Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v44i1.55262

Antimicrobial properties of lysozyme in meat and meat products: possibilities and challenges

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ABSTRACT. Meat and meat products are highly perishable as they can provide an appropriate environment for microbial growth due to their high water activity and proper pH level. Quality, safety, sensory and nutritional properties of meat products are highly influenced by pathogenic and spoilage microorganisms. To prevent microbial growth, artificial antimicrobials have been used in food matrices, however safety concerns regarding the use of synthetic preservatives is a challenging issue. Additionally, consumer's trend towards natural mildly processed products with extended shelf life necessitates the identification of alternative additives originating from natural sources of new acceptable and effective antimicrobials. Although the effectiveness of some natural antimicrobial agents has already been reported, still, there is lack of information regarding the possibility of using lysozyme as a preservative in meat and meat products either alone or in combination with other hurdles. In the present review the applications and beneficial effects of applying lysozyme in meat products, considering its limitations such as allergic problems, interactions with food constituents, reducing sensory changes and toxicity due to high required concentrations to prevent spoilage and oxidation in foods will be discussed.

Keywords: Lysozyme; product; meat; natural antimicrobial.

Received on August 15, 2020.
Accepted on September 22, 2021.

Introduction

Meat and meat products are susceptible to microbial spoilage due to their proper pH (5.5–6.5), rich nutritional composition, and high water activity (0.98–0.99), which provides favorable growth conditions for various microorganisms (Ranaii, Pilevar, Mousavi Khaneghah, & Hosseini, 2020, Pilevar, Hosseini, Beikzadeh, Khanniri, & Alizadeh., 2017, Dave & Ghaly, 2011). Moreover, some of the components in these products provide a complex growth environment which have the ability to protect microbial cells from antimicrobial access (Zhang, Kong, Xiong, & Sun, 2009). Therefore, the meat industry continues to face concerns regarding the safety and hygiene of the products. The techniques of meat processing can render it susceptible to microbiological contamination, thereby, beside the implications of microbiological deteriorations, microorganisms are responsible for many meat borne outbreaks and diseases. Although a variety of food preservation strategies such as drying, freezing, canning, chilling, fermentation, nutrition restriction, irradiation, thermal processing, and synthetic antimicrobial agents can be utilized to extend food shelf life; it is not consummate to inhibit microorganisms that may threaten consumer's health (Negi, 2012, Goodarzi, Hovhannisyan, & Barseghyan, 2016). All mentioned methods possess their own limitations principally when attempting to apply them in fresh meats. These methods are not generally designed to completely eliminate all the microorganisms (Sung et al., 2013, Quintavalla & Vicini, 2002). To prevent cross contamination in production, distribution, and sale stages and to expand the shelf life of processed or raw meats, synthetic additives should be used. Aside from their advantages, synthetic antimicrobials, such as benzoate, sulfites, sorbates, and nitrate may have life-threatening side effects such as teratogenic and carcinogenic attributes (Anand & Sati, 2013, Seetaramaiah, Smith, Murali, & Manavalan, 2011). Modern trends aim to have natural origin to protect meat from both pathogenic and spoilage microorganisms and to have minimum processing (Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007, Pilevar & Hosseini, 2013, Pilevar et al., 2017). There

are various numbers of antimicrobial agents in animals, plants, and microorganisms as a part of host defensive systems. Some of these compounds are flavonoids and saponins in spices and herbs, lysozyme as an iron chelator in figs and egg white, polysaccharide chitosan evolved from shrimp shells and nisin, the best known of all bacteriocins, from lactic acid bacteria (LAB) (Pilevar et al, 2020, Pilevar & Hosseini, 2013, Kasra-Kermanshahi & Mobarak-Qamsari, 2015, Olaoye, Onilude, & Ubbor, 2015). Meat gets spoiled during storage due to two major causes: microbial growth and oxidative rancidity (Kim, Cho, & Han, 2013). Lysozyme has shown both antibacterial and antioxidant activities in meat and meat products, inhibiting meat spoilages with other hurdles (Kozuka et al., 2015, Li et al., 2014, Zimoch-Korzycka & Jarmoluk, 2015, Cegielska-Radziejewska & Szablewski, 2013, Liberti, Franciosa, Gianfranceschi, & Aureli, 1996). Though the effectiveness of some natural antimicrobials has been well established, no reviews have been managed to study the potential of applying lysozyme in order to preserve meat products. Hence, the aim of this review is to discuss mechanism of action, advantages, limitations and different aspects of application of lysozyme, an important natural antimicrobial, in the meat industry.

Structure and enzymatic function of lysozyme

Lysozyme is a lytic enzyme (hydrolase), single peptide protein, cross-linked by four disulphide bonds, heat-stable in acidic solutions, with a molecular weight of 14600 Daltone (Proctor, Cunningham, & Fung, 1988) that consists of 6 tryptophan, 3 tyrosine, and 3 Phenylalanine amino acids residues (Wu et al., 2017). The protonated Glu35 (-COOH) and ionized Asp52 ($-COO^-$) are the key catalytic residues as a part of the lysozyme active site (Fig.1) (Johnson, Phillips, & Rupley, 1968). This valuable and economic biological catalyst consists of a well-known amino acids sequence and three-dimensional configuration (Chung & Hancock, 2000; Goodarzi et al., 2016; Abdollahzadeh, Rezaei, & Hosseini, 2014). The lysozyme exhibits strong enzymatic activity in a wide range of temperatures when dissolute in water or is influenced by solvents (Cegielska-Radziejewska & Szablewski, 2013). The lysozyme lytic action against Gram-positive bacteria was found by Fleming in 1922 (Fleming & Allison, 1922). Lysozyme's natural substrate is certain polysaccharides including the bacterial cell wall (Lesnierowski & Kijowski, 2007). Other than antimicrobial activity, lysozyme may possess radical scavenging activities and can have a role in the inhibition of calmodulin-dependent phosphodiesterase (CaMPDE) (You, Udenigwe, Aluko, & Wu, 2010). Hen egg white lysozyme or murimidase [EC 3.2.1.17] has 129 amino acids in length and consists about 3.4 % of total egg white proteins (You et al., 2010). Lysozyme is grouped into three major types according to the similarity among amino acid sequences: chicken-type, goose type, and invertebrate type (Strominger & Tipper, 1974). Hen egg lysozyme is a primary protein that has an isoelectric pH (PI) of 10-11. This protein has two domains, the α-domain and the β-domain (Mine, Ma, & Lauriau, 2004), which are dissociated by a Helix-Loop-Helix Motif (Asp 87-Arg 114), that play an important role in antimicrobial activity of the molecule (Lesnierowski & Kijowski, 2007). Egg white and albumin are the cheapest (Avramescu, Borneman, & Wessling, 2008) and major commercial (Lesnierowski & Kijowski, 2007) available sources of lysozyme, respectively. Lysozyme can be extracted from membrane of a fresh hen egg shell, which shows weaker antibacterial activity compared to lysozyme extracted from the hen egg white. However, the lysozyme extracted from hen egg shell can be used as an antifungal agent in food industry (Kozuka et al., 2015). Lysozyme in a 100% purified form can be easily obtained by NaCl (5%) at pH 9.5-9.8 using crystallization method and acetate buffer (pH=4.5) (Whitaker, Wong, & Voragen, 2003). Duck lysozyme has shown greater anti *Salmonella enteritidis* activity comparing to chicken lysozyme, and exhibits synergism effect in combination with lactoferin similarly to chicken lysozyme (Naknukool, Hayakawa, Uno, & Ogawa, 2009). In contrary to chicken and duck egg white, emu egg white contains very low levels of lysozyme (Maehashi et al., 2012). Lysozyme constitutes about 0.5 % of albumen fraction and 3.5% of total egg white proteins based on lytic activity (Malicki, Jarmoluk, & Bruzewicz, 2004, Whitaker et al., 2003). Enzymatic activity of lysozyme is measured by quantifying the turbidity loss of a suspension of lyophilized *Micrococcus lysodeikticus* cells in phosphate buffer at 25°C (Weert, Hoechstetter, Hennink, & Crommelin, 2000). The reduction of optical dispersion at 450 nm by 0.001 $min.^{-1}$ is equivalent to 1 unit (U) of enzyme activity (Proctor et al., 1988). However, turbidity loss might be related to bacterial cell rupture and is not equal to the cleavage of glycosidic bonds due to the lytic action of lysozyme enzyme (Whitaker et al., 2003).

Antimicrobial mechanism of action in meat products

Lysozyme possesses enzymatic activity against (1-4) glycosidic linkages between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) of cell wall peptidoglycan. Lysozyme is effective against Gram-

positive, while less active against Gram-negative bacteria (Chen, Han, Li, & Sheng, 2017). Antimicrobial activity of lysozyme is associated with 11% of enzymatic activity, cationic activity, and also hydrophobic acting (You et al., 2010). The application of lysozyme is limited due to the great resistance of Gram-negatives, whose cell walls are covered by lipoprotein–lipopolysaccharide layer of the outer membrane, rendering them less susceptible to disruption by lysozyme. Among *Bacillus*, *B. cereus* and *B. stearothermophilus* express higher resistance to lysozyme (Abdou, Higashiguchi, Aboueleinin, Kim, & Ibrahim, 2007). A study indicated the viability loss of Gram-positive such as *B. cereus* and *Staphylococcus aureus* cells were 69 and 80% at 1 mg mL^{-1} of lysozyme, respectively. Although, no significant reduction in viability of Gram-negatives such as *Pseudomonas fluorescens* and *Escherichia coli* even at 2 mg mL^{-1} of lysozyme occurred (Rao, Chander, & Sharma, 2008), its activity was increased in combination with membrane destroying agents or by sub-lethal injuries (Gill & Holley, 2003). Lysozyme is an antimicrobial agent with a positive charge that can be loaded onto/into materials to overcome instability and inactivation (Huang et al., 2012). By using protein engineering, lysozyme can be physically immobilized by encapsulation or layer by layer technique, ensuring stability and antibacterial activity of an enzyme. It is suggested that via electrospraying of lysozyme, losses can be avoided or controlled. Lysozyme is stable in the presence of CO_2 gas when applied to foods with acidic pH, thereby might be suitable for application in modified atmosphere packaging (MAP). Lysozyme can also be immobilized through active packaging in cellulose-based or carboxymethyl cellulose-based packages alone (Mascheroni et al., 2010) or combination with lactoferrin (glycoprotein) exhibiting synergism effects (Barbiroli et al., 2012). Cellulose acetate is an environmentally friendly polysaccharide that is modified and can be negatively charged. Lysozyme can be easily electro sprayed onto cellulose acetate which shows higher antibacterial activity, better enzyme activity, and lower amount of losses (Li et al., 2014). Antimicrobial activity of lysozyme enhances when incorporated in a layer by layer technique as combined with negatively charged pectin on the surface of nano fibrous of cellulose (Zhang et al., 2015). Spray drying of lysozyme with low concentrations of pectin improves lysozyme antimicrobial activity and decreases aggregation due to configuration and conformational changes of complexes (Amara, Eghbal, Degraeve, & Gharsallaoui, 2016).

Some investigations have reported a significant increase in lysozyme antimicrobial activity in meat products follows by the addition of certain substances including Ethylenediaminetetraacetic acid (EDTA) as the chelating agent (Branen & Davidson, 2004), trisodium phosphate, sodium lactate (Malicki et al., 2004), butylparaben (Gill & Holley, 2000), chitooligosaccharides (Rao et al., 2008), and nisin (Chung & Hancock, 2000). Cannarsi et al. (2008) have demonstrated that lysozyme alone in high concentrations of more than 0.5% is capable to suppress the growth rate of *B. thermosphacta*. A mixture of nisin and lysozyme balances the high cost of nisin and the required amount of lysozyme to inhibit bacterial growth (Cannarsi et al., 2008). A ratio of 1:3 nisin and lysozyme has shown the highest antimicrobial activity against *Bacillus thermosphacta* and *Carnobacterium* (Nattress, Yost, & Baker, 2001). The growth of *Lactobacillus curvatus* on both bologna and ham has been reduced by lysozyme-nisin treatment (Gill & Holley, 2000). The range of lysozyme antimicrobial activity against Gram-negatives can be extended by specific modifications for improving the functional properties in meat products (Rao et al., 2008). It has been reported that the mechanism of lysozyme antimicrobial activity was more widespread than muramidase activity thought before. The amphipathic helix structures in the T4 lysozyme C-terminus mediate its fungistatic and bactericidal activities (Düring, Porsch, Mahn, Brinkmann, & Gieffers, 1999). In another study, the same results were reported as it was suggested that susceptibility of bacteria to lysozyme is not dependent to enzymatic activity. It is probable that irreversibly denatured lysozyme, for example the dimeric form through polymerization, has an intrinsic structure which generally destroys the bacteria by membrane destruction (Ibrahim, Higashiguchi, Juneja, Kim, & Yamamoto, 1996). It is reported that released peptides of lysozyme also contribute in antimicrobial activity of lysozyme as well as its enzymatic activity (You et al., 2010).

Application of lysozyme alone and in combination with other hurdles in meat products

For application of lysozyme as an antimicrobial agent in food products, the source of origin in terms of being safe should be considered due to potential risks for consumer health. Lysozyme is very popular for use in food systems such as hard cheeses, and is commercially used to inhibit the growth of spore of *Clostridium tyrobutyricum* after germination (Chung & Hancock, 2000) and also meat and meat products. Lysozyme as a nitrate replacer is added to washed-curd/round-eyed cheeses to prevent late-blowing by spore-forming bacteria via hydrolysis of polysaccharides in their cell walls, controlling butyric acid fermentation (Lodi & Stadhouders, 1990). However, this enzyme is not applied to meats as a nitrate replacer which can be further

studied. In the European Union (EU) food legislation, enzymes except for lysozyme in wine and cheese and invertase in confectionary are classified as processing aids and not as food additives. Lysozyme is not harmful to human and is naturally produced in tissues and secretions of human and many animals (Cho, Bae, Ha, & Park, 2005). The concern regarding the use of lysozyme is that it can contribute to problems of susceptible individuals who suffer from lacking immunity (Malicki et al., 2004; Gill & Holley, 2000). Therefore, it should be considered that lysozyme may cause problems including allergic (Leduc et al., 1999) and antigenic aspects of ingested lysozyme (Cunningham, Proctor, & Goetsch, 1991). Thus, this enzyme is applied to meat products in combination with other antimicrobial agents and hurdles to reduce the concentrations needed to be applied for inhibition of bacteria. However, enzymatic activity of lysozyme decreases at high temperature and increased pH value, thereby other preservation methods than temperature are proposed. For example, using modern techniques such as high hydrostatic pressure (HHP) along with heat treatment can reduce the allergenic effects of lysozyme in meat products (Hildebrandt et al., 2010). These novel non-thermal hurdle approaches are used in meat processing to ensure microbiological safety in combination with natural preservatives such as lysozyme (Oliveira, Ramos, Ramos, Piccoli, & Cristianini, 2015). Using a combination of lysozyme in minimum inhibitory concentration (MIC) level (MIC against *Lactobacillus brevis*=50 mg L^{-1}) and 150-170 MPa of pressure has shown a reduction of 6 logarithmic cycles in model systems (Tribst, Franchi, & Cristianini, 2008).

The lysozyme at a concentration of 0.5-2 % without addition of EDTA cannot reduce *Pseudomonas* spp. population in buffalo meat samples (Cannarsi et al., 2008). It has been shown that lysozyme along with 2% EDTA can inhibit the other spoilage microorganisms and extend the shelflife of fresh buffalo meat. Consistent with this survey, in time-kill assay, organic acids without addition of lysozyme did not have anti listeria activity (Oh, Lee, Jeong, & Kim, 2016). Therefore in these cases, lysozyme is applied to meat products with other components exhibiting anti-listeria activity. Lysozyme has shown the same antimicrobial activity alone and in combination with other hurdles against *Listeria monocytogenes* in dairy products (Whitaker et al., 2003). As other examples of hurdle technique, combined EDTA and lysozyme exhibit higher antimicrobial activity in red meat and dairy products (Bevilacqua, Sinigaglia, & Corbo, 2010), or lysozyme in combination with essential oils such as oregano or rosemary oil in chicken meat (Ntzimani, Giatrakou, & Savvaidis, 2010) and combination with organic acids and chitosan in pork meat (Huang et al., 2012) exhibits synergistic antimicrobial activities. By checkerboard assay *in vitro*, a mixture of lysozyme with organic acids such as acetic/lactic acid and others have shown synergistic effects against *Listeria monocytogenes*. Amongst examined organic acids, malic acid and succinic acid have demonstrated higher synergistic effects against *L. monocytogenes* (Oh et al., 2016). Added lysozyme to chitosan coatings and hydrosols (with Nano silver) also can help in increasing antibacterial and antioxidant activities of theses antimicrobial agents when applied onto meat surfaces (Zimoch-Korzycka & Jarmoluk, 2015). At specific pH values lysozyme in combination with sodium caseinate and Spirulina protein forms insoluble complexes of edible films which modifies mobility and release of lysozyme (Benelhadj et al., 2016). Combination of gamma radiated chitosan (chitooligosaccharides) with lysozyme in minced meat shows synergistic effects against Gram-negative bacteria and extends the shelf life up to 15 days during storage at refrigeration temperatures (Rao et al., 2008). As other example of combined components in meat products is application of xanthan gum with lysozyme which leads to higher antimicrobial activity of lysozyme and higher quality of emulsifier in meat and meat products (Hussain et al., 2017).

Series of studies are done on the application of lysozyme in meat and meat products such as Vienna sausage (Akashi, 1971), salami (Akashi, 1970), and cooked sausages (Akashi, 1969). The results have indicated the effective combination of lysozyme with other preservatives such as salt or sodium nitrite. In a type of Italian chicken sausage, half of the nitrite amount was replaced by lysozyme (1/2 nitrite+1/2 lysozyme). The sample showed the same antibacterial activity against *Escherichia coli* and *Salmonella* and antioxidant activity compared to the control with 2/2 of applied nitrite. The sensory properties have been improved by replacing a part of nitrite with lysozyme (Herath, Priyanath, Ahn, & Abeyrathne, 2015). The addition of polyphosphates (lipase) to lysozyme has shown to significantly enhance the lytic and antibacterial activity of lysozyme on growing cultures at various temperatures and against *L. monocytogenes* in buffer solution (Liberti et al., 1996). In ground pork samples, modified lysozyme has shown stronger antibacterial activity against *Pseudomonas* species and *Enterobacteriaceae* family when compared to lysozyme monomer. Modified lysozyme in heat-treated samples have shown to exhibit higher antibacterial activity in ground pork (Cegielska-Radziejewska & Szablewski, 2013) and also in wine against lactic acid bacteria and acetic acid bacteria (Carrillo, García-Ruiz, Recio, & Moreno-Arribas, 2014).

In meat, carbohydrates and amino acids interact to form intermediate substances that are converted to flavor compounds through decarboxylation, oxidation, cyclization and condensation (Nagai, Inoue, Kanamori, Suzuki, & Nagashima, 2006). Typical examples of pronounced compounds are flavonoids and saponins (spices and herbs), lysozyme (figs, egg white), nisin (LAB), chitosan (shrimp shells), and lactoperoxidase from milk. Herbs and spices have been used in many cuisines to impart aroma and flavor in food (Kanatt, Chander, & Sharma, 2008). Aforementioned, like essential oils, lysozyme can be prepared in micro encapsulated or beads form, or in forms of films and nanoparticles in meat and meat products (Wu et al., 2017). However, lysozyme can make changes in sensory properties that can be inhibited by micro ionization, but in this method degradation of lysozyme should be controlled. The particles of lysozyme (2.8-13.8 micrometer) resulted by expanded liquid anti-solvent technique are smaller than can be felt, but integrity of lysozyme and absence of solvent in final product should also be considered to ensure the safety (Prosapio, Reverchon, & De Marco, 2016). Decontaminated chicken legs by spraying lysozyme (6000-48000 U mL^{-1}) has not shown any significant sensory differences with fresh meat samples during storage at 4°C (120h), where control leg samples were deteriorated and had darker color mainly in cutting lines (Kijowski, Marciszewska, Cegielska-Radziejewska, & Popiół, 2013). The addition of modified lysozyme into ground pork samples have not shown any adverse effect on sensory properties compared to samples without addition of lysozyme during 72h (Cegielska-Radziejewska & Szablewski, 2013).

Lysozyme is commercially obtained from hen egg white (albumen fraction), where contains about 0.5% lysozyme (with low activity) that might possess immunological problems. Except for allergic problems, extraction of lysozyme from egg white has other disadvantages such as huge amounts of needed egg white to reach an acceptable purity. Therefore, application of lysozyme extracted from egg white might be restricted, and it should be noted on product labeling that the food product contains egg. lysozyme is used in the hydrochloride form in foods, largely to preserve vegetables, tofu salad, potato, fresh fruits, semidry cheeses, and seafoods including fish cakes, bacon, meat, and sausages (Malicki et al., 2004). The commercial production of lysozyme by microbial fermentation i.e. *Micrococcus lysodeikticus* has been proposed as an alternative to the lysozyme extracted from hen egg. In this method, cheaper raw materials with uniform quality can be applied for enzyme production through surface or submerged fermentation. In this case, *Micrococcus luteus* is an obligate Gram-positive aerobe.

Conclusion

Meat and meat products are highly perishable as they have relatively high water activity and an appropriate pH level for microbial growth. Lysozyme as a natural preservative is of great interest for use in meat and meat products as it shows antibacterial activity against Gram-positive bacteria including *L. monocytogenes* and partially Gram-negative bacteria in meats. Few studies have been carried out to explain the synergistic effects of lysozyme with other antimicrobial components, therefore, more investigations are needed to obtain higher antibacterial activity against resistant Gram-negative bacteria in meat and meat products as well as Gram-positives. In conclusion, studies indicate that antibacterial and antioxidant activities of lysozyme can be improved by HHP or/and in combination with bacteriocins (nisin), glycoprotein (lactoferrin), EDTA, certain salts (phosphates), paraben derivatives, oregano or rosemary essential oils, and organic acids such as malic acid and succinic acid or by loading of enzyme with chitosan or radiated chitosan hydrosols, coatings and onto cellulose based packages. Hen egg white lysozyme is expensive and may pose a risk to individuals allergic to eggs, therefore, production of lysozyme by fermentation process has been proposed.

More investigations are required to figure out the approaches to achieve better application of lysozyme in meat products conforming to market trends.

Acknowledgements

We appreciate the 'Student Research Committee' and 'Research & Technology Chancellor' in Shahid Beheshti University of Medical Sciences for their financial support of this study.

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