THE ROLE OF PROTEINASE ENZYMES IN THE PROCESS OF CONVERSION OF MUSCLE TO MEAT

ULOJA ENZIMA PROTEINAZE U PROCESU KONVERZIJE MIŠIĆA U MESO

E. Dümen

Post mortem meat tenderization is a complex mechanism and unfortunately it has not been fully identified scientifically. It is known that endogenous proteinases have an important role in this mechanism. Detailed studies are being performed about the destructive effects of lysosomal proteinases and calcium dependent proteinases on the myofibrils and these are most common topics that are being investigated about meat tenderization processes by the scientists. The aim of this paper is to review the role of proteinase enzymes in the process of conversion of muscle to meat.

Key words: meat, texture, proteinase, calpain, calpastatin

Introduction / Uvod

Most vertebrated animals have similar muscle construction in spite of genetic diversities. In almost every species, skeleton muscles consist of approximately 75% of water, 20% of different proteins, lipids, carbohydrates and a little amount of water soluble organic compounds [43]. According to physiologic functions, muscles can differ largely. In the general aspect, it is indicated that there are 4 main groups of specified muscle fibril structures [29]:

- Slow contracting oxidative muscles (type 1),
- Fast contracting oxidative – glucolitic muscles (type 2A),
- Fast contracting glucolitic muscles (type 2B),
- Average (middle) level activity muscles (type 2C).

These muscle fibrils consist of different amount of oxidative and glucolitic enzymes, different qualitative contractile proteins, different proteinase groups
and inhibitor systems. The composition of the muscle proteins varies according to the main functions of the muscles. Excess variation of the muscle fibrils provides the muscles to be of more heterogenous structure [29]. In spite of many different factors being effective on the softening process of the meat, this review examines the role of proteinases on the softening process of the meat.

Softness, is known as one of the most important factors that affects the quality of the meat [43]. The softness factor is determined according to contractile structures of the connection tissues [47]. The toughness is the opposite process of the softness and it is identified as the result of the variations that take place in the background connection tissues [29]. Hertzman et al. [22], indicate that myofibrillar toughness exists because of the toughing of the contractile structures. Myofibrillar toughness takes shape with the help of the development of rigor mortis, and the softness process originates from the destruction of the contractile proteins with the help of the enzymatic reactions [29].

The post-mortem period and time relationship, are known to be two very important parameters in myofibrillar toughness and softness [29]. The enzymes in these two processes are the muscle proteinases and they are active at the post mortem pH’s [59].

### Development of rigor mortis / Razvoj rigor mortisa

Rigor mortis is the first step of the transformation of muscle to meat. Energy rich organic molecule and a series of biochemical reactions cause to develop the rigor mortis process. Adenosintriphosphate (ATP), creatinphosphate (Cp) and glucogen are the main organic structures that play important roles in the rigor mortis process. The organic structures that activate ATP, Cp and glucogen are ATPase, kinase and and glucoolytic enzymes. ATP is hydrolyzed by ATPase and especially myosin in living muscle tissues and in the early post mortem period and free ADP is again transformed into ATP by creatinkinase or by the glucoysis of glucose molecules [1, 29]. ATP molecules are connected to the basis part of the myosins and these type of myosins are called „charged myosins“. Thus, the possible reactions that would take shape in the thin and thick filaments of actin and myosin are blocked by the penetration of troponin and tropomyosin into actins and myosins. The contraction activities in the living muscles begin by the secretion of Ca^{2+} ions from the sarcoplasmic reticulum. During the muscle contraction period ATP is destructed into ADP and by the help of the energy that is released from destruction of ATP to ADP charged myosins are connected to actins and the action of the muscle filaments exist. The head parts of the myosin are still connected to actins at the same time of the process and releasing of the head parts of the myosins can only take shape in the presence of the ATP molecules. The head parts of the myosins are locked to the actins during the transformation process of muscles to meat because of absence of the ATP molecules and even passive fla-
ment slide actions are impossible [29]. When the pH decreases to 6.0 (the optimal level for ATPase activities in muscles) the ATP presence becomes too low and this presence level cannot activate the main contractile proteins and main contractile proteins keep their locked position to each other. Thus, the actomyosin complexes that cause the characteristic toughness of rigor mortis are formed. The pH level of the muscle tissue in the post mortem period begins to decrease due to the protons formed from the glucoysis reactions and the accumulating of the lactic acid. Decrease rate of the pH is related with the texture of the meat during the slaughtering process [29, 51]. The speed and rate of decrease of pH is closely related 3 main factors [51, 66]:

- Reserve of the glucogen and energy rich organic structures of the animal during the slaughtering process
- The rate of the ATP cycling
- The oxidative capacity of the muscle tissue
- Glucolytic potential and the contracting properties of the muscle proteins

Toughness of the meat / Tvrdoća mesa

Tenderness of meat is due to slaughtering process and / or post – mortem storing conditions. The processes that are applied by the consumers during the cooking processes are another important reasons for the toughness / tenderness condition of the meat. According to Wheeler and Koohmaraie [70], the longissimus muscle of lambs has a middle rate of shear force just after the slaughtering process. The toughness takes shape in the muscles in the first 24 hours after the slaughtering process, and just after 24 hours if the meats are stored approximately at 4°C, the softening process begins. Sarcomere length is inversely proportional with the shear force. The sarcomeres shorten during the development of the rigor mortis process. During the post mortem period, except just after the slaughtering, any difference values of the shear force could be determined in the longissimus muscle of the lambs [34]. Post-mortem toughness disappears totally or decreases to very low levels in the absence of muscle shortening. Some other factors except sarcomeres are indicated to be responsible for meat toughness, too. Goll et al. [17], indicated that the toughness of the meat formed in the first 24 hours after the slaughtering period is due to the effect of both actin and myosin molecules on each other. We think that this hypothesis explains that there is no relationship between the hardening of the meat and the shortening of the muscles in the very early periods of the post mortem process.

Plenty of data from medical literature supports that the idea of sarcomere shortening directly affects the toughness of the meat, but the relationship between sarcomere shortening and the toughness of the meat cannot be clearly identified yet. Some medical literature indicates that increasing toughness of the meat is directly related with the sarcomere shortening [3, 9, 23]. However, other
data maintain the exactly opposite of this hypothesis [6, 40, 57, 60, 61, 62]. Marsh and Leet [48] indicate the relationship between the sarcomere length and the softness of the meat. The maximum softness in the excised meat was observed while the muscle shortening level was about approximately 40% levels [48]. A different situation was reported about the muscles directly connected to the skeleton. Sixty-seven aged and unaged cattle carcasses were investigated to explain the relationship between sarcomere length and the softness of the muscles in a study made by Smulders et al. [63]. A very strong relationship between the softening process and the sarcomere length was observed at the 48th hour of the post-mortem period. However, a completely different situation was determined when the meat samples were separated according to their pH levels after the 3rd hour of the post-mortem period. The correlation between the sarcomere length and the shear force, was measured as -0.84 at the unaged samples, and -0.68 at the aged samples at the 6.3 or at the higher levels of pH values, but at the lower pH levels than 6.3, any correlation between the sarcomere length and shear force could be observed. According to these results we think that the softness of the slow glycolysis metabolised muscles is strongly related with the muscle shortening processes, and fast glycolysis metabolised muscles are aged faster than slow glycolysis metabolised muscles. In parallel with our observations, Jiang et al. [28, 29] state that the length of the post-mortem process is directly effective on the relationship between the sarcomere length and the softness of the meat.

The toughness observed and/or determined in the first 24 hour of the post-mortem process could be related to the sarcomere shortening. The shortenings and the contractions cause the toughness indicated above that can be observed in almost all carcasses, but this situation does not imply that there is no difference in the sarcomere length values among the species, even among carcasses of the same species [29].

There are a lot of parameters that affect the length of the sarcomers, like the factors that effect rigor mortis. The sarcomere length values of the same species are generally approximate to each other and the hardening process is almost parallel. The sheer force values determined at any time of the post-mortem process are under the effect of two opposite forces; these are the shortening of the sarcomeres and the softness. However much the softening processes start before the sarcomere shortening period, the same toughness values cannot be observed and the same processes cannot be observed in every carcass [12, 23, 29].

**Softness of the meat / Mekoča mesa**

Softening processes start in the post-mortem period. Storing conditions are very important for maximizing softening processes during the post-mortem period. The values to maximize the softening process for storing are 15°C 10-14 days for cattle, 15°C 7-10 days for sheep, and 15°C 5 days for pigs. Softening reactions of the meat continue differently even in the members of the same
species unlike hardening reactions. The reactions can take shape differently even in same animal if the half or quarter carcasses are stored in different conditions (pH, temperature, and the storing period are the most important parameters) [31, 32, 33, 54, 64]. On the other hand, these different results of the softening reactions cause variations of taste in the meat and these variations again cause positive results for the consumers who have different tastes. The preference of the consumers can be widely different regarding hard meat and soft meat. Some consumers choose harder meats for their meal, while others want to eat more soft meats. If the softening processes in the post-mortem period is identified clearly, meat can be produced according to the market requirements.

The softening processes during storing conditions have been studied by numerous scientists [7, 16, 19, 31, 32, 33, 34, 40, 53, 54, 65]. The role of the key myofibrils and associated proteins in the softening processes are clearly identified by these studies. It is reported that these proteins can be desmin, vinculin, which can exist in the intermyofibrillar area, and titin, nebulin, troponin which can be in the intramyofibrilar area. Moreover, it is also indicated that the muscle cells connected to the basal lamina could include laminin, fibronectin, new identified 550 kDa proteins [20]. The main role of these proteins is to provide the integrity of the myofibril structures [29]. Thus the decrease of the mentioned proteins would naturally cause myofibrillar reactions, too, and the meat would become soft. The main reaction takes shape as a result of proteolysis of the key myofibrilar proteins and the texture of the meat softens. In parallel with the studies on farm animals such as cattle, sheep, pigs, and chickens, the post-mortem softening processes are also studied in almost all species of animals. The fractures in the Z regions on the I bounds can be observed in almost all animal species [21]. However, these reactions take shape in avians much more faster than in cattle [29]. A lot of the biochemical changes start with the releasing of endogenous muscle enzymes that are active in the post-mortem pH's [59]. Most scientists accept that the softening appears as a result of the separation of the actomyosin complexes in the Z regions. This event also includes the denaturation reactions of the collagens [29, 35, 36, 37, 38, 39, 41, 59].

The role of muscle proteinases in the meat softening process/
Uloga proteaz mišća u procesu omešavanja mesu

The biochemical reactions that provide the softening of the meat are known to be enzymatic and physicochemical reactions [53]. In the living muscle cells, the decrease of the intracellular proteins is controlled by endogenous proteolytic systems [2, 29]. All the post-mortem reactions causing the softening of the meat are a part of the proteolytic mechanism. The organic molecules responsible for this mechanism are the proteinases localized in the muscle cells or in sitosols. The biochemical mechanisms that provide the post-mortem softening processes have not been clearly identified even today.
However, the proteolytic systems like calpains – calpastatins, cathepsins – cistasins and proteasome – macropains are defined in several medical sources, calpain – calpastatins are the most studied proteinases by the scientists. These organic structures are μ – m calpains and cathepsin D, B, H and L that are the members of the proteolytic systems [29]. There are 4 main events that can explain the post-mortem processes of the calpains [29, 49, 69]:

- The structural decrease of the myofibrils is very similar to the post-mortem myofibrilar decrease
- The post-mortem myofibrils that react or not react with calpains, are electrophoretically similar
- Z regions where the calpains localized, are very sensitive to calpain-catalysing reactions
- While the calpain level increase in the muscles, the post mortem softening processes are accelerated

Calcium concentration, which is one of the most important endogenous inhibitor of the calpains – calpastatins has been considered [5, 29]. Endogenous protein inhibitors, are quite effective homeostatic tampon systems for the muscle proteinases. Studies that are performed under the light of this informations indicate that, protein inhibitors present in the skeleton muscles mostly inactivate cysteine and /or serine proteinases [29, 49, 69].

There are a lot of different interpretations about the role of the specific proteases on the post mortem processes. According to Kochmarai [34], a protease must be reaction specific organic structure. Goll et al. [18] expose the first logical criteria of the post mortem processes as that the proteases must be non-specific organic molecules that are localized in the skeletal muscles. They also maintained that proteases could cause different post-mortem changes in the myofibrils under in vitro conditions, as an argument. Lastly, they indicated that proteases can react with the myofibrilar structures in the tissues. Again, according to Goll et al. [18], if a protease does not have the properties indicated above, it must not be evaluated as a factor that is responsible and/or an active organic molecule in the post-mortem processes. Scientists who consider the hypothesis indicated above identify the calpains as the primer organic molecules that are responsible for the post-mortem softening processes of meat. According to different medical data, calpains are key organic molecules responsible from the softening processes at refrigerator temperature [16, 17, 18, 19, 27, 29, 31, 32, 33]. There are also a lot of medical papers that support that calpains provide the softening process as a result of proteolysis of the Z bands [16, 17, 18, 19, 27, 29, 31, 33, 34, 40, 53, 54, 64, 67, 68].

In spite of the common belief which supports that the main reactants of the post-mortem proteolysis are the calpains, there are still some doubts. The primary reason for these doubts are the inactivation period of the μ calpains. Because the i calpains inactivate so rapidly (just 24-48 hours after the slaughtering...
process), some scientists think that they can not be the organic molecules which are the main reactant of the post-mortem proteolysis which are mechanism. These arguments were indicated after the determination of the polypeptides quantitatively existing as a result of hydrolysis of casein due to TCA precipitation of μ calpains by the help of sensitive molecular genetic methods. By using more sensitive methods as radioactive marking of caseins, scientists discovered active μ calpains at a rate of 5-10% even in carcasses stored for 14 days at 4°C [34]. As a matter of fact, autolysis and the inactivation of μ calpains are intramolecular processes. The reactions possibly do not continue until the finishing of all μ calpains, which is why a small amount of μ calpains can remain active after the autolysis reactions [14, 31, 52]. Differently, m calpains are inactivated by the autolysis reactions because they are a part of intra molecular processes [5, 14, 31]. Additionally, because m calpains are inactivated by the effect of high Ca concentration, Ca ion are very important parameters inactivating m calpains in vivo situations [37]. According to Ouali and Talmant [55] and Valin et al. [66], aging of the meat is not depends on the calpain activity, instead depends on the total rate of the enzyme / inhibitor complexes. In a study 50 oxes were used as material and it was determined that the rate of i and m calpains to calpastatins were affected due to nutrition factors [29], but calpastatin activity remains the same, while the calpain activity decrease at freezing conditions of up to -70°C and this finding shows that cold and freezing storing conditions do not affect calpastain activity [34]. In spite of some medical studies indicating that calpastatins and μ calpains lose their activity more rapidly than m calpains [13, 39], it was determined that thermal stability of calpains and calpastatins are higher than m calpains [10, 11, 29]. The resistance of myofibrillar proteins to calpains and the providing of myofibrillar proteins to remain calpains at constant levels are the main factors of meat softening processes. According to Valin et al. [66], there is not a direct relationship between muscle proteinas and meat softening processes but there is a positive correlation between the rate of proteinas / inhibitors and meat softening process.

Another different argument is that the amount of calpastatin is much more higher than the amount of μ calpain in the muscles, so μ calpains cannot be activated adequately. This hypothesis is still being discussed, because, primarily, the ratio of the calpastatins to μ calpains is different in different species. These ratios are 4:1, 2.5:1 and 1.5 in cattle, sheep and pigs, respectively. Secondly, much data indicates that m calpains are one of the main organic molecules for determining calpastatins quantitatively [42, 55]. To inhibit μ calpains, double amount of calpastatins than the required amount to inhibit m calpains is required [34].

According to the information given above, we think that the ratios of calpastatins to μ calpains are the half values of the ratios indicated in the previous paragraph. Similarly, there are medical data that report the rate of calpastatins to μ calpains as 2:1, 1.25:1 and 0.75:1 in cattle, sheep and pigs respectively. The theories about calpastain activites are not as valid now as they were in recent years.
[12, 29, 42]. According to our findings both μ calpains and μ calpains are responsible for the meat softening processes.

In addition to calpains, there are also enzymes that attack the proteins such as desmin, flamin, nebulin and connectin in the Z regions [8, 29, 46]. Cathepsins attack myosins as facultatively [26, 28, 29]. Calpains and lysosomal enzymes can attack different strategic proteins in different regions [29]. Cathepsins take one of the most important tasks in the meat softening process and cathepsin B reacts with heavy chains of myosins and cathepsin L reacts with troponin T, I and C very rapidly [24, 26]. Calpains perform optimum activity especially with cathepsin B and L in neutral pH levels [15, 24, 37, 44].

Koochmarie [34], indicates that the cathepsins are not fully responsible for the meat softening process. Actually, lysosomal proteinases can exist in the lysosomes in the myofibrills during the post-mortem period without entering directly into the cytosoles. However, the effect of low pH degrees and the weakening of the glycolysis reactions denaturates the membranes of the lysosomes, and as a result, lysosomal proteinases which are active at 5.5 - 6.5 pH degrees like cathepsin B, H, L are released directly to the sarcoplasm [15, 24, 29, 44]. Additionally, while the aged meat expands, fractures get more close to Z bands, but these closing processes are not very clear at the connection points of A and I bands [29, 45]. The increased fragility of the mentioned regions in stored meat and meat products can be related with the activations of lysosomal proteinases [53]. Myofibrillar proteins are quite sensitive to calpains and lysosomal proteins, according to in vitro studies. While heavy myosin chains, light myosin chains, α – actin, troponin C and actin, are sensitive to cathepsin activities, lysosomal proteinases decrease the level of some organic molecules like troponin T, troponin I, tropomyosin, C – proteins, desmin, titin and nebulin [18, 26, 28, 30, 50, 56, 58].

Myofibrillar proteosomes or macropains are weak substrates for the multicatalytic protease complexes, known as MCP. It is indicated that MCPs do not have any role in the decrease of protein types at the post-mortem period [24, 34, 65]. Further medical studies are required in order to identify the role / roles of MCPs in the post-mortem period.

Conclusion / Zaključak

The biochemical mechanism of the post-mortem changes could not be fully identified at the present time. In spite of most medical data relating the meat softening process to the proteasae work agonist with the Z disks, and to making these proteasae fragile of A and I bands, we think lysosomal proteinases and calpains are highly responsible for meat softening processes. We believe that sarcomer proteins are another important topic that must be studied. The total ruptures of myofibrills do not take shape in the meat softening process. Maybe this reaction can be evaluated as a weakening process.
According to the information we gathered and indicated in the review, we think that calcium independent proteinases are mainly responsible for the biochemical mechanism of the meat softening process. We also think that these complex reactions cannot be performed only by the reactions of the proteolitic enzymes. To understand the meat softening process clearly, it is necessary to perform further medical studies, especially on pH – temperature effects, released organic molecules, and the effects of pH – temperature parameters as calpains and/or lysosomal enzymes connected to the cell membrane and proteinase topics.

Literatura / References


ULOGA ENZIMA PROTEINAZE U PROCESU KONVERZIJE MIŠIĆA U MESO

E. Dūmen

Omešavanja mesa post mortem je kompleksni mehanizam koji, na žalost, nije u potpunosti naučno sagledan. Zna se da endogene proteinaze imaju važnu ulogu u ovom mehanizmu. U toku su detaljna istraživanja o destrukтивnim efektima lizaromiane
proteinaze i kalciijum-zavisnih proteinaza na miofibrale, to su teme koje naučnici najčešće ispituju kod procesa omekšavanja mesa. Cilj ovog rada je da sagleda ulogu enzima proteinaze u procesu konverzije mišića u meso.

Ključne reči: Meso, tekstura, proteinaze, kalpan, kalpastatin

**ПУСКИЙ**

РОЛЬ ЭНЗИМА ПРОТЕИНАЗЫ В ПРОЦЕССЕ КОНВЕРСИИ МЫШЬЮ В МЯСО

Э. Думан

Смягчение мяса post mortem комплексный механизм, который, к сожалению, не полностью научно уведен. Известно, что эндогенные протеиназы имеют важную роль в этом механизме. В течение детальных исследований о деструктивных эффектах лизосомальной протеиназы и кальций-зависимых протеиназ на микрофирильное, это темы, которые учёные чаще всего испытывают у процесса смягчения мяса. Цель этой работы увидеть роль энзима протеиназы в процессе конверсии мышц в мясо.

Ключевые слова: мясо, текстура, протеиназы, кальпан, кальпастатин