

Improved Antibacterial Spectrum of Hen Egg White Lysozyme with Thermal Modified

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Abstract

The aim of the research to determine the effect of thermal modification to Improve the antibacterial spectrum of hen egg white lysozyme (HEWL) particularly in gram-negative bacteria. The temperature treatment were 40 °C, 45 °C, and 50 °C for 20 minutes. Observed variables include lysozyme activity on *M.lysodeikticus* and *E. Coli* and MIC. The thermal modification with different temperature treatment gave significant effect ($P < 0.05$) to increase the antibacterial spectrum of egg white lysozyme. Treatment temperature of 50 °C for 20 minutes on the hen egg white lysozyme extract yield is 12.92 % the highest inhibition against gram-negative bacteria (*E. Coli*) with a concentration of 1.18 mg/ml.

1. Introduction

Lysozyme is a safe food preservative ingredient [6]. Lysozyme is used for food preservation because of its ability as an antibacterial [12]. Lysozyme is found in hen egg white and can be made on a commercial scale as preparations containing biological activity [6]. Egg production in the world is very large, reaching 61.111 million tons [1]. So that the egg white lysozyme production is very potential to be developed on an industrial scale.

Lysozyme has antibacterial activity bonds that hydrolyze β -1, 4 of the homopolymer of *N-acetylglucosamine (Glc Nac)* and *GLC* heteropolimer *muramik acid N-Acetyl-Nac*, a gram-positive bacterial cell lysis [12]. Limited antimicrobial activity of lysozyme against gram-positive strains, but so far many microorganisms that contaminate foods of animal origin caused by gram-negative bacteria.

Heat treatment (thermal) could lead to changes in the molecular conformation of lysozyme, which gave rise to the surface hydrophobic groups lysozyme. These changes can increase the ability of lysozyme to stick to the gram-negative bacterial lipopolysaccharide [8]. Modification is expected to enhance the antibacterial spectrum of lysozyme against gram-negative bacteria.

2. Materials and Methods

The work was carried out in the laboratories of Animal Product Technology, Faculty of Animal Husbandry, University of Brawijaya, Malang, Indonesia.

2.1. Materials

The materials used in this study include: Hen Egg White 1 year old, 1 N acetic acid (Merck), NaCl (Merck), KCL (Merck), NH₄Cl (Merck), Buffer Phosphate (Na₂HPO₄ 0.1 M) (Merck), Silica (SiO₂) (PT. Panadia Corporation Indonesia), distilled water, 30 % bis-acrylamide (Merck), 1M HCl pH 6.8 (Merck), 1M tris HCL pH 8.8 (Merck), aquabidest , 10 % APS (Merck), 10% SDS (Merck), TEMED (Merck), R-250 Coomassie blue (Merck), methanol (Merck), distilled water, glacial acetic acid (Merck), 50 % glycerol (Merck), 1% Bromophenol blue (Merck), culture of bacteria: *Micrococcus lysodeikticus* (Sigma), *E-coli* (Laboratory of Microbiology, Faculty of Medicine, UB) and phosphate buffer (pH 6.24) of NaH₂PO₄ (Merck) and Na₂HPO₄ (Merck), Peptone (Oxoid), NB (Oxoid), NA (Oxoid), Vegetable Peptone Broth (Oxoid) and Vegetable Peptone Agar (Oxoid). 0.1 mg / mL Lysozyme (Sigma Chemical), 95 % ethanol (Merck) and 85 % phosphoric acid (H₃PO₄) (Merck).

2.2 Instrumental

The Instruments used in this study include: Erlenmeyer (Pyrex) measuring glass (Pyrex) beaker glass (Pyrex), stirrer (Pyrex), pipettes (Pyrex), petridish (Pyrex), test tubes (Pyrex), aluminum foil, whatman paper no.1, analitical balance (Mettler PM 200 Switzerland), Vortex (Janke 43480), refrigerated (Panasonic), 3.5 cm magnetic stirrer (Labinco), Refrigerated Micro 22 R centrifuge (Hettich), and pH meter CG. 818T (Schoot Geräte), Water bath type digital J.26, SDS-PAGE (Bio-Rad Mini Protean 3), Eppendorf, micropipet 10 μ l - 1000 mL (Hamilton syringe), blue tip, yellow tip, cuvette, UV-2100 spectrophotometer (Unico), *nano drop* spectrophotometer (ND-1000).

2.3 Method of HEWL Extraction

HEWL extraction is done by preparing 20 ml of hen egg white and 1 N acetic acid was added to adjust the pH level of treatment appropriate. Then added some salt type and concentration of appropriate treatment for 3 times the volume of egg whites and stirrer for 15 minutes. Silica (SiO₂) added as much as 0.851 g and stirrer for 10 minutes then add in 20 ml phosphate buffer (NaH₂PO₄) and stirrer for 5 minutes. Then left overnight at 4 °C, after which stirrer for 5 minutes. Speed centrifuge with 6000 rpm at 4 °C for 20 min, then the supernatant was taken for analysis [14].

2.4 Method of HEWL Modification

Thermal modification is done by preparing distilled water into a water bath and then set the temperature on the digital display with the treatment temperature 40 °C, 45 °C and 50 °C and control, each of which was repeated 3 times. Samples were taken as much as 5 ml and put in water bath for 20 minutes. samples to cooling at the room then analyzed [14].

2.5 Lysozyme Activity Determination

M. lysodeikticus and *E. coli* suspension by mixing the bacterial suspension into the 0.067 M Phosfat Buffer (pH 6,24). Then taken as 2.98 ml and inserted into the cuvette, and the absorbance in the spectrophotometer with a wavelength of 450 nm (A 450) to read numbers from 0.6 to 0.7 on the display device. The suspension has been measured as 2.98 ml was taken and put into the cuvette, then add 20µl lysozyme sample extract and mixed it flat, then spektrofotometer with absorbance wavelength of 450 nm (A 450). Number that appears on the display device is recorded at 0 seconds, 30 seconds, 60 seconds, 90 seconds and 120 seconds. Decrease in absorbance at 450 nm wavelength (DA 450) of 0.001 / min recorded as 1 unit of enzyme activity with units units / ml, calculated by the formula: Lysozyme activity (U / ml) = (Slope A.450/min) / 0.001 / min x 0.02 ml) [5].

2.6 Lysozyme Specific Activity Determination

Lysozyme Specific activity was calculated by dividing the protein content of the sample : Lysozyme Specific Activity (U / mg) = (U / ml) / (mg protein / ml) [5].

2.7 Lysozyme Recovery Determination

Lysozyme recovery is defined as the amount of enzyme lysozyme obtained after the extraction process [5].

Lysozyme recovery (%) =

$$\frac{\text{Lysozyme activity after Extraction}}{\text{Lysozyme activity before extraction}} \times 100$$

2.8 MIC Determination

MIC of *M.lysodeicticus* and *E.coli* is determined with contact method [10].

2.9 Protein Molecular Weight Determination

Molecular weight fraction of lysozyme protein extracts was determined by SDS-PAGE [7].

2.10 Data Analysis

Data were analyzed by analysis of variance (ANOVA), if there is a difference between the treatment are LSD (Least Significant Difference) and Duncan's Multiple Range Test [16]. Data calculations performed with Excel 2007 Microsoft program, and data analysis performed using SPSS version 16.0.

3. Results and Discussion

3.1 Effect of Thermal Modified on

M. lysodeicticus

Results of analysis of variance showed that HEWL modified by thermal treatment temperature of 40 °C, 45 °C and 50 °C for 20 minutes and the controls not significant defferent (P>0.05) on the activity of lysozyme, lysozyme recovery and specific activity of lysozyme on *M.lysodeikticus* gram positif bacteria. The mean results are shown in Table 1.

Table 1. Mean Effect of Thermal Modification on Lysozyme Activity, Recovery Lysozyme, and Lysozyme Specific activities on *M. lysodeikticus*.

Treatments	Means			
	Lysozyme Activity (U/menit)	Lysozyme Recovery (%)	Lysozyme Specific Activity (U/mg)	Protein content (mg/ml)
Control	45504688,16 ^a	85,02 ^r	4084801,45 ⁿ	11,14 ^v
40 °C	52680475,76 ^a	83,47 ^r	4728947,55 ⁿ	11,14 ^v
45 °C	41737646,92 ^a	66,13 ^r	3746646,94 ⁿ	11,14 ^v
50 °C	28723013,02 ^a	45,51 ^r	2578367,42 ⁿ	11,14 ^v

Description : The same superscript in the same column indicates no significant difference (P> 0.05),

Table 1 show that the means of lysozyme activity, lysozyme recovery and specific activity of lysozyme on *M. lysodeikticus* will decrease with increasing temperature treatment although the decrease was not

significantly different ($P>0.05$). Highest average activity of lysozyme obtained from 40 °C treatment amounting 52,680,475.76 U/min. There was a decrease of 19 % lysozyme recovery at 45 °C temperature rise towards 50 °C. Highest average specific activity of lysozyme was also obtained from the 40 °C treatment amounting 4,728,947.55 U/mg and decreases with increasing temperature treatment.

Thermal modification on lysozyme oligomers cause 50 % - 70 % and more than 40 % dimer enzyme molecule. Lysozyme heat denaturation can lead to decreased activity of lysozyme against gram-positive bacteria but improve antibacterial against gram-negative bacteria [9].

3.2 MIC of HEWL Thermal Modified on

M. lysodeikticus

Inhibition of *M. lysodeikticus* greatest obtained from 40 °C treatment. This is consistent with previous variable activity of lysozyme, lysozyme recovery and specific activity of lysozyme that showed similar results. Results of the determination of minimum inhibitory concentration (MIC) of lysozyme extracts are presented in Table 2.

Table 2. MIC of HEWL Thermal Modified on *M. lysodeikticus*

Treatment	Extract Concentration (mg/ml)	The number of Colonies (Cfu/ml)		Inhibition (%)
		Early	24 hours contact	
40 °C	0,12	4,8 x 10 ⁴	1,6 x 10 ⁴	66,56
	0,29	2,1 x 10 ⁵	TBUD	TBUD
	0,59	3,9 x 10 ⁴	6,9 x 10 ³	82,08
	0,88	3,3 x 10 ⁴	4,8 x 10 ³	85,38
	1,18	2,7 x 10 ⁴	2,0 x 10 ³	92,64*

Description. * : MIC, TBUD : not determined

Table 2 shows that the inhibition of lysozyme extracts thermal modification will increase with the addition of concentration. Minimum inhibitory concentration (MIC) of HEWL on *M. lysodeikticus* 40 °C temperature treatment is 1.18 mg/ml to produce inhibition percentage of 92.64 %. The concentration 0.5 mg / ml of lysozyme Extract with thermal modified could inhibit 32 % of gram-positive bacteria *M. lysodeikticus* [8]. Increased surface hydrophobicity properties of lysozyme associated with conformational changes that play a role in increasing the antimicrobial activity of lysozyme which has been modified [4]. If the residual lysis of lysozyme reacted with phenolic aldehydes, fenilaldehyd, the lysozyme molecule undergoes a conformational change and increased

antimicrobial activity against both gram-positive and gram-negative bacteria [3].

3.3 Effect of HEWL Thermal Modified on

E. coli

Based on the analysis of variance is known that HEWL modified by thermal treatment temperature of 40 °C, 45 °C and 50 °C for 20 minutes and the control effect is highly significant different ($P<0.01$) the activity of lysozyme, lysozyme recovery and specific activity of lysozyme on gram-negative bacteria *E. coli*. The mean results are shown in Table 3.

Table 3. Effect of Thermal Modification on Lysozyme Activity, Recovery Lysozyme, and Lysozyme Specific Activities on *E. coli*.

Treatments	Means			
	Lysozyme Activity (U/ment)	Lysozyme Recovery (%)	Lysozyme Specific Activity (U/mg)	Protein Content (mg/ml)
Control	107182907,27 ^a	100,00 ^f	9621445,89 ^b	11,14 ^v
40 °C	389827113,63 ^a	363,70 ^f	34993457,24 ^a	11,14 ^v
45 °C	326171705,94 ^a	304,31 ^f	29279327,28 ^a	11,14 ^v
50 °C	502927624,92 ^b	469,22 ^e	45146106,37 ^m	11,14 ^v

Description : Different subscript letters in the same column indicate significantly different ($P<0.01$)

Based on Table 3 is known that the means activity of lysozyme, lysozyme recovery and specific activity of lysozyme on *E. coli* will increase with increasing temperature treatment. Highest means recovery of lysozyme activity and specific activity of lysozyme obtained from the 50 °C temperature treatment amounted to 502.927.624,92 U/min, and 469,22 % 45.146.106,37 U/mg. Lysozyme oligomers interaction with the cell membrane of gram-negative bacteria resulting in increased surface hydrophobicity of lysozyme [9]. Increased surface hydrophobicity of egg white protein solution occurs above the temperature of 50 °C [15].

Increased activity of lysozyme on gram-negative bacteria is also suspected due to the increase in temperature due to polymerase lysozyme [8]. Other research states that thermal modification of the lysozyme can form 27.2 % of the total polymer, monomer 72.2 %, 27.2% dimer and trimer 0% which is irreversible.

3.4 MIC of HEWL Thermal Modified on *E. coli*

Based on the test inhibition of *E. coli* is known that the 50 °C temperature treatment produces the greatest percentage of inhibition. Previous highest average variable activity of lysozyme, lysozyme recovery and specific activity of lysozyme was also obtained from the treatment temperature of 50 °C. Results of the determination of minimum inhibitory concentration (MIC) of lysozyme extracts are presented in Table 4.

Table 4. MIC of HEWL Thermal Modified on *E. coli*

Treatment	Extract Concentration (mg/ml)	The number of Colonies (Cfu/ml)		Inhibition (%)
		Early	24 hours contact	
50 °C	0,12	2,8 x 10 ⁵	2,7 x 10 ⁵	5,11
	0,29	3,0 x 10 ⁵	2,8 x 10 ⁵	7,14
	0,59	3,0 x 10 ⁵	2,9 x 10 ⁵	7,76
	0,88	3,1 x 10 ⁵	2,8 x 10 ⁵	9,72
	1,18	3,1 x 10 ⁵	2,7 x 10 ⁵	12,92

Description : no one reached ≥ 90 % inhibition

Based on Table 4 generally known that inhibition test results lysozyme modified thermal extract against *E. coli* has not yet reached the MIC (≤ 90 %). Percentage inhibition of *E. coli* will be higher with increasing concentration of lysozyme extracts. Highest inhibition results obtained from extracts of lysozyme treatment 50 °C temperature with a concentration of 1.18 mg / ml is equal to 12.92 %.

Antimicrobial activity of lysozyme can be converted into active against gram-negative bacteria through genetic hydrophobic peptide C-terminal to lysozyme [3]. Bakteriolytic lysozyme activity against gram-negative bacteria through the destruction of the function of the phosphate groups of phospholipids with lipopolysaccharide in the outer membrane of gram-negative bacteria [3]. The research results prove that egg white lysozyme thermal modification can increased the antibacterial spectrum mainly on gram-negative bacteria *E. coli*.

3.5 Protein Fraction Profiles of HEWL Thermal Modified

Based on the calculation of molecular weight is known that the different temperature treatment did not cause differences in protein bands were obtained. It is the same with protein bands that appeared on the previous treatment of the type and concentration of salt.

Profil of the protein fractions of thermal modification can be seen in Figure 1.

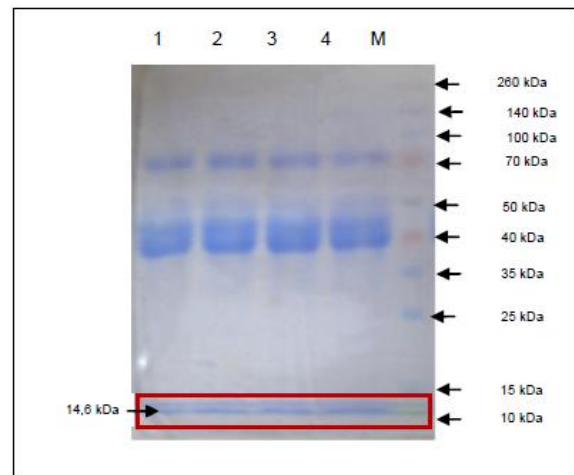


Figure 1. Protein Fraction Profiles of HEWL Thermal Modified
M = Marker, 1 = Control, 2 = 40 °C, 3 = 45 °C, 4 = 50 °C

Figure 1 shows that there are 3 protein fraction having a molecular weight of 78.5 kDa, 54.8 kDa and a target protein with a molecular weight of 14.6 kDa. The emergence of non lysozyme protein polymer forms as a result of lysozyme [7]. Extraction technique with silica (SiO₂), which modified the thermal not cause loss of protein lysozyme. Lysozyme molecule consists of four disulfide bonds (S - S), which is why lysozyme is stable at high temperatures, along with the six-part helix [8].

4. Conclusion

Thermal modification with different temperature treatment gave significant effect ($P < 0.05$) to increase the antibacterial spectrum of egg white lysozyme. Temperature treatment of 50 °C for 20 minutes on the egg white lysozyme extract yield is 12.92% the highest inhibition against gram-negative bacteria *E. coli* with a concentration of 1.18 mg / ml.

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6. References

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