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POTENTIAL POSSIBILITIES OF PRODUCTION, MODIFICATION AND PRACTICAL APPLICATION OF LYSOZYME

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ABSTRACT

Introduction. Lysozyme, taking a stand in many biological fluids and tissues of a large number of living organisms, is a strongly basic protein. Hen egg white is its rich source and from this source enzyme can be obtained on a commercial scale as a preparation of biological activity. Monomer of lysozyme is known as hydrolase cutting the b-1-4 glycosidic bond, but its dimeric form received after modification of monomer form, exerts different and new valuable properties. In this study we indicated ways of production and modification of lysozyme and possibilities of its practical application.
Material and methods. The material for producing of lysozyme was fresh egg white. Enzyme was isolated by direct crystallization, ion-exchange chromatographic and utrafiltration methods. Lysozyme received by ion-exchange method has been used for modification. Modification of enzyme was carried out by chemical, chemical-thermal, thermal and membrane methods.
Results. The presented methods of lysozyme isolation from hen egg white yielded good results. Depending on employed procedure it was recovered from 20 to 85% of enzyme. However, after modification approximately the quantity of 50-70% of polymerized enzyme was received, which contained from 30-40% of dimer.
Conclusion. The method of isolating and modifying lysozyme can be successfully used to produce high active preparation of enzyme. Lysozyme monomer, and especially its modified form, shows the possibility of wide use not only in food industry, but also in medicine, pharmacology and veterinary medicine.

Key words: lysozyme, modification of lysozyme, antibacterial activity, monomer, dimer

INTRODUCTION

Lysozyme (E.C.3.2.17) is a relatively small enzyme that catalyses the hydrolysis of specific polysaccharides contained in the cell walls of bacteria. Lysozyme is classified according to the similarity among amino acid sequences into three major types: chicken-type, goose-type and invertebrate-type. Most avians, including hens, have chicken-type lysozyme in their egg white and some avians like geese or ostriches have goose-type enzyme [Maehashi et al. 2012]. Lysozyme from hen egg white is a polypeptide of 129 amino acid residues having a molecular weight of 14 300 daltons. It is an elementary protein with the isoelectric point (pI) of 10-11 [Leśnierowski 1997, Leśnierowski and Kijowski 2007].

Molecule of lysozyme consists of two domains, linked by a long α-helix, between which lies the active site of the enzyme [Young et al. 1994]. These two domains of the molecule are separated by a helix-loop-helix motif (Asp 87-Arg 114) which plays a key role in antimicrobial function of enzyme. The molecule conforms to the principle of being hydrophobic in and hydrophilic out of protein. All of its polar groups are

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on the surface and the majority of hydrophobic groups are buried in the interior of the enzyme particle. Conformational transition in lysozyme involves relative movement of its both lobes to each other in a manner allowing free access to the substrate and providing an appropriate environment for catalysis. The formation of the so-called "hinge-bending" structure is thought to play a critical role in the enzymatic (hydrolytic) action of lysozyme [Ibrahim 1997, Masuda et al. 2001].

Lysozyme is an exceptionally abundant protein in poultry egg white (3.5% of the total egg white proteins), so hen egg albumen is the major commercial source of this enzyme. Its biological action in fowl eggs is unclear, but perhaps it serves as a defence mechanism until the embryo has reached the capability to produce immunoglobulins.

The aim of this paper was to indicate possibilities of production, modification and practical application of hen egg white lysozyme.

MATERIAL AND METHODS

The material for producing of lysozyme was fresh egg white from eggs laid by Astra S hens directly supplied from a poultry farm. For modification it lysozyme was used obtained from egg white by ion-exchange chromatographic method.

Methods of lysozyme isolation from egg white

Direct crystallization. Before isolation of enzyme the pH value of egg white was modified to 9.6, then small amount of sodium chloride and crystalline lysozyme (0.025%) was added. The crystallization of the enzyme was performed at the temperature of 4-6°C. Subsequently the sample was centrifuged for 15 min at 2500xg. The participate containing mainly lysozyme was separated from the rest of the egg white and dissolved in a diluted acetic acid (pH 5.0) and centrifuged. The obtained supernatant was further crystallized twice under the same conditions, resulting in purified lysozyme preparation freezed-dryed. Egg white remaining after crystallization was desalted with the use of ultrafiltration technique. In order to remove sodium chloride in fast and selective way, desalting was performed by diafiltration.

Ion-exchange chromatographic. The previously selected ion-exchange support type E [Leśnierowski 1997] characterized by a large sorption capacity and high selectivity towards lysozyme was used for enzyme separation. Enzyme sorption was carried out in three ways, with the use of sonication (ultrasonic wave generator UM-20 made by UNITRA-UNIMA, Olsztyn, Poland), shaking (Wrist shaker made by BURREL Comp.) and stirring (SJR-1 rotating stirrer). Sorption of lysozyme was finished after saturation of the ion exchanger with enzyme, i.e. the moment when lysozyme activity in the egg white sample was not decreasing. Next the residual egg white was decanted and resin was intensively rinsed with distilled water. Then lysozyme was washed out from the resin with several bed volumes of phosphate buffer. Finally the eluate of the lysozyme preparation was dialysed and lyophilised. On completion of each lysozyme separation cycle the sorbent was regenerated by step washing with HCl, further – with NH_4OH and finally submerged in phosphate buffer.

Ultrafiltration (UF). Before isolation egg white was filtrating and homogenizing. The ultrafiltration process was carried out using polysulphonate membranes with the cut off of 20, 30, 50 and 100 kDa. In order to optimize conditions, the UF process was performed with diluted egg white, where its amount in proportion to water ranged from 1+1 to 1+6. Different pH levels were used (from 8.0 to 11.0) as well as varying ionic strength of egg white solutions (from 0.085 to 0.85 M).

Methods of lysozyme modification

Thermal modification. Lysozyme preparation (2.5 g) was dissolved in 100 cm^3 of the acetate buffer with pH of 4.4 was heated for 20 min in a water bath at the temperature of 60°C. Next the solution was quickly cooled to 10-12°C.

Chemical-thermal modification. Lysozyme preparation (2.5 g) was dissolved in 100 cm^3 of the acetate buffer with pH of 4.4. The chemical modification was performed with a 10% share of an oxidant at the temperature of approx. 4°C for 1 and 14 days, and subsequently the solutions were heated for 20 min in a water bath at 75°C. After the modification was completed the solutions were intensively cooled to 10-12°C.

Chemical modification. Lysozyme preparation (2.5 g) was dissolved in 100 cm^3 of the acetate buffer with pH of 4.4. The modification was conducted with

the 10% share of an oxidant at the temperature of approx. 4°C for 11 and 20 days.

Membrane modification was conducted in a DDS 20-0.36 LAB ultrafiltration module with the use of membranes with the threshold permeability of 6 kDa. The lysozyme solution with pH of 7.0 was subjected to the process of diafiltration at the temperature of 50°C and the pressure of 20 Ba. The modification lasted for 150 min.

Methodology of determining physical and chemical characteristics of lysozyme

Lysozyme hydrolytic activity was determined with the use of spectrophotometry, the principle of which is based on the phenomenon of cell wall lysis in *Micrococcus lysodeikticus* bacteria by the enzyme [Leśnierowski 1997, 2007].

The content of lysozyme polymeric forms in the preparations after modification was determined by electrophoretic analysis on polyacrylamide gel using an SE-600 apparatus (Hoefer Scientific Instruments) [Leśnierowski 2007]. The application of computer software (TotalLab by Nonlinear Dynamics) made it possible to calculate the percentage of dimer in individual samples.

Chlorides contents in investigated samples was determined by the Mohr method.

RESULTS AND DISCUSSION OF ISOLATION, MODIFICATION, ANTIBACTERIAL ACTIVITY AND PRACTICAL APPLICATION OF LYSOZYME

Isolation of enzyme. Several methods of lysozyme isolation from egg white have been developed so far. Most of them are used in laboratory practice to obtain the enzyme of high activity, but only some of them could be applied on the commercial scale. The most useful methods of extracting lysozyme from egg white include the conventional procedure by direct crystallisation and precipitation, direct membrane filtration [Chiang et al. 1993, Leśnierowski 1997, Kijowski et al. 1998], affinity chromatography [Chiang et al. 1993] and ion exchange chromatography [Leśnierowski 1997, Kijowski et al. 1998].

Methods of lysozyme isolation from hen egg white presented in this paper have given good results. Depending on employed method from 15 to 85% effect was received (Table 1). The crystallization method is a classical laboratory and commercial procedure of lysozyme separation from the egg white based on direct enzyme crystallisation with sodium chloride. Our works indicate that it is possible to reduce salt in the remaining egg white after prior lysozyme separation by using ultrafiltration and diafiltration techniques (Table 1). We proved that desalted egg white demonstrated unchanged foaming ability and foam stability when compared with the native egg white [Leśnierowski 1997].

Table 1. Effect of lysozyme isolation from hen egg white

Method of isolation	Effect of isolation %	Hydrolytic activity U/mg	Amount of salt remaining in egg white %
Crystallization	60-75	15 500-17 500	below 1
Ion-exchange chromatography	70-85	20 000-24 000	–
Ultrafiltration	15-20	10 000-12 500	–

Presently there are some ways methods of separating the enzyme from the egg white. They are based on exchange chromatography technique. In our department laboratory the following methods are applied to carry out the process: batch system with sonication, mechanic shaking or stirring techniques which give very good results in the sorption of lysozyme from egg white. These methods with freeze-drying or spray drying of receiving liquid lysozyme can be very easy and successfully used for commercial obtaining of lysozyme [Leśnierowski and Kijowski 1997, Kijowski et al. 2000].

We have also exerted that it is possible to isolate lysozyme from egg white by using membrane techniques, especially ultrafiltration. Our further research proved that it is possible to broaden more extensively the antibacterial spectrum of lysozyme by its modification [Leśnierowski and Kijowski 2007, Leśnierowski et al. 2009].

Modification of enzyme. Lysozyme exists in two conformational states, between 20 and 30°C with a transition point at 25°C [Jolles and Jolles 1984].

In nature the enzyme appears 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 the intermolecular disulfide exchange) when eggs are stored for long periods. The dimer retains enzymatic activity of the monomer, means that this reaction is not responsible for the loss of hydrolytic activity. The dimerization, as well as higher polymerization process depends on pH, enzyme concentration and temperature. Modified lysozyme exhibits quite new properties and quite a novel antimicrobial activity, which are being extensively examined nowadays [Ibrahim et al. 1991, Leśnierowski et al. 2009].

The research lead in our department of original method of enzyme modification created a possibility of receiving a high quality product. Some methods such as: thermal, thermochemical, chemical or membrane method facilitate transformation of the laboratory methods to semi-technical or even to industrial scale conditions. The process of transformation would not be complicated. The preparations received as a result of an application of the above methods were a subject to numerous detailed estimations of chosen physico-chemical and antibacterial specificity [Leśnierowski et al. 2001, Leśnierowski 2009]. It showed, that modified enzyme includes 50-70% of oligomers and from 30 for over 40% of enzyme dimer (Table 2). The research showed, that heat denaturation of lysozyme results in the progressive loss of enzymatic activity, but greatly improved antimicrobial action towards Gram-negative bacteria. Partial unfolding of lysozyme can switch the antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive bacteria [Ibrahim et al. 1994, Ibrahim 1998, Cegielska-Radziejewska et al. 2003, 2009]. The possibility to extend the range of lysozyme activity in order to include Gram-negative bacteria i.e. *E. coli*, is offered by e.g. the thermal and thermochemical modification (Fig. 1), which leads to the formation of an enzyme preparation with increased content of polymeric forms [Leśnierowski et al. 2004, Leśnierowski 2009]. An effective tool that can be used in the direct production of preparations with increased quantities of the polymeric forms of lysozyme as has been mentioned above is also its modification by the membrane technique. It is shown that the temperature, pressure and time of modification, as well as acidity of the medium significantly affected the quantity of polymers in the obtained preparations [Leśnierowski et al. 2003, 2009].

Table 2. Characteristics of lysozyme preparations obtained after modification of enzyme on different way

Method of modification	Content of, %		Hydrolytic activity U/mg
	dimer	oligomers	
Thermal	27-29	27-30	7 000-12 000
Thermochemical	33-36	45-48	6 500-9 500
Chemical	32-34	65-70	2 500-5 500
Membrane	22-25	39-42	4 000-11 000

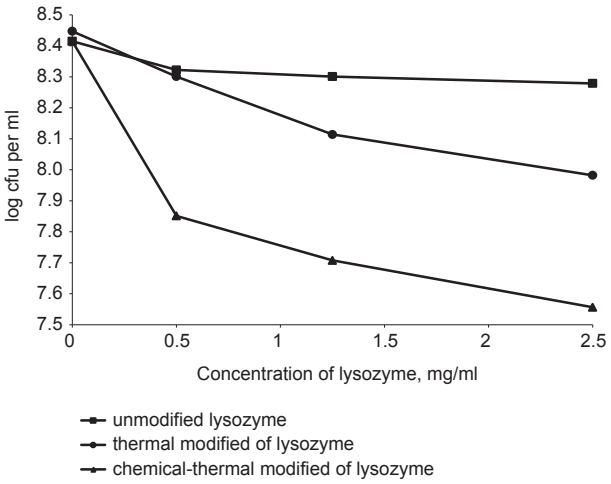

Fig. 1. Action of monomer and modified lysozyme against *Eschrichia coli*

Antibacterial activity and practical application of enzyme. The chicken-type lysozyme demonstrates a strong antibacterial potential and goose-type exhibits even 3-10 times higher than chicken one, mainly against Gram-positive bacteria [Maehashi et al. 2012]. This phenomenon has found practical applications in the food and pharmaceutical industries, as well as medicine [Proctor and Cunningham 1988,

Kijowski and Leśnierowski 1995]. Enzyme hydrolyses a number of structurally similar substrates, but the best known are the polysaccharide copolymer, i.e. N-acetyl glucosoamine (NAG) and N-acetyl muramic acid (NAM), which represent structural units of many bacterial cell walls. The action of an enzyme muramidase (hydrolase) in the cutting site is breaking the b-1-4-linkage of the glycosidic bond between NAG and NAM. The antimicrobial activity of the lysozyme monomer is limited to Gram-positive bacteria. Cell walls of those microorganisms consist mainly of the peptidoglycan layer. The Gram-negative bacteria are less susceptible to the bacteriolytic action of this enzyme since they have a more complex envelope structure. The peptidoglycan layer, being the substrate of lysozyme, is covered additionally by the outer membrane consisting of lipoproteins, lipopolysaccharides (LPS) and some hydrophobic peptides. The outer membrane serves as a barrier to the access of enzyme to its site of action. It is most probable that lysozyme can be entrapped in the outer membrane by LPS of Gram-negative bacteria and inactivated [Ibrahim et al. 1993, Nakamura et al. 1997]. Generally, the limited action of lysozyme on Gram-negative bacteria seems to be influenced by both the composition and the sequence of N-acetylamino sugars of the bacterial cell walls. Moreover, there is evidence that lysozyme devoid of enzymatic activity kills different types of bacteria. It is also stressed that its bacteriostatic, bacteriolytic and bactericidal activities seem to operate by different mechanisms. From extensive studies conducted on its binding capacity to different polysaccharides or lipids of bacteria, it is clear now that lysozyme exhibits both a direct and indirect antimicrobial action.

The bacteriostatic and bactericidal properties of lysozyme have been used to preserve various food items [Proctor and Cunningham 1988, Cunningham et al. 1991], as well as in pharmacy, medicine and veterinary medicine [Johnson 1994, Malinowski 2001]. Lysozyme incorporated into food packaging materials has the potential to extend the shelf life of non-sterile or minimally processed foods by preventing the contamination or growth of microorganisms. Edible films have also been investigated as potential antimicrobial packaging systems [Appendini and Hotchkiss 1997, Seacheol et al. 2005, Péréz-Péréz et al. 2006].

In the pharmaceutical industry, hen egg white lysozyme can protect the body against bacterial, viral or inflammatory diseases [Leśnierowski and Kijowski 2007]. It has been used in aerosols for the treatment of bronchopulmonary diseases, prophylactically for dental caries, for nasal tissue protection and is incorporated into various therapeutic creams for the protection and topical reparation of certain dystrophic and inflammatory lesions of the skin and soft tissues.

Lysozyme is used to preserve meat and processed meats, fish and processed fish products, milk, vegetables and fruits [Cunningham et al. 1991, Johnson 1994, Nattress et al. 2001]. In cheese making, lysozyme has been used to prevent growth of *Clostridium tyrobutyricum*, which causes off-flavours and late blowing in some cheeses, in particular those made from pressed and cooked curds [De Roos et al. 1998, Danyluk and Kijowski 2001, Sinigaglia et al. 2008]. Another application of lysozyme may be the possible antimicrobial agent use in brewing for the control of lactic acid bacteria in beer [Daeschel et al. 1999] and the control of lactic acid bacteria in wine making is essential in order to obtain wines of consistent high quality [Gerbaux et al. 1997, Delfini et al. 2004]. An advantageous effect of lysozyme on the quality of meat and meat products has been shown [Malicki et al. 2003, 2004, Rao et al. 2008, Ntzimani et al. 2010]. The bactericidal and bacteriostatic properties of lysozyme have been examined against the saprophytic and pathogenic bacteria on poultry carcasses and chicken legs with skin. The experimental results suggest that treatment with lysozyme solution could be used as an effective antimicrobial means in the extension of shelf life of poultry meat under refrigerated storage [Cegielska-Radziejewska et al. 2009, 2011].

Antimicrobial activity of lysozyme can be enhanced by preservatives or other substances including nisin, sodium lactate, EDTA, butylparaben, trisodium phosphate [Carneiro de Melo et al. 1998, Gill and Holley 2000, 2003, Nattress et al. 2001, 2003, Cannarsi et al. 2008, Mangalassary et al. 2008].

CONCLUSION

The presented examples of lysozyme using distinctly indicate that the modified enzyme as substance of natural origin is highly attractive and has potential

capabilities of utilization in food industry. However, in the first place it is necessary to receive a monomer of enzyme. Among the presented methods for isolation of lysozyme from egg white the best results were obtained by ion exchange chromatography. Other techniques such as crystallization or membrane method gave also good results. Using these methods up to 85% recovery of enzyme from egg white was obtained. After producing the obtained monomer must then be modified to improve its properties. It was shown that such modifications can be made using the thermal, thermochemical and chemical methods.

It appears that the proposed methods for isolation and modification of lysozyme can be easily adopted by the industrial egg processing plants. It also seems that fabricating such products could be very attractive, in economical terms, for these plants.

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MOŻLIWOŚCI PRODUKCJI, MODYFIKACJI I PRAKTYCZNEGO WYKORZYSTANIA LIZOZYMU

STRESZCZENIE

Wstęp. Lizozym, białko enzymatyczne, powszechnie występujące w przyrodzie, charakteryzuje się użytecznymi właściwościami. Bogatym źródłem lizozymu, z którego można pozyskiwać enzym na szerszą skalę, jest białko jaja kurzego. Monomer, znany jako hydrolaza, rozkłada wiązanie b-1-4-glikozydowe, a dimer enzymu uzyskiwany w wyniku modyfikacji wykazuje nowe, cenne właściwości. W prezentowanej pracy pokazano sposoby produkcji i modyfikacji lizozymu oraz możliwości jego praktycznego wykorzystania.
Materiał i metody. Surowcem do badań było świeże białko jaj kurzego, którego lizozym izolowano metodą krystalizacji, jonowymiennej chromatografii i ultrafiltracji. Do modyfikacji użyto lizozym otrzymany metodą chromatograficzną. Modyfikację enzymu przeprowadzono metodami termiczną, termiczno-chemiczną, chemiczną i membranową.
Wyniki. Przedstawione w pracy sposoby izolowania enzymu z białka jaja kurzego dały dobre wyniki. W zależności od zastosowanej metody odzyskiwano od 20 do 85% lizozymu zawartego w białku jaja. Natomiast po modyfikacji otrzymano ok. 50-70% spolimeryzowanego enzymu, który zawierał 30-40% dimeru.
Wnioski. Przedstawione metody izolowania i modyfikowania enzymu mogą być wykorzystane do produkcji wysokoaktywnego preparatu lizozymu. Monomer lizozymu, a zwłaszcza jego zmodyfikowana postać wykazuje możliwości szerokiego wykorzystania nie tylko w przemyśle spożywczym, ale także w medycynie, farmakologii i weterynarii.

Słowa kluczowe: lizozym, modyfikacje lizozymu, aktywność przeciwbakteryjna, monomer, dimer

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