EFFECTS OF TREATMENT WITH LYSOZYME AND ITS POLYMERS ON THE MICROFLORA AND SENSORY PROPERTIES OF CHILLED CHICKEN BREAST MUSCLES

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Abstract

The aim of the study was to assess the effect of lysozyme monomer and dimer as well as modified lysozyme preparations on the shelf life of chicken carcass elements (breast muscles) cold stored at 3°C±1°C. Total counts of aerobic bacteria were determined and sensory examination of the product was conducted. Analyses showed that surface application of dimer solutions and modified lysozyme preparations on chicken breast muscles makes it possible to extend the shelf life of the tested product by approx. a factor of 2 at the storage temperature used. It may be stated that lysozyme dimer and preparations containing both the dimer and monomer forms exhibit a more effective antibacterial action in comparison to the monomer form of this enzyme.

Key words: poultry meat, lysozyme, bacteriostatic activity.

Lysozyme (E.C.3.2.17, N-acetyl-muramidase) is a bacteriolytic enzyme commonly found in nature. A rich and easily-available source of lysozyme is the egg white. In the hen egg white, lysozyme accounts for 3.5% of total egg white proteins. 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.

The extension of shelf life and the control of pathogenic and spoilage organisms in refrigerated foods are of great importance to the food industry. There is a considerable interest within the food industry in using natural antimicrobial agents that are non-toxic to humans and exert inhibitory effects on undesirable microorganisms. Lysozyme is known as a natural preservative with strong antimicrobial activity, especially against Gram-positive bacteria, rather than Gram-negative ones, due to the differences in the structure of their cell walls. This phenomenon has found practical applications in the food and pharmaceutical industries as well as in medicine (3, 6). In case of Gram-negative bacteria an additional barrier for lysozyme is the inner membrane composed of protein, phospholipids, and lipopolysaccharides (15). This enzyme is widely used as a preservative for meat, meat products, fish and their products, for milk and dairy products, as well as fruit and vegetables. A special role is played by lysozyme in cheese ageing, thanks to the reduction of butyric fermentation bacteria (Clostridium tyrobutyricum, Clostridium butyricum), which adversely affect cheese quality (1, 6, 7).

Lysozyme demonstrates antimicrobial activity against a limited spectrum of bacteria and fungi; however, its activity can be enhanced by other preservatives, especially nisin, sodium lactate or certain substances including EDTA, butylparaben, and trisodium phosphate (2, 5, 9, 22).

It has been found that the spectrum of lysozyme antibacterial action can be broadened by its modification and formation of polymeric forms. It has also been found that the chemical and thermal modification of lysozyme increases its antimicrobial properties towards Gram-negative bacteria. Such a 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 (4, 14). It is now evident that, aside from the lysozyme bacteriolytic action, the dimeric form of lysozyme exhibits therapeutic, antiviral and anti-inflammatory properties (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 (15). Investigations conducted by Ibrahim et al. (14) also indicated the possibility of extending the range of lysozyme activity to include Gram-negative bacteria, using thermal modification. It has also been found that heat denaturation of lysozyme caused by increasing temperatures results in the progressive loss of enzymatic activity, while its antimicrobial action towards Gram-negative bacteria is greatly enhanced.

Modification of lysozyme by the membrane technique broadened the spectrum of enzyme antibacterial action, especially against *Pseudomonas fluorescens* and *Proteus mirabilis* Gram (+) bacteria. In the case of Gram (-) bacteria the most effective antibacterial action was observed in the lysozyme preparation containing the highest quantity of dimer and the lowest of monomer (20).

For this reason the aim of the study was to determine the effect of lysozyme monomer and dimer as well as modified lysozyme preparations on the shelf life of chicken carcasses (breast muscles) cold stored.

### Material and Methods

The experimental material consisted of skinless chicken breast muscles (chicken fillets) obtained from a chicken slaughterhouse and a processing plant. Samples were collected at random from two batches of production. The external surface of each fillet was covered under sterile conditions with lysozyme solutions at a concentration of 1mg/mL, in the amount of 1 ml. Water solutions of lysozyme monomer (M), dimer (D), and preparations produced as a result of thermo-chemical modification (P4 and P10). Two different preparations were obtained, P4 and P10, formed from the combination of lysozyme samples modified at 0°C, 5°C, and 10°C for 24, 72, and 144 h, at pH 4 and pH 10. In the analyses lysozyme monomer by Belovo and Lydium-KLP, a lyophilised dimer preparation by NikaTM Health Products were used. The lysozyme forms differed in their enzymatic activity and dimer content. The characteristics of modified lysozyme preparations are presented in Table 1. Fillets covered with lysozyme solutions were placed on trays with a water-absorbing lining and, after being covered with polyethylene film, they were stored under refrigeration at 3°C ±1°C. The control (K) comprised chicken breast muscles with no lysozyme addition. During the cold storage of the samples, microbiological tests were conducted (determination of total bacterial counts) and sensory examination was performed. Microbiological analyses and sensory examinations were conducted after 1, 24, 72, 120, and 168 h of storage.

The hydrolytic activity of the lysozyme preparations was determined with the use of the spectrophotometric method, the principle of which is based on the phenomenon of cell-wall lysis caused by the enzyme in *Micrococcus lysodeicticus* bacteria (19). The lytic activity of lysozyme was determined by monitoring the decrease in the turbidity of suspension of *M. lysodeicticus* cells at 450 nm. The activity was presented as the rate of decrease in absorbance per minute of the initial rate of reaction.

The content of lysozyme polymeric forms in the preparations after modification was determined by electrophoresis on polyacrylamide gel using an SE-600 apparatus (Hoefer Scientific Instruments). Electrophoresis in SDS-PAGE was carried out according to Laemmli (17) and Lesnierowski (18) using 12.5% acrylamide separating gel and 6% stacking gel containing 0.1% SDS. The following standards were used: Lysozyme 14.6 kDa (Sigma), Lydium KLP 28 kDa (Nika Health Product), and Hen Albumen 45 kDa (Sigma). The amounts of polymer in individual samples were calculated using Quanti Scan 2.0 computer software (Biosoft).

Samples for microbiological analyses were collected, using swabs and sterile stencil plates of 5x5 cm USDA Template (Noack) from four different locations on the fillet surface and next transferred to 100 ml diluent, obtaining a bacterial sample from 1 cm²/mL. A ten-fold dilution was prepared. The total bacterial count was determined on agar medium using the flooding method (Standard Plate Count Agar Oxoid). Incubation was run at 30°C ±1°C for 72 h. Bacterial counts were given in cfu/cm² area.

Moreover, sensory examination was performed on analysed chicken fillets, taking into consideration such attributes as: overall appearance, colour, and aroma. Examination was conducted by a 6–member trained panel on a 5–point scale: the score of 5.0 was equivalent to very good, 4.0 – good, 3.0 – satisfactory, 2.0 – unsatisfactory, 1 – poor. Table 2 shows the applied criteria for evaluation.

### Table 1

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Proportion of monomer (%)</th>
<th>Proportion of dimer (%)</th>
<th>Proportion of trimer (%)</th>
<th>Hydrolitic activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>39.2</td>
<td>34.4</td>
<td>26.6</td>
<td>4,500</td>
</tr>
<tr>
<td>P10</td>
<td>39.4</td>
<td>35.5</td>
<td>25.4</td>
<td>3,100</td>
</tr>
</tbody>
</table>
Table 2
Criteria for the sensory examination of chicken breast muscles using a 5-point scale

<table>
<thead>
<tr>
<th>Quality attributes</th>
<th>Score (5-point scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Overall appearance</td>
<td>wet surface</td>
</tr>
<tr>
<td>Colour</td>
<td>light pink or creamy, uniform</td>
</tr>
<tr>
<td>Aroma</td>
<td>characteristic of poultry meat</td>
</tr>
</tbody>
</table>

Statistical calculations were performed using Statistica 7.1. The Weibull model was applied to describe changes in the overall acceptability of the tested products, while the Gompertz model (Bacterial Growth Kinetics) was used to determine the dynamics of changes in bacterial counts.

Results

During cold storage of chicken breast muscles an increase was observed in the total count of aerobic microorganisms in case of all analysed samples; however, it was most rapid in the control. Counts of aerobic bacteria after 1 h storage of the muscles were 2.2–2.9 log cfu/cm². As early as after 24 h storage a higher bacterial count was recorded in the control in comparison to the count of bacteria in samples subjected to the action of monomer, dimer, and modified preparations of lysozyme. After 120 h storage the total bacterial count in samples with no lysozyme added was 5.4 log cfu/cm², while in samples with an addition of lysozyme dimer it was 3.9 log cfu/cm². In the final stage of the study (168 h) total bacterial counts in samples with an addition of lysozyme dimer were by 2.4 log cycles lower than in samples with no lysozyme added. In order to describe the increase in the total bacterial counts for all analysed samples the Gompertz models were applied (Fig. 1). Approximation of models to empirical data was high.

![Fig. 1. The Gompertz model of the increase in aerobic bacterial count for chicken breast muscles.](image-url)
Kinetic parameters of bacterial growth assessed on the basis of the Gompertz function are given in Table 3. The growth-rate index calculated from the Gompertz curve was the lowest for samples of chicken breast muscles covered with a dimer solution. The longest bacteria generation time was found in samples of the muscles covered with a lysozyme dimer solution. In the case of muscle samples covered with the P4 lysozyme preparation solution, the recorded bacterial growth rate was comparable to that of the control; however, the maximum population density was the lowest among the tested samples. Based on kinetic parameters of the Gompertz curve, it may be stated that the bacterial growth rate was the highest in the case of breast muscles with no lysozyme added.

The results of the sensory examination were adequate to changes in the total bacterial count on the surface of the fillets. During cold storage of poultry fillets a gradual decrease was observed in the scores for overall desirability, aroma, and colour, depending on the added lysozyme form. Up to 72 h storage the overall appearance of all tested samples received high scores, as was the case with colour and aroma. After 120 h storage samples with no lysozyme addition received the lowest score of 3.0, by 0.5 points lower than samples with the addition of lysozyme monomer. In the final stage of storage (168 h), fillet samples with an addition of lysozyme dimer and modified preparations received higher scores in comparison to samples with the addition of lysozyme monomer. Samples with the addition of lysozyme dimer were given scores by 1.4 points higher than samples with no lysozyme.

Similar changes were observed for colour, which considerable deterioration was also found after 120 h storage. In the final stage of the storage, the lowest scores for colour (2.2–2.8) were given to samples with no lysozyme added and to those with an addition of lysozyme monomer. After 168 h samples with an addition of dimer and modified preparations of lysozyme were given scores higher than 3.0 points.

All analysed samples were characterised by high scores for aroma (over 4 points) after 72 h storage. A considerable deterioration of this attribute, in the case of all tested samples, was found after 120 h storage of chicken breast muscles. In the final stage of storage (after 168 h) scores for aroma in samples with no lysozyme added were markedly different from those of the other samples. Samples with no addition of lysozyme received scores of 1.0 point, whereas the others were given 2.9 - 3.0 points.

Results of organoleptic analysis indicate that in case of the investigated attributes breast muscle samples with no lysozyme added were characterised by inferior quality in comparison to samples covered with the enzyme solutions. Samples with an addition of dimer and modified preparations of lysozyme in each of analysed periods received higher scores in comparison to samples with the addition of lysozyme monomer. Adverse changes in colour in samples with no lysozyme added resulted in the appearance of a grey, greenish colour.

The storage time of chicken breast muscle fillets depending on the added lysozyme form, required to obtain scores for overall desirability of three points, was calculated from the Weibull model (Table 4). A high correlation was shown between storage time of chicken breast muscles calculated from the Weibull model and one of the parameters of the Gompertz curve, i.e. maximum population density (Table 5).

It may be stated that the application of dimer and modified preparations of lysozyme makes possible an approx. 2-fold extension of shelf life of the tested product at storage temperature of 3°C ±1°C.

### Table 3

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Parameters of Gompertz growth curve</th>
<th>Parameters of growth rate calculated from Gompertz curve</th>
<th>Fit $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td>K</td>
<td>3.03</td>
<td>4.12</td>
<td>0.026</td>
</tr>
<tr>
<td>M</td>
<td>2.65</td>
<td>4.98</td>
<td>0.015</td>
</tr>
<tr>
<td>D</td>
<td>2.24</td>
<td>2.33</td>
<td>0.019</td>
</tr>
<tr>
<td>P4</td>
<td>2.78</td>
<td>3.15</td>
<td>0.019</td>
</tr>
<tr>
<td>P10</td>
<td>2.68</td>
<td>2.03</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Max growth = BC/e

** Time of 1 generation = ln(2)e/BC
Table 4
Parameters of the Weibull curve $y = a - b \times \exp(-c \times x^d)$ for changes in overall desirability of chicken breast muscles depending on the type of lysozyme

<table>
<thead>
<tr>
<th>Packaging method</th>
<th>Initial value</th>
<th>Curve parameters</th>
<th>R</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>5.00</td>
<td>289.7</td>
<td>-0.23</td>
<td>0.9672</td>
</tr>
<tr>
<td>M</td>
<td>5.00</td>
<td>6.33</td>
<td>-0.85</td>
<td>0.9582</td>
</tr>
<tr>
<td>D</td>
<td>5.00</td>
<td>3.27</td>
<td>-1.27</td>
<td>0.9822</td>
</tr>
<tr>
<td>P4</td>
<td>5.00</td>
<td>3.37</td>
<td>-1.23</td>
<td>0.9430</td>
</tr>
<tr>
<td>P10</td>
<td>5.00</td>
<td>2.72</td>
<td>-1.63</td>
<td>0.9578</td>
</tr>
</tbody>
</table>

R - correlation coefficient between experimental data and data calculated from the model
D - storage time required to obtain overall acceptability score of 3 (h)

Table 5
Coefficient of linear correlation between sensory shelf life of chicken breast muscles and parameters of the Gompertz model

<table>
<thead>
<tr>
<th>$\lambda$ - lag time</th>
<th>N - max density of population</th>
<th>$\mu$ - max rate of growth coefficient</th>
<th>GT - generation time</th>
<th>D - storage time</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$</td>
<td>1</td>
<td>1</td>
<td>-0.9788</td>
<td>-0.2448</td>
</tr>
<tr>
<td>N</td>
<td>0.151</td>
<td>0.0162</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>$\mu$</td>
<td>0.951</td>
<td>0.9564</td>
<td>0.5685</td>
<td>1.0</td>
</tr>
<tr>
<td>GT</td>
<td>-0.9788</td>
<td>-0.2448</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>-0.5253</td>
<td>-0.9058*</td>
<td>-0.3527</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*statistically significant differences at the level $P \leq 0.05$

Discussion
Most patent-protected food preservation technologies using lysozyme have been developed in Asian countries. This enzyme is used to preserve meat and processed meats, fish and processed fish products, seafood, milk and dairy products, vegetables, fruits, and wine, as well as typical Oriental dishes, such as kimchi, sushi or Japanese salad. In Europe, a wide range of commercial applications has been developed for lysozyme in cheese making and as an agent preventing swelling of ripening cheeses as a result of the action of bacteria (1, 6).

The potential application of lysozyme as a food preservative has been focused on, acting synergistically with other substances such as EDTA, nisin, trisodium phosphate, or sodium lactate (10, 22, 23). An advantageous effect of lysozyme on the quality of stored raw meat, processed meats, and processed fish products has been shown (9, 21, 24). The ability of lysozyme and nisin to control meat spoilage bacteria was evaluated. An effective action of antimicrobials was shown in inhibiting the growth of Brochothrix thermosphacta and Carnobacterium sp. It was stressed that the antibacterial action of lysozyme and nisin is directed first of all against Gram (+) bacteria, which results in their considerably limited application to preserve raw meat, feasible only in case of modified-atmosphere packaged food (24). However, lysozyme has a minor effect on Gram (-) bacteria isolated from meat. It was indicated that modifications of lysozyme make it possible to extend the range of antibacterial action of this enzyme (4, 20). The antibacterial activity of modified lysozyme is not connected only with its enzymatic function, but also catalytically-independent activity (8, 13, 16). It was found that pressure sensitises the bacteria to different forms of lysozyme (25).

Our results indicate that in the case of breast-muscle samples covered with lysozyme solutions, their shelf-life was extended approx. 2-fold in comparison to the control. It is consistent with literature data confirming the applicability of lysozyme in the extension of shelf-life of cold-stored meat and meat products (10, 12, 24). In our previous studies, it was also found that the application of a lysozyme monomer solution spray to the surface of chilled cut-up poultry significantly prolongs its shelf-life (12). However, it needs to be stressed that studies conducted to date by other authors concerned only the application of lysozyme monomer or a combination of this form of the enzyme with other preservatives, particularly nisin (5, 10, 24), EDTA (9), and sodium lactate (22). The shelf-life of breast muscle fillets was estimated on the basis of organoleptic examination and total counts of aerobic
bacteria. Samples with the addition of modified lysozyme preparations received higher scores for examined attributes, such as overall appearance, colour, and aroma, in comparison to the control samples. In studies by other authors no adverse effect was found for the action of lysozyme in combination with other antimicrobials on organoleptic attributes of meat products (22).

In the course of cold storage of breast-muscle samples, the total count of aerobic bacteria in samples with the addition of lysozyme was lower than in samples with no enzyme added. Reduced counts of aerobic bacteria were also observed in the case of the application of lysozyme to preserve steamed sausage (22). Among the analysed forms of lysozyme the most effective action was found for dimer. After 168 h storage bacterial counts in samples with the addition of this lysozyme form were by 2.4 log cycles lower in comparison to the control samples. It may be stated that a more effective action of dimer and modified lysozyme preparations in comparison to monomer results from a wider spectrum of its antibacterial action. Earlier studies showed that the modification procedure facilitates the extension of antibacterial spectrum of lysozyme, particularly against *Pseudomonas fluorescens* and *Proteus mirabilis* (4, 20). Microflora of poultry meat, apart from Gram (+) bacteria, also comprises Gram (-) bacteria, which in most cases are not sensitive to the action of lysozyme monomer. Effective antibacterial action has so far been found in case of the combined action of lysozyme monomer with other substances (5, 9, 10, 22). The application of sodium lactate (2%) with lysozyme (200 ppm) made it possible to obtain in the final stage of storage of minced steamed sausage (28 d) an aerobic bacteria count lower by 2.5 log cycles in comparison to control samples. However, the synergistic action of sodium lactate and lysozyme was shown first of all in relation to lactic acid bacteria. Such an action was not found for total bacterial count (22).

It needs to be stressed that many antimicrobials have been shown to be effective in culture media, but they lose their activity when applied to a natural product. Results of experiments indicate that preparations produced as a result of lysozyme modification and containing polymeric forms of the enzyme maintain their effect on fresh meat, which facilitates their applicability to extend its shelf life. This is consistent with literature data pointing to the advantageous action of lysozyme and nisin in the preservation of raw meat (23). No studies were conducted on the effect of modified lysozyme preparations on shelf-life of meat products. In the case of lysozyme monomer, its thermostability and maintenance of antibacterial properties under the influence of thermal processing, particularly in an acid medium, are stressed (6, 21).

Studies conducted to date have concerned only the use of lysozyme monomer to preserve different types of foodstuffs. No investigations have been carried out to determine the effect of modified preparations or lysozyme dimer on the microbiological or sensory quality of food. It seems that the application of modified lysozyme to preserve meat and its processed products may be advantageous due to the potential inhibition of growth in case of Gram (-) food spoilage bacteria. It needs to be stressed that lysozyme, being a natural preservative, may replace chemicals used to extend shelf life. However, studies need to be conducted on different products and the effectiveness of action against pathogenic bacteria should be evaluated for other than monomer forms of lysozyme. Production of bigger amounts of polymeric forms of lysozyme will facilitate studies concerning their effect on the shelf life of other products.

**References**