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PROPERTIES AND APPLICATION OF EGG WHITE LYSOZYME AND ITS MODIFIED PREPARATIONS – A REVIEW

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Lysozyme monomer exhibits strong antibacterial activity against Gram-positive organisms. This phenomenon has found a practical application in the food processing industry, in medicine and pharmaceutical industry. The use of lysozyme in the food processing industry is connected primarily with its application as a natural preservative. The enzyme is widely used as a preservative for meat, fish and their products, for milk and dairy products, as well as for fruit and vegetables. The pharmaceutical industry uses this enzyme in the manufacture of adjuvant drugs for antibiotics and analgesics in viral and bacterial infections, in the treatment of leukemia and neoplastic diseases. Lysozyme is also used as a diagnostic agent, being an indicator of the occurrence and the progression of pathological changes in humans and animals. The range of the practical applications of lysozyme may be considerably extended as a result of its modification. The enzyme after modification exhibits a new specific activity in relation to Gram-negative bacteria, being a result of dimerization, with no loss of activity against Gram-positive bacteria, characteristic for the monomer, as it was indicated in studies by Ibrahim *et al.* [1991;1996], Lesnierowski *et al.* [2004] and Kijowski *et al.* [2006]. The dimeric form of lysozyme has been used in the treatment of bacterial and viral animal diseases. A drug produced on the basis of lysozyme dimer shows immunostimulating and immunocorrective activity.

INTRODUCTION

Lysozyme (E.C.3.2.17, N-acetyl-muramic-hydrolase) is a bacteriolytic enzyme commonly found in nature. It is a globular basic protein characterised by MW of approx. 14.4 kDa. It is found as a single polypeptide chain consisting of 129 amino acids, in which lysine is the N-end amino acid and leucine is the C-end one. In a lysozyme molecule there are four disulfide bridges (S-S), which cause high thermal stability of the enzyme, together with six helix regions. The enzyme molecule is a compact complex in the shape similar to an ellipsoid with dimensions of 4.5 x 3.0 x 3.0 nm.

A rich and easily available source of lysozyme is the egg white of birds. In the hen egg white, lysozyme accounts for 3.5% of the total egg white proteins. The activity of egg white lysozyme is affected by numerous factors such as management system of laying hens, feed modification and egg storage [Kopeć *et al.*, 2005; Świerczewska *et al*., 2003a]. Immunostimulating preparations applied in poultry breeding have been found to increase the activity of lysozyme [Świerczewska *et al.*, 2003b].

Numerous methods are used in laboratory practice to separate lysozyme from hen egg white, but only some of them have been used in industry. A group of methods to separate lysozyme includes its direct crystallization from egg white, techniques based on the phenomenon of the adsorption of the enzyme by certain substances (chromatographic techniques), or membrane techniques (especially ultrafiltration).

Bacteriostatic and bactericidal properties of lysozyme have been used both to preserve various kinds of food, as well as in pharmacy, human and veterinary medicine [Kopeć & Trziszka, 1997; Lesnierowski & Kijowski, 1995; Rosiak & Kołożyn–Krajewska, 2003].

In nature lysozyme is found mainly as a monomer, and like many other natural compounds, might be even more active in a dimeric or polymeric form. This enzyme has been reported to exist as a reversible dimer, which can be evoked by the pH, concentration and/or temperature dependent phase transition of the molecule [Sophianopoulos, 1969]. It was investigated that diamines (1,3-diaminopropane, 1,4-diaminobutane and 1,5-diaminopentane) suppress thermal aggregation and inactivation of lysozyme whereas no diols or monoamines prevented its thermal aggregation [Okanojo *et al.*, 2005].

It has also been found that the chemical and thermal modification of lysozyme increases its antimicrobial properties towards Gram-negative bacteria. Such modified lysozyme exhibits a novel, but not completely defined antimicrobial action. Recent reports suggest that this unique antimicrobial action of unfolded lysozyme is attributed to membrane binding and the subsequent perturbation of its functions [Ibrahim *et al*., 1996].

It is now evident that, aside from the lysozyme bacteriolytic action, a dimeric form of lysozyme exhibits therapeutic, antiviral and anti-inflammatory properties. The studies conducted so far show that it induces the activity of phagocytizing cells, influences the immunological processes by the stimula-

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tion of immunoglobin synthesis, stimulates alpha-interferon synthesis, and most importantly – modulates TNF (Tumor Necrosis Factor) generation [Kiczka, 1994].

SEPARATION AND APPLICATION OF LYSOZYME MONOMER

Due to increasing demand for natural food preservatives, lysozyme has become increasingly important in food processing and therefore, there is a need to develop an efficient and simple methodology for its production. Several methods of lysozyme isolation from egg white have been developed until now. The classic method for the preparation of commercial lysozyme is direct crystallization from hen egg white [Alderton & Fevold, 1946; Lesnierowski *et al*., 1993]. Other methods such as adsorption, ion exchange chromatography [Ahvenainen *et al*., 1979; Li-Chan *et al*., 1986; Banka *et al*., 1993; Weaver & Carta, 1996; Lesnierowski & Kijowski, 1997], affinity chromatography [Weaver *et al*., 1977; Muzzarelli *et al*., 1978; Yamada *et al*., 1985; Chiang *et al*., 1993], membrane separation [Chang *et al*., 1986; Chiang *et al*., 1993; Chiu *et al*., 2007; Kijowski *et al*., 1998; Lesnierowski & Kijowski, 2001, 2002], molecular imprinted particles (Lys-MIP) [Odabaşi *et al.*, 2007; Shen & Cao, 2007] and an aqueous two-phase extraction of lysozyme [Ratajczak *et al.*, 2004] have been investigated and their separation efficiencies have been reported. Most of these methods are only used in laboratory practice because they suffer from major deficiencies, *i.e.* a long time or high cost of lysozyme production. Thus, an efficient and rapid method for the separation of lysozyme from egg white is needed.

Lysozyme exhibits an antibacterial activity against a certain number of food spoilage bacteria and pathogens. There is a considerable interest within the food industry in using natural antimicrobial agents that are non-toxic to humans and show inhibitory activity to undesirable microorganisms. The extension of shelf life and the control of pathogenic as well as spoilage organisms in refrigerated foods is of great importance to the food industry.

The enzyme is antibacterial because it degrades the polysaccharide that is found in the cell walls of many bacteria. It does this by catalyzing the insertion of a water molecule. This hydrolysis breaks the chain at that point.

The bacterial polysaccharide consists of long chains of alternating amino sugars: N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM).

These hexose units resemble glucose except for the presence of the side chains containing amino groups. Lysozyme is a globular protein with a deep cleft across part of its surface. Six hexoses of the substrate fit into this cleft. X-ray crystallography has shown that as lysozyme and its substrate unite, each is slightly deformed. The fourth hexose in the chain (ring 4) becomes twisted out of its normal position. This imposes a strain on the C-O bond on the ring-4 side of the oxygen bridge between rings 4 and 5. It is just at this point that the polysaccharide is broken. A water molecule is inserted between these two hexoses, which breaks the chain.

Lysozyme is present in almost all secretion body fluids and tissues of human and animal organisms. It has also been isolated from some plants, bacteria and bacteriophages. It exhibits more effective antibacterial activity towards Gram-positive rather than Gram-negative bacteria due to the differences in the structure of their cell walls. Cell walls of Gram-positive bacteria consist mainly of a peptidoglycan layer. The peptidoglycans of some Gram-positive bacteria are resistant to hydrolysis by lysozyme. This is the case for *Staphylococcus aureus* [Bera *et al.*, 2005] and for *Bacillus subtilis* [Atrih *et al*., 1999]. In the case of Gram-negative bacteria an additional barrier for lysozyme is the inner membrane composed of proteins, phospholipids and lipopolysaccharides [Ibrahim *et al*., 1991]. Generally, the limited action of lysozyme on Gram-negative bacteria seems to be influenced both by the composition and the sequence of N-acetyloamino sugars of the bacterial cell walls.

In the study on the degree of susceptibility of Gram-positive strains it was found that isolated cell walls of the tested bacteria were sensitive to lysozyme. The most sensitive were bacteria of the *Micrococcus*, *Sarcina* and *Bacillus* genera. Cell wall isolates indicated the following percent turbitity reduction in 24 h at 37°C (the higher lysozyme activity the higher reduction): *Micrococcus* 72 to 98%, *Sarcina lutea* 98%, *Bacillus* 51 to 95%, *Sporosarcina urea* 98%, *Corynebacterium* 92%, *Staphylococcus* 9 to 91%, *Streptococcus faecalis* 73% and *Lactobacillus arabinosus* 22% [Proctor & Cunnigham, 1988].

It was found that lysozyme isolated from chicken egg white showed bacteriostatic properties not only against saprophytic bacteria, but also against important food pathogens, such as *Listeria monocytogenes* and *Clostridium botulinum* [Hughey & Johnson, 1987]. Among the 15 examined bacteria species, *Clostridium tyrobutyricum*, *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* were completely inhibited by lysozyme hydrochloride in the concentration of 20 or 200 mg per litre of complex media. *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum* types A, B and E, *Yersinia enterocolitica* and *Listeria monocytogenes* were among the bacteria moderately inhibited, whereas *Clostridium perfringens*, *Escherichia coli* O 157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* were not inhibited [Hughey & Johnson, 1987]. Public health depends on the control of *Clostridium botulinum* to prevent botulinal toxin formation in low acid foods. Lysozyme was effective in controlling toxin formation by *Clostridium botulinum* in fish, poultry and certain vegetables [Johnson, 1994]. Lysozyme exhibits considerable activity against the *Clostridium tyrobutyricum* contaminant causing late gas defect (blowing cheese) resulting from butyric acid fermentation in Edam, Gouda and other types of cheese. In our study, the bacteriostatic activity of lysozyme monomer against *Clostridium tyrobutiricum* causing inferior cheese quality was established. It was found that lysozyme was a highly effective agent inhibiting the growth of *Clostridium tyrobutiricum* bacteria. A conspicuous bacteriostatic effect was observed after using lysozyme with the activity of 250 to 500 U/mL of bacteria suspension. No bacteriostatic effect against *Clostridium tyrobutiricum* was found only in the case of lowering lysozyme activity to 100 U/mL. The effective and recommended activity for lysozyme monomer of 99% purity is 250 U/mL [Danyluk & Kijowski, 2001].

Lysozyme inhibitory activity against *Listeria monocytogenes* depends on the physiological status of the bacterium,

medium or food in which it was suspended and also on temperature. Cells grown at a refrigeration temperature were found to be more sensitive than those grown at a room temperature [Smith *et al*., 1991; Johnson, 1994]. These results were important for they suggested that lysozyme is most effective in refrigerated foods. In certain foods lysozyme killed or prevented the growth of *Listeria monocytogenes* and was more active against this pathogen in vegetables than in animal-derived foods [Hughey & Johnson, 1987]. Chander *et al*. [1984] studied the antibacterial activity of lysozyme against some common food-poisoning organisms and reported inhibition of some bacteria that reached 77% for *Bacillus cereus*, 56% for *Escherichia coli*, 45% for *Salmonella typhosa*, 19% for *Shigella dysenteriae*, 63% for *Proteus vulgaris* and 60% for *Pseudomonas aeruginosa*.

Lysozyme demonstrates antimicrobial activity against a limited spectrum of bacteria and fungi; however, its enzyme activity can be enhanced by certain substances including EDTA, butylparaben, tripolyphosphate, as well as some naturally occurring antimicrobial agents [Durance, 1994]. Chelating agents (EDTA, disodium pyrophosphate, pentasodium tripolyphosphate) were observed to affect the inhibition of the growth of *Escherichia coli* O157:H7 by lysozyme. The results indicate that inhibition occurred witch each lysozyme-chelator combination [Boland *et al.*, 2003]. Trisodium phosphate increases sensitivity of Gram-negative bacteria to lysozyme but *Staphylococcus aureus* is resistant to lysozyme, even after TSP treatment. The TSP-lysozyme treatment was effective in killing cells attached to chicken skin [Carneiro *et al.*, 1998]. Lysozyme itself has a weak activity against yeast growing in foods, but its activity was enhanced by lysolecithin or poly-L-lysine compounds [Johnson, 1994]. To increase the antibotulinal activity it is necessary to use chelators or other synergistic agents, including DTPA, EDTA, cysteine, tripolyphosphate and naturally occurring agents: nisin and avidin. In a water suspension of turkey meat, lysozyme hydrochloride at 100 μg/g delayed botulinal toxin formation for 11-14 days at 20°C. The delay in toxin formation was also extended for two additional weeks by the synergic action of cysteine, proline or 2% sodium lactate [Johnson, 1994]. Therefore, certain substances may enhance lysozyme activity, and using chemical synergists or physical treatments that render microorganisms susceptible can broaden its spectrum against target organisms. Permeabilization of the outer membrane of bacteria can be accomplished by physical stresses such as a high-pressure treatment [Masschalck *et al.*, 2002]. The inactivation of Gram-negative bacteria by high hydrostatic pressure treatment was examined in the presence of hen egg-white lysozyme, denaturated lysozyme and lysozyme derived peptides. None of these compounds had a bactericidal or bacteriostatic effect on the tested bacteria at atmospheric pressure. It was indicated that pressure sensitized the bacteria to lysozyme [Masschalck *et al*., 2001]. A comparison of bactericidal activity of lysozymes at atmospheric pressure and under high hydrostatic pressure indicated that *Micrococcus lysodeicticus* and *Pseudomonas aeruginosa* became sensitive to all tested lysozymes under high pressure. The other bacteria (*Enterococcus faecalis*, *Bacillus subtilis*, *Listeria inocua*, *Yersinia enterocolitica, Shigella flexneri*) showed sensitisation to some of the lysozymes, whereas *Salmonella typhimurium* remained insensitive to all lysozymes [Nakimbugwe *et al.*, 2006].

The effect of lysozyme of our own production on the microbiological stability and sensory attributes of commercial cut-up poultry was evaluated in recent studies [Kijowski *et al*., 2002]. The contamination of samples on average total bacteria counts attained 1.2-1.6 $\times$ 10^5 cfu/g. The samples were sprayed with a constant volume of lysozyme solution from 3-48 $\times$ 10^3 U/mL and stored at 4°C for the period of 144 h. The total bacteria count; coliform bacteria, enterococci, spore-forming anaerobes, pathogenic staphyloccoci and *Salmonella* were determined. It was found that the growth of bacteria of public health significance was substantially reduced. The used lysozyme inhibited, to the highest degree, the growth of aerobic microorganisms, as well as coliforms and enterococci. Lysozyme of 12-24 $\times$ 10^3 U/mL activity was found effective and the storage life of cut-up poultry was extended from 48 to 72 h for control and enzyme-treated samples, respectively. The used doses of lysozyme did not reduce the growth of *Salmonella.* The application of a lysozyme solution to spray the surface of chilled cut-up poultry significantly prolongs its shelf life and the period of its commercial suitability. Additionally, in our previous study [Kijowski *et al*., 2002] we analyzed the influence of a different activity of lysozyme on the growth of Gram-positive and Gram-negative bacteria (*Staphylococcus* sp.*, Clostridium tyrobutiricum, Salmonella enteritidis*, *Proteus mirabilis, Pseudomonas fluorescens*). The activity of lysozyme against bacteria was expressed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). It was found that the most sensitive were *Clostridium tyrobutyricum* and *Staphylococcus*. Lysozyme had a weak activity against *Proteus mirabilis* isolated from meat and was not effective towards *Salmonella enteritidis* and *Pseudomonas fluorescens* [Marciszewska, 2000]. It was indicated that the addition of lysozyme, nisin and EDTA to ham and bologna prior to cooking may restrict the growth of *Brochothrix thermosphacta*, *E.coli* O157:H7 and *Lactobacillus curvatus* [Gill & Holley, 2000].

MODIFIED LYSOZYME AND ITS APPLICATION

Lysozyme was found to exist in two conformational states between 20 and 30°C with a transition point at 25°C [Jolles & Jolles, 1984]. The enzyme is normally present as a reversible dimer between pH 5.0 and 9.0. It is also known that hen egg white lysozyme tends to associate in an irreversible dimeric form (presumably through intermolecular disulfide exchange) when eggs are stored for long periods. This conformation-dependent, novel antimicrobial activity of lysozyme was extensively examined by means of its gradual thermal inactivation at neutral pH and at different temperatures [Ibrahim, 1998]. Heated lysozyme showed also a several-fold increase in surface hydrophobicity over the native enzyme.

Another study of lysozyme irreversible heat denaturation gave also evidence for the accumulation of multiple chemical reactions, such as isomerisation, cyclic imide formation, deamidation and racemization of some residues [Tomizawa *et al*., 1994a, b].

Research indicates that the range of lysozyme activity may be extended by means of modifications leading to changes in the

conformation of enzyme molecules and as a consequence – the production of its polymeric forms. It was found that a modified form of lysozyme showed a significantly wider range of bacteriostatic activity, including also Gram-negative bacteria, than the active lysozyme. It needs to be emphasized that the enzyme after modification retains antibacterial activity towards Gram--positive bacteria, characteristic for the monomer [Ibrahim *et al*., 1991, 1994, 1996; Kijowski *et al*., 2000; Lesnierowski & Kijowski, 2007; Pellegrini *et al.*, 1992; Proctor & Cunningham, 1988; Baron & Réhault, 2007]. Any modification of lysozyme properties that could render it useful on both Gram-negative and Gram-positive bacteria would be an important contribution. Lysozyme can be lethal to Gram-negative bacteria if the interaction with the bacterial membrane is strengthened by modifying the enzyme surface hydrophobicity *e.g.* chemically modified with palmitate or stearate residues [Ibrahim *et al*., 1991, 1993] or genetically fused with a hydrophobic pentapeptide [Ibrahim *et al*., 1994; Kato *et al*., 1998]. All these derivatives exhibited a strong bactericidal action against *E.coli* K12. They are capable of inserting into the lipid belayer and subsequently disrupting the electrochemical potential by forming ion pores in the membrane. The data suggest that the lysozyme molecule can be lethal to bacteria if its structure supplies a hydrophobic domain on the surface of a molecule.

One of the methods leading to an increase in the efficiency of enzyme activity is to create lysozyme conjugates with substances active towards Gram-negative bacteria. The studies carried out so far have concerned the formation of complexes with palmitic acid, perillaldehyde or dextran [Ibrahim *et al*., 1991, 1994; Pellegrini *et al*., 1992]. Lysozyme modified with perillaldehyde residues exerted a considerably enhanced antimicrobial activity against both Gram-negative bacteria (*Escherichia coli* K12) and Gram-positive bacteria (*Staphylococcus aureus*), as compared to the activity of perillaldehyde or lysozyme alone or even to their mixture [Ibrahim *et al.*, 1994]. Lipophilization of lysozyme by short- and middle-chain fatty acids broadened the spectrum of enzyme antibacterial action on the Gram-negative bacteria [Liu *et al.*, 2000]. Investigations conducted by Ibrahim *et al*. [1996] indicated also a possibility to extend the range of lysozyme activity to include Gram-negative bacteria using thermal modification. It was found that the incubation of such bacteria as *Escherichia coli K12, Salmonella enteritidis,* and *Pseudomonas aeruginosa* in the environment of the modified enzyme resulted in the inhibition of their growth or their inactivation. It has also been found that heat denaturation of lysozyme caused by increasing temperatures resulted in the progressive loss of enzymatic activity, while its antimicrobial action towards Gram-negative bacteria was greatly promoted [Ibrahim *et al.*, 1996, 1997]. It was shown that the antimicrobial action of egg white lysozyme was independent from its enzymatic activity because enzyme lacking catalytic activity still retained its bactericidal properties. It was indicated that the antimicrobial action of lysozyme was due to structural factors and suggested that a specific bactericidal domain may be involved in the antimicrobial action of lysozyme [Düring *et al.*, 1999; Ibrahim, 2001; Ibrahim, 2003]. In addition, it was found that a dimeric form of lysozyme exhibited therapeutic, antiviral and anti-inflammatory properties [Kiczka, 1994].

CONCLUSIONS

Our investigations indicated a possibility to extend the range of lysozyme activity using thermal and chemical-thermal modification [Lesnierowski *et al.*, 2005]. It was observed that lysozyme concentration in the solution subjected to thermal modification, the pH value of the solution, the temperature and time of modification had a significant effect on the content of the forming polymers. The time of oxidation influenced also the amounts of polymers in the case of the chemical-thermal modification. The investigation indicated also a possibility to extend the range of lysozyme activity to include Gram-negative bacteria, *i.e. Escherichia coli* [Lesnierowski *et al*., 2004]. Modification of lysozyme by the membrane technique also broadened the spectrum of enzyme antibacterial action especially against *Pseudomonas fluorescens* and *Proteus mirabilis* bacteria [Cegielska-Radziejewska *et al*., 2003]. It may be stated that increased antibacterial activity against Gram–negative bacteria is not connected with a decrease in the activity of modified lysozyme preparations against Gram--positive bacteria. Studies showed that the applied lysozyme preparations showed a varying activity, depending on the type of bacteria [Kijowski *et al*., 2006].

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WŁAŚCIWOŚCI I ZASTOSOWANIE LIZOZYMU Z BIAŁKA JAJA I JEGO MODYFIKOWANYCH PREPARATÓW – ARTYKUŁ PRZEGLĄDOWY

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Monomer lizozymu wykazuje silne działanie przeciwbakteryjne w stosunku do bakterii Gram-dodatnich. To zjawisko znalazło praktyczne zastosowanie w przemyśle spożywczym, w medycynie i przemyśle farmaceutycznym. Wykorzystanie lizozymu w przemyśle spożywczym jest związane głównie z jego zastosowaniem jako naturalnego środka konserwującego. Ten enzym jest szeroko stosowany jako środek utrwalający mięso, ryby, ich przetwory, mleko i produkty mleczne, jak również owoce i warzywa. Przemysł farmaceutyczny wykorzystuje lizozym w produkcji adiuwantów dla antybiotyków i środków przeciwbólowych w infekcjach wirusowych i bakteryjnych, w leczeniu białaczki i chorób nowotworowych. Lizozym jest także wykorzystywany jako środek diagnostyczny, będący wskaźnikiem występowania i zaawansowania zmian patologicznych u ludzi i zwierząt. Zakres praktycznych zastosowań lizozymu może się znacznie zwiększyć w wyniku jego modyfikacji. Enzym wykazuje wtedy nową specyficzną aktywność w stosunku do bakterii Gram–, będącą rezultatem dimeryzacji; właściwości w stosunku do bakterii Gram+, właściwe dla monomeru, są zachowane – o czym świadczą wyniki badań: Ibrahim *et al.* [1991, 1996]; Lesnierowski *et al.* [2004] oraz Kijowski *et al.* [2006]. Lizozym w formie dimeru wykorzystuje się w leczeniu chorób bakteryjnych i wirusowych zwierząt. Lek wyprodukowany na bazie dimeru lizozymu wykazuje działanie immunostymulujące i immunokorekcyjne.