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Enzymatic and Antibacterial Activity of Low-pH and Pepsin-Modified Freeze-Dried Lysozyme from Local Chicken Egg White

Syahrizal Nasution[1)]*, Eni Kusumaningtyas[2)], Didah Nur Faridah[3)], Zakiah Wulandari[4)], Harsi Dewantari Kusumaningrum[3)]

[1)] Department of Food Technology, Sumatera Institute of Technology, Lampung Selatan, Indonesia
[2)] Research Center for Veterinary Science, National Research and Innovation Agency, Cibinong Science Center, Bogor, Indonesia
[3)] Division of Food Science and Technology, Faculty of Engineering and Technology, IPB University, Bogor, Indonesia
[4)] Department of Animal Production and Technology, Faculty of Animal Science, IPB University, Bogor, Indonesia

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ABSTRACT

The research aimed to isolate and modify lysozyme from local chicken egg white to improve the antibacterial activity of lysozyme hydrolysates, and to characterize the isolated and modified lysozyme. Lysozyme was isolated from local chicken egg white using an ion-exchange resin. The lysozyme isolate was then modified with pepsin in a low-pH solution, and the pepsin-modified samples were neutralized to pH 7 before testing. The enzymatic activity of lysozyme was measured spectro-photometrically using *Micrococcus lysodeikticus*. The antibacterial activity of lysozyme was tested using the microdilution method. The lysozyme was characterized by SDS-PAGE and RP-HPLC. The yield of lysozyme isolate was 5.35±0.23% (n= 3) with a lysozyme concentration of 41.07±4.44 mg/g (n= 3). After dialysis, the lysozyme concentration was 143.24±8.32 mg/g (n= 3). The enzymatic activity of the lysozyme isolate was 4,778±1,835 unit/mg (n= 3). The modification of lysozyme improved its antibacterial activity by decreasing its MIC value from 6 mg/mL (or >6 mg/mL) to 3 mg/mL and increasing its antibacterial spectrum to include *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Escherichia coli*. However, all low-pH treatments against *E. coli* reduced the MIC value only from >6 mg/mL to 6 mg/mL. The SDS-PAGE and RP-HPLC profiles of the lysozyme isolate showed that it had the same molecular weight (MW) and retention time (RT) as the standard lysozyme, with values of approximately 14.70±0.43 kDa (n= 3) and 43.59±0.09 min (n= 3), respectively. The modification of lysozyme also affected its enzymatic activity and SDS-PAGE and RP-HPLC profiles, explaining why the antibacterial activity increased.

Keywords: antibacterial, egg white, enzyme activity, lysozyme, MIC

INTRODUCTION

Lysozyme is a protein derived from egg white and is a hydrolase enzyme commercially used in the food industry. Lysozyme is classified as an in ovo antimicrobial, which is non-toxic, so it was recognized as a food additive with generally recognized as safe (GRAS) status. Lysozyme can be used directly, in combination with other antimicrobials, and added to edible food packaging. Lysozyme has been investigated for its use in foods such as cheese, vegetables, meat, fish, and milk to improve food safety. In addition, lysozyme can be used as a feed additive in livestock (Biswas *et al.,* 2016; Kusumaningtyas *et al.,* 2025).

Lysozyme can be used as an antibacterial agent because it degrades peptidoglycan in the cell walls of Gram-positive bacteria. The antibacterial activity of lysozyme is limited to Gram-positive bacteria, even though food contaminants can originate from Gram-negative bacteria (Nasution *et al.,* 2018). Therefore, it is necessary to modify lysozyme to enhance its antibacterial activity and enable it to damage or kill Gram-negative bacteria, which are more resistant due to their cell membranes coated with lipopolysaccharide.

Chicken egg white is known as one of the richest sources of lysozyme. In Indonesia, chicken egg white as a source of lysozyme can be obtained from both commercial and local chicken eggs. Even though free-range chicken eggs are more expensive than commercial chicken eggs due to production costs, demand for these eggs remains high because they are believed to be healthier. The yolk of local chicken eggs is often used in local drinks, while the egg white is discarded; therefore, using local chicken egg white as a source of lysozyme can add value to egg waste as a poultry byproduct.

*Corresponding Author: E-mail: syahrizal.nasution@tp.itera.ac.id

Indonesian local chicken eggs were chosen because their egg white is presumed to contain higher levels of hydrophobic amino acids than that of commercial chicken eggs, which may enhance antibacterial activity against Gram-negative bacteria. Hydrophobic amino acids in lysozyme facilitate interactions with the outer membrane of Gram-negative bacteria, thereby enhancing membrane disruption and antibacterial efficacy (Wulandari, 2018). This study was based on the hypothesis that hydrolysis treatment could generate bioactive peptides with enhanced antibacterial properties by increasing the exposure of hydrophobic and positively charged amino acids. Previous research by Carrillo and Ramos (2018) showed that the antibacterial activity of lysozyme-derived peptides increased when dominated by hydrophobic and positively charged amino acids, such as lysine, arginine, tryptophan, and tyrosine. Sentul chicken eggs were selected to obtain a more uniform lysozyme composition, as their egg white characteristics were expected to support the formation of antibacterial hydrolyzed peptides.

Although some studies have focused on lysozyme modification (Nasution *et al.,* 2020; Nasution *et al.,* 2021), studies covering lysozyme isolation, modification, and characterization using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC), including measurements of lysozyme's enzymatic and antibacterial activities, have not been fully reported. In addition, this research uses low-pH modification as a simple and inexpensive method, compared with enzymatic and other modification methods, to enhance the antibacterial activity of the lysozyme isolate (Nasution *et al.,* 2018). Importantly, isolating lysozyme from local chicken eggs, combined with pepsin and pH treatment, is critical for enhancing its activity and broadening its antimicrobial spectrum. Such modification strategies are expected to improve both enzymatic performance and antibacterial effectiveness. Previous studies have also demonstrated that protein hydrolysates exhibit improved functional properties (Kwon *et al.*, 2017; Lee *et al.*, 2019; Rathnapala *et al.*, 2021). Therefore, this research aimed to isolate and modify lysozyme from local chicken egg white, evaluate the effects of pepsin and low-pH treatment on its antibacterial activity, and characterize the modified lysozyme using SDS-PAGE and RP-HPLC.

MATERIALS AND METHOD

Materials

The local eggs were Sentul chicken eggs obtained from the Indonesian Poultry and Miscellaneous Livestock Assembly and Testing Centre (Ciawi, Bogor), and the commercial chicken eggs were obtained from the egg wholesaler (Kurnia Jaya, Bogor). The physicochemical characteristics of the eggs used in this study are shown in Table 1. Both chicken egg types were 1–2 days old. The eggs were cleaned before use.

Table 1. The characteristics of egg white from local and commercial chicken eggs

The Characteristics of Egg White	CET	
	LCE	CCE
Dry weight (g/egg, n= 3)	4.93±0.00[a]	5.47±0.00[b]
Volume (mL/egg, n= 20)	26.75±3.26[a]	34.70±2.67[b]
Specific gravity (g/mL, n= 20)	0.95±0.07[a]	0.99±0.02[a]
Water content (%, n= 3)	86.86±2.17[a]	88.58±0.93[b]
Protein concentration (mg/mL, n= 3)	1.54±0.02[a]	1.74±1.44[a]
pH (n= 3)	8.76±0.07[a]	8.99±0.04[b]

Note: The data are presented as means ± standard deviation. The values followed by the same letters are not significantly different (p>0.05) between the two types of eggs. CET= chicken egg types, LCE= local chicken egg, CCE= commercial chicken egg

Isolation of lysozyme

Lysozyme was isolated with an Amberlite FPC3500 ion-exchange resin (styrene-divinylbenzene, 0.3–1.18 mm, >2.6 mEq/mL; Thermo Scientific Acros Organics, Thermo Fisher Scientific, France) in both preparative and batch systems (Abeyrathne *et al.,* 2014; Lesnierowski and Stangierski, 2018). A total of 250 mL of local chicken egg white was mixed with 250 mL of sterile distilled water (1:1, v/v) and homogenized using a magnetic stirrer (Nuova II, speed scale 4, 4 °C, 10 min). Then, 25 g of resin was added to the previous mixture (the resin was equilibrated in 250 mL of 0.1 M glycine-NaOH buffer, pH 10, before use). The mixture was then stirred with a magnetic stirrer (speed scale 4, 4 °C, ±18 h). The mixture was centrifuged using a Centra MP4 refrigerated centrifuge (Thermo Fisher Scientific, USA) (3400 × *g*, 4 °C, 15 min). Resin pellet-bound lysozyme was separated from the supernatant by 100 mL of sterile distilled water and rinsed 9 times with 100 mL of 0.1 M glycine-NaOH buffer (pH 9.3). Each rinsing and lysozyme release step was conducted in the Schott bottle and mixed with a magnetic stirrer (speed scale 4, 4 °C, 3 min). Afterwards, a 0.1 M glycine-NaOH buffer containing 0.1 M NaCl (pH 9.3) was used to rinse the resin to release lysozyme. The solution containing the lysozyme isolate was confirmed with SDS-PAGE. Once confirmed, the lysozyme solution was freeze-dried, weighed to the desired mass, modified, tested for enzymatic and antibacterial activity, and characterized by SDS-PAGE and RP-HPLC.

The isolated lysozyme solution was dialyzed using a Viva Flow 50R 5000 MWCO Hydrosart membrane (Sartorius, Germany), a peristaltic pump (2.5 bar, 25 °C, 5 h), and double-distilled water (1:5, v/v) (Wulandari, 2018). The dialyzed lysozyme was freeze-dried, weighed to determine its yield, and characterized by SDS-PAGE and RP-HPLC (Shimadzu, Japan).

Each lysozyme yield, both dialyzed and non-dialyzed, was weighed after freeze-drying. The weight of lysozyme obtained was divided by 250 mL of egg white to obtain the amount of lysozyme per mL of egg white. The percentage yield of lysozyme was calculated by dividing the yield weight of lysozyme by the specific gravity of egg white, then multiplying by 100%.

Modification of lysozyme by pepsin treatment

Pepsin modification of lysozyme was carried out following the method of Memarpoor-Yazdi *et al.* (2012). A lysozyme solution (6 mg/mL), prepared in 10 mL of 10 mM potassium phosphate buffer at pH 1.5, was mixed with 1 mL of a pepsin solution (1.2 mg/mL), also prepared in 10 mM potassium phosphate buffer. The mixture was incubated in an incubator (Yamato Scientific Co, Japan, at 37 or 52 °C) for 1 h, and the enzymatic reaction was stopped by heating (80 °C) for 15 min. After completing the modification, the lysozyme solution was put into the refrigerator (4 °C). The modified lysozyme solution was tested for enzymatic and antibacterial activities and characterized by SDS-PAGE and RP-HPLC.

Modification of freeze-dried lysozyme by low-pH treatment

The low-pH modification followed the same procedure as the pepsin modification, except that pepsin was not used. This modification used 10 mL of 10 mM potassium phosphate buffer (pH 1.5), prepared with a pH meter (Laqua PH1100, Horiba Scientific, Japan). The mixture was incubated in an incubator (at 37 or 52 °C) for 1 h, and the enzymatic reaction was stopped by heating (80 °C) for 15 min. After modification treatments were completed, the lysozyme solution was put into the refrigerator (4 °C). The modified lysozyme solution was tested for enzymatic and antibacterial activities and characterized by SDS-PAGE and RP-HPLC.

Measurement of freeze-dried lysozyme enzymatic activity

Lysozyme was characterized by enzymatic activity referring to the Shugar method (Carrillo and Ramos, 2018). *Micrococcus lysodeikticus* ATCC 4698 was used as the substrate for enzymatic activity. A substrate suspension (0.015%) was prepared by mixing 0.15 mg/mL of dried *M. lysodeikticus* ATCC 4698 in 50 mM potassium phosphate buffer (pH 6.24) at 25 °C. The substrate was adjusted to read 0.6–0.7 against a blank at 450 nm. A 0.7 mg/mL lysozyme solution was prepared in 50 mM potassium phosphate buffer (pH 6.24, 8 °C). 1.5 mL of the substrate and 0.1 mL of the lysozyme solution were put into a cuvette. In another cuvette containing the substrate, 0.10 mL of buffer was added as a blank. Samples in the cuvette were homogenized and measured at 450 nm using a spectrophotometer (UV Mini 1240, Shimadzu, Japan) for 5 min. One unit of lysozyme was defined as a 0.001 decrease in absorbance per min. The enzymatic activity of lysozyme can be calculated using Equation 1.

$$EA = \frac{\left(\frac{\Delta A_{450}}{\text{min}}\text{sample} - \frac{\Delta A_{450}}{\text{min}}\text{blank}\right)}{\text{lysozyme (mg/mL)}} \frac{\left(\frac{\text{unit}}{\text{mL}}\right)}{0.001 \times 0.1} \quad \ldots\ldots\ldots (1)$$

EA = Enzymatic activity of lysozyme (unit/mg)

The bacterial strains tested

The antibacterial activity of lysozyme was measured using the microdilution method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (Balouiri *et al.,* 2016). A total of 50 *µ*L of Mueller Hinton Broth (MHB) was added to each well of a microplate. Then, 50 *µ*L of a 6 mg/mL solution of isolated or modified lysozyme was added as the initial lysozyme solution (100%). The pepsin-modified and low-pH-modified lysozymes were tested in two forms: direct (pH 1.5) and neutralized (pH 7). Furthermore, the initial lysozyme solution was diluted 4-fold in MHB to a final concentration of 0.38 mg/mL.

This research used Gram-positive bacteria (*Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Salmonella* Typhimurium ATCC 14028 and *Escherichia coli* ATCC 25922) to test the antibacterial activity of lysozyme. Before incubation, 50 *µ*L of each bacterial culture was added to separate wells (the bacterial stock was prepared in Mueller Hinton Agar (MHA) and incubated at 37 °C for 24 h, then transferred to MHB and incubated for an additional 24 h at 37 °C). 150 *µ*L of control solutions (MHB, tested bacteria, and standard lysozyme solutions) were also put into separate wells in the microplate. The 96-well microplate was closed and incubated (37 °C) for 24 h before the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lysozyme were determined.

Determination of MIC and MBC of lysozyme

The MIC of lysozyme was defined as the concentration that resulted in no visible bacterial growth in each well after 24 h of incubation (no cloudiness or deposits). The MIC was determined

using a microplate reader (Bio-Rad, USA, 655 nm). The MBC was determined by scratching the MIC results onto the MHA in a Petri dish. The non-turbid mixture of lysozyme and medium was taken from the wells and etched onto the MHA, then incubated (37 °C) for 24 h. The MBC of lysozyme was determined as the concentration at which no growth was observed on the medium.

Characterization of freeze-dried lysozyme by SDS-PAGE

Lysozyme was characterized with SDS-PAGE, following the Laemmli method (Carrillo *et al.,* 2014). The electrophoresis gel consisted of 12.5% separating gel and 4% stacking gel. The sample was dissolved in 10 mM potassium phosphate buffer (1:2 w/v). Then, it was heated (95 °C) for 5 min. 50 mL of isolated or modified lysozyme was treated by running the electrophoresis equipment for 3.5 h at 60 V. After reaching the lower limit, the gel was soaked in a fixation solution, cleaned with a washing solution, rinsed with double-distilled water, soaked in a sensitizing solution, washed with double-distilled water, stained with silver nitrate, washed with double-distilled water, soaked in developing solution, added to a stop solution, and finally cleaned with double-distilled water. The resulting gel was scanned to measure lysozyme molecular weight by comparing lysozyme bands with the marker and standard lysozyme bands. The purity was calculated by comparing the lysozyme bands with the total number of bands in the gel using ImageJ 1.52 software (National Institutes of Health, Bethesda, MD, USA).

Characterization of freeze-dried lysozyme by RP-HPLC

Lysozyme was characterized using LC-20A RP-HPLC with a Diode Array detector (Shimadzu, Japan), and a Zorbax Eclipse C-18 column (particle size 5 *μ*m, inner diameter 4.6 mm, column length 150 mm) (Carrillo *et al.,* 2014; Kusumaningtyas *et al.,* 2016). 10 *μ*L of isolated or modified lysozyme was injected into the C-18 column using an RP-HPLC system at a flow rate of 1 mL/min for 50 min, with an oven temperature of 29.3 °C and detection at 215 nm. Before injection, the sample was filtered with a 0.45 *μ*m nylon membrane. Solvent A was 0.37% trifluoroacetic acid in double-distilled water, while solvent B was 0.27% trifluoroacetic acid in acetonitrile. The HPLC system was balanced with 95% A solvent (5 min), followed by a gradient from 5% to 45% B solvent (20 min), and then rebalanced with 95% A solvent (5 min). The concentration of isolated and modified lysozyme was measured by comparing the peak area of lysozyme isolation to the peak area of the lysozyme standard. The concentration of peptides was measured by comparing the peak area at each retention time (RT) of the peptide to the peak area of the lysozyme isolate or to the total peak area on the chromatogram, and multiplying by 100% to obtain the percentage.

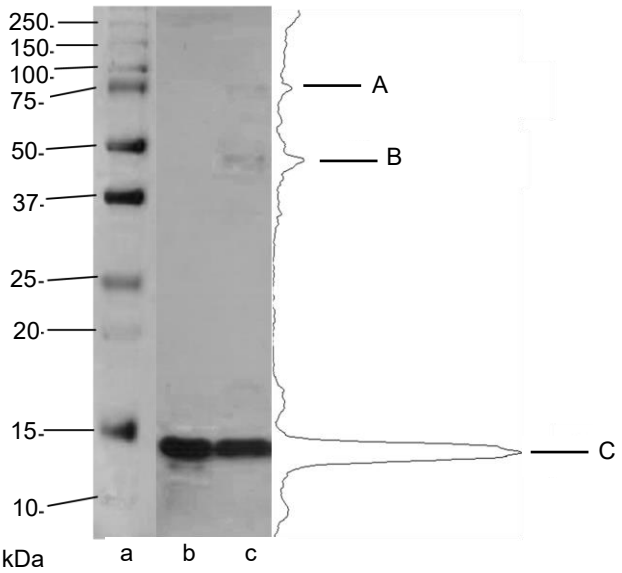
Data analysis

The isolation and modification process was performed in triplicate to obtain the isolated and modified lysozyme. The collected data were analysed using one-way ANOVA and then displayed as mean ± standard deviation. The analysis results were used to compare the significance of the data.

RESULTS AND DISCUSSION

Isolated freeze-dried lysozyme from local chicken egg white

The isolated lysozyme that was confirmed by SDS-PAGE showed a molecular weight of approximately 14.70±0.43 kDa, which was the same as the standard lysozyme (a) and the protein marker (b) (Figure 1). The SDS-PAGE profile showed that the isolated lysozyme was not as pure as the standard lysozyme because there were still thin ovalbumin (45.25±1.33 kDa) and ovotransferrin (74.58±2.19 kDa) bands. Both ovalbumin and ovotransferrin bands appear likely, given that they are the most abundant proteins in egg white (Table 2), and may still have been physically bound to the resin during the isolation process.



Note: a= marker 10–250 kDa, b= standard of lysozyme, c= isolated lysozyme, A= ovotransferrin, B= ovalbumin, C= lysozyme

Figure 1. The SDS-PAGE profile of isolated lysozyme from local chicken eggs

The molecular weights of all studied chicken egg white proteins fell within the range of the protein marker, 10–250 kDa (Figure 1), except for ovostatin (approximately 780.00 kDa), which had a molecular weight above the marker. Some proteins with very low concentrations, less than that of the lysozyme, did not appear as in the reference (Table 2). Based on the protein bands in the SDS-PAGE profile, the isolated lysozyme can be considered sufficiently pure due to its minimal presence of ovalbumin and ovotransferrin (Figure 1). In addition, ImageJ confirmed that the isolated lysozyme had 93% purity by comparing the detected bands (Figure 1). This purity level was higher than that reported in Wulandari's research (Wulandari, 2018), which was only 68.62%, and was similar to the findings of Abeyrathne *et al.* (2013), which reached 97%. The dry weight of the lysozyme studied was similar to that obtained from commercial chicken egg white, which was isolated with the same resin (Table 3). The lysozyme obtained from local chicken egg white after being dialyzed was approximately 1.79±0.04 g from 250 mL of egg white (7.18±1.63 mg/mL), which was almost double the yield reported in previous study (Emami *et al.,* 2018), in which 0.96 g was obtained from 250 mL of egg white (3.84 mg/mL), using a membrane twice the size of the MWCO 5000 membrane in this study.

The weight of lysozyme was lower after dialysis compared to lysozyme without dialysis (Table 3). This was because the dialysis process, using a 5000 MWCO Hydrosart membrane, removed all components except lysozyme. In the dialysis process, all components with a molecular weight of less than 5000 Da (5 kDa) in the dissolved 10 mM potassium phosphate buffer were separated from lysozyme, which has a molecular weight of 14700 Da (14.70±0.43 kDa). Nevertheless, lysozyme can be used with or without dialysis. Various lysozyme products are available on the market, such as those from Pharma Food International, which contain 50% glycine and 50% lysozyme (Wulandari, 2018), and lysozyme from Sigma with product codes L2879 and L6876, which contain NaCl and NaCOOH. However, other components contained in isolated lysozyme also need to be considered.

Enzymatic activity of freeze-dried lysozyme

The enzymatic activity of lysozyme was less than that of standard lysozyme (Table 4). This was presumably because the concentration of lysozyme with a tertiary structure (globular) was reduced in lysozyme. This condition likely altered the active sites of glutamate 35 and aspartate 52 on the surface of lysozyme, thereby reducing its ability to hydrolyze the substrate. This condition shows that enzymatic activity will be high if lysozyme maintains its globular structure, even though this structure will affect its antibacterial activity (Table 5 and 6). Therefore, it was important to consider the isolation process and the modification that would be used.

Table 2. The molecular weight and composition of local chicken egg white proteins

The Proteins of Egg White	Molecular Weight (kDa)		Composition[3] (%)
	MA[1]	MB[3]	
Ovalbumin	45.25±1.33	45.00	61.60
Ovotransferrin	74.58±2.19	76.00	14.30
Ovomucoid	29.84±0.88	28.00	12.60
Ovomucin	211.23±6.22	210.00	4.00
Lysozyme	14.70±0.43	14.30	3.50
Ovoinhibitor	ND	49.00	1.70
Ovoglobulin G2	ND	47.00[2]	1.00
Ovoglobulin G1	ND	50.00[2]	1.00
Ovostatin	ND	780.00[2]	0.50
Avidin	ND	68.30	0.06
Cystatin	ND	12.70	0.05

Note: The data are presented as mean ± standard deviation from three independent experiments. [1)]The research data, [2)]Steven (1991), [3)]Lesnierowski and Stangierski (2018). ND= not detected, MA= the molecular weight of isolated lysozyme, MB= the molecular weight of lysozyme from references

Table 3. The type of isolated lysozyme from local and commercial chicken eggs

The Weight of Lysozyme	CET			
	LCE		CCE	
	(mg/mL)	(%)	(mg/mL)	(%)
LA1	51.00±2.19[a]	5.35±0.23[a]	40.55±6.24[a]	4.10±0.63[a]
LA2	7.18±1.63[a]	0.19±0.08[a]	9.98±6.45[b]	0.73±0.16[b]

Note: Each value represents the mean ± standard deviation, with a sample size of n= 3. The values followed by the same letter do not differ significantly (p>0.05) between the two types of eggs. LA1= lysozyme (pre-dialysis), LA2= lysozyme (post-dialysis), CET= chicken egg types, LCE= local chicken egg, CCE= commercial chicken egg

Table 4. Enzymatic activity of isolate and modified lysozyme from local chicken egg

Lysozyme Types and Treatments Modifications	Enzymatic Activity (unit/mg)
	LCE
LA	37571±11631[a]
LB	4778±1835[bcd]
LC	3445±2117[cd]
LD	2000±1155[d]
LE	3333±2185[cd]
LF	8889±3686[b]

Note: The data are presented as mean ± standard deviation from three independent experiments. The values followed by the same letter do not differ significantly (p>0.05) among the lysozymes. LA= standard of lysozyme, LB= isolated lysozyme (unmodified), LC= pepsin-modified lysozyme 52 °C, LD= pepsin-modified lysozyme 37 °C, LE= low-pH modified lysozyme 52 °C, LF= low-pH modified lysozyme 37 °C, LCE= local chicken egg

Table 5. The minimum inhibitory concentration (MIC) value of lysozyme from local chicken eggs

Lysozyme Types and Treatments Modifications	The MIC of Lysozyme (mg/mL, n= 3)			
	LCE			
	GP (P1)	GP (P2)	GN (N1)	GN (N2)
LA	6[a]	6[a]	>6[a]	>6[a]
LB	6[a]	6[a]	>6[a]	>6[a]
LC1	3[b]	3[b]	3[b]	3[b]
LC2	3[b]	3[b]	3[b]	3[b]
LD1	3[b]	3[b]	3[b]	3[b]
LD2	3[b]	3[b]	3[b]	3[b]
LE1	3[b]	3[b]	3[b]	6[b]
LE2	3[b]	3[b]	3[b]	6[b]
LF1	3[b]	3[b]	3[b]	6[b]
LF2	3[b]	3[b]	3[b]	6[b]

Note: The values followed by the same letter do not differ significantly (p>0.05) for each bacterium. LA= standard of lysozyme (pH 7), LB= isolated lysozyme (unmodified, pH 7), LC1= pepsin-modified lysozyme, 52 °C + 80 °C (pH 1.5), LC2= pepsin-modified lysozyme, 52 °C + 80 °C (pH 7), LD1= pepsin-modified lysozyme, 37 °C + 80 °C (pH 1.5), LD2= pepsin-modified lysozyme, 37 °C + 80 °C (pH 7), LE1= low-pH modified lysozyme, 52 °C + 80 °C (pH 1.5), LE2= low-pH modified lysozyme, 52 °C + 80 °C (pH 7), LF1= low-pH modified lysozyme, 37 °C + 80 °C (pH 1.5), LF2= low-pH modified lysozyme, 37 °C + 80°C (pH 7), LCE= local chicken egg, GP= Gram-positive bacteria, GN= Gram-negative bacteria, P1= *Bacillus cereus* ATCC 10876, P2= *Staphylococcus aureus* ATCC 25923, N1= *Salmonella* Typhimurium ATCC 14028, N2= *Escherichia coli* ATCC 25922

Table 6. The concentration of isolated and modified lysozyme from local chicken eggs by RP-HPLC

Lysozyme Types and Treatments Modifications	Lysozyme Concentration (mg/g)
	LCE
LB1	41.07±4.44[b]
LB2	143.24±8.32[a]
LC	4.20±0.45[d]
LD	ND
LE	28.41±3.07[c]
LF	ND

Note: Each value represents the mean ± standard deviation, with a sample size of n= 3. The values followed by the same letter are not significantly different (p>0.05) among the isolated and modified lysozymes. LB1= lysozyme (pre-dialysis), LB2= lysozyme (post-dialysis), LC= pepsin-modified lysozyme 52 °C, LD= pepsin-modified lysozyme 37 °C, LE= low-pH modified lysozyme 52 °C, LF= low-pH modified lysozyme 37 °C, LCE= local chicken egg, ND= not detected

All modified lysozymes from local chicken egg white exhibited changes in their enzymatic activity (Table 4). Pepsin and low-pH modification reduced enzymatic activity against *M. lysodeikticus* compared with the unmodified isolated lysozyme. Pepsin modification reduced lysozyme enzymatic activity relative to isolated lysozyme. This is consistent with previous study (Carrillo and Ramos, 2018; Mine e*t al.,* 2004), which reported that pepsin modification of lysozyme at 37 °C for 1 h resulted in the near-loss of lysozyme's enzymatic activity. In addition, other studies (Carrillo *et al.,* 2014; Carrillo *et al.,* 2016) showed that pepsin modification under similar conditions decreased lysozyme enzymatic activity by up to 41.8%. In the pepsin modification, temperature did not significantly affect enzymatic activity.

In contrast to pepsin modification, the significant temperature difference between 52 and 37 °C on low-pH modification changed enzymatic activity. The low-pH modification at 37 °C increased the enzymatic activity of lysozyme, presumably because under low-pH modification at 37 °C, lysozyme does not undergo hydrolysis as much as in pepsin modification, which was different from what occurred at 52 °C. Although the band of lysozyme on the SDS-PAGE profile appears the same at both low-pH modification temperatures (Figure 2), the enzymatic activities were

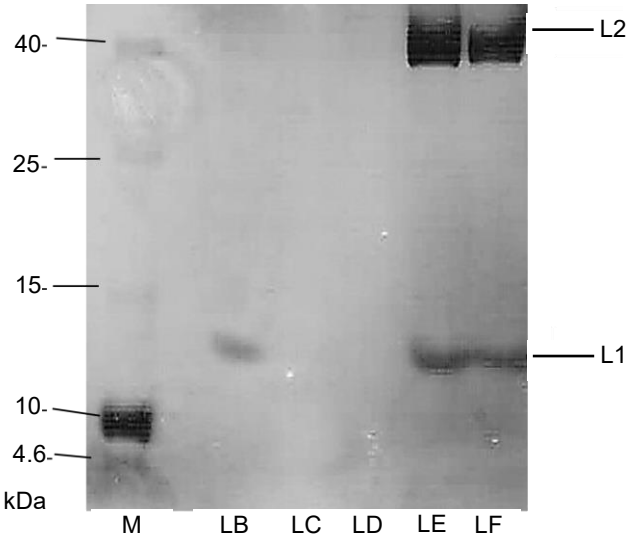
different. Lysozyme at low-pH modification was polymerized (Figure 2) due to the heating process (80 °C for 15 min) after low-pH modification for 60 min. In addition, the different monomers of lysozyme at low-pH modification may have caused this to occur.

Antibacterial activity of freeze-dried lysozyme

The MIC values of isolated and modified lysozyme from local chicken egg white were 3 and 6 mg/mL, respectively. These values represented 50 and 100% concentrations of the lysozyme solution tested (Table 5). The MIC value of isolated lysozyme against all bacteria was the same as that of standard lysozyme, although the standard lysozyme was purer than the isolated lysozyme. This can happen because the SDS-PAGE profile of isolated lysozyme differed from that of the standard lysozyme (Figure 2). The results also showed that all modified lysozymes could inhibit both Gram-positive and Gram-negative bacteria but have slightly different MIC values (Table 5). The modification of lysozyme improved its antibacterial activity by reducing its MIC from 6 mg/mL (or >6 mg/mL) to 3 mg/mL and expanding its antibacterial spectrum to include *B. cereus, S. aureus, S.* Typhimurium, and *E. coli*. However, all low-pH treatments against *E. coli* reduced the MIC value only from >6 mg/mL to 6 mg/mL. In addition, none of the modified lysozymes at a concentration of 6 mg/mL exhibited MBC values. This may have occurred because the lysozyme concentration was still insufficient to stop bacterial growth.

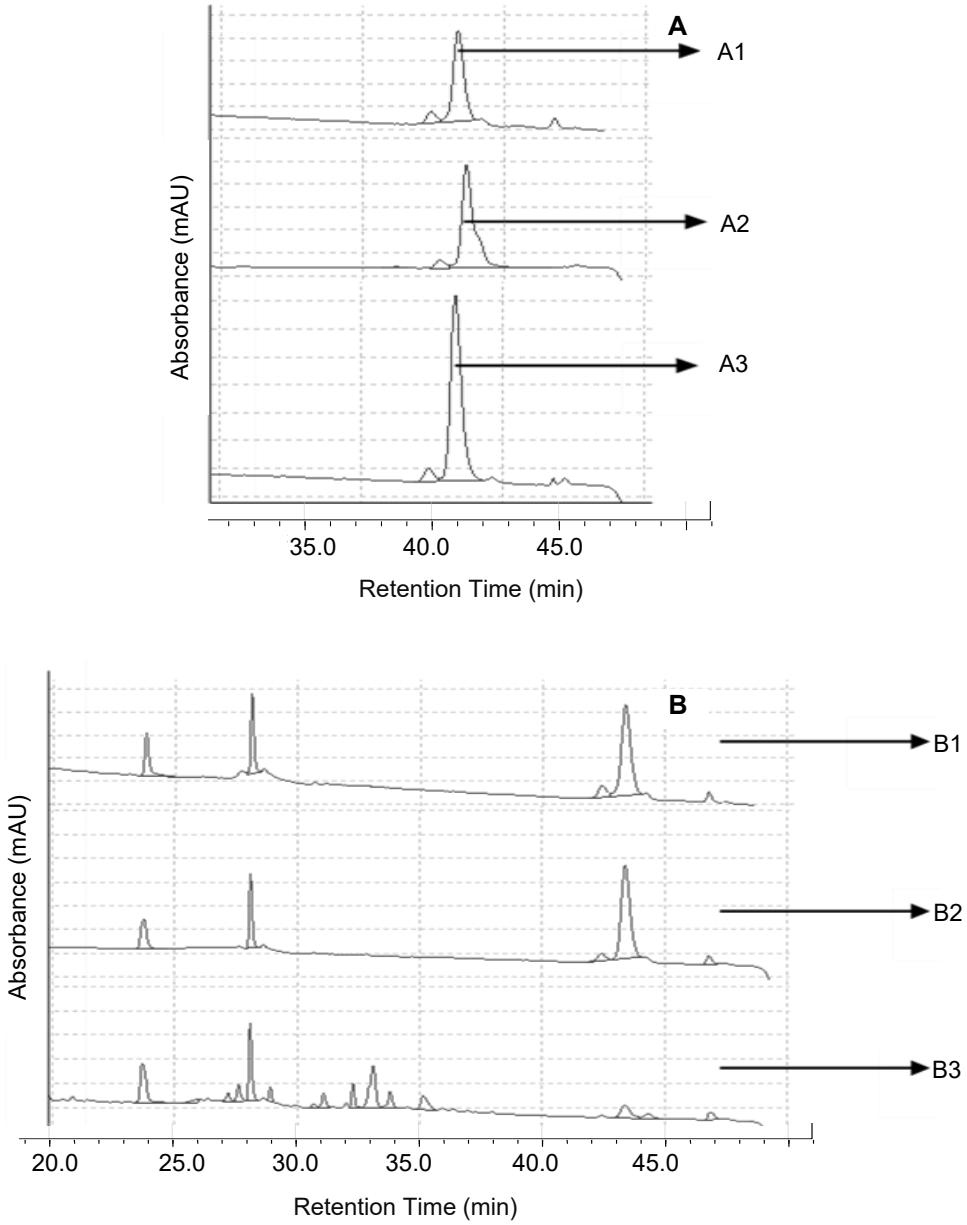
Pepsin-modified lysozyme, when compared to standard and isolated lysozyme, showed a decrease in MIC against both Gram-positive and Gram-negative bacteria. This happened because lysozyme was hydrolyzed by pepsin, producing a lysozyme hydrolysate containing bioactive peptides, including antibacterial peptides. The antibacterial activity of pepsin-modified lysozyme improved because the peptides in lysozyme can inhibit bacterial growth, thereby decreasing the MIC. This study aligns with prior report (Priyadarshini and Kansal, 2002), which showed that peptides in lysozyme hydrolysates have antibacterial activity against *E. coli* and *Leuconostoc mesenteroides*. The peptides in the hydrolysate of pepsin-modified lysozyme were detected on the RP-HPLC chromatogram (Figure 3b), but they were not detected in the SDS-PAGE profile up to 4.6 kDa (Figure 2), suggesting that the peptides had a molecular weight of less than 4.6 kDa. Although not yet tested in this study, previous study (Carrillo and Ramos, 2018) reported that the antibacterial activity of peptides from lysozyme hydrolysates was higher when the peptides were dominated by hydrophobic AAs, positively charged, and formed into an amphiphilic helical molecule.

The amino acids components that affected the antibacterial activity included lysine (positive), arginine (positive), tryptophan (hydrophobic), and tyrosine (hydrophobic). The tryptophan residue was found to influence the antibacterial activity of heat-modified lysozyme (Ibrahim *et al.,* 1996). Apart from its antibacterial activity, previous studies (Abdou *et al.,* 2007; Rao *et al.,* 2012; You *et al.,* 2010) have shown that pepsin modification of lysozyme produces a hydrolysate containing a multifunctional peptide with multiple activities, including ACE-inhibitory, free-radical scavenging, and antioxidant activities. This multifunctional peptide was an advantage of the pepsin modification methods.



Note: M= marker 4.6-40 kDa, LB= isolated lysozyme (unmodified), LC= pepsin-modified lysozyme 52 °C, LD= pepsin-modified lysozyme 37 °C, LE= low-pH modified lysozyme 52 °C, LF= low-pH modified lysozyme 37 °C, L1= lysozyme (monomer), L2= lysozyme (after polymerization)

Figure 2. The SDS-PAGE profile of the isolate and modified lysozyme from local chicken eggs



Note: A= the profile of lysozyme before modification treatments, A1= standard of lysozyme, A2= isolated lysozyme (pre-dialysis), A3= isolated lysozyme (post-dialysis), B= the profile of lysozyme after pepsin/low-pH modification treatments, B1= isolated lysozyme (unmodified), B2= low-pH modified lysozyme 52°C, B3= pepsin-modified lysozyme 52°C

Figure 3. The RP-HPLC profile of isolated and modified lysozyme from local chicken eggs

Low-pH modified lysozyme, when compared to standard lysozyme and isolated lysozyme, showed a decrease in the MIC value against all Gram-positive and Gram-negative bacteria. The low-pH modified lysozyme had the same antibacterial activity as pepsin-modified lysozyme, except for *E. coli*. This improvement in antibacterial activity resulted from denaturation, which causes both hydrolysis and polymerization of lysozyme (Figures 2 and 3). The fact that the MIC value of lysozyme against *E. coli* did not increase is presumably because, under low-pH conditions in the absence of pepsin, hydrolysis occurred randomly. The low-pH modification could not hydrolyze lysozyme in the specific manner that pepsin does at the active site, so it did not produce peptides. Additionally, the data demonstrated that *E. coli* was more resistant than the other strains tested.

This low-pH-modified lysozyme represents a cost-effective alternative for enhancing its anti-bacterial activity. Low-pH modification can reduce the need for pepsin in pepsin modification. Based on this result, the effect of pepsin or low-pH modification on

the antibacterial activity of lysozyme cannot yet be determined clearly, as the pepsin- or low-pH modified lysozyme in this study was modified in combination with heat treatment. The antibacterial activity of the low-pH-modified lysozyme, which was not adjusted to neutral pH (pH 7), may have resulted from low-pH conditions that disrupted bacterial metabolism. The final pH after mixing lysozyme with MHB was 4.91, while the final pH of the neutralized mixture was 7.22 (data not shown). The antibacterial activity observed after neutralization to pH 7 suggests that the inhibitory effect was not solely caused by acidic conditions but may also be associated with structural modifications or peptide formation resulting from the treatment process. Therefore, further studies are needed to evaluate the specific contribution of low-pH conditions, peptide formation, and heat treatment to the antibacterial activity of lysozyme. However, the mechanism underlying lysozyme's antibacterial activity has not been further tested using other methods.

Characteristics of freeze-dried lysozyme with SDS-PAGE

The SDS-PAGE profile with silver staining showed that low-pH modification (1.5) did not eliminate the lysozyme with a molecular weight of approximately 14.70±0.43 kDa, unlike pepsin modification (Figure 2). The pepsin modification hydrolyzed the lysozyme band, but the low-pH modification did not completely hydrolyze lysozyme. Even so, a low-pH of 1.5 indicated that the primary structure of lysozyme is highly stable, which is why it is used in various industries.

Pepsin modification significantly influenced the SDS-PAGE profile of lysozyme (Figure 2). Pepsin modification at a temperature of 52 and 37 °C for 60 min caused all bands in the gel to disappear, which showed that lysozyme and other proteins in local chicken egg white were hydrolyzed into smaller proteins (peptides) and were not polymerised, even after the 80 °C stop-heating treatment. This was different from low-pH modified lysozyme (Figure 2). The research by Mine *et al.* (2004) showed that lysozyme can be hydrolyzed to produce peptides. Other research (Carrillo *et al.,* 2016) also reported that peptides produced by pepsin hydrolysis for 60 min at 37 °C were smaller than 10 kDa.

The peptides in hydrolysates of lysozyme after pepsin modification were responsible for the improvement of antibacterial activity of pepsin-modified lysozyme. This is supported by research (Memarpoor-Yazdi *et al.,* 2012), which showed that pepsin modification of lysozyme can produce peptides, including one of about 1.75±0.5 kDa, with antibacterial activity against Gram-positive and Gram-negative bacteria. The characteristics of modified lysozyme after pepsin modification showed that although the primary structure of lysozyme was stable at low pH, lysozyme can be hydrolyzed by pepsin as a digestive enzyme.

The hydrolysis process in the low-pH modification differed from that in the pepsin modification because the lysozyme was only slightly hydrolyzed randomly and underwent polymerization under low pH (Figure 2). Even so, low pH modification at both temperatures produced thicker polymer forms of lysozyme (42.60±1.47 kDa) than the monomer form (Figure 2). This was due to the low-pH modification with a 10 mM potassium phosphate buffer at pH 1.5, combined with heating at 80 °C for 15 min, which induced lysozyme polymerization.

Characteristics of freeze-dried lysozyme with RP-HPLC

The results showed that the modification decreased the lysozyme concentration, as detected by globular protein analysis of lysozyme from local chicken egg white, with the decrease calculated by comparison with standard lysozyme (Table 6). The reduction of lysozyme concentration detected at a wavelength of 215 nm indicates that there was still a small amount of ovotransferrin and ovalbumin, as also shown on the SDS-PAGE profile (Figure 2). The RP-HPLC profile of the modified lysozyme chromatogram decreased the peak area of lysozyme at RT 43.59±0.09 min, indicating a reduction in the concentration of lysozyme.

The highest concentration of isolated lysozyme was found in lysozyme obtained by dialysis compared to that without dialysis, because the dialysis process can increase its concentration. The reduced concentration was also attributed to lysozyme denaturation and hydrolysis caused by structural modifications during RP-HPLC column processing. This condition prevented detection of all globular proteins at 43.59±0.09 min RT, as observed for the standard lysozyme. Some lysozyme concentration underwent dimerization at an oven temperature of 29.3 °C during the running process, because the dimer conformation can form between 20 and 30 °C (Cegielska-Radziejewska *et al.,* 2008). Some modified lysozyme was also detected at an RT of less than 43 min, which was suggested to be peptides, and at an RT of more than 43 min, which was thought to be a polymer form of lysozyme.

The lysozyme concentration measured by RP-HPLC at 43.59±0.09 min RT can be used to evaluate the lysozyme isolation process. The 0.1 M glycine-NaOH buffer (pH 9.3) used during the isolation process was suggested to cause the gradual dimerization of lysozyme. This occurs because lysozyme normally exists as a reversible dimer between pH 5 and 9 (Cegielska-Radziejewska *et al.,* 2008), but the higher pH of 9.3 used may have promoted dimerization, resulting in a lower amount of lysozyme detected in its globular form in isolated lysozyme.

This was also evidenced by the lysozyme concentration not matching the standard, even after dialysis. The appropriate pH must be considered during lysozyme isolation to obtain high concentrations of lysozyme in its globular form, especially for enzymatic purposes. Nevertheless, this isolation process was quite effective in yielding the antibacterial agent, as evidenced by lysozyme's antibacterial activity (Table 5). The isolated lysozyme still contained small amounts of ovotransferrin and ovalbumin, detected on the SDS-PAGE profile. The proteins were suggested to appear at RT values greater than 43.59±0.09 min due to their higher molecular weight compared to lysozyme.

Based on other research of Wulandari (2018), pepsin modification of lysozyme can trigger the formation of new proteins or peptides with different retention times. The research showed that peptide formation was detected at retention times shorter than lysozyme's RT (less than 38 min). This was consistent with the findings of this study, which showed several molecules detected at a faster RT than that of lysozyme (43.59±0.09 min) on a pepsin-modified lysozyme chromatogram (Figure 3). These molecules were thought to be peptides in the lysozyme hydrolysate after pepsin modification, because peptides have a lower molecular weight than lysozyme. It was also the reason for the changes and improvements in lysozyme's antibacterial activity.

Even though the pepsin modification was able to hydrolyze the lysozyme band, the pepsin-modified lysozyme was still detected by the RP-HPLC method (Figure 3). Lysozyme was detected by RP-HPLC after pepsin modification because spectrophotometric detection of RP-HPLC absorbance was more sensitive than silver staining, although silver staining was more sensitive than Coomassie Brilliant Blue staining on SDS-PAGE. Pepsin modification did not completely hydrolyze lysozyme (as evidenced by its detection on the RP-HPLC chromatogram), possibly because the 60-min incubation time was insufficient.

Suggested peptides of lysozyme after pepsin modification appear on chromatograms, as seen in Table 7. Suggested peptide concentrations were obtained by comparing the peak area of isolated lysozyme with the peak area of peptides formed at each retention time. Some of the highest concentrations of peptide were observed at retention times of 23.68 min (19.26%), 33.09 min (21.23%) and 28.09 min (20.51%) (Table 7). The amino acids sequences of the peptides are not yet known because they are difficult to compare with existing standards or references. Nevertheless, the peptides can be further analyzed by isolating them with a cation-exchange membrane and identifying their amino acids components, as reported by Carrillo *et al.* (2018), or by other methods.

Table 7. The peptides from the hydrolysate of lysozyme from local chicken egg after pepsin modification

Peptides	RT (min)	Peptides of Lysozyme Hydrolysate (%)
P1	23.68	19.26
P2	25.88	1.81
P3	27.19	2.72
P4	27.61	4.92
P5	28.09	20.51
P6	28.91	3.16
P7	30.66	1.52
P8	31.08	4.54
P9	32.28	6.27
P10	33.09	21.23
P11	33.80	5.94
P12	35.14	8.10

Note: P1–P12= peptides obtained from retention time less than 43 min on LC-20A RP-HPLC with a Diode Array detector and a Zorbax Eclipse C-18 column, RT= retention time

CONCLUSION

In conclusion, the lysozyme with a molecular weight of 14.70±0.43 kDa was successfully isolated. Modification of lysozyme with pepsin and low-pH increased its antibacterial activity. These modifications enhanced the antibacterial spectrum of lysozyme against Gram-negative bacteria while maintaining its activity against Gram-positive bacteria, especially with the low-pH-modified lysozyme in the 37 °C treatment. The improvement in lysozyme's antibacterial activity can be explained using SDS-PAGE (primary structure), RP-HPLC (tertiary structure), and lysozyme's enzymatic activity against *M. lysodeikticus*. The changes in bands of the SDS-PAGE profile, peaks on the RP-HPLC chromatogram, and the enzymatic activity of local chicken egg white lysozyme were the reasons for the improvement in the antibacterial activity of lysozyme. This study also showed that isolated and modified local chicken egg white lysozyme retains enzymatic activity. The suggestion is that further research should focus on identifying the amino acid composition of lysozyme and peptides to determine which specific amino acids contribute to enhanced antibacterial activity. In addition, lysozyme activity needs to be monitored to ensure it does not decline.

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