2008; Zork *et al*., 2009]. The problem of tenderness concerns mostly beef which requires at least 14 days of storage in cooling conditions to obtain the final tenderness, while pork requires 5–7 days, and lamb 7–10 days [Koohmaraie & Geesink, 2006]. Consumers are willing to pay higher prices for beef which has guaranteed appropriate tenderness [Boleman *et al*., 1995, 1997; Lusk *et al*., 2001; Shackelford *et al*., 2001; Feldkamp *et al*., 2005]. Various pre-slaughter and post-slaughter factors and their mutual effects influence tenderness of meat [Destefanis *et al*., 2008]. The most important pre-slaughter factors include: age, species, sex, breed, feeding of animals, degree of stress prior to slaughter, *etc.* Post-slaughter transformations, including *rigor mortis* and ageing, have a crucial influence on meat tenderness. It was observed that in the ageing process one can distinguish changes in the micro and ultrastructure of muscle fibers (weakening of myofibrils, fragmentation, changes in the area of the Z-line and the I-band) and degradation of myofibrillar and cytoskeletal proteins: T troponins, I troponins, titins, desmins, dystrophins, nebulins, vinculins, meta-vinkulins [Koohmaraie, 1992, 1994; Taylor *et al*., 1995a; Takahashi, 1996; Tornberg, 1996; Korzeniowski *et al*., 1998; Kołczak, 2000; Koohmaraie & Geesink, 2006]. These changes lead to obtaining the final meat tenderness.

Many studies have shown that tenderness is dependent on such enzymes as: cathepsins and calpains. Some researchers suggest that proteasomes may be responsible for the final tenderness, while others that caspases [Bernard *et al*., 2007; Kemp *et al*., 2010]. However, many studies have assigned this role to the calpains system [Neth *et al*., 2007; Kemp *et al*., 2010]. Therefore, in this paper most attention is dedicated to calpains. This paper includes the characteristics of calpains as well as their localization, structure, activity of calpain system, and research in this field. Recently, genetic tests have provided much information. Markers are used commercially to assess meat quality. At present, we are observing great interest in the caspases which may contribute to *postmortem* proteolysis and tenderization of meat. This paper mentions this fact as well.

ENZYMES IN TENDERIZATION

The mechanism of meat tenderization is complicated. Numerous investigations are being conducted which are aimed at explaining the mechanism of meat tenderization and factors responsible for the initiation and course of this process. There are theories of enzymatic and non-enzymatic meat tenderization. Many studies have shown that enzymes (proteases) are responsible for tenderization. It is considered that the protease system can be involved in *postmortem* proteolysis and meat

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tenderization if it meets certain basic criteria: proteases must be endogenous to skeletal muscle cells; they must be able to mimic *postmortem* changes in myofibrils *in vitro* and have access to myofibrils in a tissue. Investigations conducted by many researchers determined proteolytic systems present in a muscle which can participate in the *postmortem* proteolysis and tenderization: the system of calpains, cathepsins and proteasomes (mainly MPC - multicatalytic proteinase complex, called 20S proteasome) [Dransfield *et al*., 1992a,b; Koohmaraie, 1996; Korzeniowski *et al*., 1998; Koohmaraie & Geesink, 2006; Kemp *et al*., 2010].

Cathepsins are a group of enzymes comprising exo- and endo-peptidases and categorized into: cysteine (cathepsins B, H, L, X), serine (cathepsin G), and aspartic (cathepsins D, E) peptidase families [Sentandreu *et al*., 2002; Kemp *et al*., 2010].

The participation of cathepsins in tenderization is doubtful, as there are no proofs that during the *postmortem* storage of meat cathepsins are freed from lysosomes. Moreover, cathepsins have the ability to disintegrate myosin, actin and α-actinin, and during normal ageing of a muscle a small quantity of these proteins is degraded [Koohmaraie, 1988, 1996; Whipple & Koohmaraie, 1991; Purslow *et al*., 2001; Koohmaraie & Geesink, 2006; Geesink *et al*., 2006].

Initially it was thought that the participation of MCP in tenderization is very unlikely [Koohmaraie, 1992b; Taylor *et al*., 1995b]. Subsequent studies have provided evidence about the possibility of proteasomes contribution to meat tenderization [Dutaud *et al*., 2006; Kemp *et al*., 2010]. Proteasomes are very large protein complexes. They are located in the muscles and cytoplasm. The 26S proteasome is a large intracellular protease that identifies and degrades proteins tagged for destruction by the ubiquitin system. The 26S proteasome consists of a 19S regulatory subunit and a 20S multicatalytic structure possessing the proteolytic enzyme activities. The 20S proteasome is built of 4 rings, which form a cylindrical structure. Each ring consists of 7 different subunits with the molecular weight of *ca*. 20–35 kDa. The 20S proteasome was named MPC (multicatalytic proteinase complex) [Dahlmann *et al*., 2001]. Studies have shown specific structural changes including an increase in the Z-line, stretching into the I-band in muscle fibres incubated with 20S [Dutaud *et al*., 2006; Kemp *et al*., 2010]. However, the degradation of myofibrilar proteins pattern in incubations with 20S is not the same as that seen *postmortem* in a muscle, but this does not exclude the participation of the 20S proteasome in the *postmortem* proteolysis [Koohmaraie & Geesink, 2006; Houbak *et al*., 2008; Kemp *et al*., 2010].

At present, the system of calpains is thought to be responsible for the *postmortem* proteolysis of the key proteins and in result meat tenderization [Goll *et al*., 2003; Koohmaraie & Geesink, 2006; Neath *et al*., 2007; Bernard *et al*., 2007].

CONSTRUCTION AND FUNCTIONING OF THE SYSTEM OF CALPAINS

Calpains are a superfamily of 14 cysteine proteases, but the system of calpains in a skeletal muscle consists of at least 3 proteases: calpain I (μ), calpain II (m) and calpain 3 (p94), as well as calpastatin – being a calpain inhibitor [Goll *et al*., 2003; Koohmaraie & Geesink, 2006; Moudilou *et al*., 2010].

Neutral proteases activated by Ca^{+2} ions, called calpains, occur universally in animal cells. In a muscle, calpains are located in the cytoplasm and cell membranes. The Calpain II is in majority located in the cytosol, whereas the calpain I is in 70% bonded to myofibrils [Xian-Xing *et al*., 2009].

Earlier investigations showed that calpain I is in 66% situated on the Z-line, and the rest is in the I-band (20%), and A-band (14%). However, the calpain II is in 52% situated on the Z-line and also in the I-band (27%), and A-band (21%) [Koohmaraie, 1994; Kumamoto *et al*., 1992]. Whereas, calpain 3 in the largest degree is situated in a sarcomere near the Z- and M-line [Ilian *et al*., 2004c]. The localization of calpastatine (a specific inhibitor of calpains) in the muscle cell is similar to that of calpains.

The structure and functioning of these enzymes was also fathomed. Calpain I encoded by CAPN1 gene, and calpain II encoded by CAPN2 gene, are activated *in vitro* by micromolar and millimolar calcium concentrations, respectively. Both calpains consist of a specific large catalytic subunit (molecular mass 80 kDa) and an invariant small regulatory subunit (30 kDa), encoded by CAPN4 gene [Goll *et al*., 2003; Moudilou *et al*., 2010]. In the large subunit one can distinguish four domains (I - IV), and in the small subunit two domains (V and VI). The I domain acts as the inhibitor of proteolytic activity. A partial removal of this domain through the autolysis method activates the enzyme. The II domain is catalytic and similar to other well-known cysteine proteases such as B, H, and L cathepsins. Whereas, four areas were affirmed in the IV domain which can be responsible for bonding calcium ions. The V domain is strongly hydrophobic and rich in glycine residues. A large part of this domain is consumed during the autolytic activation of an enzyme. The VI domain, similarly as the IV, contains four areas binding calcium ions [Jakubiec-Puka, 1993; Carragher & Frame, 2002]. Calpain 3 is encoded by the CAPN3 gene [Moudilou *et al*., 2010]. The calpain 3 is a single polypeptide of the molecular mass of 94 kDa whose large sub-unit is homologous with the large sub-unit of the μ- and m-calpain [Koohmaraie & Geesink, 2006]. The calpains I and II are negatively regulated by the calpastatin – a specific endogenous inhibitor [Moudilou *et al*., 2010]. Calpastatin is a protein with a molecular mass of 60–70 kDa. Calpastatin contains 4 inhibitory domains, each of which can inhibit calpain activity. Within these domains there are three regions (A, B, C) predicted to interact with calpain. The region between A, and C, region B, then blocks the active site of calpain [Goll *et al*., 2003; Kemp *et al*., 2010].

So far, the mechanism of the proteolytic functioning of calpains has not been entirely elucidated. Very often, the knowledge on the subject of this mechanism is acquired by way of intermediate proofs on the basis of the measurement of activity of a cleaned enzyme towards individual proteins, functioning of inhibitors and activators, changes of concentration and localization of the intracellular calpain and calpastatin, effects of calpain activation in isolated cells or tissue scraps. While studying products of degradation of proteins present in a muscle during ageing and products which are produced during the *in vitro* incubation of myofibrils with calpains one can determine the influence of calpains on the process of tenderization.

The activity of the system of calpains depends on many factors such as: pH, temperature, and first of all concentration of calcium ions [Steen *et al*., 1997; Goll *et al*., 2003].

The first mechanism of tenderization was proposed by Dransfield [1992a, c, 1994a, b]. According to this author, the calpain I in meat after slaughter is inactive due to a low concentration of calcium ions which in the sarcoplasm reaches as few as 10^{-7} mol. Activation of the calpain I occurs around 6 hours after slaughter (pH around 6.1–6.3) as a result of calcium ions concentration increase in the sarcoplasm to the value of 10^{-4} mol. The increase of calcium ions concentration in the sarcoplasm takes place through the activation of the calcium pumps or through the proteolytic attack on the sarcoplasmic reticulum [Dransfield, 1992a,c, 1994a,b].

According to other investigations the calpain I needs 3–50 μmol/L calcium ions to be activated and the calpain II 0.4–0.8 mmol/L calcium to achieve half its maximum activity. The concentration of calcium ions in a living muscle reaches 0.2 μmol/L and it is a level much lower than that required to activate the calpains I [Kurebayashi *et al*., 1993; Goll *et al*., 2003]. However, after slaughter the concentration of free calcium in a cell increases and accounts for 100 μmol/L [Jeacocke, 1993]. Hopkins & Thompson [2001] proved that the concentration of calcium ions after slaughter at pH 5.5 in *longissimus lumborum* (LL) and *longissimus thoracis* (LT) muscles of sheep reached 110 μmol/L and was sufficient to activate the calpains I.

The activation of the enzyme under the influence of calcium ions (bonding to the end of every sub-unit) causes hydrolysis of both sub-units. In result of the hydrolysis the large sub-unit alters its mass from 80 kDa to 76 kDa, while the smaller subunit from 30 kDa to 18 kDa [Jakubiec-Puka, 1993; Dransfield, 1999; Moudilou *et al*., 2010]. Further autolysis causes the formation of fragments from the large sub-units, which have even smaller mass, and the loss of enzymatic activity.

The optimum conditions of calpains activation have been recognized as well. When determined *in vitro* they included pH 7.2–7.8 and a temperature of 25°C [Dransfield, 1999; Kanawa *et al*., 2002]. However, during ageing, meat is kept in a much lower temperature (cooling conditions), and its pH reaches around 5.5–5.7. Investigations have shown that cleaned myofibrils are degraded by the μ-calpain at the temperature of 4°C and pH 5.6 in the presence of 100 μmol/L calcium chloride [Huff-Lonergan *et al*., 1996]. Thomson *et al*. [1996] proved that the activity of the calpain I was decreasing within the first several days after slaughter and was correlated with tenderization increase.

By studying products of protein degradation present in a muscle during ageing, and also products which are formed during the *in vitro* incubation of myofibrils with calpains one can determine the influence of calpains on the process of tenderization. The effects of caplains on muscle myofibrils were understood quite well. At first, calpain contributes to the removal from myofibrils of the N_2 line - a gentle structure running parallel to the Z line. Then, the Z line is removed, breaks appear in its continuity and gradually the Z line disappears entirely. The removal of the Z line causes the degradation of myofibrils. Calpain frees the α-actinine from the Z line without causing its degradation. It also does not cause the degradation of actin nor myosin. However, it causes the degradation of tropomyosine, T and I troponin and basic cytoskeletal proteins: titin, desmin, nebulin, dystrophin, vinculin, and meta-vinculin [Koohmaraie, 1992, 1994; Jakubiec-Puka, 1993; Homma *et al*., 1995; Taylor *et al*., 1995a; Takahashi, 1996; Tornberg, 1996; Steen *et al*., 1997; Korzeniowski *et al*., 1998; Kołczak, 2000; Koohmaraie & Geesink, 2006; Kemp *et al*., 2010]. The degree of cytoskeletal protein degradation determines the tenderness of meat. Degradation of T troponin causes the appearance of fragments with the molecular mass of 27 kDa or 30 kDa [Geesink *et al*., 2001]. Similar changes can be observed during incubation of myofibrils with the μ-calpain at the temperature of 4°C and pH 5.6 [Huff-Lonergan *et al*., 1996]. Hydrolysis of T troponin through μ-calpain causes release of 8 peptides with the molecular mass from 14 to 26 kDa [Hughes *et al*., 2001]. The majority of components of muscle fibres, especially cytoskeletal proteins were recognized as a potential biological parent substance of calpains [Sentandreu *et al*., 2002]. The level of calpains depends on the species of animals (beef, pork, sheep), their breed, and the type of muscle and its activity [Northcutt *et al*., 1998]. Similar observations were made in relation to calpastatin. The activity of calpain and calpastatin is correlated with tenderness [Geesink & Koohmaraie, 1999; Neath *et al*., 2007]. For example, meat of callipyge sheep, which is hard, is characterized by a high level of calpastatin and by a slow degradation of proteins [Geesink & Koohmaraie, 1999].

Some researchers affirmed that calpain 3 can play an important role in meat tenderization [Ilian *et al*., 2001, 2004a,b,c]. Ilian *et. al*. [2004a,b] showed that the calpain 3 influenced proteolysis of titin and nebulin. However, some other researchers deny that the calpain 3 participates in *postmortem* meat tenderization [Geesink *et al*., 2005].

It is assumed that the system of calpains in a skeletal muscle responsible for meat tenderization can also be created by different enzymes which are still unknown.

Numerous investigations conducted so far have shown that the majority of the activated calpain is bond by calpastatin. The required concentration of calcium ions to inhibit calpain I and II by calpastatin is: 40 μmol/L and 250–500 μmol/L, respectively. Crystallographic observations identify the nature of the interaction of calpastatin with calpain [Hanna *et al*., 2008; Moldoveanu *et al*., 2008].

The binding of calcium to calpain causes changes in a calpain molecule enabling it to become active but also allowing calpastatin to interact with the enzyme.

Quantification of calpastatin as a meat tenderness biomarker has been performed using different methods such as: ELISA, a surface plasmon resonance or the fluorescence resonance energy transfer-based immunosensors [Zór *et al*., 2009]. Recently, the development of optical fiber and capillary-based biosensor for calpastatin detection in heated meat samples has been reported [Zór *et al*., 2009]. The latest studies have focused on determining the factors that regulate calpastatin gene expression. They have shown that it is regulated *via* several promoters associated with the 5'exons: 1xa, 1xb, which are in tandem and 1u, which is the 3' and distal to 1xa and 1xb [Meyers & Beever, 2008]. The difference between transcriptional activity of the calpastatin gene promoters between species and their different response to stimuli is probably partly responsible for the variation in calpastatin expression that contributes to variations in meat tenderness [Kemp *et al*., 2010]. This, however, requires further research.

INFLUENCE OF ES AND Ca^{2+}-IONS ON THE ACTIVITY OF CALPAINS

The influence of calcium chloride and electric stimulation on the activity of calpains were also studied as well as the influence of these factors on the growth of meat tenderness. Many investigations were conducted on the influence of electric stimulation (ES) on meat tenderness. A part of these investigations affirmed a positive influence [Soares & Areas, 1995; Morton *et al*., 1999; Maribo *et al*., 1999; Lee *et al*., 2000; Hope-Jones *et al*., 2010], the other part affirmed a negative one [Olsson *et al*., 1994; Tornberg, 1996]. The positive influence concerned high-voltage ES. Electric stimulation causes an increased inflow of calcium ions to cytoplasm and this way it can cause the activation of μ-calpain [Veeramuthu & Sams, 1999]. ES should not be executed soon after slaughter. Hwang *et al*. [2001] claimed raised hardness of meat when ES was applied after 3 minutes in comparison to that conducted after 40 minutes.

Electrical stimulation improved loin tenderness of both β-agonist supplemented and non-supplemented animals. ES advances the tenderness by reducing the activity of calpastatin [Hope-Jones *et al*., 2010]. Introduction of calcium ions to a muscle by way of an injection, infusion or pickling can improve the tenderness of meat through the activation of the system of calpains [Koohmaraie, 1992; Whipple & Koohmaraie, 1991; Boleman *et al*., 1995; Rees *et al*., 2002]. The best results are obtained by applying an injection in the possible shortest time postmortem and applying 0.3 mol/L solutions of $CaCl_2$ [Morgan *et al*., 1991; Boleman *et al*., 1995]. This way tenderness of meat can increase by 50% only after 24 h [Morgan *et al*., 1991]. Ca and Mg ions are in favour of calpains activity and Zn ions are the inhibitors of calpains. This was confirmed by the conducted investigations in which meat injected with Ca and Mg ions (0.3 mol/L solution of chloride) was more tender (on the basis of shear force measurements) than in the check test made at the temperature of 4ºC [Nowak & Korzeniowski, 2004; Nowak, 2005].

CALPAINS OR/AND CASPASES IN TENDERIZATION?

However, present investigations suggest that the calpain I (μ) is responsible for the degradation of myofibrils structure and in result for the beginning of meat tenderization [Huff-Lonergan *et al*., 1996; Koohmaraie *et al*., 2002; Geesink, 2006; Neath *et al*., 2007]. The calpain II (m) and other proteases can contribute to further proteolysis in the later period of posthumous ageing [Boehm *et al*., 1998; Dutaud *et al*., 2006; Neath *et al*., 2007]. Calpastatine participates in meat tenderization and is negatively correlated with tenderness [Kent *et al*., 2004; Neath *et al*., 2007]. Therefore it is claimed that calpains I and II and calpastatine play an important role in meat tenderization [Camou *et al*., 2007].

Currently, we have found the polymorphisms of the calpain gene. Several studies have demonstrated the calpastatin gene polymorphism [Casas *et al*., 2006]. Differences in the arrangement of genes responsible for the activity of the calpain I and calpastatin allow predicting what will be the rate of the tenderization process during the ripening of meat *postmortem*. It is known that the calpains I, II and III are encoded by the CAPN1 gene, CAPN2 gene, and CAPN3 while calpastatin is encoded by the CAST gene. Several markers have been developed to determine the CAST gene and three markers (316, 530, 4751) to determine the CAPN1 gene [White *et al*., 2005; Casas *et al*., 2006; Frylinck *et al*., 2009]. Another studies have shown an association of indyvidual markers at with meat tenderness in beef cattle [Moudilou *et al*., 2010]. Currently, markers of the calpain I and calpastatin are used commercially (genetic tests) to identify beef cattle to produce tender meat [Casas *et al*., 2006; Kemp *et al*., 2010]. However, this is a material for another paper.

There are also new proteolytic systems that may be responsible for *postmortem* proteolysis and meat tenderization - caspases. Caspases may contribute to *postmortem* proteolysis and meat tenderization [Ouali *et al*., 2006; Bernard *et al*., 2007; Chen *et al*., 2011]. Caspases are a family of specific cysteine proteases. These highly specialized enzymes create the intracellular network signaling proteolysis. Proteolysis and activation of caspases may occur with the participation of enzymes such as: granzyme B (a strong activator of procaspases 3 and 7), cathepsin G, calpains, cathepsin D [Korzeniewska-Dyl, 2007]. Currently, the expression of caspases was investigated in different skeletal muscle types and it is proposed that these proteases could have caused meat tenderization. Presumably, the caspase 3 plays the most important role. Kemp *et al*. [2006] demonstrated that the caspase 3 activity increased during the early stages *postmortem* but then decreased with time and was negatively related to Warner-Bratzler shear force. A recent study provided further evidence of the potential role of caspases in meat tenderization. Selective inhibitors of caspases significantly hinder the degradation of the skeletal proteins: titin, nebulin, desmin and troponin-T, whereas the activity of the calpains was not influenced. Therefore, it is proposed that the degradation of muscle proteins should not be exclusively attributed to the calpains system and that the caspase-3 may be a protease involved in *postmortem* tenderization [Huang *et al*., 2009; Chen *et al*., 2011]. This hypothesis requires further studies. However, many researchers attribute the major role in *postmortem* proteolysis and meat tenderization to the calpains system. Presumably, caspases will modify the activity of calpastatins [Kemp *et al*. 2010]. It is also possible that both calpains and caspases may be responsible for the conversion of muscle into meat and final tenderness.

CONCLUSIONS

Final meat tenderness is determined by the rate and extent of *postmortem* proteolysis of key myofibrillar proteins in the muscle. The calpains system is the principal contributor to *postmortem* proteolysis which is related to meat tenderness. Numerous studies have shown that the main role among the calpain system plays a calpain I and calpastatin. However, a number of subsequent studies have provided evidence that proteasome could contribute to meat tenderisation. Also, it is possible that caspases are responsible for *postmortem* proteolysis and meat tenderization. This requires further investigation and confirmation in next works.

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