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ANTIBACTERIAL ACTIVITY OF THERMALLY MODIFIED LYSOZYME

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ABSTRACT

Lysozyme (EC 3.2.1.17, mucopeptyde N-acetylmuramic-hydrolase) exhibits the ability to destroy cell walls of Gram-positive bacteria. The range of activity for this enzyme may be extended through modifications, as a result of which polymerized forms of lysozyme are obtained. It was found that lysozyme dimer exhibits bacteriostatic properties towards both Gram-positive and Gram-negative bacteria. The aim of the study was to determine the conditions of thermal modification of lysozyme and to assess the antibacterial action of this enzyme after modification. The dimer content in the modified lysozyme preparations depended on the pH and concentration of a given solution. It was found that modified lysozyme was characterized by higher antibacterial activity towards *Micrococcus luteus* and *Escherichia coli* in comparison to the unmodified form of the enzyme.

Key words: lysozyme monomer, lysozyme dimer, pH value, hydrolytic activity

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INTRODUCTION

Lysozyme (E.C. 3.2.1.17, mucopeptide N – acetylmuramic-hydrolase), found in the monomer form e.g. in hen egg whites, is a natural compound with an antibacterial action, which makes it possible to use this enzyme in the food and pharmaceutical industries [4, 13].

The antibacterial activity of lysozyme consists in the hydrolysis of β (1→4) glycoside bonds formed between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell walls. 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. In case of Gram-negative bacteria an additional barrier for lysozyme is the inner membrane composed of protein, phospholipids and lipopolysaccharides [1,12].

Research indicates that the range of lysozyme activity may be extended thanks to the 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 shows a significantly wider range of bacteriostatic activity, including also Gram-negative bacteria, than active lysozyme. It needs to be emphasized that the enzyme after modification retains antibacterial activity towards Gram-positive bacteria, characteristic for the monomer [1, 2, 3, 5, 10, 11].

One of the methods leading to the 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 [1, 2, 10]. Investigations conducted by Ibrahim et al. [3] 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 results in the inhibition of their growth or their inactivation.

The aim of the study was to establish optimum conditions for lysozyme thermal modification and to determine the antibacterial activity of the enzyme after modification.

MATERIALS AND METHODS

The experimental material was lysozyme obtained from the whites of fresh hen eggs coming from Astra S laying hens from the breeding farm of the Poultry Research Division of the Department of Animal Husbandry in Zakrzewo near Poznań. The ion-exchange method [7] was used to isolate lysozyme from hen egg whites. Lysozyme preparations obtained in such a way were modified thermally.

Thermal modification of lysozyme was conducted at the temperature of 80°C for 20 min by heating its water solutions of varying concentrations in a water bath (type 1083 by Gesellschaft fur Labortechnik) at pH of 4 – 8. Solutions of varying lysozyme concentrations were coded as C1 – C5 to protect the data. After modification lysozyme was dried using the spray-drying method with the application of a Büchi spray drier.

In the obtained preparations base composition and hydrolytic activity were determined and electrophoretic analysis was performed. Lysozyme hydrolytic activity was determined with

the use of the spectrophotometric method, the principle of which is based on the phenomenon of cell wall lysis in *Micrococcus lysodeicticus* bacteria by the enzyme [8].

The content of lysozyme polymeric forms in the preparations after modification was determined by electrophoretic analysis on polyacrylamide gel using the SE-600 apparatus (Hoefer Scientific Instruments) [7]. The application of computer software (Quanti Scan 2.0 by Biosoft) made it possible to calculate the percentage of dimer in individual samples.

Antibacterial action of the modified lysozyme was established towards *Micrococcus luteus* bacteria (Calbiochem Corporation 475817) and *Escherichia coli* (ATCC 259220). The amount of 1 cm^3 of bacterial suspension from prepared dilutions was introduced to a lysozyme solution with a specific concentration. Consecutive dilutions of bacterial suspension constituted control samples. The MacConkey medium (Oxoid) was applied for *Escherichia coli*, whereas Agar APHA (Oxoid) was used for *Micrococcus luteus.*

Chemical analysis were repeated six times and microbiological examination were repeated four times. The obtained results of the determination were subjected to statistical analysis using STATISTICA™ software.

RESULTS AND DISCUSSION

Lysozyme obtained from egg whites was subjected to thermal modification at the temperature of 80°C for the period of 20 minutes. In all the obtained lysozyme preparations the formation of lysozyme dimer was observed after modification (Figs 3 and 4).

The obtained results indicate that the content of the forming dimer as well as the hydrolytic activity of the preparations depend on the pH values of the thermally modified solution (Fig. 1). Under the adopted modification conditions, a significant amount of dimer in this range is formed in a slightly acid environment, whereas its highest percentage, equal to 31.3 – 33.8%, was observed at pH of 5.0 and 6.0. However, at pH of 7.0 and 8.0 the amount of the produced dimer was small and did not exceed 10%.

It was shown that a significant factor during the thermal modification of lysozyme, which may affect the final results of polymerization, is its concentration (Fig. 2). Depending on the lysozyme concentration in the solution subjected to modification the obtained enzyme preparations varied in dimer contents, ranging from 22.3% to 33.4%. It was found that at higher lysozyme concentrations C4 and C5 in the solution being modified the dimer content lowers. The maximum dimer content was observed for concentration C3.

The purity of enzyme preparations obtained after thermal modification is presented in Figures 3 and 4. It was found that in case of all the preparations the hydrolytic activity of the enzyme decreased after modification. The activity of unmodified lysozyme was approx. 22 500 U/mg, whereas for enzyme preparations with the highest dimer content the hydrolytic activity ranged from 6 500 to 9 000 U/mg. A similar phenomenon was observed in the studies by other authors [1, 2, 4]. It results from literature that along with the decrease in hydrolytic activity a new specific antibacterial activity appeared [2, 3, 12]. This phenomenon may lead to the extension of the range of the enzyme antibacterial activity also towards Gram-negative bacteria. Thus, in further investigations the bacteriostatic action of lysozyme was determined both towards Gram-positive and Gram-negative bacteria.

Fig. 1. The percentage of lysozyme dimer and the hydrolytic activity of thermally modified preparations within the pH range of 4.0 – 8.0
a-c: different letters used for mean values concerning the influence of pH on amounts of dimer denote statistically significant differences at the level of p = 0.05
A-C: different letters used for mean values concerning the influence of pH on hydrolytic activity denote statistically significant differences at the level of p = 0.05

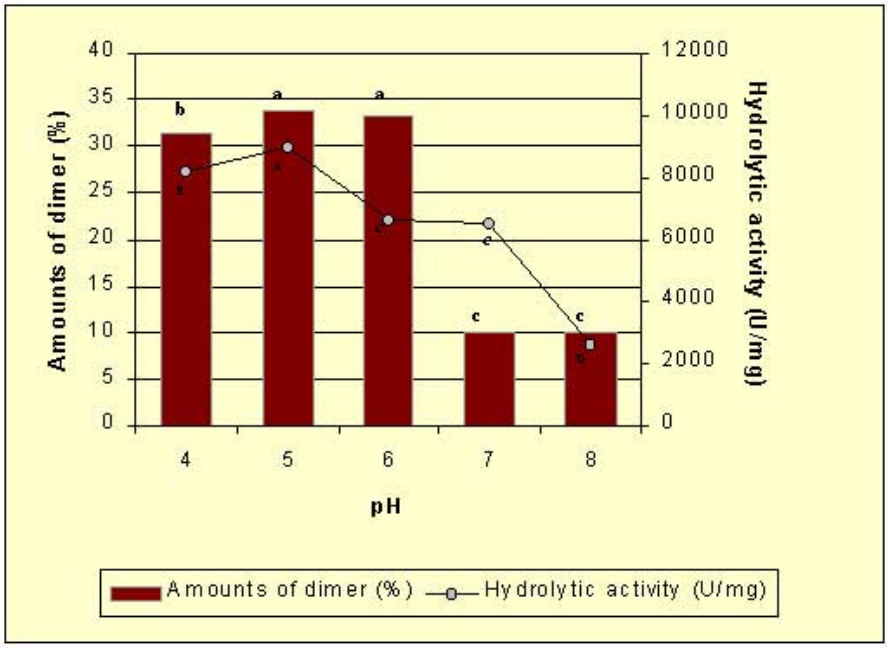


Fig. 2. The percentage of lysozyme dimer and the hydrolytic activity of thermally modified preparations with varying lysozyme concentrations
a-c: different letters used for mean values concerning the influence of concentration of lysozyme on amounts of dimer denote statistically significant differences at the level of p = 0.05
A-C: different letters used for mean values concerning the influence of concentration of lysozyme on hydrolytic activity denote statistically significant differences at the level of p = 0.05

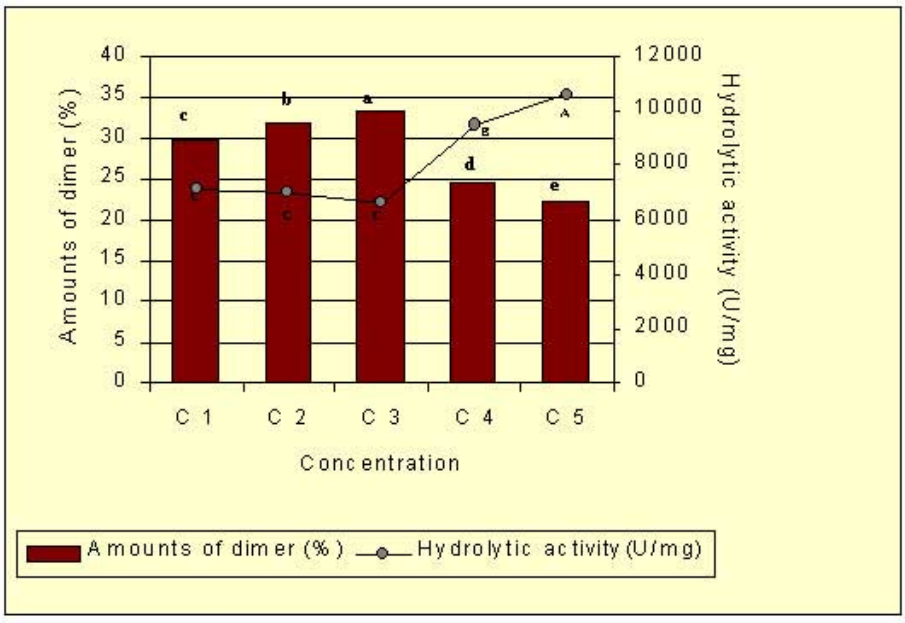


Fig. 3. Electrophoretic analysis of modified lysozyme preparations at various pH values

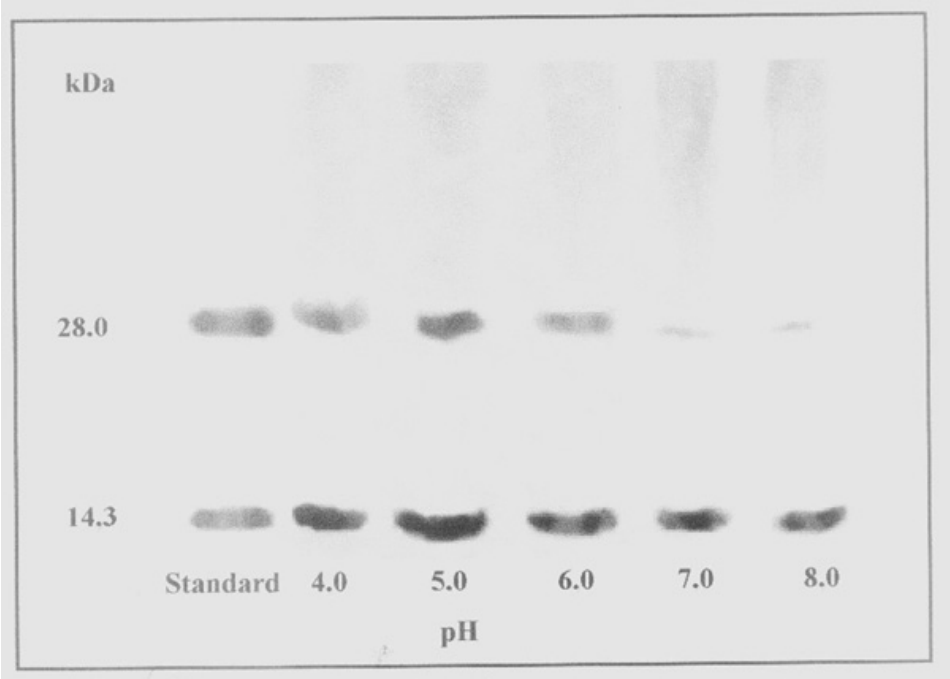
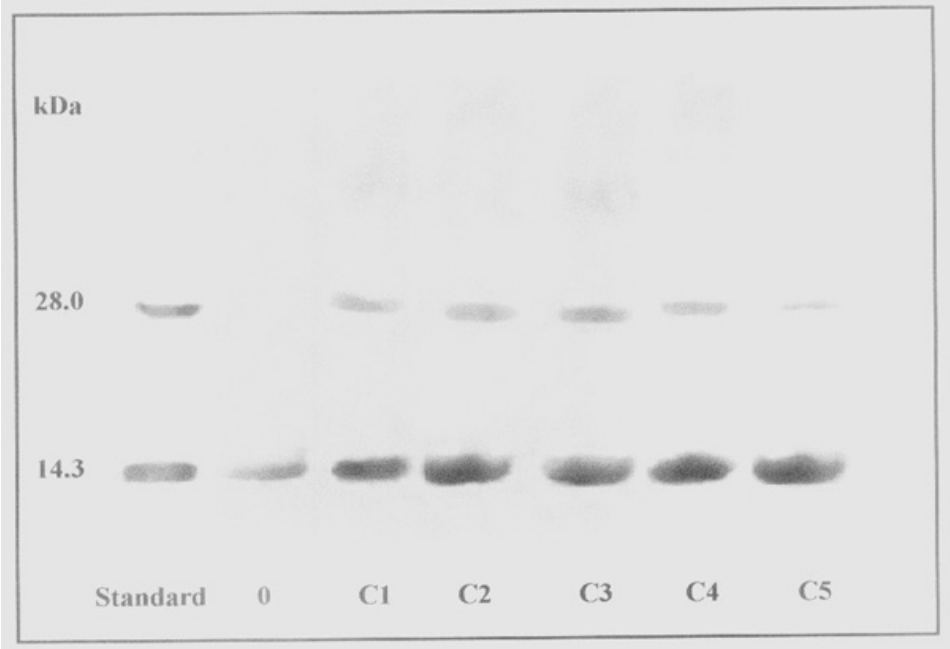


Fig. 4. Electrophoretic analysis of preparations of modified lysozyme at various concentrations



The enzyme antibacterial activity was determined for the optimum conditions of thermal modification, under which the highest dimer content was obtained. It was found that the application of lysozyme thermal modification leads to an increase in its antibacterial activity in comparison to the activity of lysozyme not subjected to modification. An advantageous effect of the action of the modified lysozyme was observed both towards Gram-positive bacteria, i.e. *Micrococcus luteus*, and Gram-negative bacteria, i.e. *Escherichia coli*. While investigating the effect of lysozyme on selected bacteria, preparations of the modified enzyme were used in the concentrations of 0.50 mg/ml, 1.25 mg/ml and 2.50 mg/ml, respectively.

Studies show that the bacterial reduction depends both on the enzyme concentration and the type of bacteria. As a result of applying lysozyme at the concentration of 2.50 mg/ml, a 74% reduction of *Micrococcus luteus* and a 66% reduction of *Escherichia coli* were obtained. It may be stated that the efficiency of lysozyme activity after modification is much higher in comparison to that of the unmodified lysozyme (Table 1). Moreover, the range of antibacterial activity of the modified enzyme was also found to be extended, as a considerable efficacy of the enzyme activity was observed towards Gram-negative bacteria, i.e. *Escherichia coli.*

Table 1. The effect of unmodified and modified lysozyme on *Micrococcus luteus* and *Escherichia coli*
Data are presented as means for 4 repetitions

Bacterial strain	Bacterial reduction (%)			Lysozym
	Concentrate of lysozyme (mg/ml)			
	0.5	1.25	2.5	
Micrococcus luteus	27	39	45	unmodified
	32	47	74	modified
Escherichia coli	17	22	28	unmodified
	47	62	66	modified

CONCLUSIONS

The conducted studies indicate that the thermal modification of lysozyme leads to the formation of enzyme preparations with an increased content of polymeric forms. It was found that under the adopted conditions of thermal modification a weakly acid environment (pH 5 – 6) promotes increased polymerization of lysozyme and makes it possible to obtain approx. 34% of dimer. It was observed that both lysozyme concentration in the solution subjected to thermal modification and the pH value of the solution have a significant effect on the content of the forming dimer. The highest percentage of the dimer was found in preparations obtained at concentration C3 of lysozyme at pH 5.0-6.0. The lysozyme thermal modification performed under the above mentioned conditions makes it possible to obtain a higher antibacterial activity towards *Micrococcus luteus* and *Escherichia coli* in comparison to that of the unmodified enzyme. It was stated that bacterial reduction depends both on the concentration of the enzyme and on the type of bacteria.

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