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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 method used is the experiment with completely randomized design (CRD) that treatment 40^{o} C, 45^{o}C, and 50^{o}C for 20 minutes and control each was repeated 3 times. Observed variables include lysozyme activity, lysozyme recovery and specific activity of lysozyme on *M.lysodeikticus* and *E. coli*, MIC and molecular weight protein fractions. The results showed that 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^{o} 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.

KEYWORDS

Hen egg white lysozyme;
Thermal modified;
Lysozyme activity.

INTRODUCTION

Lysozyme is a safe food preservative ingredient[6]. Lysozyme is used for food preservation because of its ability as an antibacterial[12].

The main 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[11]. Modification is expected to enhance the antibacterial spectrum of lysozyme against gram-negative bacteria.

MATERIALS AND METHODS

Material

The materials used in this study include: Hen Egg White 1 year old, 1 N acetic acid (Merck), NaCl (Merck), KCL (Merck), NH_4Cl (Merck), Buffer Phosphate (Na_2HPO_4 0.1 N) (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 Coomasie 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 NaH_2PO_4 (Merck) and Na_2HPO_4 (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 (H_3PO_4) (Merck).

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, micropippet 10μl - 1000 mL (Hamilton syringe), blue tip, yellow tip, cuvette, UV-2100 spectrophotometer (Unico), *nano drop* spectrophotometer (ND-1000).

Methode 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 distirer for 15 minutes. Silica (SiO2) added as much as 0.851 g and distirer for 10 minutes then add in 20 ml phosphate buffer (NaH_2PO_4) and distirer for 5 minutes. Then left overnight at 4 ° C, after which distirer for 5 minutes. Speed centrifuge with 6000 rpm at 4 ° C for 20 min, then the supernatant was taken for analysis[14].

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 40^0 C, 45^0 C and 50^0 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].

Method of determining lysozyme activity

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 dispektrofotometer 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].

Method of determining specific activity of lysozyme

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

Method of determining lysozyme recovery

Lysozyme recovery is defined as the amount of enzyme lysozyme obtained after the extraction process[2]:

$$\text{Lysozyme recovery (\%)} = \frac{\text{activity of lysozyme after Extraction}}{\text{lysozyme activity before extraction}} \text{X100}$$

Method of determining *MIC*

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

Method of determining molecular weight protein fraction

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

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 (UJBD)[16]. Data calculations performed with Excel 2007 Microssoft program, and data analysis performed using SPSS version 16.0.

RESULT AND DISCUSSION

Effect of HEWL thermal modification on *M.lysodeikticus*

Results of analysis of variance showed that HEWL modified by thermal treatment temperature of 40^{o} C, 45^{o} C and 50^{o} 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 and the difference presented in Figure 1.

TABLE 1 and Figure 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>.05). Highest average activity of lysozyme obtained from 40oC treatment amounting 52,680,475.76 U / min. There was a decrease of 19% lysozyme recovery at 450C temperature rise towards 500C. Highest average specific activity of lysozyme was also obtained from the 40oC treatment amounting 4,728,947.55 U / mg and decreases with increasing temperature treatment.

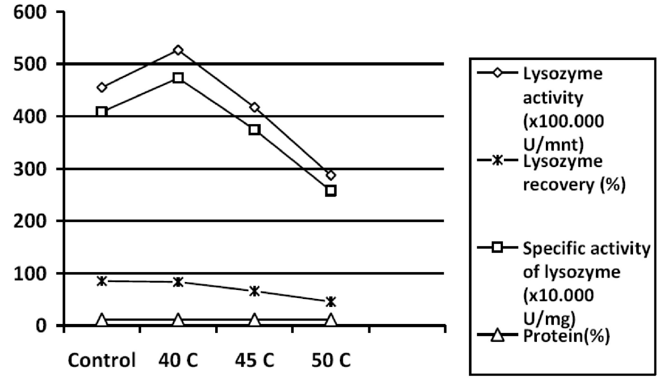

Figure 1 : Comparison of mean effect of thermal modification on lysozyme activity, recovery lysozyme, specific activity of lysozyme on *M.lysodeikticus.*

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 its activity against gram-negative bacteria[3,4,9].

MIC of HEWL thermal modification on *M.lysodeikticus*

Inhibition of *M.lysodeikticus* greatest obtained from 40^{o} 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 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^{0} C temperature treatment is 1.18 mg / ml to produce inhibition percentage of 92.64%[8]. showed that 0.5 mg / ml

TABLE 1 : Mean effect of thermal modification on lysozyme activity, recovery lysozyme, lysozyme specific activities on *M.lysodeikticus.*

Treatments	Means			
	Lysozyme Activity (U/menit)	Lysozyme Recovery (%)	Specific Activity of Lysozyme (U/mg)	Protein (mg/ml)
Control	$45504688,16^{a}$	$85,02^{r}$	$4084801,45^{n}$	$11,14^{v}$
40^{0} C	$52680475,76^{a}$	$83,47^{r}$	$4728947,55^{n}$	$11,14^{v}$
45^{0} C	$41737646,92^{a}$	$66,13^{r}$	$3746646,94^{n}$	$11,14^{v}$
50^{0} C	$28723013,02^{a}$	$45,51^{r}$	$2578367,42^{n}$	$11,14^{v}$

Description: The same subscript in the same column indicates no significant difference (P> 0.05); Different subscripts in the same row are not defined as the difference

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TABLE 2 : MIC of HEWL thermal modification on *M.lysodeikcticus*

Treatment	Ecxtract Volume (ml)	Culture of bacteria (ml)	Vagetable pepton (ml)	End Volume (ml)	Ecxtract Concentration (mg/ml)	The number of Colonies (Cfu/ml)		Inhibition (%)
						Early	24hours contact	
	0,2	1	3,8	5	0,12	$4,8 \times 10^4$	$1,6 \times 10^4$	66,56
	0,5	1	3,5	5	0,29	$2,1 \times 10^5$	TBUD	TBUD
40°C	1	1	3	5	0,59	$3,9 \times 10^4$	$6,9 \times 10^3$	82,08
	1,5	1	2,5	5	0,88	$3,3 \times 10^4$	$4,8 \times 10^3$	85,38
	2	1	2	5	1,18	$2,7 \times 10^4$	$2,0 \times 10^3$	92,64*

Description. * : MIC, TBUD : not determined

concentration of lysozyme modified thermal extract could inhibit 32% of gram-positive bacteria M.lysodeicticus. 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, fenilaldehid, the lysozyme molecule undergoes a conformational change and increased antimicrobial activity against both gram-positive and gram-negative bacteria[3].

Effect of HEWL thermal modification on *E.coli*

Based on the analysis of variance is known that HEWL modified by thermal treatment temperature of 40oC, 45oC and 50oC 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 and the comparison reratanya presented in Figure 2.

Based on TABLE 3 and Figure 2 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

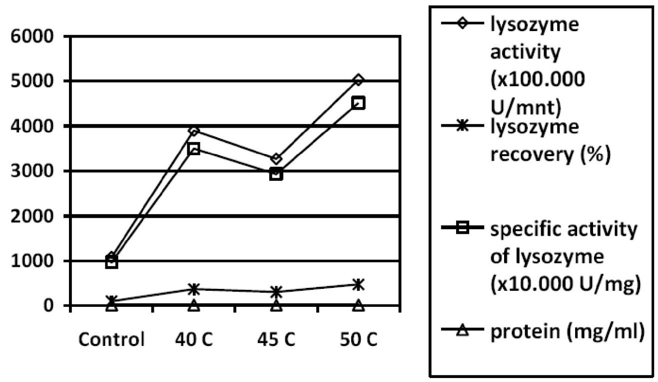


Figure 2 : Comparison of mean effect of thermal modification on lysozyme activity, recovery lysozyme, specific activity of lysozyme on *E.coli.*

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 50oC[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

TABLE 3 : Mean effect of thermal modification on lysozyme activity, recovery lysozyme, lysozyme specific activities on *E.coli.*

Treatments	Means			
	Lysozyme Activity (U/menit)	Lysozyme Recovery (%)	Specific Activity of Lysozyme (U/mg)	Protein (mg/ml)
Control	$107182907,27^a$	$100,00^r$	$9621445,89^n$	$11,14^v$
40^0 C	$389827113,63^a$	$363,70^r$	$34993457,24^n$	$11,14^v$
45^0 C	$326171705,94^a$	$304,31^r$	$29279327,28^n$	$11,14^v$
50^0 C	$502927624,92^b$	$469,22^s$	$45146106,37^m$	$11,14^v$

Description: Different subscript letters in the same column indicate significantly different (P <0.01); Subscript different figures in the same column indicate significantly different (P <0.05); Different subscripts in the same row are not defined as the difference

27.2% of the total polymer, monomer 72.2% 27.2% dimer and trimer 0% which is irreversible

MIC of HEWL thermal modification on *E.coli*

Based on the test inhibition of E.coli is known that the 500C 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 500C. Results of the determination of minimum inhibitory concentration (MIC) of lysozyme extracts are presented in TABLE 4.

Based on TABLE 4 generally known that inhibition test results lysozyme modified thermal extract against

TABLE 4 : MIC of HEWL thermal modification on *E.coli*

Treatment	Ecxtract Volume (ml)	Culture of Bacteria (ml)	Vagetable Pepton (ml)	End Volume (ml)	Ecxtract Concentration (mg/ml)	The number of Colonies (Cfu/ml)		Inhibition (%)
						Early	24hours contact	
	0,2	1	3,8	5	0,12	$2,8 \times 10^5$	$2,7 \times 10^5$	5,11
	0,5	1	3,5	5	0,29	$3,0 \times 10^5$	$2,8 \times 10^5$	7,14
50°C	1	1	3	5	0,59	$3,0 \times 10^5$	$2,9 \times 10^5$	7,76
	1,5	1	2,5	5	0,88	$3,1 \times 10^5$	$2,8 \times 10^5$	9,72
	2	1	2	5	1,18	$3,1 \times 10^5$	$2,7 \times 10^5$	12,92

Desctiption : no one reached ≥ 90% inhibition

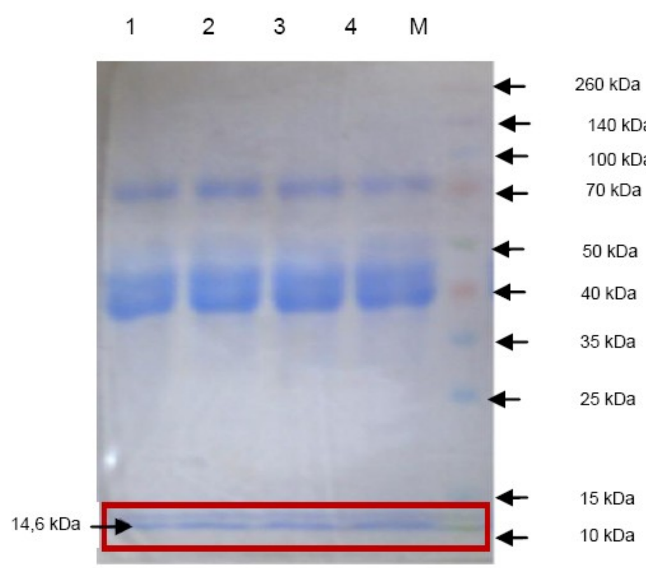
E. coli has not yet reached the MIC (≤ 90%). Percentage inhibition of E. coli will be higher with increasing concentration of lysozyme extracts. Highest inhibition results obtained from extracts of lysozyme treatment 50oC temperature with a concentration of 1.18 mg / ml is equal to 12.92%.

Antimicrobial activity of lysozyme can be converted into active against gram-negative bacteria through genetic hydrophobic peptide C terminal to lysozyme[3]. Bakteriolitic 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 increase the antibacterial spectrum mainly on gram-negative bacteria *E.coli*.

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 protein fractions garam. Profil thermal modification can be seen in Figure 3.

Figure 3 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].



M = Marker, 1 = treatment 0°C, 2 = treatment 40°C, 3 = treatment 45°C, 4 = treatment 50°C

Figure 3 : Protein fraction profiles of HEWL thermal modified

CONCLUSION

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 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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