



Investigation of the antibacterial activity against *Cutibacterium acnes* of lysozyme purified from “Co Co” duck egg whites

Tran Khoa Nguyen and Vo Van Song Toan *

Biotechnology Research and Development Institute, Can Tho University, Viet Nam

*Correspondence: Vo Van Song Toan (email: vvstoan@ctu.edu.vn)

Article info.

Received 01 Mar 2021

Revised 22 May 2021

Accepted 19 Jul 2021

Keywords

Antibacterial activity, *Cutibacterium acnes*, lysozyme, storage, “Co Co” duck

ABSTRACT

Lysozyme has been applied in various fields such as food technology, medicine, and diagnostics because it can resist many types of bacteria. In this research, lysozyme from duck egg whites was studied to evaluate the antibacterial activity against *Cutibacterium acnes* (*C. acnes*) often causing acne on human skin. Lysozyme was purified from duck egg whites by ion-exchange chromatography and gel-filtration chromatography. After that, this enzyme was used to investigate the resistance to *C. acnes* at different pH, temperature, concentration, and storage conditions. The results presented that lysozyme exhibited the best resistance to *C. acnes* at pH 6.0 and 6.5 on trypticase - yeast extract - heart extract - glycerol agar (TYEG) medium, at 30°C and 35°C. Additionally, these conditions had the least effect on lysozyme antibacterial activity. The minimal inhibitory concentration (MIC₈₀) and minimum bactericidal concentration (MBC) of lysozyme to *C. acnes* were 0.55 mg/mL and 1.11 mg/mL, respectively. Lysozyme could keep up the best antimicrobial activity when stored at -20°C and -10°C; After 30 days, it still kept nearly 80% of its activity. These findings will offer a basis for larger-scale production of lysozyme powder for further research and commercial purposes, especially skin-care products.

1. INTRODUCTION

Lysozyme, also known as N-acetylmuramide glucanhydrolase, is an antibacterial enzyme that can hydrolyze the β (1,4)-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) of the peptidoglycan layer in the bacterial cell walls (especially Gram-positive bacteria) thereby destroying the cell wall of bacteria (Cotterill, 1954). Lysozyme is considered as one of the enzymes having diverse applications in the food industry, medicine, and diagnostics. Although lysozyme can be found in different sources such as milk, saliva, tears, urine, stomach mucus, or egg whites (Jollès et al., 1990; Maroni &

Cuccuri, 2001), lysozyme-powder in Viet Nam has been mainly imported from abroad at very high prices.

“Co Co” duck is an egg-laying breed imported from Zhejiang, China through the quota. It is the second-largest duck-head breed in Viet Nam after Tau duck. “Co Co” duck has an early laying age, about 90 - 120 days, the average egg yield per year reaches 260 - 300 eggs, higher than both grass duck and Khaki Campbell duck (Nguyen Duc Trong et al., 2006). “Co Co” duck eggs are rich sources of proteins as well as many essential amino acids, fats, and minerals necessary for the body. The protein content of a fresh duck egg is 11.8%,

of which lysozyme accounts for 3.5% of the 11 proteins in egg whites (Kovacs-Nolan et al., 2005).

Many previous publications have figured out that the antibacterial ability of lysozyme was markedly affected by temperature and pH conditions of the agar medium or buffer solution. Lysozyme is a heat-resistant enzyme, with a denaturing temperature up to 81.5°C (Campbell et al., 2003). Majid (2015) also performed research on lysozyme extracted from cow's milk and indicated that lysozyme at 37°C gave the best anti-*Micrococcus luteus* activity. Lysozyme is acid-soluble and thermally stable in a neutral medium because it can persist at 100°C for 30 minutes and loses its activity at pH values higher than 7.0. Besides, many studies have been conducted to determine the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of lysozyme extracted from different sources for the bacteria that cause foodborne-diseases such as *Escherichia coli* and *Clostridium botulinum* or acne and skin inflammation such as *Staphylococcus aureus*, *Staphylococcus epidermis*. However, the information on MIC and MBC values of lysozyme for *C. acnes* have not been published yet.

Therefore, the study was conducted to take advantage of cheap and plentiful duck eggs in Viet Nam to obtain lysozyme high values, serving in research and human needs in many areas of life.

2. MATERIALS AND METHODS

2.1. Materials

“Co Co” duck eggs were purchased in Hau Giang province, Viet Nam. SP-Streamline and Sephadex G-50 resins were bought from Pharmacia Fine Chemicals (Sweden). Bovine Serum Albumin and Coomassie Brilliant Blue G-250 were obtained from Merck (Germany). The *C. acnes* ATCC 11827 strain was purchased from Microbiologics company (USA). *Micrococcus lysodeikticus* ATCC 4698 and commercial lysozyme were obtained from Sigma-Aldrich (USA). Other reagents were of analytical grade from local sources.

2.2. Preparation of crude protein extract and lysozyme from duck egg whites

The procedure was implemented based on the research by Vo Thi Truc Ngan (2018) with small modifications. Duck egg whites was diluted with 0.1 M sodium phosphate buffer at pH 7.0 (with the ratio of 1 egg whites:2 buffer solution) for crude sample extraction. The filtrate was centrifuged at

5,000 rpm for 10 minutes at 4°C to remove residues. The supernatant was continuously treated with Streamline-SP and Sephadex G-50 resins for lysozyme purification. The absorbance measurement at 280 nm (A_{280}) was conducted with Hitachi U-1500 spectrophotometer (Japan) and 100-QS quartz cuvette (10 mm) (Hellma Analytics, Germany) to identify target protein fractions.

The lysozyme solution obtained from the purification process was immobilized with maltodextrin in the form of physical adsorption and drying. The lysozyme powder was store at -20°C and dissolved in buffer solution before using in the experiments.

2.3. Investigation of the impact of the external medium pH on the antibacterial effect of duck egg-white lysozyme against *C. acnes*

2.3.1. Impact of the TYEG medium pH on the antibacterial effect of duck egg-white lysozyme against *C. acnes* using the agar diffusion method

Bacterial colonies were suspended in 0.9% saline solution and then the bacterial suspension was adjusted to a density equivalent to 10^6 cells/mL. For *C. acnes*, with an absorbance of 0.132 at 600 nm, the bacterial suspension is equivalent to 10^8 cells/mL (Crane et al., 2013), 10^6 cells/mL in density can be obtained with 100 times diluting. The pH values of the TYEG medium (6.0, 6.5, 7.0 and 7.5) were prepared using NaH_2PO_4 and Na_2HPO_4 .

A volume of 30 μL of the bacterial suspension was inoculated onto the agar plates, then a 6-mm-in-diameter glass punch was used to make wells on the agar surfaces. Next, 20 μL of lysozyme solution was pipetted into the wells above (negative control was lysozyme inactivated at 100°C, positive control was commercial lysozyme). All the agar plates were then incubated at 35°C and measured the diameter of inhibition zones after 14 hours.

2.3.2. Impact of liquid medium pH on the change of antibacterial activity of duck egg-white lysozyme

Micrococcus lysodeikticus powder was dissolved in 0.06 M potassium phosphate solution at different pH values and the bacteria suspension was then measured spectroscopically at 450 nm to adjust the absorbance values in the range of 0.6 - 0.7. The buffer solutions at investigated pH values (6.0, 6.5, 7.0 and 7.5) were prepared using KH_2PO_4 and K_2HPO_4 at different ratios. 100 μL of lysozyme

sample was added to a cuvette containing 2.5 mL of the bacterial suspension of pH to be tested. A 2-minutes-decrease in an absorbance value at 450 nm using 10 mm glass cuvette (Hellma Analytics, Germany) was recorded to calculate the lysozyme activity presenting in the lysis reaction.

2.4. Investigation of the impact of the external medium temperature on the antibacterial effect of duck egg-white lysozyme against *C. acnes*

*2.4.1. Impact of the TYEG medium temperature on the antibacterial effect of duck egg-white lysozyme against *C. acnes**

The procedure was conducted similarly to the previous experiments that followed Kirby's method (1956) on TYEG medium with the pH was chosen from the previous experiment. All the plates were incubated at 25°C, 30°C, 35°C, and 40°C. The diameter values of inhibition zones were recorded after 14 hours.

2.4.2. Impact of liquid medium temperature on the change of antibacterial activity of duck egg-white lysozyme

The experimental design was implemented based on the research by Miyazaki (1998) with small modifications. The suspension of *Micrococcus lysodeikticus* was prepared as described in 2.3.2 section. pH value of the potassium phosphate solution used to dissolve the bacteria powder was chosen from the result of the previous experiment.

A volume of 100 µL of lysozyme sample was added to a cuvette containing 2.5 mL of the bacterial suspension. Record the absorbance value at 0 min. Then incubate the mixture of bacteria and enzyme solutions at the temperatures to be examined (25°C, 30°C, 35°C, and 40°C). After 90 minutes of incubation, re-measure the absorbance value and record the results to calculate the percentage of remaining enzyme activity compared to the initial one.

2.5. Determination of the MIC and MBC values of the duck egg-white lysozyme for *C. acnes*

Broth microdilution and colonies forming units counting assay (Somasegaran & Hoben, 1994) were carried out for MIC and MBC determination. A volume of 100 µL of bacterial suspension (10^6 cells/mL) was added to 900 µL of trypticase - yeast extract - heart extract - glycerol broth medium. 50 µL of this bacterial solution was respectively transferred into wells of a 96-well microplate, which was then mixed with 50 µL of lysozyme in increas-

ing concentration as well as control samples. The plate was incubated at 35°C for 14 hours. Bacterial counting was carried out by serially diluting the samples in the 96-well microplate with 0.9% saline solution to give a bacterial suspension within the countable range on agar plates. The inhibition level of lysozyme to *C. acnes* growth was calculated from the bacterial density in the sample wells and in the negative control after the incubation period.

2.6. Impact of storage conditions on the antibacterial activity of duck egg-white lysozyme

The experiment was a two-factor completely randomized design, including temperature (-20°C, -10°C, 4°C, and 25°C) and time (1, 15, 30, and 60 days). Lysozyme powder samples after being immobilized were stored at the storage temperatures above and determined the antibacterial activity after each timeline required to be examined. The results of this experiment were also evaluated based on the diameter of *C. acnes* inhibition zones (Kirby et al., 1956) and the lytic action of lysozyme in potassium phosphate buffer solution (Shugar, 1952). The remaining lysozyme activity percentage was calculated based on its lytic activity at the time to be tested versus initially.

2.7. Statistical analysis

The data were entered, stored, and processed using Microsoft Excel 2016 software. Statistical analysis, mean value, and standard deviation were calculated using Minitab 16. The mean values were compared with the Tukey test and the experiments were performed with three replicates.

3. RESULTS AND DISCUSSION

3.1. Impact of the external medium pH on the antibacterial effect of duck egg-white lysozyme against *C. acnes*

As can be seen in Table 1, duck egg-white lysozyme (at 3,000 U/mL) inhibited the growth of *C. acnes* in pH agar from 6.0 to 7.5. At pH 6.0, the largest inhibition zone was obtained, reaching 12.50 ± 0.50 mm, but the difference was not statistically significant at 5% level compared to the inhibition zone at pH 6.5. Meanwhile, the smallest one was obtained from the medium of pH 7.5, reaching 3.67 ± 0.58 mm. This result was also in line with the positive control when using commercial lysozyme with the same activity (3,000 U/mL), the antibacterial zone diameter reached the highest value at pH 6.0 and lowest at pH 7.5. These results

are in line with some early works. Hjelmeland et al. (1983) studied lysozyme from the skin mucus of rainbow trout (*Oncorhynchus mykiss*) and showed that pH 6.0 of the medium had the least effect on lysozyme activity, where its antibacterial activity was strongest. Besides, Sonomi et al. (2001) also concluded that lysozyme extracted from the kidneys of Japanese flounder (*Paralichthys olivaceuscho*) kept the best antibacterial activity at pH ranging from 5.0 to 6.5.

In this experiment, when observing the growth of *C. acnes* on TYEG agar, the bacteria grew quickly on the agar surface after only 12 hours. Besides, Achermann et al. (2014) also demonstrated that *C. acnes* grows best in media with pH between 6.0 and 7.0, better than an acidic or more alkaline medium. Therefore, although pH 6.0 and 6.5 were the most suitable pH for the *C. acnes* growth, lysozyme in this experiment still illustrated very clear resistance to *C. acnes* at these pH values.

Table 1. Antibacterial ability of duck egg-white lysozyme at different external pH medium

pH	Diameter of <i>C. acnes</i> inhibition zone (mm)	Antibacterial activity in phosphate buffer (U/mL)
6.0	12.50 ± 0.50 ^a	9,227 ± 377.54 ^a
6.5	10.70 ± 0.58 ^{ab}	9,127 ± 100.66 ^a
7.0	10.30 ± 1.15 ^b	8,107 ± 930.02 ^{ab}
7.5	3.67 ± 0.58 ^c	7,500 ± 200.33 ^b
P-value	0.00	0.01
CV (%)	11.11	6.06

*Note: The data was an average of 3 replicates. Means in the same column that do not share a letter are significantly different.

The positive control was commercial lysozyme (3,000 U/mL) giving inhibition zone diameters at pH 6.0: 6.33 ± 0.58^a mm; pH 6.5: 5.67 ± 1.00^a mm; pH 7.0: 5.00 ± 0.58^a mm; pH 7.5: 1.00 ± 0.00^b mm.

The influence of the pH values of agar plates and buffer solution on lysozyme activity was relatively similar. Specifically, at the buffer of pH 6.0, lysozyme showed the highest lysis activity on the substrate, reaching 9,227 ± 377.54 U/mL, not statistically different compared with the buffer of pH 6.5 and 7.0 (Table 1). The smallest value was also obtained from the buffer medium with pH 7.5.

According to Chang and Charles (1971), the decreasing activity of lysozyme in phosphate buffers when the medium pH increased from 6.0 to 8.0 was mainly due to the sharp increase in ionic strength. The ionic strength required for suitable lysis action is abided by a principle that at the lower pH the optimum lies at the high ionic strength, while at a higher pH value the optimum is converted to relatively low ionic strength (Davies et al., 1969). In this experiment of the study, the ionic strength of buffer solution at pH 6.0 was $I_1 = 0.124\text{ M}$ and that of buffer solution at pH 7.5 was $I_2 = 0.268\text{ M} > I_1$. From these values, it can be explained why lysozyme put in the pH 6.0 of phosphate buffer exhibited higher antibacterial activity than at pH 7.5.

From the analyzed results, it can be seen that the pH of the external medium influences the expression of lysozyme antibacterial activity. When performing this experiment, the conductors took into account the possibility that the environmental pH

will simultaneously affect the growth of bacteria and lysozyme activity. This is consistent with the practical conditions if lysozyme is applied in skin-care products. Because everyone's skin has a different pH, acne and skin cleansing solutions can change the pH of facial skin in a long enough period of time (Gfatter et al., 1997; Lambers et al., 2006; Prakash et al., 2017), the results of this study can help propose recommendations to the consumer who will suit this lysozyme product and which skincare solution is suitable to be used together.

3.2. Impact of the external medium temperature on the antibacterial effect of duck egg-white lysozyme against *C. acnes*

As evidence from the second column in Table 2, it can be noted that the inhibition zone reached the largest value at 30°C (10.67 ± 0.58 mm) and was not statistically different at 5% level compared with at 35°C (10.00 ± 0.00 mm). A similar propensity was also observed from the reactions in the potassium buffer solution (the third column in Table 2) in which lysozyme was lost only 36% and 39% of its activity at 30°C and 35°C, respectively, while lost until 80% of activity at the other two temperatures. Kawahara and Kusuda (1988) also showed that lysozyme from the slime of Japanese eel (*Anguilla japonica*) at pH 6.0 also acted best at 30°C of the external medium. Similarly, Miyazaki

(1998) performed a study on plasma-derived lysozyme of pink dot salmon (*Salverinus leucomaenis*) and reported that lysozyme exhibited optimum activity at medium treated with 30°C.

When incubated at 40°C, no growth of *C. acnes* was observed resulting in an inability to assess enzyme activity. However, the measurement of lysozyme activity in potassium buffer solution illustrated that such high temperature made lysozyme lose up to 80% of its activity within 90 minutes.

In 1978, Frasco et al. used infrared spectroscopy to study the mechanism of lysozyme heat denaturation. As the temperature increases, water molecules are released from the outer polar amino acid-base.

Water moves to the peptide-peptide bonds within the structure, encouraging the transformation of peptide-peptide bonds into water-peptide bonds, causing proteins to begin to swell and reduce the degree of coils, from there affecting the expression of lysozyme activity. However, many previous publications have shown that lysozyme can even show a relatively strong activity at higher than 40°C at suitable external pH conditions. In 2013, Venkataramani et al. presented that egg-white lysozyme has a temperature range of 25°C to 95°C in an acidic solvent. Cunningham and Lineweaver (1965) found that lysozyme was 50 times more thermally stable in phosphate buffer (pH 6.2) than in egg whites (pH 9.0) at 62.5°C.

Table 2. Antibacterial ability of duck egg-white lysozyme at different external pH medium

Temperature (°C)	Diameter of inhibition zone (mm)	Decrease in lysozyme activity after 90 minutes of reaction (%)
25	9.00 ± 1.00 ^b	81.36 ± 10.54 ^b
30	10.67 ± 0.58 ^a	36.21 ± 3.64 ^a
35	10.00 ± 0.00 ^{ab}	39.11 ± 4.38 ^a
40	0.00 ± 0.00 ^c	80.95 ± 10.52 ^b
P-value	0.00	0.00
CV (%)	7.78	13.41

*Note: The data was an average of 3 replicates. Means that do not share a letter are significantly different.

The positive control was commercial lysozyme (3,000 U/mL) giving inhibition zone diameters at 25°C: 8.00 ± 0.58^b mm; 30°C: 10.00 ± 0.58^a mm; 35°C: 7.00 ± 0.58^c mm; 40°C: 0.00 ± 0.00^d mm.

3.3. MIC and MBC values of duck egg-white lysozyme for *C. acnes*

MIC₈₀ value is defined as the lowest concentration of a reagent in which bacterial colonies still develop but are inhibited by 80% of their density (Kumar & Pandey, 2013). Table 3 illustrated that duck egg-white lysozyme at the concentration of 0.55 mg/mL (corresponding to 5,000 U/mL) inhibited more than 80% of the *C. acnes* colonies compared with results from negative control (inactivated lysozyme gave 0% in inhibitory ability), so lysozyme concentration of 0.55 mg/mL was considered to be the MIC₈₀ for *C. acnes*. Because lysozyme from the concentration of 0.66 mg/mL onwards could inhibit more than 90% of the bacteria, so the MIC₉₀ value of the duck eggs lysozyme for *C. acnes* was 0.66 mg/mL. MBC value is defined as the lowest reagent concentration that can kill bacterial growth by up to 99.9% (Levison, 2004). Therefore, the MBC value of lysozyme was chosen for *C. acnes* was 1.11 mg/mL. In addition, according to Levison (2004), if the MBC/MIC ratio is bigger than 4.00, the reagent used has bacteriostatic effects, while if this ratio is smaller than 4.00, the reagent is con-

sidered to be effective bactericidal. The MBC/MIC ratio of the duck egg-white lysozyme in this study was 2.00 < 4.00, so it can be concluded that this lysozyme was effective bactericidal against *C. acnes*.

Until now, there have been not many studies related to MIC and MBC of lysozyme from egg whites. Xiu et al. (2008) used lysozyme from marine microorganisms to treat *Staphylococcus aureus* and *Staphylococcus epidermis* and found MIC values of 0.5 mg/mL to 1.0 mg/mL; MBC values were also reported to be 1.0 mg/mL to 4.0 mg/mL. Antibiotics have long been also used to treat acne, such as tetracycline, minocycline, and azithromycin (Farrah & Tan, 2016). Cha et al. (2020) studied the MIC of some antibiotics and compounds commonly used to treat *C. acnes*, the results demonstrated that the minimum concentration of erythromycin and salicylic acid to inhibit the *C. acnes* growth was both about 1.25 mg/mL, which was equivalent to MBC values of duck egg lysozyme (1.11 mg/mL) and ampicillin concluded from the present study. In addition, many studies have also shown that long-term use of these antibiotics often cause

side effects such as mood disturbances, dry skin, dermatitis, burning (Eichenfield, 2015).

Side effects of antibiotics and the emergence of multidrug-resistant bacteria have become a threat to the health of consumers, so the main concern today is finding compounds or proteins derived from nature that possess strong antibacterial, anti-inflammatory activity and cause fewer side effects

(Neamsuvan et al., 2015), such as natural compounds from herbal plants, tea or grapefruit essential oils, and even some brown algae extract. Lysozyme is also a protein that can be obtained from abundant, easy-to-find, and inexpensive ingredients like egg whites. Therefore, lysozyme is believed to be a potential protein that can replace antibiotics in the treatment of skin problems.

Table 3. Percentage of inhibited C. acnes density by the concentration of duck egg-white lysozyme

Lysozyme concentration (mg/mL)	Lysozyme activity (U/mL)	Percentage of inhibition (%)	MIC/MBC
0.11	1,000	4.00 ± 2.35 ^c	
0.22	2,000	11.85 ± 5.59 ^c	
0.44	4,000	36.30 ± 6.68 ^b	
0.55	5,000	86.37 ± 1.05 ^a	MIC ₈₀
0.66	6,000	93.78 ± 0.44 ^a	MIC ₉₀
0.88	8,000	94.52 ± 0.26 ^a	
0.99	9,000	99.08 ± 0.25 ^a	
1.11	10,000	99.99 ± 0.00 ^a	MBC
1.66	15,000	100.00 ± 0.00 ^a	
Lysozyme TM - 0.1 mg/mL	14,500	100.00 ± 0.00 ^a	
Ampicillin – 1.0 mg/mL		100.00 ± 0.00 ^a	
Inactivated lysozyme		0.00 ± 0.00 ^c	
P-value			0.00
CV (%)			7.42

3.4. Impact of storage conditions on the antibacterial effect of duck egg-white lysozyme against C. acnes

Observing in Table 4, from 15 days to after 30 days of storage, the inhibition zone values obtained at all temperatures were almost not statistically different at 5%, but they were all significantly smaller than the first day. In addition, at 4°C and 25°C, the reduction in inhibition zone diameters was more than

the first two temperatures, which decreased 3.5 times and 4.1 times after 30 days. This is consistent with Wasserfall’s (1977) conclusion, more decrease in the activity of lysozyme was observed when increasing storage temperature and the more stable lysozyme activity was when stored at low temperature. After 60 days of storage, the values in all treatments were 0.00 mm, however, lysozyme has still partly retained its initial activity, which is proved in Table 5.

Table 4. The C. acnes inhibition zone of duck egg-white lysozyme at different storage temperatures and times

Storage time (days)	Storage temperature (°C)			
	-20	-10	4	25
1	23.00 ± 0.00 ^a	21.00 ± 0.00 ^a	22.00 ± 0.58 ^a	22.00 ± 0.58 ^a
15	8.67 ± 1.53 ^b	7.00 ± 1.00 ^{bc}	6.00 ± 3.61 ^{bc}	5.33 ± 0.58 ^c
30	7.67 ± 0.58 ^{bc}	7.00 ± 1.00 ^{bc}	6.33 ± 0.58 ^{bc}	5.33 ± 0.58 ^c
60	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d
P-value				0.19
CV (%)				12.23

*Note: The antibacterial zone diameter figure is an average of 3 replicates. Means that do not share a letter are significantly different.

The results in Table 5 reflected the causes of data of Table 4. Accordingly, lysozyme after the first 30

days of storage retained 70% to 79% of its activity when stored at -20°C to 4°C; whilst 25°C only maintained more than 30% of lysozyme activity.

After 30 to 60 days, at -20°C, lysozyme retained up to 63% of the activity, while at the remaining temperatures, lysozyme activity kept only 15% to 35%. Uddin et al. (2017) also reported that lysozyme had almost unchanged activity in the first 30 days when stored under cold conditions (from ≤ 0°C - 4°C) and low humidity (from 12% - 25%), the activity of lysozyme would decrease slightly when stored at room temperature and higher humidity (about 65%).

There have been many studies demonstrating that lysozyme activity during storage also depends on how the enzyme is immobilized, the material holding the enzyme, or the substance dissolving enzymes. In 1972, Uchida et al. used lysozyme in a mirin wine sample and found that more than 95% of the lysozyme activity was remained after one year, meanwhile, a combination of mirin with glutentannin and activated carbon reduced lysozyme activity by 15%. Zi-xuan et al. (2012) reported that free lysozyme almost lost 100% of its activity after one month, whereas, under the same storage conditions, the decrease in the activity of immobilized lysozyme was 2 to 3 times lower. In 2017, Uddin et al. immobilized lysozyme in aerogel and then

found that lysozyme activity almost unchanged after 30 days. Lysozyme in the present study when immobilized with 5% maltodextrin also presented a good protective effect, maintaining more than 60% of the activity after 2 months.

Types of containers used to store lysozyme solutions also influence its activity. Goldblum et al. (1981) reported that when breast milk was contained in flasks made of pyrex and polypropylene, lysozyme concentrations decreased by 40% after 24 hours of refrigeration. Kravchenko et al. (1967) found that the lysozyme adhered to the glassware and causing a poor ability to maintain enzyme activity. Preservation of lysozyme samples in a glass capillary tube for 9 days at -4°C also reduced lysozyme activity by about 20% to 50% (Copeland et al., 1982). Lysozyme in this experiment was preserved in plastic Eppendorf tubes, compared with the data in Table 5 and the references above, it can be assumed that the plastic material helped stabilize the activity. Lysozyme properties were better and longer when it was stored after two months at minus temperature, retaining up to 65% of the activity.

Table 5. The activity maintenance level of the egg-white lysozyme stored at different temperatures and times

(Unit: %)

Storage time (days)	Storage temperature (°C)			
	-20	-10	4	25
1	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a
15	97.67 ± 3.94 ^a	81.33 ± 14.01 ^{ab}	100.00 ± 0.00 ^a	38.67 ± 0.68 ^{cde}
30	78.67 ± 37.09 ^{ab}	70.67 ± 9.47 ^{abc}	78.67 ± 15.40 ^{ab}	30.67 ± 7.53 ^{de}
60	63.00 ± 2.94 ^{bcd}	34.67 ± 0.14 ^{de}	15.33 ± 0.41 ^e	21.67 ± 2.39 ^e
P-value				0.00
CV (%)				16.00

*Note: The antibacterial zone diameter figure is an average of 3 replicates. Means that do not share a letter are significantly different.

4. CONCLUSIONS

Lysozyme from “Co Co” duck eggs was most effectively resistant to *C. acnes* at pH of 6.0 and 6.5, between 30°C and 35°C. At pH 7.5 and 40°C, lysozyme exhibited weak antibacterial ability. Lysozyme stored at -20°C or -10°C retained the most effective and longest lysozyme activity, after 60 days still retained up to 63% of the activity. The authors strongly recommended that before preserving, lysozyme can be investigated with some kinds of carriers for the immobilization, compounds that can have synergistic effects with lysozyme, or materials holding lysozyme.

The MIC₈₀, MIC₉₀, and MBC values of the duck egg-white lysozyme for *C. acnes* were 0.55 mg/mL, 0.66 mg/mL, and 1.11 mg/mL, respectively. This study expressed that the effects of duck egg-white lysozyme were in line with or better than many antibiotics used for skin treatments nowadays. Therefore, in the future, lysozyme is hoped to be studied more and produced on an industrial scale, creating a base for acne treatment products with many good properties, efficient and safe for consumers.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Prof. Cao Ngoc Diep and Dr. Tran Thi Giang, Biotechnology Research and Development Institute (BiRDI) of Can Tho University, for their valuable and constructive suggestions during the planning and development of this research work. We also immensely appreciate Ms. Le Thi Truc Mai, Ms. Phan Thi Hong Tiep, and Mr. Nguyen Minh Thuan, BiRDI of Can Tho University, for their comments on an earlier version of the manuscript.

REFERENCES

- Campbell, L., Raikos, V., & Euston, S. R. (2003). Modification of functional properties of egg-white proteins. *Food/Nahrung*, 47(6), 369-376.
- Cha, H., Su-Kyung, K., MooChang, K., & Tae-Hoo, Y. (2020). *Lactobacillus paraplantarum* THG-G10 as a potential anti-acne agent with anti-bacterial and anti-inflammatory activities. *Anaerobe*, 64, 102243.
- Chang, K. Y., & Charles, C. W. (1971). Studies on the structure and function of lysozyme: I. The effect of pH and cation concentration on lysozyme activity. *Biochimica et Biophysica Acta*, 229(2), 0-503.
- Copeland, J., Lamberts, D., & Holly, F. (1982). Investigation of the accuracy of tear lysozyme determination by the Quantiplate method. *Investigative Ophthalmology & Visual Science*, 22(1), 103-110.
- Cotterill, O. J. (1954). Egg-white lysozyme: 3. The effect of pH on the lysozyme - ovomucin interaction. *Poultry Science*, 34(3), 679-686.
- Crane, J. K., Hohman, D. W., Nodzo, S. R., & Duquin, T. R. (2013). Antimicrobial susceptibility of *Propionibacterium acnes* isolates from shoulder surgery. *Antimicrobial Agents and Chemotherapy*, 57(7), 3424-3426.
- Cunningham, F. E., & Lineweaver, H. (1965). Stability of egg-white proteins to pasteurizing temperatures above 60°C. *Food Technology*, 19, 136-141.
- Davies, R. C., Neuberger, A., & Wilson, B. M. (1969). The dependence of lysozyme activity on pH and ionic strength. *Biochimica et Biophysica Acta*, 178(2), 0-305.
- Eichenfield, L. (2015). Evolving perspective on the etiology and pathogenesis of Acne Vulgaris. *Journal of Drugs in Dermatology*, 14(3), 263-268.
- Farrah, G., & Tan, E. (2016). The use of oral antibiotics in treating acne vulgaris: a new approach. *Dermatology and Therapy*, 29(5), 377-384.
- Frasco, C. B., Karmas, E., & Gilbert, S. G. (1978). Water binding patterns of native and denatured lysozyme as determined by infrared spectroscopy. *International Congress of Food Science Technical Abstracts*, p. 170.
- Gfatter, R., Hackl, P., & Braun, F. (1997). Effects of soap and detergents on skin surface pH, stratum corneum hydration and fat content in infants. *Dermatology*, 195(3), 258-262.
- Goldblum, R. M., Garza, C., Johnson, C. A., Harrist, R., Nichols, B. L., & Goldman, A. S. (1981). Human milk banking. I. Effects of container upon immunologic factors in mature milk. *Nutrition Research*, 1(5), 449-459.
- Jollès, J., Prager, E. M., Alnemri, E. S., Jollès, P., Ibrahim, I. M., & Wilson, A. C. (1990). Amino acid sequences of stomach and nonstomach lysozymes of ruminants. *Journal of Molecular Evolution*, 30(4), 370-382.
- Kawahara, I., & Kusuda, R. (1988). Properties of lysozyme in cultured eel. *Nippon Suisan Gakkaishi*, 54(6), 976-973.
- Kirby, W. M., Yoshihara, G. M., Sundsted, K. S., & Warren, J. H. (1956). Clinical usefulness of a single disc method for antibiotic sensitivity testing. *Antibiotics Annual*, pp. 892-897.
- Kovacs-Nolan, J., Phillips, M., & Mine, Y. (2005). Advances in the value of eggs and egg components for human health. *Journal of Agricultural and Food Chemistry*. 53(22), 8421-8431.
- Kravchenko, N. A., Kagramanova, K. A., & Kuznetsov, Y. D. (1967). Effect of salts and other factors on lysozyme activity. *Biokhimiya*, 32(3), 618-23.
- Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, 2013, 1-16.
- Lambers, H., Piessens, S., Bloem, A., Pronk, H., & Finkel, P. (2006). Natural skin surface pH is on average below 5, which is beneficial for its resident flora. *International Journal of Cosmetic Science*, 28(5), 359-370.
- Levison, M. E. (2004). Pharmacodynamics of antimicrobial drugs. *Infectious Disease Clinics of North America*, 18(3), 451-465.
- Majid, H. (2015). Isolation and characterization of buffalo milk lysozyme. *International Journal of Food Properties*, 18(6), 1288-1297.
- Maroni, P., & Cuccuri, C. (2001). Relationship between mammary gland infections and some milk immune parameters in Sardinian breed ewes. *Small Ruminant Research*, 41(1), 1-7.
- Miyazaki, T. (1998). Influences of pH and temperature on lysozyme activity in the plasma of Japanese founder and Japanese char. *Fish Pathology*, 33(1), 7-10.
- Neamsuvan, O., Abdulmalek, K., Abeedah, S., Suraiya, L., & Nasreen, W. (2015). A survey of herbal formulas for skin diseases from Thailand's three southern border provinces. *Journal of Herbal Medicine*, 5(4), 190-198.
- Nguyen Duc Trong, Hoang Van Tieu, Nguyen Thi Minh, & Ho Khac Oanh. (2006). Study on the production

- capacity of Zhejiang duck. Scientific report. National Institute of Livestock Production (in Vietnamese).
- Prakash, C., Bhargava, P., Tiwari, S., Majumdar, B., & Bhargava, R. K. (2017). Skin surface pH in acne vulgaris: Insights from an observational study and review of the literature. *The Journal of Clinical and Aesthetic Dermatology*, 10(7), 33-39.
- Shugar, D. (1952). The measurement of lysozyme activity and ultraviolet inactivation of lysozyme. *Biochimica et Biophysica Acta*, 8(3), 302-309.
- Somasegaran, P., & Hoben, H. J. (1994). Quantifying the Growth of Rhizobia. In: *Handbook for Rhizobia*. Springer. New York, pp. 47-57.
- Sonomi, M., Jun-ichi, H., Ikuo, H., Takashi, A., & Hajime, M. (2001). Expression of Japanese flounder c-type lysozyme cDNA in insect cells. *Developmental and Comparative Immunology*, 25(5-6), 439-445.
- Uchida, M., Yokomura, S., & Nagahama, G. (1972). Studies on Lactobacilli isolated from mirin liquor. V. Antiseptic effects of egg-white lysozyme on the growth of *Lactobacillus*. *Journal of Fermentation Technology*, 50, 292-297.
- Uddin, K. M. A., Orelma, H., Mohammadi, P., Borghei, M., Laine, J., Linder, M., & Rojas, O. J. (2017). Retention of lysozyme activity by physical immobilization in nanocellulose aerogels and antibacterial effects. *Cellulose*, 24(7), 2837-2848.
- Venkataramani, S., Jeremy, T., & Denis, C. (2013). Thermal stability of high concentration lysozyme across varying pH: A fourier transform infrared study. *Journal of Pharmacy and Bioallied Sciences*, 5(2), 148-153.
- Vo Thi Truc Ngan. (2018). Investigation of conditions for extraction and purification of lysozyme from *Anas Platyrhynchos* domesticus. Bachelor thesis. Can Tho University. Can Tho (in Vietnamese).
- Wasserfall, F. (1977). Principles of the effect of lysozyme in the manufacture of semi-hard cheese. I. Accuracy of determination recovery and stability of lysozyme. *Milchwissenschaft*, 32, 654-657.
- Xiu, Z., Fa-xing, W., Mi, S., Qing-yin, W., Yue-jun, W., & Xiang-ke, Y. (2008). Study on antimicrobial and antiviral activities of lysozyme from marine strain S-12-86 *in vitro*. *Agricultural Sciences in China*, 7(1), 112-116.
- Zi-Xuan, L., Zhong-Su, M., Jing, W., & Huan, L. (2012). Preparation and characterization of immobilized lysozyme and evaluation of its application in edible coatings. *Process Biochemistry*, 47(2), 201-08.