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Inhibition of food-borne bacteria by thermo-chemically modified egg white lysozyme

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This study investigates the effect of different concentrations (0.50 to 1.50%) of thermo-chemically modified lysozyme on the growth of selected bacteria, that is, Gram-negative *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Escherichia coli*, *Proteus mirabilis* and Gram-positive *Listeria innocua*, *Leuconostoc mesenteroides* in nutrient broth. The content of lysozyme polymeric forms after modification was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The dimer and trimer fractions of the modified lysozyme were 35.9 and 33.1%, respectively. The modified lysozyme exhibited higher surface hydrophobicity and lower hydrolytic activity than monomer. No inhibitory action of lysozyme monomer was observed in relation to the tested Gram-negative bacterial strains. The application of modified lysozyme at a concentration of 0.75% and higher, caused a complete reduction of bacterial counts as early as after 1 h incubation in case of *P. fragi*, *P. fluorescens* and *E. coli* or after 6 h incubation in case of *P. mirabilis*. After 6 h incubation both in samples with an addition of monomer and those with modified lysozyme a complete growth inhibition of *L. mesenteroides* was recorded. Modified lysozyme showed a stronger antibacterial activity against *L. innocua* than native lysozyme. Conducted analyses indicate that changes in the structure of enzyme molecules leads to an increase in the antibacterial action mainly towards Gram-negative bacteria.

Key words: Antibacterial activity, modified lysozyme.

INTRODUCTION

Lysozyme is an enzyme commonly found in various biological fluids and tissues. Egg white is a rich and readily available source of lysozyme containing from 0.3 to 0.4 g enzyme per egg. Lysozyme is a natural presser-vative used in food industry (Cunningham et al., 1991; Losso et al., 2000; Nattress and Baker, 2003; Wellman-Labadie et al., 2007). It is indicated that the application of natural antibacterial agents combined with good manufac-turing practice provide food stabilization and prolong shelf life of food. Antibacterial action of lysozyme concerns first of all Gram-positive bacteria and is primarily ascribed to its hydrolase enzyme activity. Such an action of the enzyme results in peptidoglycan hydrolysis and cell lysis. Antibacterial activity of lysozyme monomer was shown effective particularly in relation to such bacteria as *Bacillus stearothermophilus*, *Clostridium tyrobutyricum*, *Clostridium thermosaccharolyticum* and to a lesser extent against *Clostridium sporogenes*. Also, lysozyme monomer has been used with other compounds such as nisin, Ethylenediaminetetraacetic acid (EDTA), lactoferrin and trypsin in *Enterococcus faecalis*, *Brochothrix thermosphacta*. *Lactobacillus* or *Listeria monocytogenes*

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(Losso et al., 2000; Branen and Davidson, 2004). The narrow spectrum of the enzyme antibacterial action considerably limits its applicability. Effective antibacterial action of lysozyme covers few genera of bacteria connected with food. A significant application for this enzyme is found mainly to prevent late blowing in semihard cheese caused by the fermentation of lactate by butyric acid bacteria (Corbo et al., 2009). Thus, it seems essential to search for strategies facilitating an extension of the antibacterial spectrum of the enzyme action. Such a possibility is provided by a modification of lysozyme by covalent attachment of fatty acids, poly-saccharides, C-terminal hydrophobic peptides and the use EDTA or polycations. Another method is to apply high hydrostatic pressure treatment (Ibrahim et al., 1996a; Ibrahim et al., 2001; Masschalck et al., 2001). Analyses indicate that an increase in the antibacterial action may also be provided applying thermal, thermo-chemical or membrane modification. Action of such modified enzyme is not dependent on hydrolytic activity, but on a novel, still not completely defined action (Ibrahim et al., 1996a; Leśnierowski, 2007).

For this reason the aim of the present study was to assess antibacterial activity of thermo-chemically modified lysozyme in relation to selected bacteria, both Gram-negative and Gram-positive, which are characteristic of food.

MATERIALS AND METHODS

Lysozyme modification

Lysozyme for the experiments was obtained from albumin of chicken eggs, freshly laid by ASTRAS layers kept at the Broiler Breeding Farm of the Institute of Animal Science at Zakrzewo near Poznan (Poland). The lysozyme preparation used in the investigations was produced by the authors using the ion-exchange method (Leśnierowski, 1997) and subjected to thermo-chemical modification. Lysozyme solution of pH 4.0 was heated for 15 min at the temperature of 70°C in a water bath type 1083 (Gesellschaft fuer Labortechnik, Germany). After heating was completed, the solution was immediately cooled in ice water. An adequate amount of H_2O_2 was added to a cooled solution to provide its 2% concentration in the solution. The solution was stored at 7 ± 1°C for 6 days. The preparation produced using thermo-chemical modification was denoted as ML. After modification the obtained lysozyme preparation was lyophilized in a GT3 Leybold-Heraeus freeze dryer (Heraeus Instruments, Hanau, Germany).

Lysozyme polymeric forms

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, Holliston, USA) by SDS-PAGE (Laemmli, 1970; Leśnierowski, 1997). The following standards were used: Lysozyme 14.6 kDa (Sigma-Aldrich, Munich, Germany), Lydium KLP 28 kDa (Nika Health Product, Poland) and Hen Albumen 45 kDa (Sigma-Aldrich, Munich, Germany). Quantitative proportions of individual forms of lysozyme were determined densitometrically using a TotalLab programme by Nonlinear Dynamics Ltd.

Hydrolytic activity

Lysozyme hydrolytic activity was determined by spectrophotometry, based on the the phenomenon of cell wall lysis caused by the enzyme in *Micrococcus lysodeikticus* bacteria (Leśnierowski and Kijowski, 1995). The lytic activity of lysozyme was determined by monitoring the decrease in turbidity of a suspension of *Micrococcus lysodeikticus* cells at 450 nm. The activity was presented as the rate of decrease in absorbance per min of the initial rate of reaction (Δabs/min).

Surface hydrophobicity

Surface hydrophobicity was determined using ANS (aniline 1-naphthalenesulfonic acid, Sigma-Aldrich, Munich, Germany) with the use of a LS Fluorescence Spectrometer (PerkinElmer, Norwalk, USA), applying output wave length of λ=390 nm and emitter wave length λ=470 nm, according to the procedure described by Kato and Nakai (1980) and Li-Chan et al., (1984). Lysozyme solutions (0.01%) and its dilutions were prepared in a phosphate buffer of pH 6.0. The volume of 3 mL was collected from each dilution and 15 µL ANS dissolved in methanol were added. Fluorescence intensity was measured after 15 s. The value of determined surface hydrophobicity is equal to the coefficient of slope for the curve of fluorescence intensity versus protein concentration.

Microbiological tests

Analyses were conducted on bacterial strains, characteristic of food, i.e. *Escherichia coli* 2057/ATCC 25922/, *E. coli* PCM 318 O2:K1(L): H4, *Proteus mirabilis* PCM 543/NTCT 5887/, *Pseudomonas fluorescens* PCM 2123/NTCT 3756/, *Pseudomonas fragi* PCM 1856, *Listeria innocua* F ATCC /33090/ and *Leuconostoc mesenteroides* PCM 2253. They were delivered by the Institute of Immunology and Experimental Therapy in Wrocław (Poland) and the Department of Biotechnology, the Poznań University of Life Sciences. Strains of analyzed bacteria were transferred to 10 mL broth medium (Brain Heart Infusion Oxoid) and incubated at a temperature of 30°C (*P. fluorescens, P. fragi, Listeria innocua, Leuconostoc mesenteroides*) and 37°C (*E. coli, P. mirabilis*). From 24-h cultures bacterial suspensions were prepared in 0.85% NaCl Medium (Biomérieux), of a density of 0.5 in the McFarland scale using a Densimat apparatus (Biomérieux). The cell suspensions were cultured in broth in the presence of different concentrations of lysozyme (0.50, 0.75, 1.00 and 1.50%). The solution of required lysozyme concentration was prepared by dissolving dry lysozyme in sterile water. To test tubes containing 4 mL broth and 5 mL prepared solutions of monomer or modified enzyme, 1 mL bacterial suspension dilution was added so that the resulting bacterial count was 10^4 cfu/mL. Samples were incubated at 30 or 37°C for 72 h. During incubation, at specified time intervals (initially -0, 1, 6, 9, 24, 48 and 72 h), with the use of the classical method, counts of analyzed bacteria were determined on Oxoid culture media: *P.* Agar Base CM 0559 supplemented with Pseudomonas CFC Selective Agar Supplement (SR 0103), Mc Conkey Agar CM 0115, MRS Agar (De Man, Rogosa, Sharpe) CM 0361, Palcam Agar CM 0877, (Oxoid, England). Results were given in cfu/mL. The control comprised samples with no lysozyme solutions. Lysozyme sterility was examined in every case.

Statistical analysis

Results were subjected to statistical analysis using the STATISTICA Version 9.1 software package. For the inoculation results (cfu/mL)

Table 1. Characteristic of lysozyme.

Lysozyme	Enzymatic activity (U/mg protein)	Surface hydrophobicity	Share of monomer (%)	Share of dimer (%)	Share of trimer (%)	Share of oligomers (%)
Monomer	17,750[a]	920[b]	100[a]			
ML	1,080[b]	40400[a]	31.0[b]	35.9	33.1	69.0

a-b: different superscripts in the separate column denote statistically significant difference at p≤0.05, ML- modified lysozyme.

logarithmic transformation log (cfu/mL+1) was applied in order to prevent potential zero values. Basic descriptive statistics were calculated for the transformed logarithmic values. Experiments were replicated with three samples. The replications were separate from each other. Values are presented as means ± standard deviation. The one-way analysis of variance (ANOVA) was performed. The significance of results was verified with the use of the NIR Fisher test. Bacterial growth curves were calculated using the Bacterial Growth Kinetics programme and the Gompertz model: $y = a*\exp(-\exp(b-cx))$, where: y - log bacterial count, a - theoretical maximum contamination level, $N_{max,}$ b, c - parameters of the growth curve, x - incubation time. Bacterial growth curve were used to calculate incubation time $t_{(2*N0)}$ required for the doubling of the initial population log ($2*N_0$), i.e. lag-phase + 1 generation. Growth dynamics in the logarithmic phase was calculated from the slope between the maximum contamination level and the value of contamination at the population doubling time, in accordance with the formula: $(\log N) /h = (\log N_{max} - (\log (2*N_0)) / (72 - t_{(2*N0)})$.

RESULTS

Antibacterial activity of lysozyme monomer and its potential applicability have been described relatively extensively in literature, whereas the amount of data concerning to antibacterial action of modified lysozyme is very limited. In this study antibacterial activity of lysozyme obtained as a result of thermo-chemical modification, as well as that of lysozyme monomer, in relation to selected Gram-negative and Gram-positive bacterial strains were compared under model conditions in broth. Analyses were conducted on lysozyme produced as a result of thermo-chemical modification, selected in earlier experiments conducted by the authors (Cegielska-Radziejewska et al., 2010).

In thermo-chemically modified lysozyme preparation, apart from the enzyme monomer, also dimer and trimer were detected (Table 1). After modification lysozyme is also characterized by lower hydrolytic activity in comparison to lysozyme monomer. Thermo-chemical modification causes an increase in surface hydro-phobicity for the modified preparation amounting to 40400 and for monomer to 920, respectively. An increase in surface hydrophobicity of lysozyme after modification is explained by a change in conformation of enzyme molecules and exposure of hydrophobic residues (Ibrahim et al., 1996b; Ibrahim et al., 1997; Chung and Hancock, 2000; Chang and Li, 2002). Conducted investigations indicate a modified lysozyme in relation to tested bacterial strains, diverse effect of antibacterial action of monomer and dependent on the applied concentration of modified lysozyme as well as bacterial strain. Despite varying sensitivity of bacteria to the action of modified lysozyme, growth inhibition was observed in all tested strains (Tables 2 and 3).

No inhibitory action of lysozyme monomer was observed against tested Gram-negative bacterial strains. In the investigated incubation time in most cases no statistically significant differences were found between the numbers of bacteria in samples with varying levels of added lysozyme monomer. After 6-hour incubation the counts of bacteria of *P. mirabilis* and *P. fluorescens* in the sample with the highest concentration of lysozyme monomer were by 0.99 and 0.65 log cfu/mL lower than the bacterial count in the control sample. In the final incubation period the differences between the bacterial counts in the samples with an addition of lysozyme monomer and the control sample were lower. It may be stated that the application of higher concentrations of lysozyme monomer is ineffective in relation to tested strains of Gram-negative bacteria.

The application of modified lysozyme at a concentration of 0.75 and 1.00% made it possible to completely reduce the number of bacteria as early as after 1 hour of incubation in case of strains *P. fragi*, *P. fluorescens* and *E. coli* 2057 or after 6 h incubation in case of *P. mirabilis*. Inhibitory action of modified lysozyme was maintained until the end of the incubation time (72 h). At the application of a lower concentration (0.50%) of modified lysozyme complete bacterial growth inhibition was not observed. The most effective action was recorded in case of *P. fragi* and *E. coli* 2057. In case of *P. fluorescens* after 48 and 72 h incubation bacterial growth was recorded (Figure 1). However, the count of bacteria was by 4.84 and 2.30 log cfu/mL lower than that in the control sample. An increase was found in the counts of *P. mirabilis* and *E. coli* PCM 318. After 6 and 9 h of incubation the number of *P. mirabilis* in the sample with an addition of the lowest concentration of modified lysozyme was by 3.62 and 5.32 log cfu/mL lower than the respective counts in the control sample. A weaker inhibitory action of the lowest concentration of modified lysozyme was observed in case of *E. coli* (Table 2).

Antibacterial action of modified lysozyme was also tested in relation to Gram-positive bacteria such as *Listeria innocua* and *Leuconostoc mesenteroides*. Since the action of lysozyme monomer is limited first of all to

Table 2. The effect of modified lysozyme and time of incubation on Gram-negative bacteria growth.

Sample	log cfu/ml ±SD						
	0 h	1 h	6 h	9 h	24 h	48 h	72 h
***Escherichia coli* PCM 318**							
Control	$4.73^{a} \pm 0.18$	$4.76^{b} \pm 0.13$	$7.55^{c} \pm 0.29$	$8.55^{c} \pm 0.04$	$10.08^{b} \pm 0.12$	$12.04^{b} \pm 0.16$	$12.44^{d} \pm 0.13$
M1	$4.73^{a} \pm 0.18$	$4.63^{b} \pm 0.13$	$6.76^{b} \pm 0.05$	$7.85^{b} \pm 0.05$	$10.12^{b} \pm 0.14$	$11.96^{b} \pm 0.07$	$12.22^{c} \pm 0.03$
M2	$4.73^{a} \pm 0.18$	$4.69^{b} \pm 0.31$	$6.78^{b} \pm 0.04$	$7.86^{b} \pm 0.02$	$10.08^{b} \pm 0.09$	$11.92^{b} \pm 0.28$	$12.05^{b} \pm 0.08$
M3	$4.73^{a} \pm 0.18$	$4.62^{b} \pm 0.21$	$6.72^{b} \pm 0.05$	$7.83^{b} \pm 0.04$	$10.03^{b} \pm 0.12$	$11.91^{b} \pm 0.47$	$12.04^{b} \pm 0.03$
ML1	$4.73^{a} \pm 0.18$	$3.98^{a} \pm 0.09$	$6.07^{a} \pm 0.09$	$7.60^{a} \pm 0.16$	$9.63^{a} \pm 0.30$	$9.94^{a} \pm 0.03$	$9.98^{a} \pm 0.05$
ML2	$4.73^{a} \pm 0.18$	ND	ND	ND	ND	ND	ND
ML3	$4.73^{a} \pm 0.18$	ND	ND	ND	ND	ND	ND
***Escherichia coli* 2057**							
Control	$4.44^{a} \pm 0.30$	$4.70^{b} \pm 0.10$	$7.37^{b} \pm 0.09$	$8.41^{a} \pm 0.08$	$9.71^{ab} \pm 0.06$	$10.28^{b} \pm 0.20$	$10.43^{ab} \pm 0.25$
M1	$4.44^{a} \pm 0.30$	$4.83^{b} \pm 0.05$	$7.44^{b} \pm 0.15$	$8.42^{a} \pm 0.09$	$9.72^{b} \pm 0.10$	$10.34^{b} \pm 0.26$	$10.51^{b} \pm 0.09$
M2	$4.44^{a} \pm 0.30$	$4.64^{b} \pm 0.08$	$7.25^{a} \pm 0.06$	$8.39^{a} \pm 0.06$	$9.66^{ab} \pm 0.06$	$10.38^{b} \pm 0.29$	$10.29^{ab} \pm 0.44$
M3	$4.44^{a} \pm 0.30$	$4.63^{b} \pm 0.07$	$7.22^{a} \pm 0.09$	$8.43^{a} \pm 0.04$	$9.62^{a} \pm 0.13$	$10.39^{b} \pm 0.07$	$10.17^{a} \pm 0.19$
ML1	$4.44^{a} \pm 0.30$	$3.98^{a} \pm 0.20$	ND	ND	ND	ND	ND
ML2	$4.44^{a} \pm 0.30$	ND	ND	ND	ND	ND	ND
ML3	$4.44^{a} \pm 0.30$	ND	ND	ND	ND	ND	ND
Proteus mirabilis							
Control	$4.20^{a} \pm 0.10$	$4.33^{c} \pm 0.05$	$6.94^{c} \pm 0.19$	$8.67^{c} \pm 0.06$	$9.64^{b} \pm 0.06$	$10.35^{c} \pm 0.23$	$10.36^{c} \pm 0.16$
M1	$4.20^{a} \pm 0.10$	$4.25^{bc} \pm 0.05$	$6.16^{b} \pm 0.10$	$7.54^{b} \pm 0.10$	$9.64^{b} \pm 0.03$	$10.02^{b} \pm 0.26$	$10.19^{bc} \pm 0.17$
M2	$4.20^{a} \pm 0.10$	$4.18^{bc} \pm 0.13$	$5.97^{b} \pm 0.28$	$7.55^{b} \pm 0.07$	$9.58^{b} \pm 0.08$	$9.94^{b} \pm 0.10$	$10.20^{bc} \pm 0.14$
M3	$4.20^{a} \pm 0.10$	$4.26^{bc} \pm 0.13$	$5.95^{b} \pm 0.23$	$7.52^{b} \pm 0.08$	$9.58^{b} \pm 007$	$10.02^{b} \pm 0.14$	$10.16^{b} \pm 0.15$
ML1	$4.20^{a} \pm 0.10$	$4.11^{b} \pm 0.03$	$3.32^{a} \pm 0.11$	$3.35^{a} \pm 0.05$	$6.39^{a} \pm 0.07$	$9.57^{a} \pm 0.13^{b}$	$9.95^{a} \pm 0.02$
ML2	$4.20^{a} \pm 0.10$	$3.38^{a} \pm 0.12$	ND	ND	ND	ND	ND
ML3	$4.20^{a} \pm 0.10$	ND	ND	ND	ND	ND	ND
Pseudomonas fragi							
Control	$3.52^{a} \pm 0.04$	$3.48^{b} \pm 0.11$	$5.21^{b} \pm 0.21$	$5.83^{b} \pm 0.15$	$8.44^{c} \pm 0.28$	$9.35^{b} \pm 0.09$	$9.76^{d} \pm 0.10$
M1	$3.52^{a} \pm 0.04$	$3.15^{a} \pm 0.05$	$4.64^{a} \pm 0.28$	$5.29^{a} \pm 0.09$	$8.13^{ab} \pm 0.22$	$9.04^{a} \pm 0.06$	$9.65^{cd} \pm 0.14$
M2	$3.52^{a} \pm 0.04$	$3.23^{a} \pm 0.05$	$4.47^{a} \pm 0.11$	$5.28^{a} \pm 0.03$	$8.04^{a} \pm 0.11$	$9.29^{b} \pm 0.12$	$9.59^{b} \pm 0.12$
M3	$3.52^{a} \pm 0.04$	$3.16^{a} \pm 0.08$	$4.43^{a} \pm 0.14$	$5.25^{a} \pm 0.07$	$8.29^{bc} \pm 0.13$	$9.01^{a} \pm 0.11$	$9.37^{a} \pm 0.13$
ML1	$3.52^{a} \pm 0.04$	ND	ND	ND	ND	ND	ND
ML2	$3.52^{a} \pm 0.04$	ND	ND	ND	ND	ND	ND
ML3	$3.52^{a} \pm 0.04$	ND	ND	ND	ND	ND	ND

a-d: different superscripts in the separate column denote statistically significant difference at $p \leq 0.05$, ND - not detected in dilution 10^{-1}; M1 (monomer 0.50%), M2 (monomer 0.75%), M3 (monomer 1.00%), M4 (monomer 1.50%), ML1 (modified lysozyme 0.50%), ML2 (modified lysozyme 0.75%), ML3 (modified lysozyme 1.00%)

Gram-positive bacteria, it was essential to assess the effect of modified lysozyme on strains belonging to this group of bacteria. In case of *L. mesenteroides* the anti-bacterial action of monomer and modified lysozyme was comparable. After 6 h incubation both in the samples with an addition of monomer and with modified lysozyme a complete bacterial growth inhibition was stated. Bacterial growth was not observed to the end of the sample incubation period. In the control sample the count of bacteria after 72 h incubation was 9.46 log cfu/mL. In case of a Gram-positive *Listeria innocua* both lysozyme monomer and modified lysozyme were found to have an antibacterial effect. Lysozyme monomer exhibited an inhibitory action against the tested strain *Listeria innocua* up to 9 h incubation. A more effective antibacterial action of the enzyme in relation to the tested bacterial strain was recorded as a result of the application of modified lysozyme. When applying higher concentrations of lysozyme (0.75 to 1.50%) already after 1 h incubation a complete growth inhibition was observed in *Listeria innocua* (Table 3).

The Gompertz model was used for the description of

Table 3. The effect of modified lysozyme and time of incubation on *Listeria innocua* growth.

Sampie	log cfu/ml±SD						
	0 h	1 h	6 h	9 h	24 h	48 h	72 h
Listeria innocua							
Control	$3.87^{a} \pm 0.33$	$4.15^{d} \pm 0.23$	$6.97^{d} \pm 0.22$	$7.86^{d} \pm 0.23$	$9.29^{d} \pm 0.18$	$9.19^{c} \pm 0.11$	$9.18^{c} \pm 0.13$
M1	$3.87^{a} \pm 0.33$	$3.27^{bc} \pm 0.31$	$1.57^{a} \pm 0.25$	$2.36^{a} \pm 0.05$	$8.67^{c} \pm 0.05$	$8.67^{b} \pm 0.05$	$9.06^{c} \pm 0.05$
M2	$3.87^{a} \pm 0.33$	$2.68^{a} \pm 0.33$	$2.48^{c} \pm 0.17$	$3.12^{c} \pm 0.25$	$8.53^{c} \pm 0.19$	$8.64^{b} \pm 0.08$	$8.55^{b} \pm 0.09$
M3	$3.87^{a} \pm 0.33$	$2.79^{a} \pm 0.33$	$2.47^{c} \pm 0.26$	$2.79^{b} \pm 0.31$	$8.17^{b} \pm 0.12$	$8.78^{b} \pm 0.14$	$8.52^{b} \pm 0.15$
M4	$3.87^{a} \pm 0.33$	$3.16^{b} \pm 0.20$	$2.05^{b} \pm 0.11$	$2.51^{a} \pm 0.18$	$8.17^{b} \pm 0.21$	$8.65^{b} \pm 0.01$	$8.36^{b} \pm 0.33$
ML1	$3.87^{a} \pm 0.33$	$3.54^{c} \pm 0.16$	$2.69^{c} \pm 0.18$	$2.39^{a} \pm 0.07$	$2.90^{a} \pm 0.28$	$2.95^{a} \pm 0.22$	$3.48^{a} \pm 0.11$
ML2	$3.87^{a} \pm 0.33$	ND	ND	ND	ND	ND	ND
ML3	$3.87^{a} \pm 0.33$	ND	ND	ND	ND	ND	ND
ML4	$3.87^{a} \pm 0.33$	ND	ND	ND	ND	ND	ND

a-d: different superscripts in the separate column denote statistically significant difference at p≤0.05; ND - not detected in dilution 10^{-1}; M1 (monomer 0.50%), M2 (monomer 0.75%), M3 (monomer 1.00%), M4 (monomer 1.50%), ML1 (modified lysozyme 0.50%), ML2 (modified lysozyme 0.75%), ML3 (modified lysozyme 1.00%), ML4 (modified lysozyme 1.50%)

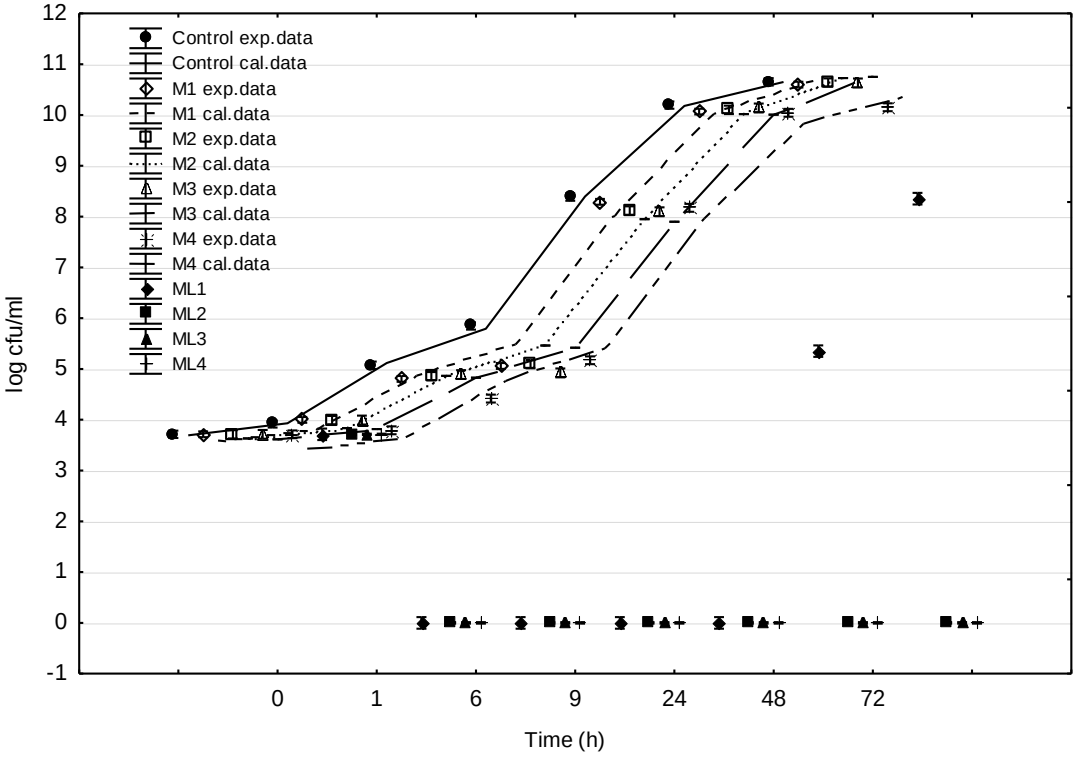

Figure 1. The Gompertz growth model for *Pseudomonas fluorescens* in samples with monomer and modified lysozyme.
M1 (monomer 0.50%), M2 (monomer 0.75%), M3 (monomer 1.00%), M4 (monomer 1.50%), ML1 (modified lysozyme 0.50%), ML2 (modified lysozyme 0.75%), ML3 (modified lysozyme 1.00%), ML4 (modified lysozyme 1.50%).

bacterial growth and survival in all samples. Approximation of models to empirical data was high. Kinetic parameters of bacterial growth assessed on the basis of the Gompertz function are presented in Table 4. The calculated growth dynamics for tested bacteria in all the control samples and in those with added lysozyme was comparable. Considerable differences were found in case of incubation time required for doubling of the initial population, calculated from the Gompertz function. In case of all the tested bacterial strains the incubation time

Table 4. Parameters of the Gompertz growth curve and growth rate calculated from the curve for tested bacteria in samples with monomer and modified lysozyme.

Bacterial species	Sample	Parameters of Gompertz curve				Parameters of growth rate calculated from Gompertz curve	
		a	b	c	Fit R^2	$t_{(2*N0)}$	(log (N))/h
Escherichia coli PCM 318	Control	12.107	-0.079	0.092	0.973	0.55	0.10
	M1	12.185	-0.036	0.077	0.993	1.12	0.10
	M2	12.061	-0.050	0.079	0.991	1.06	0.10
	M3	12.053	-0.044	0.078	0.989	1.16	0.10
	ML1	10.045	-0.116	0.114	0.973	2.21	0.07
Escherichia coli 2057	Control	10.230	-0.146	0.158	0.995	0.71	0.08
	M1	10.290	-0.157	0.156	0.990	0.60	0.08
	M2	10.210	-0.140	0.155	0.993	0.78	0.08
	M3	9.670	-0.055	0.171	0.990	1.63	0.07
Proteus mirabilis	Control	10.222	-0.054	0.165	0.985	0.9	0.08
	M1	10.148	-0.068	0.118	0.993	1.2	0.08
	M2	10.119	-0.060	0.115	0.989	1.2	0.08
	M3	10.144	-0.067	0.113	0.995	1.2	0.08
	ML1	12.260	-0.028	0.960	0.992	9.25	0.12
Pseudomonas fluorescens	Control	10.805	0.071	0.060	1.000	1.3	0.10
	M1	10.940	0.107	0.053	0.992	1.9	0.10
	M2	11.043	0.106	0.051	0.993	1.9	0.10
	M3	11.095	0.115	0.050	0.990	1.9	0.10
	M4	10.547	0.116	0.058	0.989	2.5	0.09
Pseudomonas fragi	Control	9.678	0.051	0.083	0.994	1.5	0.08
	M1	9.594	0.101	0.073	0.989	2.4	0.08
	M2	9.696	0.110	0.070	0.994	2.5	0.08
	M3	9.415	0.110	0.078	0.987	2.6	0.08
Listeria innocua	Control	9.254	0.089	0.192	0.990	0.65	0.07
	M1	9.571	0.384	0.061	0.781	9.87	0.09
	M2	9.095	0.302	0.069	0.840	8.48	0.08
	M3	9.175	0.303	0.063	0.838	9.13	0.08
	M4	9.084	0.298	0.061	0.796	9.65	0.08

a-theoretical maximum contamination level N_{max}; b,c-parameters of the growth curve; (log N)/h -growth dynamics; $t_{(2*N0)}$ - incubation time required for the doubling of the initial population; M1 (monomer 0.50%), M2 (monomer 0.75%), M3 (monomer 1.00%), M4 (monomer 1.50%), ML1 (modified lysozyme 0.50%).

required for doubling of the initial population, was the shortest for the control samples with no lysozyme addition. For the bacterium *L. mesenteroides* the Gompertz model was not applied, due to a lack of bacterial growth in samples with an addition of lysozyme. The longest lag phase time was observed in case of samples with an addition of modified lysozyme. The lowest concentration of modified lysozyme cause 4 to 10-fold extension of the lag-phase time, dependent on the type of bacteria. For higher concentrations the time of the lag phase was not calculated, due to a complete bacterial growth inhibition in samples with an addition of modified lysozyme.

DISCUSSION

Effective action of lysozyme monomer against tested strains of *Listeria innocua* and *L. mesenteroides* confirms antibacterial activity of lysozyme monomer in relation to Gram-positive bacteria. Johnson (1994) reported that sensitivity of *L. monocytogenes* to the action of egg white

lysozyme monomer is dependent on the growth environment. The application of 20 mg or 200 mg/L causes a delay in bacterial growth, but not its inhibition. A more effective antibacterial action of lysozyme monomer may be obtained when EDTA, lactic acid, conalbumin or lactoferrin are used simultaneously with lysozyme. Results of conducted experiments indicate that antibacterial action against the tested strain of Gram-positive bacteria is also provided by thermo-chemically modified lysozyme despite a much lower, in comparison to lysozyme monomer, hydrolytic activity. Thus, it may be assumed that the mechanism of action of such modified enzyme is more complex. It is indicated that the antibacterial action of partially denatured lysozyme results from additional mechanisms, independent of muramidase activity (Düring et al., 1999). In a study by Ibrahim et al., (1996b) heat-denatured lysozyme showed the same bactericidal effects as native lysozyme against Gram-positive bacteria: *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*.

Recorded results confirm a lack of effective antibacterial action of lysozyme monomer against Gram-negative bacteria, which is connected with a considerable difference in the cell wall structure between Gram-positive and Gram-negative bacteria (Masschalck and Michiels, 2003). In case of Gram-negative bacteria access of lysozyme to the relatively thin murein layer is considerably hindered due to the presence of the outer membrane composed of phospholipids, protein and lipopolysaccharides (Ohno and Morrison, 1989). Disruption of the bacterial outer membrane may be provided by chemical or physical treatments (Chung and Hancock, 2000; Masschalck et al., 2001; Iucci et al., 2007). In the presence of EDTA lysis was found in Gram-negative bacteria, insensitive to the action of lysozyme monomer, that is *Enterobacter aerogenes*, *E. coli* or *P. aeruginosa* (Boland et al., 2003; Branen and Davidson, 2004). Iucci et al. (2007) reported that both heat treatment and HPH treatment may cause an immediate increase in antibacterial activity against *L. monocytogenes* in BHI.

Results of these experiments confirm that this way of lysozyme modification may be an effective method to extend the spectrum of antibacterial action of this enzyme against Gram-negative bacteria. Earlier studies conducted by the authors indicate a potential effective antibacterial action of chemically and thermo-chemically modified lysozyme in relation to *P. fluorescens* (Cegielska-Radziejewska et al., 2010). Ibrahim et al. (1996b) showed antibacterial activity of mild heat treatment lysozyme against *E. coli* K12, *P. aeruginosa* and *Salmonella* Enteritidis. As a result of the performed thermo-chemical modification a considerable reduction was shown in hydrolytic activity, at a simultaneous increase in antibacterial activity against Gram-negative bacteria. It was shown that the degree of reduction in hydrolytic activity is dependent on the applied modification method and it is correlated with enzyme modification conditions 2007; Cegielska-Radziejewska et al., 2010). According to Ibrahim et al. (1996b), heat treatment of native lysozyme at a temperature of 80°C at pH 6 makes it possible to retain 50% enzymatic activity of native lysozyme. Reduction of hydrolytic activity of the enzyme is connected with a change in cell structure, primarily the enzyme active centre and the formation of dimer and oligomers. Leśnierowski (2007) indicated dependence between hydrolytic activity and the propor-tion of monomer in the enzyme preparation after modify-cation. At the same time as a result of modification in enzyme molecules changes occur in the structure of the polypeptide chain, as well as unfolding of protein, exposure of hydrophobic groups and as a consequence an increase in surface hydrophobicity is observed (Ibrahim et al., 1996a; Touch et al., 2003). The formation of a new cell conformation is responsible for an increase in antibacterial activity of the enzyme. It was suggested that antibacterial action of denatured lysozyme is not only dependent on its hydrolytic activity but also to its hydrophobic and cationic properties (Ibrahim et al., 2002). An increase in hydrophobicity leads to an increase in the binding capacity of the enzyme to components of the bacterial outer membrane and a distortion of lipid layer integrity. Ibrahim et al. (1996b) reported that heat treatment of lysozyme provides higher reactivity with the lipopolysaccharide fraction of *E. coli* in comparison to native lysozyme, which indicates the capacity of the enzyme to interact with the outer membranes of bacteria.

Conclusion

Conducted analyses indicate that changes in the structure of enzyme molecules leads to an increase in the antibacterial action mainly towards Gram-negative bacteria. The thermo-chemically modified egg white lysozyme may reduce or eliminate spoilage caused by Gram-negative species in different foods. However, the data presented here were carried out in model conditions and it would be essential to determine the action of such modified lysozyme toward different bacterial strains and validate these data in food.

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