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THE ROLE OF ENZYMES IN THE FORMATION OF MEAT AND MEAT PRODUCTS

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Abstract

The meat industry is one of the most dynamic and competitive sectors of the food industry. As the global population keeps on growing and the demand for protein does the same, the consumers define ever higher standards of quality for the meat producers. One of the key quality criteria is the tenderness and juiciness of meat, which directly affects its taste and texture characteristics. In order to satisfy the expectations of the modern consumers and to ensure the stable quality of the meat product, meat processing enterprises actively introduce the innovative technologies. In recent decades, proteolytic enzymes have been increasingly used to improve the quality characteristics of the meat products, which is a more progressive method in comparison with to mechanical methods of processing due to less impact on other consumer properties. This article overviews the role and importance of enzymes in the meat industry. We will consider various aspects of the application of these enzymes for the meat products, their effect on the level of tenderness, juiciness and other characteristics of meat, as well as prospects for the further development of their using.

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Introduction

The meat industry is one of the biggest sectors of the food industry, and the quality of meat products plays an important role in the consumers' satisfaction [1]. Tenderness and juiciness of meat are considered to be the key characteristics that determine its taste and quality [2,3]. It has been shown that tenderness, defined as "the ease, perceived by consumers, with which meat structure is disorganized during mastication" [4], plays the largest role in making decision on the purchase of the particular product [5,6].

It is not an exaggeration to say that the meat obtained from each animal is the "unique meat" with its own evolution [7]. The meat industry must ensure that quality standards for the meat it produces are complied with. The meat industry has developed a variety of approaches to achieve this purpose, including both traditional methods of curing or aging, as well as the new technologies [8]. In modern conditions the methods of mechanical processing with the needles and blades or by massaging are widely used as preparation of the raw meat for its subsequent sale [9,10]. The main effect is aimed to the softening of the meat product during such processing. So the mechanical processing softens the muscle component of the product, which results in loosening of the meat surface, rupture of muscle fibers and release of myofibrillar proteins [11]. An alternative to mechanical processing is tenderization with proteolytic enzymes [12], which is a more progressive method compared to mechanical ones due to less impact on other consumer properties [13]. Many enzymes that make changes to the connective tissue structure or myofibril integrity were extracted from plant, bacterial, and fungal sources and were thoroughly studied (Figure 1).

The special feature of this review is the comparative characteristics of enzymes involved in the formation of meat and meat products. The results of these enzymes application are illustrated in the article with examples taken from the publications of the recent years, including our paper too. The analysis presented in this review will undoubtedly be useful for proper orientation in the development of the modern approach to improving the food industry processes, in particular improving the consumer qualities of the meat products.

Objects and methods

The developments of domestic and foreign scientists on application of enzymes for meat products, presented in the articles, are the object of the study. The search for the data was run in the databases ScienceDirect, PubMed, Google Scholar, eLibrary and other open electronic sources. Combinations of keywords such as enzyme, protease, meat tenderness, meat industry, fermented sausages, microbial transglutaminase (applied for cross-linked meats) were used. Keywords were used in English and Russian.

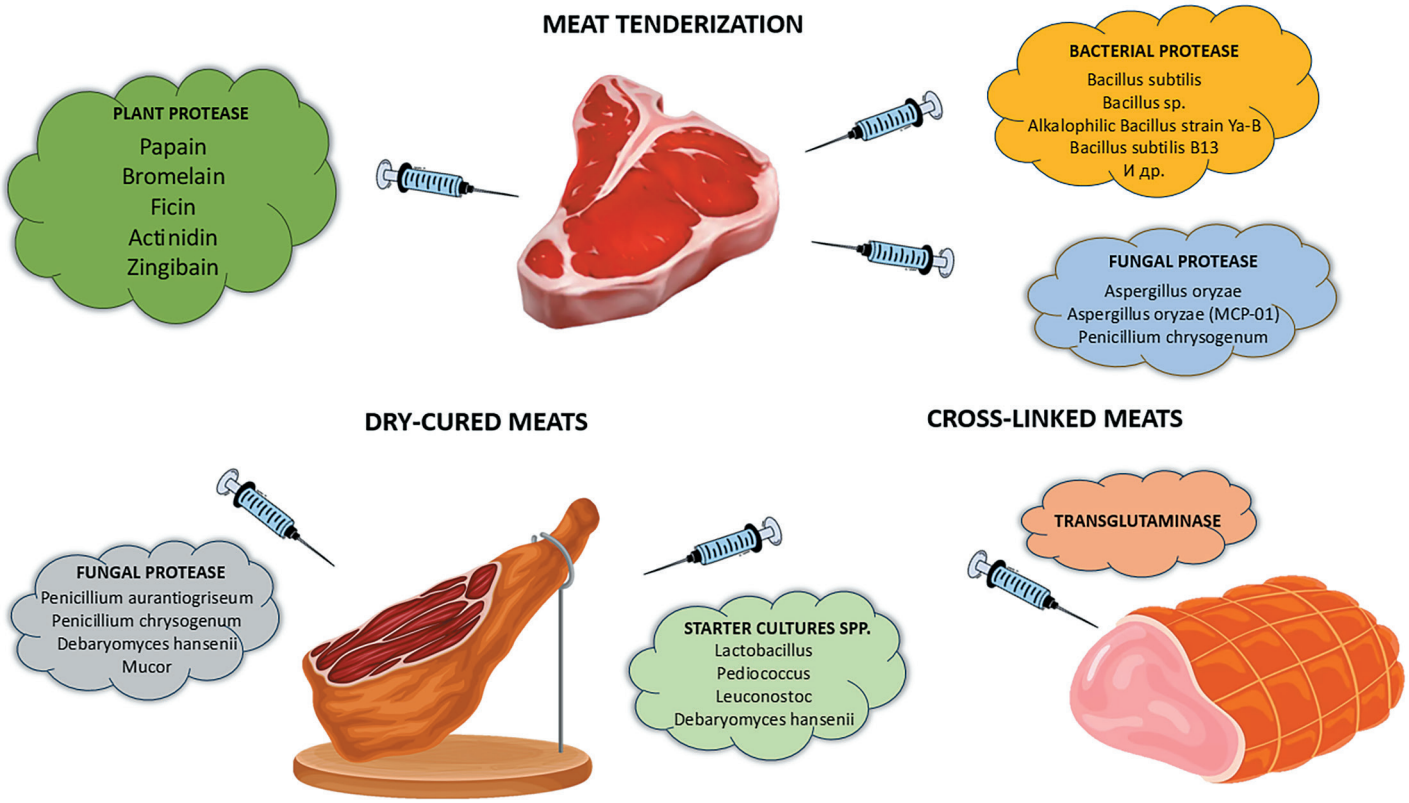

Figure 1. Enzymes used to cure steaks and other meat products

In addition, thematically similar articles were searched for using the citation chains. Non-peer-reviewed, uninformative and duplicate sources were excluded from the search results, as well as the sources included in the search patterns that were not related or were just indirectly related to the topic of the research.

Proteolysis during post-mortem aging

Quite soon after the slaughter of the animal the homeostasis gets lost, and biochemical changes and a number of physicochemical reactions in the muscles start [14]. To provide the required amount of ATP without the oxygen, glycogen is decomposed in the muscles through anaerobic glycolysis. This leads to the formation of lactic acid with a simultaneous decrease in pH from approximately 7.2 in a live muscle down to approximately 5.5 in the slaughtered meat. Stress or the pre-slaughter treatment are the key factors that influence the process of post-mortem glycolysis, which in its turn determines the final level of pH and therefore the quality of meat [15]. Decrease of pH and temperature leads to a decrease of the glycolytic enzymes activity, which, in turn, leads to a gradual decrease of ATP levels [16]. As a result, the main contractile proteins — actin and myosin — become irreversibly linked to each other, thus leading to a phenomenon known as *rigor mortis*, which heightens the meat toughness through a phase of sarcomere shortening. Meat with very short sarcomeres tends to be tougher [17].

Animal muscles have fibrous structure necessary to ensure their strength, contractibility and stretchability. As a result, even in case of exposure to intensive tenderization, the inherent fibrous texture of the meat is still preserved. The conversion of muscle to meat is shown schematically in the recent articles [7,15]. Decreasing of the raw meat toughness during its storage after slaughter are generally recognized as enzymatic process, it involves intracellular proteolytic systems capable of post-mortem proteolysis [18]. Several endogenous proteolytic systems found in meat, including calpains, cathepsins and lysosomal proteases, are capable to degrade the myofibrillar and cytoskeletal proteins [19].

Calpains

The calpain system, responsible for the proteolysis of cytoskeletal proteins (titin and nebulin) and intermediate filaments (desmin), as part of the cysteine protease system, is now widely recognized as critical factors promoting muscle tenderization during postmortem maturation [20]. The calpain system includes endogenous proteases (calpains), which are considered the main candidates for the muscle degradation that starts in the first 24 hours after slaughter, and their inhibitor — calpastatin [21]. Studies show that the degree of tenderization in the meat of different animal species is inversely related to the calpastatin: calpain ratio [22]. These enzymes have been reported to be capable of degrading specific muscle proteins, including intermediate filament proteins like desmin, and structural proteins like titin [21] with minimal effects on myosin and actin. Postmortem proteolysis, as measured by loss of intact desmin and troponin-T, was limited in transgenic mice expressing calpastatin [23]. These results were later confirmed by a similar study in calpain knockout mice [24]. More than

30 years ago, it was found that the administration of calcium chloride (CaCl2), which activates calpains, accelerates postmortem proteolysis in the muscles [25]. On the contrary, the introduction of calpain inhibitors prevents postmortem proteolysis and, accordingly, prevents the tenderization of meat. Numerous studies have again confirmed the crucially important role of calpains in the proteolysis and tenderization of lamb, beef and pork [20].

Cathepsins — caspases

Calpains are not the only proteases in muscle, and there is evidence that other proteases are either directly involved in the meat maturation, or interact with calpain. Cathepsins are proteases that digest cellular components in lysosomes and that are active at the acidic pH of meat [26]. However, they gain the access to myofibrils only under conditions of lysosomal membranes rupture. Cathepsins also proteolyze actin and myosin, and during the meat maturation only very limited destruction of these proteins is observed [27]. Becila et al.'s work views early maturation as a process of apoptosis or controlled cell death [28]. Caspases are key cysteine-dependent aspartate-targeting enzymes, activated during apoptosis. There is evidence that caspase 3 is able to reproduce many of the characteristics of postmortem proteolysis in the myofibers [16]. The work examined the application of human recombinant caspase 3 (rC3), which was expressed in *Escherichia coli* bacteria, in order to degrade myofibrillar proteins in pork muscle after a pig slaughter. The work showed that recombinant caspase is capable of degrading a variety of proteins, including alpha-actin, troponin T, myosin, myofibrillar proteins, desmin and troponin I [16]. Caspases were also suggested to be the target of serine peptidase inhibitors, also called as SERPINs, which correlate with beef strength [29].

There are lot of evidences that caspases interact with calpains and are able to proteolyze the calpastatin inhibitor [30]. Thus, the current view of postmortem maturation of meat focuses on calpains, but also includes their interactions with several other groups of proteases. Cathepsins are more thermostable than calpains [31], as the latter are reported to be completely inactivated at the temperatures above 55 °C. While cathepsins, especially B and L, remain active even after 24 hours of exposure to the temperature of 55 °C [32]. Recent studies have shown that mitochondria-mediated apoptosis also affects post-mortem muscle tenderization by promoting the degradation of myofibril proteins and cytoskeletal structures. In result mitochondria provide their influence on the formation of color, tenderness and taste of meat through the effects on oxidative stress, changes in the redox state of myoglobin, glycolysis and apoptosis [33].

Enzymes as texture and tenderness modifiers

Meat is primarily muscle protein, present as bundles of protein fibers clustered into blocks surrounded by connective tissue. This connective tissue also contains structural proteins including collagen and elastin. In order to give the meat a more tender structure, it is possible to run preliminary splitting of connective tissue proteins and some muscle fiber proteins. Given the structural features of muscle and fibrous tissue that affect the tenderness of meat, it is necessary to strive for an integrated approach that affects both muscle and connective tissue.

The tenderness of meat is the result of combination of several factors that can be considered in certain sequence [34]. "Background toughness" is determined by the characteristics of the muscle, in particular the connective tissue (collagen), its quantity and type [35]. This in its turn depends on factors such as species, age of the animal, nutritional status, sex and muscle type. Different types of muscle fibers differ in their collagen content, and therefore muscles with different fiber compositions also vary in level of their tenderness [36]. There is also a correlation between the tenderness and the diameter of a fiber. Small-fiber muscles are considered more tender in cattle and pigs [37]. There is also data on correlation of tenderness with total content of lipids, intramuscular fat levels [38] and content of water in the cell [39]. Differences in tenderness still exist even when controlling for the above-listed parameters, and this has proven to be still a very difficult aspect to predict and control. The introduction of exogenous enzymes for meat tenderization has recently gained the increasing interest among food technologists and scientists (Table 1). This promotes consistent production of tender meat and increases the nutritional value of lower quality meat cuts.

The toughness of meat is determined by two structural components. The first structural component is connective tissue in meat, mainly composed of structural proteins that provide support to muscles at different levels. Structural aspects and the contribution of connective tissue to the tenderness of raw and cooked meat are considered in the work [58]. The contribution of connective tissue to the toughness of meat depends on the structure and/or amount of various collagens and elastin in the meat. This part of meat toughness is mainly influenced by "on-farm" factors like breed, sex, age, physical activity, and so on. Processing and post-mortem handling have a negligible effect on "background toughness". The second structural component that influences the toughness of meat after slaughter is changes in the contractile apparatus of the muscle (sarcomere), which becomes shorter (the phase of *rigor mortis* development). The stiffness caused by muscle contraction is primarily dependent on the conditions of the processing. Thus, by changing processing conditions, a significant improvement in meat tenderness can be achieved [15].

Plant proteases have already been proven to improve meat tenderness through a proteolytic degradation mechanism. Common exogenous plant proteases used for the processing of the meat are bromelain extracted from pineapple (*Ananas comosus*), papain from papaya (*Carica papaya*), ficin from figs (*Ficus carica*), actinidin from kiwi (*Actinidia Lindl*) [59] and zingybaine from ginger root (*Zingiber offi-*

Table 1. Application of enzymes for meat tenderisation

Application	Source	Optimal conditions	Active agents group	Type of enzyme
Hydrolysis of connective tissue and myofibrillar proteins, beef (tough meat)	Papaya (*Carica papaya*)	4.0–8.0 pH 50–70 °C	Sulfhydryl group of cysteine	Papain [15]
Hydrolysis of myofibrillar proteins, beef (tough meat) and beef steak	Pineapple (*Ananas comosus*)	3.0–7.0 pH 35–70 °C	Sulfhydryl group of cysteine	Bromelain [15,40,41]
Hydrolysis of myofibrillar proteins, beef steak	Fig (*Ficus carica*)	5.0–8.0 pH 45–60 °C	Thiol group	Ficin [42]
Hydrolysis of myofibrillar proteins, beef steak	Kiwi (*Actinidia Lindl*)	7.5 pH 2–45 °C	Thiol group	Actinidin [43–46]
Hydrolysis of connective tissue and myofibrillar proteins, beef (tough meat)	Ginger root (*Zingiber officinale*)	6.0 pH 60 °C	Proline	ZIngibain [15,47]
Hydrolysis of myofibrillar proteins and collagen, beef steak	*Bacillus subtilis*	7.0 pH 60 °C	Subtilisin and neutral protease	Bacterial (*Bacillus subtilis*) [48]
Hydrolysis of collagen and myofibrillar proteins, beef tenderloin	*Bacillus* sp. (EL31410)	5.5–6.0 pH 10–50 °C	Alkaline elastase	Bacterial (*Bacillus* sp. (EL31410)) [49]
Hydrolysis of elastin and collagen, beef (tough meat)	*Alkalophilic Bacillus strain Ya-B*	5.5–6.0 pH 50 °C	elastase	Bacterial (*Alkalophilic Bacillus strain Ya-B*) [50]
Hydrolysis of elastin and collagen, beef (tough meat)	*Bacillus subtilis B13*	7.5 pH 60 °C	Serine/metalloprotease	Bacterial (*Bacillus subtilis* B13) [51]
Hydrolysis of elastin and collagen, beef (tough meat)	*B. siamemsis S6*	7.5 pH 60 °C	Serine/metalloprotease	Bacterial (*B. siamemsis S6*) [51]
Collagen hydrolysis, beef steak	*Clostridium histolyticum*	6.0 pH 60 °C	Collagenase	Bacterial (*Clostridium histolyticum*) [52]
Collagen hydrolysis, synthetic substrate	*Pseudoalteromona* ssp. (SM9913)	9.0 pH 40 °C	Collagenolytic protease (MCP-01) (serine protease)	Bacterial (*Pseudoalteromonas* sp. (SM9913)) [53]
Hydrolysis of elastin and collagen, beef (tough meat)	*Aeromonas salmonicida*	6.0 pH 4–30 °C	Metallopeptidase, M9	Recombinant metallopeptidase (*Aeromonas salmonicida*) [54]
Hydrolysis of myofibrillar proteins, beef (tough meat)	*Aspergillus oryzae*	6–10 pH 50 °C	Protease	Fungal (*Aspergillus oryzae*) [55]
Hydrolysis of myofibrillar proteins and collagen, beef steak	*Aspergillus oryzae* (MCP-01)	2.5–6.0 pH 75 °C	Aspartic protease	Fungal (*Aspergillus oryzae* (MCP-01)) [48]
Hydrolysis of myofibrillar proteins and collagen, muscle of pork loin	*Penicillium chrysogenum* (EPg222)	3.0–5.0 pH 30–60 °C	Serine protease	Fungal (*Penicillium chrysogenum* (EPg222)) [56,57]

cinale) [60]. Thus, plant proteases can be obtained from a wide range of untaped plant resources via suitable extraction technologies. These proteases decompose the connective tissue proteins of the muscle by hydrolyzing peptide bonds in proteins into peptides, and finally into amino acids, thereby reducing meat toughness [59,61]. The classification of plant proteases is detailed in the chapter "Use of Plant Proteolytic Enzymes for Meat Processing" [62].

In practice the mixture of plant enzymes and bacterial collagenase is often used [59], or a combination of two or more plant proteases is used. The use of more than one protease has a synergistic effect. The proteolytic effect of exogenous plant proteases on muscle protein increases the nutritional value of the product due to the availability of essential amino acids, improving digestibility and taste [63,64].

Controlling the plant proteases action plays an important role, because the excessive exposure of meat under uncontrolled conditions leads to loss of consumer qualities and spoils of the product. This affects texture and flavor, increases bitterness due to the formation of basic amines and some amino acids. In order to be able to control the process of meat tenderization, it is necessary to determine their enzymatic kinetics and characteristics, as well as an understanding of the influence of their surrounding conditions (pH, temperature) on enzyme function. This will create the optimal conditions for tenderization of the fresh meat and eliminate or reduce any negative impact on the other quality characteristics.

Enzyme activity is influenced by processing conditions like combination of duration and temperature, pH, and the contact area of the enzyme with the surface. In the meat industry, there is an acute demand for proper regulation of enzyme activity, diffusion of enzymes in the meat matrix to obtain the desired effect on tenderness, texture, color, flavor and juiciness of meat [65]. The cooking method also contributes to achievement of the required tenderness of the dishes. For example, studies have shown that slow cooking of meat increases protease activity, and improves tenderness [66]. Thus, slow cooking requires lower concentrations of papain and bromelain than fast cooking to achieve the same level of the meat tenderness.

It is possible to highlight the main advantages of plant enzymes application:

- Plant proteases are produced naturally and are found in most plant sources, in contrast to microbial proteases, which primarily are the by-products of microbial fermentation. In addition, the hydrolytic activity of plant

proteases on meat myofibrillar proteins is more profound than the same of bacterial proteases.
- Consumers prefer to use plant-based proteases over animal-based ones due to the potential risk of disease transmission, environmental concerns, sustainability, lower cost, religious and ethical concerns. Also, in some countries, enzymes obtained by recombinant technology are not allowed into the food industry.
- Plants are also used as a natural preservative in the meat industry.
- Plant extracts are a rich source of polyphenols, essential oils, minerals and other biologically active compounds. Thus, the use of plant extracts may provide more advantages than the commercial preparations of enzymes, like higher nutritional value, wider product variety, and positive effects on the organoleptic properties of the meat products.

The main disadvantages of using plant enzymes are as follows:
- Plant proteases typically lack substrate specificity for the meat proteins, and extensive and non-selective hydrolysis of myofibrillar and connective tissue proteins results in a soft texture and "unpleasant" sensory notes such as "grainy" texture and "bitter" taste. Microbial proteases, in its turn, exhibit more specific activity towards meat substrates;
- There might be allergic reactions caused by the use of or exposure to papain, ficin, bromelain and actinidin, as well as thaumate-like protein extracted from kiwi;
- Issues related to composition, stability and control of enzymes after their processing. Many of these issues are related to the fact that commercial protease preparations contain many complex proteins and proteases that feature variable and uncontrollable hydrolytic activity and can lead to excessive tenderization;
- Another issue with plant protease extracts is that they may have some inherent flavor that may be acceptable to some kinds of meat and unacceptable to others, like ginger extracts containing zingibaine;
- Tenderization of meat with the help of plant proteases is quite time-consuming process; thus, there is a possibility of deterioration of the meat color. The use of new technologies such as high pressure treatment, ultrasound, shock waves, hydrodynamic pressure, pulsed electric field, etc. can increase the efficiency of enzymes by improving the enzyme-substrate ratio due to their better penetration/diffusion of the enzyme within the meat tissue.

New meat processing technologies such as ultrasonication, high pressure processing, reverse phase micellar processing, shock waves and pulsed electric field are increasingly being used today [15]. These methods help to increase the efficiency and activity of plant proteases due to deep penetration, and also increase the efficiency and reactivity of plant proteases due to better enzyme-substrate interaction [67].

The optimal and controlled tenderization of meat with plant proteases will help reduce batch-to-batch variability in meat and meat products and will improve their acceptability for the consumers. The significant reduction in meat hardness by plant proteases makes these products suitable for consumption by older people (who are not able for the full energy chewing), as meat is necessary for a complete balanced diet and the provision of quality proteins.

At the current moment in the profile literature there is no sufficient information on the effect of exogenous proteases on the tenderization rate, depending on different periods of time elapsed after slaughter and on cooking methods (speed of cooking, final temperature of cooking), which requires further research to find the optimal mode of these proteases application. The method of treatment with proteases (coating, pickling, injection or tumbling) can also have a significant impact on the degree of tenderization.

Enzymes of fungal and bacterial origin

In addition to plant proteases, microbial enzymes can serve as sources for meat tenderization as they cause degradation of connective tissue and induce changes in the muscle fibers of tough meat [55,68]. The chemicals like organic acids and phosphates, used in tenderizing meat, can greatly affect the health of the consumers. The acute necessity of their replacement with environmentally friendly substances to improve food safety urges the scientists to find ways to use various proteases, especially obtained from microbial sources, in order to decompose and tenderize meat in the meat industry (Table 1).

Bacterial proteases are reported to be more effective for degrading meat connective tissue than papain enzyme. They can be targeted and used to tenderize meats with a high number of connective veins [69]. Proteases of bacterial and fungal origin are widely used in the food industry and biotechnology. They have a lot of advantages in comparison with proteases of the plant origin. Microorganisms can be cultured comparatively quickly under stringent conditions that provide better control of protease production. Protease expression and activity can be managed with the help of modifying the conditions of production or cloning.

To improve the tenderness of meat, peptidases of fungal (aspartic protease from *Aspergillus oryzae*) and bacterial (neutral protease from *Bacillus subtilis*) origin are used; however, the bacterial peptidase has wider range of application in the meat industry because it has higher specificity and lower temperatures of inactivation. However, in the fungi *Rhizomucor miehei* CAU432 the process of cloning the aspartic protease gene (RmproA) resulted in a protease with the same efficiency as papain, used for pork tenderization [70]. Fungal protease obtained from *Penicillium chrysogenum* (EPg222) is also used to tenderize meat. It has an optimum temperature of 45 °C, it is effective between 30 °C and 60 °C and it is still active at pH below 4.5. EPg222 exhibits better activity in myofibrillar proteins than papain and *A. oryzae* [56].

Microbial proteases are grouped in reference to their acidic or basic properties. They can also be classified based on the present functional groups and the position of the peptide bond [55,71]. Bacterial enzymes such as subtilisin and neutral proteases are produced mainly by *B. subtilis*, *B. licheniformis, B. alcalophilus* and *B. lentus* [72]. Usually, bacterial proteases are specific in their activities. They are dependent on temperature, which makes them the best tool for the meat tenderization due to their ability to self-regulate. Proteases derived from *Bacillus amyloliquefaciens* and *Bacillus subtilis* are denatured at higher temperatures. This property gives them an advantage in catalytic action in the meat hydrolysis, and their activity can be easily controlled.

In this article [69] it was reported that meat products treated with *Aspergillus oryzae* have higher water retention capacity than the meat products treated with papain and bromelain. This means that treatment with *A. oryzae* preserves meat freshness, color characteristics, and shows better tenderizing potential than bromelain and papain. Most fungal proteases provide degradative effects on elastin and collagen, and effect the myofibrillar proteins to a lesser extent [73]. Aspartic protease produced by *A. oryzae* operates optimally at pH 2.5–6.0 and loses 80% of its activity at 75 °C after cooking, it affects collagen, however it provides less impact on meat myofibrillar proteins in comparison with the action of plant proteases [12]. The strain of alkaliphilic rod *Ya-B*, which produces alkaline elastase, dramatically degrades elastin and collagen in comparison with collagenase of *C. histolyticum* [50].

Raw meat is a complex object, where the ratio of muscle and connective tissue of different types can strongly vary depending on the type of meat, physiological and anatomical pecualirities of the animal. Therefore, the specificity of the enzyme preparations action plays an important role. Collagen is an insoluble fibrillar protein that provides strength and elasticity to connective tissue. Collagen is the most abundant protein found in the body of mammals and accounts for 25 to 35% of the total mass of proteins. Meanwhile collagen is difficult to digest, which makes it one of the main factors influencing the toughness of meat [11]. It is known that the wide range of proteases specifically affect the collagen molecules [11,74,75]. The work of Zhao et al researched the softening effect and effect of the cold-adapted collagenolytic enzyme MCP-01 on beef. At 4 °C, meat shear force decreased by 23% and relative myofibril fragmentation index increased by 91.7%, while meat freshness and moisture content remained unchanged [53].

The particular interest is raised in regards to M9 family of metallopeptidases [11] from among the microorganisms *Clostridium histolyticum* [76] and *Vibrio alginolyticus* [77]. It is known that the collagen filament features a specific sequence "G-X-Y", where "G" is glycine, "X" is often proline, and "Y" is hydroxyproline or one of the hydrophobic acids. *Vibrio* and *Clostridium* collagenases have specific ability to digest native triple-helix collagens of I, II and III types into a mixture of small peptides, cutting on the bond "Leu-Gly" under physiological conditions. Since the toughness of meat is partly caused by the presence of collagen, it is possible to use these collagenases to tenderize meat. However, the introduction of such enzymes into the technological process is associated with a number of challenges. Industrial use of microbial collagenases is limited by safety concerns related to their potential toxicity and the other adverse effects [78]. One of possible approaches involves the production of recombinant collagenases [54] in non-pathogenic microorganisms to avoid contamination by associated virulence factors. For example, we previously obtained the recombinant metallopeptidase from *Aeromonas salmonicida* using the transformation of *Pichia pastoris* for subsequent softening of meat. Histological examination of beef shank samples found a pronounced separation of the perimeter from the muscle bundles and the breakdown of collagen fibers, while the muscle fibers remained unchanged [54].

Bacterial and fungal proteases have proven to be more acceptable and beneficial than plant proteases, including their ability to act at a lower temperature, thereby preventing excessive tenderization of meat (myofibrillar and connective tissues) during its fermentation. Their hydrolytic activity depends on the substrate. Meanwhile the cold-active peptidases are isolated from *Chryseobacterium soli* and used to tenderize meat (for fragmentation of myofibrils) [79]. Proteases are also used to separate meat from bones for its further use in animal feed. In this particular case, peptidases must affect primarily the connective tissue, hydrolyzing collagen and elastin. The enzymes of microbial origin are commercially available from non-pathogenic sources. However, some consumers feel uncomfortable with the concept of bacterial or fungal food additives. The good strategy to overcome this negative perception is to use probiotic bacteria as sources of effective proteases that can be used for the dual function of maintaining the intestines health and for meat tenderization [80].

Food proteases are not commonly found in genetically modified animal/plant forms, probably because the universal properties of native proteases are suitable for most food processing temperatures and pH and their mechanism of action is well known (Table 1). This may also be due to the abundant availability of microbial proteases for industrial use. Recent studies of engineered food proteases from *Aspergillus* and *Bacillus* species demonstrated that engineered metalloproteases obtained through site-directed mutagenesis of His224 feature the improved substrate affinity [81]. Acid protease obtained from a mutant strain of A. oryzae was obtained using solid-state fermentation with potato pulp powder with increased glycine release activity [82]. Also, neutral protease from *A. oryzae* had an optimal pH of 8.0 and an optimal temperature of 55 °C, and its enzymatic characteristics showed that it was efficient in producing antihypertensive peptides and elimination of bitterness [83]. The alkaline protease of *Bacillus*

alcalophilus is active at 10 °C and alkali-resistant, but these properties have been improved through directed evolution using error-prone PCR [84] for its use in food processing at lower temperatures. Similarly, an alkaline serine protease obtained from mesophilic *Bacillus pumilus* was created that has increased hydrolytic efficiency at 15 °C without compromising its thermal stability [85].

Dry-cured meats

Dry-cured meats are the uncooked meat products that are being manufactured for more than 8 months, during which period the certain microorganisms multiply on the surface of the meat. When processing dry-cured meat products, sarcoplasmic and myofibrillar proteins undergo the process of proteolysis, which provides the significant effect on the taste of the product. The changes that take place during proteolysis are associated with the action of both endogenous and microbial enzymes. Microbial proteolytic activity is initiated predominantly by the lactic acid bacteria (LAB), mainly belonging to the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc*, and to a lesser extent from micrococci (*Micrococcus/Kocuria spp.*). The main species that develop during the process of natural fermentation are *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Staphylococcus xylosus*. Amino acids play the decisive role in determining the taste of the food [86,87], Moreover, this effect is clearly enhanced by the activity of proteolytic enzymes. The application of purified proteinases (PrA and PrB) and aminopeptidases (arginyl aminopeptidase and prolyl aminopeptidase) extracted from *Debaryomyces hansenii* CECT 12487 allows obtaining the desired sensory quality. These enzymes catalyze the hydrolysis of sarcoplasmic proteins to form ammonia, increase pH and accelerate the proteolytic pathway [88].

A lot is known about the proteolytic activity of enzymes in the meat products [89], however, there are practically no studies on the proteolysis of the cured ham. The role of molds in dry-cured ham is also important. Various species of Penicillium contain enzymes with proteolytic activity capable to produce peptides and amino acids [56,90]. Certain strains of *Penicillium* and *Mucor* isolated from minced meat have proteolytic activity against meat proteins both in vitro and in processed minced meat [91,92]. *Penicillium aurantiogriseum* has high proteolytic activity against dry-cured meat. The enzyme *Penicillium chrysogenum EPg222* is very active against myofibrillar proteins. At the same time, collagen, being the dominant protein of connective tissue, remains relatively unchanged [56]. An extracellular protease obtained from *Penicillium chrysogenum* isolated from dry-cured ham is also of interest for the taste of dry-cured meat products [93], because it hydrolyzes proteins and reduces processing time.

As far as the yeasts are concerned, strains of *Debaryomyces hansenii* isolated from Iberian ham feature significant aminopeptidase and proteolytic activity [94,95]. Although the activity of these enzymes has mainly been assessed in regards to specific substrates such as myosin [95].

Cross-linked meats

The enzymes for proteins cross-linking like transglutaminases (EC2.3.2.13) are used to improve the texture, flavor and shelf life of the meat products. Transglutaminase is able to adsorb on the surface of meat and, through the acyl-transferase reaction, to cross-link the ε-amino group of the lysine residue with the γ-carboxamide group of the glutamine residue in proteins [96,97]. In industrial production, transglutaminase is predominantly obtained from the bacterium *Streptoverticillium mobaraense* [98]. One of the largest producers of transglutaminase (TG) from *Streptomyces mobaraensis* is the Japanese company Ajinomoto ("ACTIVA" TG) [99]. This enzyme is a monomeric protein with a mass of 38 kDa, that contains 331 amino acids [97]. Since transglutaminase remains active even when refrigerated, it is able to bind raw meat during its storage at temperatures close to 0 °C. For this reason, transglutaminase is widely used in the production of sausages, raw smoked ham and fish products. The effect of TG on meat is significantly increased by the presence of sodium chloride [100]. Adding of salt (more than 2%) increases the solubility of fibrillar muscle proteins, which then become available to TG for their cross-linking. In the last two decades the researches have been run on the use of TG in meat products to improve their properties, such as gelation, water binding, emulsion stability, cleaning losses, cooking losses, etc. The recent review considers the various possibilities for using TG to control the functional characteristics of meat and its products, including the processes of restructuration and value-adding [101].

Cross-linked meats are interesting because it allows using the lower quality cuts of meat, such as trimmings vie their assembly into integral lump meat products, which shape is more appealing to the consumers. However, the process of creating the cross-linked meat often involves its freezing and can cause the product to lose its color, which can make its sale pretty challenging [102]. To avoid consumers' misunderstanding the meat, meat products and meat semi-finished products that may look like a single lump of meat but are actually made up of different pieces mixed together should be labeled as "formed meat" (Annex VI, Part A No.7 Regulation (EU) No 1169/2011[1]). There is some evidence that transglutaminase (TG) in combination with dietary proteins can form proteins that are structurally similar to gluten [103]. This can create problems for people who suffer from celiac disease. Additionally, using parts from different animals in one lump can make it difficult to trace the origin of a product and identify potential sources of diseases outbreaks [104].

Nowadays the sensitive analytical method has been developed to verify the labeling of meat products, including the products restructured with the help of transglutaminase, and to protect the consumers from possible misrep-

[1] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006. Retrieved from https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169 Accessed January 16, 2024

resentation. High performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) is used to detect TG obtained from *Streptomyces mobaraensis* in the restructured meat products. Six tryptic marker peptides are used for this purpose [100]. Among these six marker peptides two peptides (VTPPAEPLDR and SPFYSALR) are suitable for the detection of all three types of TG in the meat and meat products and have been successfully inter-laboratory validated by the method HPLC–MS/MS [105].

In addition to transglutaminase, the other oxidative enzymes were researched as probable candidates for meat cross-linking, in particular: tyrosinases [106] and laccases [107]. Tyrosinase proved to have advantages in improving water holding capacity in comparison with transglutaminase (TG) [108]. However, none of these enzymes are currently used in the meat processing industry [109]. Microbial transglutaminase is recognized as an effective "protein maker", capable to catalyze the cross-linking reactions between protein and peptide molecules within the meat products, thus improving their functional characteristics [101]. Based on the above, it is obvious that TG can be successfully used to develop new products with improved properties [110]. It can be concluded that in the future TG will play an important role in creation of restructured meat products with increased value.

Identified gaps and future trends

Many researches are devoted to the ability of exogenous enzymes to improve meat tenderness [12,111]. However, the problem and issues with exogenous enzymes are rooted in the regulation of their specific activities. Many kinds of proteases have a tendency to indiscriminately degrade the essential proteins in muscle due to their broad substrate specificity [1,111], which leads to extensive damage to the fiber microstructure and affects the color, taste and moisture content of the meat.

Bromelain, ficin extracted from *Ficus insipida*, papain, and peptidase obtained from *Bacillis subtilis* are currently approved by the Food Safety Inspection Service of the US Department of Agriculture as "generally recognized as safe" and are allowed for their use in improving meat tenderness [112]. However, the excessive tenderization of meat by plant proteases under uncontrolled conditions, due to the non-specificity of their proteolytic activity, still leads to loss of quality characteristics and promote the accelerated spoilage of meat, which still remains a challenge for the meat industry [111]. The use of plant proteases affects the taste, increases bitterness due to the formation of basic amines and amino acids, spoils texture and deteriorates the consumer appeal of the processed meat products. This fact can be explained by the general principles of interaction of the enzymes with the substrate [11]. In raw meat, which consists mainly of muscle tissue, the proteases predominantly affect the soluble globular proteins due to the overwhelmingly larger contact area. Several studies have noted that papain causes a mushy texture and foreign flavors [113]. Increased concentrations of non-plant peptidases may also provide negative effects on the sensory characteristics of meat, in particular making the processed product bitter [49]. To improve the methods of meat tenderization, it is necessary to solve the problem of obtaining safe enzymes with different specificities depending on the target component of the meat raw material, regardless of its being muscle or connective tissue.

However the application of microbial proteases also faces another challenge: native enzymes often have limitations that require certain modification to fit the specific conditions of the food processing. The use of genetic modification of enzymes became the solution. This modification aims to rationally improve enzyme characteristics such as purity, yield, specificity, catalytic efficiency, stability and versatility of functions. The purpose of those modifications is to ensure cost-effective production and sustainable development of the food industry.

On the other hand, the use of enzymes in food production and processing has been a representative example of the practical application of biotechnology for a long time. The reasons for this long-term success are explained by the specificity, rapid action and biocompatible nature of the enzymes. These features of the enzymes enable efficient chemical modification of the substrates and food processing to preserve nutrients along with the compliance with the public health and safety requirements. Most of the applied enzymes are derived from microbial sources, which can be attributed to the large number of transferred catalytic activities, plasticity and the relatively undemanding requirements for its growth along with the high productivity, provided by microorganisms. Moreover, recombinant DNA technology has made it possible to express the enzymes, being the subject of interest, from the higher organisms or from the slow-growing or pathogenic microorganisms in fast-growing microbial strains that comply with health safety regulations. As a result of market limitations that require increasing the production capacity as well as production of the new products, there is a necessity of research to improve the performance of known enzyme catalysts or to find the new ones. In the first case, a better understanding of the catalytic mechanisms and protein configuration at the molecular level allows for a better understanding of the behavior pattern and stability of enzymes. Along with this, a more rational approach to application of enzyme compositions, including immobilization techniques, is required. These strivings in their nature are interdisciplinary, and this trend is expected to keep on going in the future. Screening for the new or more efficient enzymes is increasingly using more and more microorganisms obtained from extreme environmental conditions, as they are likely to provide enzymes capable of operating under the production conditions more suitable for given processes where they typically use the enzymes obtained from mesophilic organisms. This approach has achieved the significant success with recent developments in metagenomics, proteomics, and the identification of efficient systems of expression. Moreover the creation or development of en-

zymes with improved properties has become possible due to the development of directed evolution strategies combined with reliable computational methods. The use of multifunctional catalysts and the development of *de novo* enzymes, capable of performing any assumed chemical reaction, will allow multi-step reactions to be run in one step. All of these new interesting developments are highly likely to be implemented in the nearest future and therefore will improve and expand the role of enzymes application in the food industry.

Conclusion

The use of enzymes in the meat industry is an efficient way to improve the tenderness, juiciness and quality of meat products. These enzymes provide manufacturers with the new tools to create food products that meet the consumers' expectations and comply with the quality requirements. Moreover they help optimize production processes and increase the competitiveness of the industry in the food market.

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