Antibacterial Activity and RP-HPLC Characteristic of Lysozyme from Local Chicken Egg White after Modification Treatments

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Abstract: Lysozyme is a globular protein and is a hydrolase enzyme. Lysozyme isolated from chicken egg whites can damage the membrane of bacteria hence it can be used as an antibacterial. The purpose of the study was to modify, to measure antibacterial activity, and to characterize the lysozyme from local chicken egg whites. The isolate of lysozyme was modified by heat modification treatments at 60°C, 75°C, and 90°C in pH seven buffer solution. The antibacterial activity of lysozyme was measured by the micro-dilution method. The isolate and the modified lysozyme were characterized by reversed phase-high performance liquid chromatography (RP-HPLC). The results showed that heat modification treatments decreased the minimum inhibitory concentration of lysozymes to Gram-positive bacteria up to 2-fold from 6 mg/mL to 3 mg/mL. Heat modification also improved antibacterial spectrum of lysozyme against Gram-negative bacteria. The RP-HPLC chromatograms of modified lysozyme showed the peak of lysozyme at 43.59 ± 0.09 minutes retained time had decreased. It was suggested that any concentration of lysozyme was retained on the different retained times doe to the modification of lysozyme. The characteristic of RP-HPLC had explained the reason for increasing the antibacterial activities of lysozyme after modification treatments.

1 INTRODUCTION

Lysozyme is a hydrolase enzyme in the form of antibacterial protein that can be isolated from the chicken egg white (Gyawali and Ibrahim 2014). The chicken egg white has excellent potential as a source of lysozyme because it contains about 2500-3000 µg/mL of lysozyme and can be obtained from some of the industrial waste (Lesnierowski and Kijowski 2007). Lysozyme can be used directly, combined with antimicrobials on food, also added to edible packaging so that it can improve food safety (Kijowski et al. 2002; Herath et al. 2015).

The food industry had been using lysozyme because it has a stable primary structure at heat and low pH treatments. The mechanism of antibacterial activity of lysozyme occurs through peptidoglycan destruction on bacterial cell wall membrane by active site of the lysozyme, which breaks the glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid (Susanto et al. 2013).

The antibacterial activity of isolate lysozyme in native form is still limited to Gram-positive bacteria, whereas Gram-negative bacteria could be food contaminants (Lesnierowski et al. 2001). Therefore, it is necessary to modify lysozyme to improve its antibacterial activity against Gram-negative bacteria that are more resistant because the bacterial cell wall is coated by lipopolysaccharide (Cegielska-Radziejewska et al. 2008; Susanto et al. 2013). One of simple methods that used to modify lysozyme was heat treatments (Nasution et al. 2018). Besides, after heat modification treatments, the characterization of lysozyme by RP-HPLC used to explained the changed of antibacterial activity of modified lysozyme that have never been reported.

2 MATERIALS AND METHODS

2.1 Lysozyme

Respectively, a local and commercial isolate of lysozyme were obtained from Sentul chicken eggs at

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the Research Institute for Animal Production (Balitnak, Ciawi-Bogor) and commercial chicken eggs wholesaler (Kurnia Jaya, Dramaga-Bogor). Both of lysozyme was isolated with Amberlite FPC3500 resin. The standard lysozyme (L-6876, from chicken egg white, 47,000 unit/mg solid) was obtained from Sigma-Aldrich. The lysozyme sample was prepared in 10 mL of 10 mM potassium phosphate buffer pH seven.

2.2 Modification of Lysozyme

The lysozyme was modified by heat treatment (Carrillo et al. 2018). As much as 6 mg/mL of each isolate lysozyme in 10 mL of 10 mM potassium phosphate buffer pH seven was heated in a water bath in each heat temperature of treatments (60°C, 75°C, and 90°C) for 20 minutes. The solution was placed in the refrigerator (4°C) after having heated.

2.3 Measurement of Antibacterial Activity

Antibacterial activity of lysozyme was measured based on the micro-dilution method concerning on Clinical and Laboratory Standards Institute (CLSI) (Balouiri et al. 2016). As much as 50 µL MHB was put into each 96-well microplate. Then, 50 µL from 6 mg/mL of each isolate lysozyme or modified as initial lysozyme was added. Afterward, the initial lysozyme was diluted four times to the Mueller Hinton Broth (MHB) until it reached 0.38 mg/mL of lysozyme dilution concentration. Before having incubated, it was added 50 µL tested bacteria culture having prepared to each well. The bacteria stock was made in sideways MHA media and incubated at 37°C as long as 24 hours in MHB when it will be utilized. Control as much as 150 µL used was put in separated well as MHB media, tested bacteria, and standard lysozyme solution. Then, a 96-well microplate in covered condition was incubated (37°C) for 24 hours before the MIC of lysozyme being evaluated.

The MIC lysozyme was considered as a concentration that triggers the viewless growth of bacteria in well after growing as long as 24 hours (not turbid and no precipitate). Determining MIC, it was confirmed with a microplate reader at 655 nm. The minimum bactericidal concentration (MBC) was obtained by scratching the MIC result to the MHA in the dish. The not turbid mixture in the well was taken and scratched to MHA in the dish, then incubated (37°C) for 24 hours. MBC of lysozyme was considered as a concentration having no growth of bacteria in media of each scratch.

2.4 The RP-HPLC Characterization

The lysozyme was characterized by RP-HPLC (Carrillo et al. 2014 and Kusumaningtyas et al. 2015). The sample of the isolate or the modified of lysozyme was injected as much as ten µL utilizing Colom C-18, possessing reverse-phase high-performance liquid chromatography (RP-HPLC) system in 215 nm wavelength for 50 minutes (1 mL/minutes speed at 29.3°C). Before being injected, the sample was refined with nylon membrane 0.45 µm. Dissolver A contains 0.37% trifluoroacetic acid in double distilled water, while dissolver B consists of 0.27 % trifluoroacetic acid in acetonitrile grade HPLC. The HPLC system was balanced with 95% solution A (5 minutes), followed by gradient 5-45% of solution A (5 minutes). The concentration of isolate lysozyme was calculated by comparing the peak of local isolate lysozyme to the peak of standard lysozyme.

2.5 Data Analysis

The data was portrayed as the average result ± standard deviation and also analyzed with Microsoft Excel, XLSTAT, and SPSS. The result of the analysis was used to compare the significance of the data.

3 RESULTS AND DISCUSIONS

3.1 Antibacterial Activity of Lysozyme

The research on improving the antibacterial activity of lysozyme by modification of heat on 60°C, 75°C, and 90°C through measurements of MIC value of lysozyme from the local chicken have never been published ultimately. The MIC value was the minimum inhibitory concentration of lysozyme against bacteria or a concentration that does not show bacterial growth in the well (not cloudy and no deposits) after 24 hours of incubation. The results showed that the MIC values of isolate lysozyme were the same as standard lysozyme in all four bacteria. All of modified lysozyme from local chicken could inhibit all bacteria from Gram-positive and Gram-negative but have slightly different in MIC values. The MIC values of isolate and modified lysozyme from local chicken were 3 mg/mL and 6 mg/mL, which were the concentrations of 50% and 100% of the solution of lysozyme tested (Table 1).

Table 1: The minimum inhibitory concentration of lysozyme towards Gram-positive and Gram-negative bacteria.

Lysozyme types and treatments modifications	The minimum inhibitory concentration of lysozyme (mg/mL)			
	Gram-positive bacteria		Gram-negative bacteria	
	Staphylococcus aureus ATCC 25923	*Bacillus* cereus ATCC 10876	*Escherichia* coli ATCC 25922	*Salmonella* Typhimurium ATCC 14028
Standard lysozyme (7)	6 [a]	6[a]	> 6[a]	> 6[a]
Before modification treatments				
Isolate lysozyme (*native*) (7)	6[a]	6[a]	> 6[a]	> 6[a]
After heat modification treatments				
Heat-modified lysozyme 60°C (7)	6[a]	6[a]	6[b]	6[a]
Heat-modified lysozyme 75°C (7)	3[b]	6[a]	6[b]	6[a]
Heat-modified lysozyme 90°C (7)	3[b]	3[b]	6[b]	6[a]

Numbers followed by the same letter were not significantly different (p>0.05) on each bacteria

The improvement of antibacterial activity of lysozyme from local chicken egg white by heat modification at pH seven towards Gram-positive and Gram-negative bacteria can be seen in Table 1. The heat-modified lysozyme at 75°C and 90°C, when compared to standard and isolate lysozyme, can decrease MIC of lysozyme from local chicken egg white against *S.* aureus ATCC 25923, and lysozyme modified by 90°C also decreased MIC of lysozyme against *B.* cereus ATCC 10876, whereas at all heat-modified temperatures, also decreased MIC of lysozyme against *E.* coli ATCC 25922 and *S.* Typhimurium ATCC 14028.

The MIC values of lysozyme in Gram-negative bacteria were still higher than in Gram-positive bacteria. This condition showed that the antibacterial activity of isolate and modified lysozyme was higher in Gram-positive bacteria than Gram-negative bacteria, which were more resistant due to the presence of lipopolysaccharide as protection (Lesnierowski et al. 2001). The results f the improvement of antibacterial activity were in accordance with other research (Carrillo et al. 2014), which showed an increasing in the antibacterial spectrum.

The difference of MIC values at each heat modification at 60°C, 75°C, and 90°C for the two Gram-positive bacteria tested was suggested to be due to changes different in the lysozyme sample. The improvement of antibacterial activity by heat modification occurs because it was triggered by changes in the conformation of lysozyme molecules from the monomers form to dimers or polymers (Lesnierowski et al. 2001; 2004; Carrillo et al. 2014; Vilcacundo et al. 2018). The research conducted by Cegielska-Radziejewska et al. (2008) reported that dimer forms in heat-modified lysozyme make the lysozyme easily attach to lipopolysaccharide in the bacterial membrane of Gram-negative cell due to changes in the composition and hence increased the hydrophobicity of the outside part of lysozyme molecule. The lysozyme attaches membrane will disrupt the electrochemical process and the stability of the lipid bilayer so that it can form holes in the bacterial membrane. Heat modification can increase the antibacterial activity against Gram-negative bacteria through opening the folds of the lysozyme and expanding the lysozyme bonds in the membrane.

This condition was explained by Ibrahim et al. 1996 that the globular structure of lysozyme has been dominanted by hydrophobic amino acids inside the molecule and hydrophilic amino acids outside the molecule. Heat modification causes the denaturation process of the globular structure of lysozyme doe to the two cysteine (Cys) as lysozyme bridges were cut off so that the hydrophobicity of lysozyme increases. Breaking disulfide (Cys64-Cys80 bond) and (Cys76-Cys94 bond) by heat modification causes the tryptophan 62, 63, and 108 (hydrophobic) in the globular structure of lysozyme to be exposed and will come to outside and then contact with bacterial membranes. The denaturation process causes some or all of the globular structure of lysozyme to change the tertiary and secondary lysozyme structures into primary structures. Heat modification will cause the hydrophobicity of the outside part of the lysozyme molecule, and the attach ability of lysozyme to the bacterial membrane increased. This condition was the reason for the improvement of the antibacterial activity of lysozyme against Gram-negative bacteria and still maintains against Gram-positive bacteria.

3.2 RP-HPLC Characteristic of Lysozyme

The characterization of isolate and modified lysozyme by RP-HPLC method aims to see the effect of heat modification treatments on the lysozyme structure from local chicken egg white based on the tertiary structure of lysozyme as globular protein and its relation to the antibacterial activity of isolate and modified of lysozyme. The results showed that the lysozyme was detected on 43.59 ± 0.09 minutes retained time close to researched by Carrillo et al. (2016) that detected the lysozyme on 38 minutes retained time.

Table 2: The concentration of lysozyme.

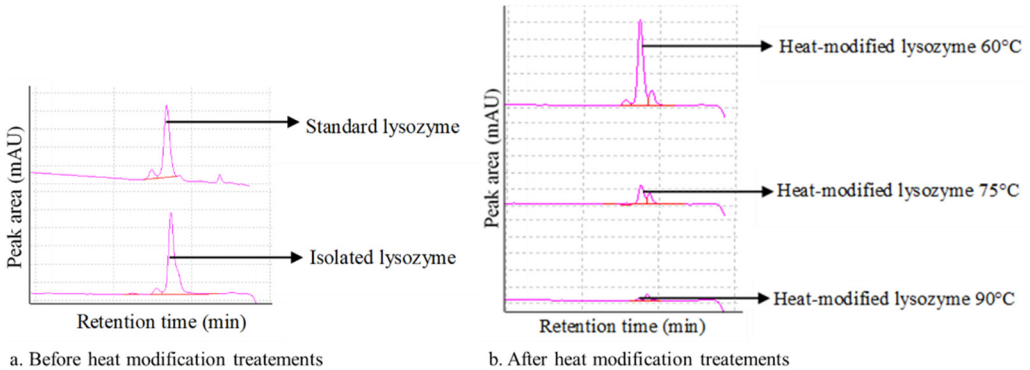
Lysozyme types	Concentration (mg/g)
Before heat modification treatments	
Isolated lysozyme	41.07 ± 4.44[a]
After heat modification treatments	
Heat-modified lysozyme 60°C	38.87 ± 4.20[ab]
Heat-modified lysozyme 75°C	8.53 ± 0.92[c]
Heat-modified lysozyme 90°C	0.99 ± 0.11[c]

Numbers followed by the same letter were not significantly different ($p>0.05$) among the isolate and modified lysozyme.

The modification decreased the concentration of lysozyme as globular proteins detected in lysozyme from local chicken egg white, which was calculated based on comparison with standard lysozyme (Table 2). The reduction of isolated lysozyme concentration detected at 215 nm wavelength indicates that isolated lysozyme from local chicken egg white was not as same as the concentration of standard lysozyme. The increasing of heat temperature makes lowering the concentration of lysozyme. The highest concentration of isolate lysozyme was found in lysozyme without heat modification treatments compare to heat-modified lysozymes.

The reduction of concentration was suggested because by the structure of lysozyme from local chicken egg white underwent reactions in running process through the RP-HPLC column such as denaturation due to the modifications that caused changes the lysozyme so that only some concentration of lysozyme was detected at 43.59 ± 0.09 minutes retained time as same with the standard lysozyme. Some concentration of lysozyme from local chicken egg white was thought to undergo dimerization at an oven temperature of 29.3°C during running RP-HPLC because dimer conformation can also be formed between 20°C to 30°C as the reversible character of lysozyme at (Cegielska-Radziejewska et al. 2008).

Based on Table 2, although the detected lysozyme concentration was reduced, it is evident in the lysozyme sample that there were other proteins of modified lysozyme, which were the reason for the increasing of antibacterial activity of lysozyme after modification. That protein might be detected at other retention times. Research Cosentino et al. (2015) showed that the concentration of lysozyme in the type of milk detected by the HPLC method still had the same concentration after pasteurization (63° C) for 30 minutes. This condition showed that although the modification of lysozyme heat in this study caused the lysozyme concentration to decrease, the concentration of lysozyme contained in the food system can survive after heat treatment.



a. Before heat modification treatements b. After heat modification treatements

Figure 1: The profile of RP-HPLC chromatograms of lysozyme.

The heat modification to the lysozyme showed that decreasing the peak of lysozyme on (Figure 2). The increasing of heat temperature makes the peak of lysozyme to be lower. This condition showed that the structure of lysozyme was changed that changing the composition of amino acid of lysozyme from some of the concentrations of lysozymes. So in the highest heat temperature of modification treatments made the peak of lysozyme was the lowest among others. Besides, column C-18 in RP-HPLC will attach the modified lysozyme based on the hydrophobicity of modified lysozymes. This condition showed that the heat modification treatments can decrease the concentration of lysozyme from the local chicken egg white and change the hydrophobicity of modified lysozymes.

4 CONCLUSIONS

The lysozyme from local chicken egg white was modified by heat modification treatments. The heat modification treatments improved antibacterial activity of lysozyme by decreasing the MIC value. Otherwise, the isolate and the modified lysozyme were characterized by RP-HPLC, and the RP-HPLC chromatograms explaining the reason for the increasing of antibacterial activity of lysozyme after modification treatments.

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