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Characterization of Egg White Lysozyme from Local Duck Mojosari and Alabio

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ABSTRACT

Lysozyme, a natural antibacterial, has potential use in several applications. Generally, it is obtained from chicken egg whites. However, the idea of using local duck eggs as a source of lysozyme is not well-known. This study aimed to isolate and purify the egg white of Mojosari and Alabio ducks to characterize lysozyme. Additionally, the study analyzed the effect of temperature treatment on lysozyme enzymatic activity. Three different temperature treatments were used: Low Temperature Long Time (63 °C, 30'), High Temperature Short Time (72 °C, 15"), and sterilization (121 °C, 15'). The Amberlite resin cation exchange chromatography method was successfully used to isolate and purify Mojosari and Alabio duck egg whites, achieving purity levels of 94.92-96.09%. The presence of lysozyme was confirmed by the existence of bands that were primarily composed of lysozyme. The results showed that each aspect of the characteristics of lysozyme and enzymatic activity were significantly affected ($p<0.05$) by the type of egg white. Enzymatic activity indicated that Alabio duck lysozyme was thermosensitive and could only survive at LTLT temperature, while Mojosari duck lysozyme could survive at LTLT and HTST pasteurization temperatures. However, both lysozymes did not survive the sterilization temperature.

Keywords: alabio duck, characterization, egg white lysozyme, mojosari duck

ABSTRAK

Lisozim memiliki potensi sebagai antibakteri alami. Pada umumnya, lisozim diperoleh dari putih telur ayam ras dan hingga saat ini, penggunaan telur itik lokal sebagai sumber lisozim belum banyak diketahui. Penelitian ini bertujuan untuk mengisolasi dan mempurifikasi putih telur itik Mojosari dan Alabio untuk mengkarakterisasi lisozim dan menganalisis pengaruh perlakuan suhu terhadap aktivitas enzimatis lisozim. Tiga perlakuan suhu yang berbeda digunakan: Low Temperature Long Time (63 °C, 30'), High Temperature Short Time (72 °C, 15"), dan sterilisasi (121 °C, 15'). Metode kromatografi penukar kation resin Amberlite berhasil digunakan untuk mengisolasi dan memurnikan putih telur itik Mojosari dan Alabio, dengan tingkat kemurnian 94.92-96.09%. Keberadaan lisozim dikonfirmasi dengan adanya pita-pita yang sebagian besar terdiri dari lisozim. Hasil penelitian menunjukkan bahwa setiap aspek karakteristik lisozim dan aktivitas enzimatik dipengaruhi secara signifikan ($p<0.05$) oleh jenis putih telur. Aktivitas enzimatis menunjukkan bahwa lisozim itik Alabio bersifat termosensitif dan hanya dapat bertahan pada suhu LTLT, sedangkan lisozim itik Mojosari dapat bertahan pada suhu pasteurisasi LTLT dan HTST. Namun, kedua lisozim tersebut tidak dapat bertahan pada suhu sterilisasi.

Kata kunci: itik Alabio, itik Mojosari, karakterisasi, lisozim putih telur

INTRODUCTION

Indonesia exhibits notable biodiversity exemplified by the diverse array of local duck species distributed across its various regions, each characterized by distinct traits. The population of ducks in the country escalated from 48,367,545 individuals in 2021 to 49,876,959 in 2022, marking a 3.1% increase (BPS 2023). Among these local duck varieties are the Tegal ducks from Tegal (Central Java), Cirebon ducks from Cirebon (West Java), Mojosari ducks from Mojosari (East Java), Alabio ducks from Sungai Pandan District (South Kalimantan), Cihateup ducks from Cihateup Village (Tasikmalaya, West Java), Bali ducks from Bali (Matitaputty and Suryana 2013). The development of Indonesian local ducks in the community has a broad role, both from an economic aspect and the fulfillment of animal food needs. The role of local ducks in meeting animal protein needs is as a source of meat and eggs that have high nutritional value, especially protein content with a complete essential amino acid composition. Although ducks can be used as a source of meat and eggs, people prefer duck eggs relatively more than duck meat. The greater availability of various processed duck egg products in the community indicates this. Duck egg production in 2021 was 313,844.76 tons/year and increased by 2.7% in 2022, amounting to 322,610.88 tons/year (BPS 2023)

Duck eggs have a similar structure to other poultry eggs, with three main components: eggshell and eggshell membrane (11-13%), egg white (45-58%), and yolk (28-35%) (Huang and Lin 2011). Egg white is the component of the egg that is rich in nutrients, particularly protein. Ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.5%) are the main proteins found in egg white, with small amounts of avidin (0.05%), cystatin (0.05%), ovomacroglobulin (0.5%), ovoflavoprotein (0.8%), ovoglycoprotein (1%), and ovoinhibitor (1.5%) (Chang *et al.* 2018). Egg white has a high protein content, is easily digestible, and has a perfect biological value (100%), indicating that humans can easily digest these proteins. This means that duck egg white is a valuable food ingredient. In addition to their nutritional value, egg white proteins have bioactive properties and a wide range of medical functionalities. For example, ovalbumin is an immunomodulator (He *et al*. 2021), ovotransferrin is an antibacterial with the ability to bind iron (Fe3+) (Giansanti *et al*. 2015), duck egg white ovomucoid is a protease inhibitor (Nurliyani *et al.* 2023), and lysozyme is an antibacterial and natural sweeteners (Wulandari 2018). This demonstrates that local Indonesian duck egg white can potentially be a source of bioactive components for various uses.

Lysozyme is a protein found in egg whites that has many useful properties. It is a monomeric glycoside hydrolase with a molecular weight of 14,300 Da and is made up of 129 amino acids (Wu *et al.* 2017). One of its main functions is its antibacterial ability, which allows it to break the β.1-4 N-acetylglucosamine (NAG) glycosidic bond in the peptidoglycan of the bacterial cell wall, effectively killing bacteria. Additionally, lysozyme has a sweet taste and can be used as a low-calorie sweetener to replace sugar (Saraiva *et al.* 2020). It is reported to have a sweetness level 100 to 2000 times sweeter than sucrose and is, therefore, a useful substitute for synthetic sweeteners (Maehashi *et al*. 2007). Studies of Tiwari (2009) and Wu *et al*. (2017) show that the application of lysozyme to raw and processed meat, dairy products, fruits, and vegetables can extend their shelf life. It is also considered safe for human consumption and has been approved as a food additive by the Food Agriculture Organization (FAO).

The sweetness and antibacterial ability are reported to have different mechanisms and are not related to each other. The antibacterial property of lysozyme is due to its ability to degrade the glycosidic bond between NAM and NAG sugars in the peptidoglycan of bacterial cell membranes. Meanwhile, the sweet taste of lysozyme is triggered by the protein's ability to interact with TR2/TR3 sweet receptors on the tongue's surface. These two functional properties of lysozyme indicate that lysozyme has broad beneficial value. Wulandari (2018) showed that lysozyme from local Indonesian poultry also has good antibacterial properties and sweetness, so it could potentially be used to replace purebred chicken eggs as a source of lysozyme.

Chicken eggs, as a source of lysozyme, face a significant obstacle due to competition with the need for egg consumption. To avoid disrupting the availability of chicken eggs as a primary source of egg consumption, local poultry eggs can be used as an alternative source of lysozyme. The industry commonly selects egg white as a source of lysozyme since it produces the most amount and is easier to handle for application purposes than other sources. A report by Business Research Insights (2024) states that most commercial lysozyme available in the market is derived from broiler eggs, followed by recombinant lysozyme from microorganisms. According to the report, the global market value of lysozyme reached USD 490.1 million and is expected to continue growing positively to reach USD 672 million by 2028. The largest markets for lysozyme are the food, pharmaceutical, and animal feed industries. Interestingly, the largest producers of lysozyme are still mainly located in the European region, including the Netherlands, Italy, Spain, and Russia, followed by Canada and China. China is the leading producer of lysozyme in Asia, and there is currently no lysozyme-producing industry in the Southeast Asian region.

The diversity of local duck species and populations in Indonesia has the potential not only as a source of animal protein but also as a source of bioactive proteins that can be utilized in various industries such as food, pharmaceutical, and feed. The market needs that shift towards natural ingredients for various life purposes also encourage the increasing need for non-synthetic preservatives and food additives. The danger of using synthetic materials is that there is a need to look for new natural antibacterial alternatives in the food and health fields. However, efforts to produce and characterize the bioactivity of lysozyme from various local birds have yet to be widely studied. Wulandari (2018) research is still limited to native chickens and Cihateup ducks. The production and characterization of lysozyme

from egg whites of other local poultry needs to be done not only to open up opportunities for exploration of local poultry eggs as a source of lysozyme, but also to provide basic information on the best local poultry eggs as a source of lysozyme. On the other hand, although general studies on the thermostability of lysozyme have been widely reported, no research has focused on the effects of the thermal processing of food ingredients on lysozyme. According to Chiozi *et al.* (2022), pasteurization and sterilization are the two central thermal processing of foodstuffs involving high temperatures.

This study focuses on Alabio and Mojosari ducks to represent local ducks with lysozyme production and characteristics that have not been studied. Alabio and Mojosari ducks are two local duck species known for their potential as egg producers. The Indonesian Ministry of Agriculture designated them as national germplasm, with reference numbers 2921/Kpts/OT.140/6/2011 (Alabio ducks) and 2837/Kpts/LB.430/8/2012 (Mojosari ducks) (Ministry of Agriculture 2011; Ministry of Agriculture 2012). Utilizing local poultry eggs as a source of lysozyme could provide added economic value for the development of local Indonesian livestock and the possibility of Indonesia becoming a new lysozyme production center in the ASEAN region. This study aimed to characterize the lysozyme of Mojosari and Alabio ducks and analyze the stability of lysozyme activity when exposed to thermal heat at pasteurization and sterilization temperatures.

MATERIAL AND METHODS

Material

The equipment used in the experiment included a centrifuge, 15 mL Eppendorf tube, 2.2 cm diameter column, viva flow 50 R 5000 MWCO hydrosart membrane, heat block, UV Vis spectrophotometer (Agilent 8453, Germany) and water bath shaker. The protein separation patterns and protein purity were determined using ImageJ 14.7 software. The materials used in the lysozyme isolation process were Alabio duck eggs and Mojosari ducks, aquademineral, Amberlite FPC 3500 resin, glycine, NaCl, and NaOH, Na_2CO_3 2%, NaOH 0.1 N, $CuSO_4$ 0.5%, NaK Tartrate 1%, Folin ciocalteau, and Bovine Serum Albumin (BSA), 1.5 M Tris pH 8.8, acrylamide, Sodium Dodecyl Sulfate (SDS), Ammonium Persulfate (APS), Tetramethylethylenediamine (TEMED), Comassie Brilliant Blue (CBB), and ColorBurst™ Electrophoresis Marker, and Micrococcus luteus ATCC 4689 (Sigma, USA).

Methods

Preparation of Duck Eggs and Resin

Mojosari duck and Alabio duck eggs (<3 days old) were collected from BALITNAK, Ciawi Bogor. Egg selection was done based on several aspects, including; not containing embryos, clean shells, egg contents free of blood spots and meat spots, and high viscosity. Eggs were washed using warm water (35 °C-40 °C), and the egg white and yolk were separated. A total of 150 mL of egg white was collected and then homogenized using a stirrer. Amberlite FPC 3500 resin was used for the isolation and purification process of lysozyme by cation exchange chromatography method (Abeyrathne *et al.* 2014). A total of 30 grams of amberlite FPC 3500 resin was calibrated using 2 N NaOH (Merck, Germany) to reach pH 9.3.

Isolation and Purification Lysozyme

A total of 150 mL of egg white was dissolved using sterile distilled water with a volume ratio of 1:1. Next, Amberlite FPC 3500 resin was added to the egg white solution at a ratio of 1 g Amberlite FPC 3500 resin: 10 mL of egg white that has been diluted with sterile distilled water. The solution was stirred at low speed at 4 °C for one night. Next, the solution was centrifuged at 3400 g (15 min, 4 °C). The resin was washed three times using sterile distilled water, then washed using 0.1 M glycine NaOH pH 9.3, and finally using 0.5 M glycine NaOH NaCl pH 9.3. The resin that is bound with lysozyme is eluted with glycine NaOH pH 9.3, 0.1 M NaCl, 0.3 M NaCl, and 0.5 M NaCl using a column with a diameter of 2.2 cm. The elution results were collected as much as 7.5 mL in 59 tubes, and the absorbance was measured using an Agilent UV Vis 8453 spectrophotometer at a wavelength of 280 nm.

Lysozyme Protein Concentration

To perform the measurement, reagent solutions such as Na_2CO_3 2% in NaOH 0.1 N as reagent I and $CuSO_4$ 0.5% in NaK Tartrate1 1% solution as reagent II are used. Bovine Serum Albumin (BSA) reagent was used as a standard solution. A standard curve was prepared for seven concentrations of BSA (0; 2.5; 5; 10; 15; 20; and 25 ppm). The BSA solution was reacted with 4 mL of distilled water, and 5.5 mL of reagent III was added. The solution was allowed to stand for 10-15 minutes at room temperature. Next, folin reagent, as much as 0.5 mL, was added to the mixture and homogenized. The solution was left for 30 minutes until a blue color was formed. The absorbance was measured using a UV-Vis 8453 Agilent spectrophotometer with a wavelength of 650 nm. A sample solution of 0.1-1 mL was put into a test tube and reacted the same as in the standard.

SDS-PAGE

The electrophoresis gel used in SDS-PAGE had a formulation consisting of a 12% separating gel and a 4% stacking gel. To perform the electrophoresis process, lysozyme samples up to 10 µL were added to the wells of the electrophoresis gel, along with a comparison marker called ColorBurst™ Electrophoresis Marker (8-220 kDa). The sample migration process was carried out at a voltage of 200 volts and 20 mA for 20 minutes, followed by an increase in voltage to 300 volts and 40 mA until the sample reached the bottom. After the process was completed, Coomasie Brilliant Blue (CBB) was used to stain the gel for 40 minutes. The dye was then removed by soaking the gel in a destaining solution made up of methanol and acetic acid for 24-48 hours. The results of the gel were scanned and the protein molecular weight was measured based on the marker standard curve.

Dialysis

The lysozyme fraction released from the resin using 0.1 M glycine NaOH with the elution of 0.1 M NaCl, 0.3 M NaCl and 0.5 M NaCl was selected and collected based on the results of chromatogram. Furthermore, the dialysis process was carried out using viva flow 50R 5000 MWCO hydrosart membrane (Sartonet) with aquabides buffer (1:5).

Enzymatic Activity of Lysozyme

The substrate in the form of 0.015% suspension was prepared by mixing 0.15 mg/mL of dry Micrococcus luteus ATCC 469 bacteria in 50 mM potassium phosphate buffer pH 6.24 at 25 ℃. Substrate adjustment was carried out to obtain an absorbance of 0.6-0.7 at a wavelength of 450 nm. Lysozyme was prepared as a solution of 0.7 mg/mL in potassium phosphate buffer (pH 6.24, 8 ℃). Micrococcus luteus as much as 1.5 mL and 0.1 mL of lysozyme solution were put into the cuvette. The cuvette containing Micrococcus luteus was added with 0.1 mL buffer solution as a blank. The sample in the cuvette was homogenized and then measured the decrease in absorbance with a wavelength of 450 nm for 5 minutes. One unit of lysozyme is defined as a decrease in Micrococcus luteus per minute by 0.001.

$$Lysozyme\ activity\ \ (unit\ /\ mg) = \frac{\frac{\Delta A450}{minute} sample\ - \frac{\Delta A450}{minute}\ blank}{0.001\ \times 0.1} \frac{(unit}{mL)}{lysozyme\ (mg\ /\ mL)}$$

Temperature Treatment of Enzymatic Activity

Alabio Duck and Mojosari Duck lysozyme were given three different temperature treatments; Low Temperature Long Time (63 ℃, 30'), High Temperature Short Time (72 ℃, 15"), and sterilization temperature (121 ℃, 15'). The solution was then put into a refrigerator (4℃) immediately after heating. An amount of 0.1 mL of each treated lysozyme was added to 1.5 mL of Micrococcus luteus bacteria suspension. Lysozyme activity was determined as described previously.

Data Analysis

The experimental design used was a completely randomized design with 3 temperature treatments; Low Temperature Long Time (63 ℃, 30'), High Temperature Short Time (72 ℃, 15"), and sterilization temperature (121 ℃, 15') using two types of duck eggs, namely Alabio ducks and Mojosari ducks. The study was conducted in three replicates. The linear model used is as follows:

$$Yij = \mu + \tau i + Ɛij$$

Where:

Yij = Observation value of lysozyme stability treatment against heating at i-th level and j-th replication

μ = General mean

τi = Effect of treatment of lysozyme stability to heating at level i-th

Ɛij = Error of treatment of lysozyme stability to heating at i-th level and j-th replication

The data acquired were analyzed with analysis of variance (ANOVA) test to determine the effect of treatment on the observed variables.

RESULTS AND DISCUSSION

The characteristics of lysozyme in the form of protein content, lysozyme weight, yield and enzymatic activity were significantly affected at ($p<0.05$) by the type of egg white.

Lysozyme Purification

The method of separating lysozyme from egg white is generally carried out using ion exchange chromatography. This is closely related to the isoelectric point (pI) difference principle. Lysozyme has a much higher pI than other egg white proteins, and only avidin has a pI close to lysozyme (Rao *et al.* 2013). Cummins *et al*. (2017) elaborated that ion exchange chromatography is based on the ability of the target molecule to be purified to bind to the stationary phase on the chromatography column through ionic bonds. This bond is formed because the target molecule and the stationary phase have opposite charges. Previously, Aberathne *et al.* (2013 and 2014) successfully purified broiler egg white lysozyme via ion exchange chromatography using Amberlite FPC 3500 resin as the stationary phase. Wulandari (2018) also reported the method's success in purifying lysozyme from egg whites of native chickens and Cihateup ducks. In the chromatography, glycine buffer NaOH NaCl pH 9.3 was used as the mobile phase, which causes lysozyme to be positively charged. In contrast, other proteins (except avidin) are negatively charged (Wan *et al.* 2006). Amberlite FPC 3500 is known as a cation exchange resin, which means that this resin will be negatively charged so that it can bind to positively charged lysozyme (cations) (Abeyrathne *et al.* 2014). Based on this, the ion exchange chromatography technique with Amberlite FPC 3500 resin was used in this study to purify lysozyme from Mojosari and Alabio duck egg whites.

The chromatographic elution of Mojosari duck egg (Figure 1) reveals that the egg white protein elutes in 59 fractions, with three main peaks. The first peak is between fractions 1-7, while the second and third peaks are between fractions 12-16 and 18-26, respectively. The third peak has the highest intensity compared to the other two, indicating that the protein concentration in that area is greater than in the other areas. The appearance of peaks in chromatography suggests the presence of proteins that have successfully eluted from the column resin during the purification process (Broeckhoven and Desmet 2022). The appearance of different peaks is caused by the strength of the protein that binds to the resin, resulting in different levels of ease when eluted using NaCl. Generally, proteins interacting strongly with the resin will require more NaCl to elute. This indicates that the protein in the third peak has a stronger bond with the resin than in the second and first peaks. The first peak was obtained by elution using glycine NaOH NaCl 0.1 M pH 9.3 (fractions 1-7), while the second and third peaks were obtained with glycine NaOH NaCl concentrations of 0.3 M and 0.5 M, respectively. The elution process using NaCl as

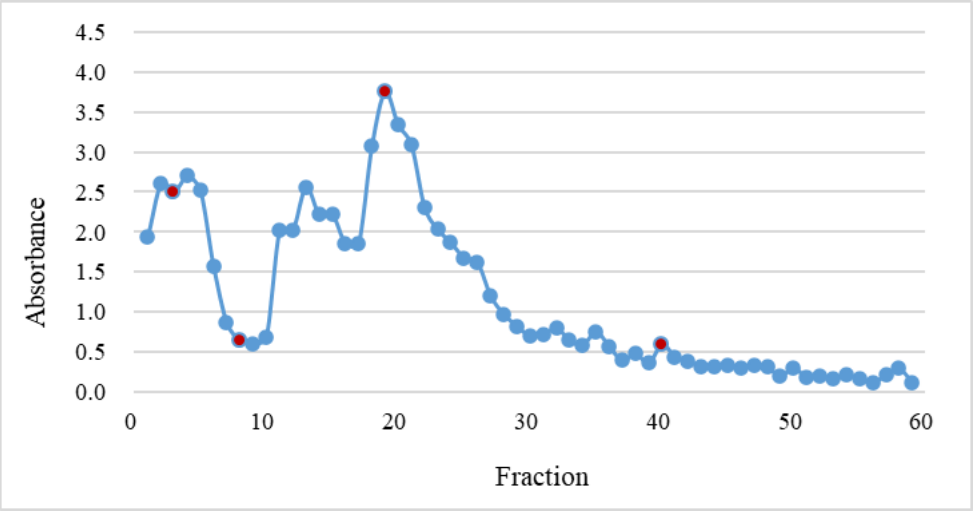

Figure 1. Chromatogram of Mojosari DEWL isolation and purification at 280 nm

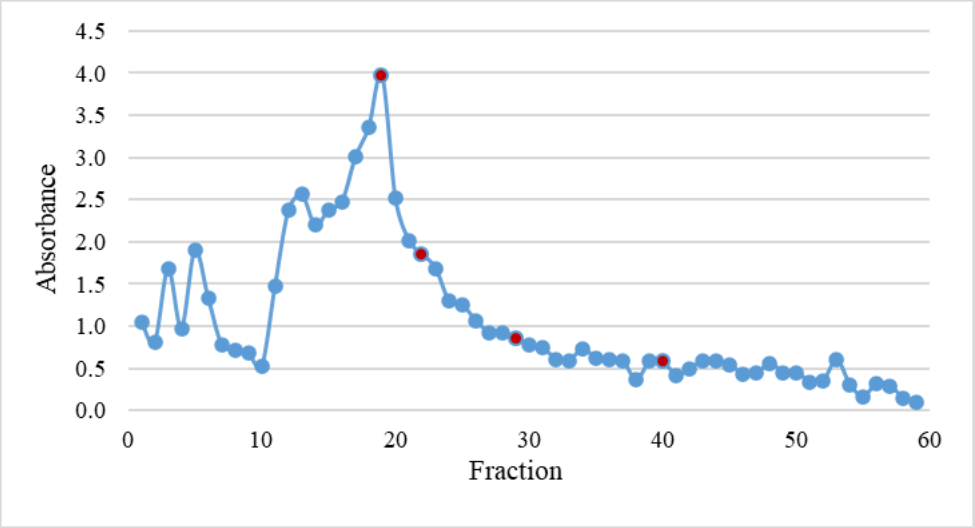

Figure 2. Chromatogram of Alabio DEWL isolation and purification at 280 nm

an ionic solution aims to separate the lysozyme complex from other proteins with the mechanism of Na+ disrupting resin bonds and Cl-binding lysozyme, enabling lysozyme to be released from the resin and dissolved in the buffer for further dialysis (Acikara 2013). Marked fraction numbers were then subjected to SDS-PAGE analysis.

The chromatographic elution results for Alabio duck eggs (Figure 2) show the distribution of eluted proteins in 59 fractions. In the chromatogram, elution produced two main peaks, peak 1 (fractions 2-7) and peak 2 (fractions 10-26). The second peak has a shoulder peak between fractions 10-14. Shoulder peaks in chromatography are triggered by low separation ability, so the molecules cannot be appropriately separated (Risum and Bro 2019). The presence of the shoulder peak indicates that peak two should have been divided into two peaks, namely the initial peak (fractions 10-14) and the rest is the final peak (fractions 15-26).

The protein in the early peak has a lower affinity to the resin than in the late peak. Meanwhile, the protein in the first peak is also confirmed to have the lowest affinity compared to the proteins in the other peaks. As with the elution results of Mojosari duck egg, the second peak in Alabio duck has the highest intensity compared to the other peaks. This indicates that the protein eluted at that peak has a higher concentration. Adhikari *et al.* (2010) generally indicated that in chromatographic techniques, fractions with high absorbance intensity contain higher protein than fractions with low absorbance intensity.

Compared with the chromatogram profiles of lysozyme from native chicken eggs and Cihateup ducks previously reported by Wulandari *et al.* (2015), the chromatogram profiles of Alabio and Mojosari duck lysozyme in this study showed different patterns. This is likely because the lysozyme in different eggs does not have the same affinity, so the strength of its interaction with the resin is also different. This is in line with the findings of Khorsidian *et al*. (2022), who indicated that lysozyme from different sources has different physicochemical properties. The difference in affinity will result in different elution positions of the protein and produce different chromatogram patterns.

Referring to Wulandari (2018), the peaks that appeared during the ion exchange chromatography process using Amberlite FPC3500 were thought to contain lysozyme from the egg. Peaks that appear earlier during the chromatography process are thought to contain lysozyme with lower affinity to the resin, and vice versa. On the other hand, peaks with higher intensity are thought to contain higher concentrations of lysozyme. However, the protein concentration and the presence of lysozyme protein in each fraction still have to be confirmed by Lowry and SDS-PAGE, respectively.

Protein Concentration of Local DEWL

Confirmation of protein concentration in each eluted fraction was carried out using the Lowry method. Measurements were made on several fractions representing the chromatogram peaks. Based on Table 1, the highest concentration, total, and yield of protein from Mojosari duck egg white was obtained in fraction F19. Meanwhile, fractions 40 and after have lower concentrations and total and protein yields than the previous fractions. This is in accordance with Figure 1, which shows the highest peak between fractions 19-20, while the fraction after it has a decreasing intensity.

Meanwhile, the highest concentration, total, and yield of Alabio duck egg protein was also found in fraction 19, which decreased in the fractions afterward. This also aligns with the Alabio duck egg protein elution pattern, as presented in Figure 2. The closer to the final fraction, the smaller the yield produced due to the decrease in the amount of protein (presumably lysozyme) bound to the Amberlite FPC 3500 column. In general, Adhikari *et al.* (2010) indicated that the chromatogram peak was formed because, at that time, most of the proteins bound to the column eluted out. The exit of the protein is due to the ability of NaCl to break the ionic interactions between the protein and resin in the column. Most proteins eluted simultaneously bind to the resin at similar affinities. However, some proteins have weaker or higher affinity, although the proportion of proteins is negligible. Proteins with lower affinity will elute earlier because they require less NaCl than proteins with higher affinity. In the chromatogram, the elution profile of such proteins appears as peaks with earlier elution times.

SDS-PAGE

Although Table 1 indicates the presence of protein in the eluted fractions, lysozyme in each fraction still needs to be confirmed. The presence of lysozyme was confirmed by SDS-PAGE, which visualizes the protein in the gel. The identity of lysozyme on the gel can be easily distinguished from other proteins in eggs because it has a much different molecular weight (about 14 kDa) than other egg white proteins. SDS-PAGE of chromatography fractions from Mojosari and Alabio duck eggs are presented in Figures 3 and 4, respectively. In Figure 3, a band of about 14 kDa in the chromatography results of Mojosari duck eggs appeared in all fractions checked for SDS-PAGE. This band is believed to be lysozyme because its size is the same as the theoretical size of egg white lysozyme, which is 14.3 kDa (Li *et al.* 2022). This indicates that lysozyme was successfully eluted in the chromatography process using Amberlite FPC 3500. However, in fraction 3, bands other than lysozyme were seen with sizes around 77 kDa, 45 kDa and 28 kDa. Based on these sizes, these bands are thought to be ovotransferrin, ovalbumin and ovomucoid, respectively, because they have the appropriate size (Li *et al.* 2022). This indicates that the lysozyme in fraction 3 is still contaminated with other proteins from egg white. Meanwhile, contamination of the three proteins was absent in the lysozyme in fractions 8, 19, and 40. This indicates that Mojosari duck lysozyme in fractions 8 to 40 is categorized as free from contamination (pure).

Meanwhile, the SDS-PAGE results of the Alabio duck egg chromatogram fractions showed that all four selected fractions (19, 22, 29, and 40) contained lysozyme bands based on their size (14 kDa) (Figure 3). However, only fraction 40 contained the lysozyme band without the presence of other bands. In fractions 19, 22, and 29, two contamination bands were suspected to be ovomucin and ovalbumin based on their respective sizes. This indicates that pure lysozyme from Alabio duck egg chromatography is obtained starting from fraction 40. This differs from Mojosari duck egg lysozyme, whose purity can be obtained from an earlier fraction (fraction 8). This difference could be due to the following reasons: (1) The concentration of contaminant proteins in Alabio duck eggs is higher than that of Mojosari duck eggs so that their presence is still detected

Table 1. Protein concentration of lysozyme isolated from duck egg white

Fraction Stage	Mojosari duck			Alabio duck		
	Protein Concentration (mg/mL)	Total protein (mg)	Yield (%)	Protein Concentration (mg/mL)	Total protein (mg)	Yield (%)
F3	0.132 ± 0.048	0.990	0.06	0.102 ± 0.045	0.762	0.08
F8	0.102 ± 0.010	0.766	0.05	0.092 ± 0.025	0.687	0.07
F14	0.355 ± 0.072	2.660	0.18	0.320 ± 0.019	2.399	0.26
F19	0.396 ± 0.024	2.969	0.2	0.450 ± 0.001	3.375	0.37
F22	0.339 ± 0.001	2.540	0.17	0.245 ± 0.074	1.836	0.2
F29	0.165 ± 0.024	1.241	0.08	0.164 ± 0.011	1.227	0.13
F40	0.077 ± 0.006	0.575	0.04	0.051 ± 0.009	0.385	0.04
F57	0.051 ± 0.002	0.384	0.03	0.018 ± 0.003	0.135	0.01

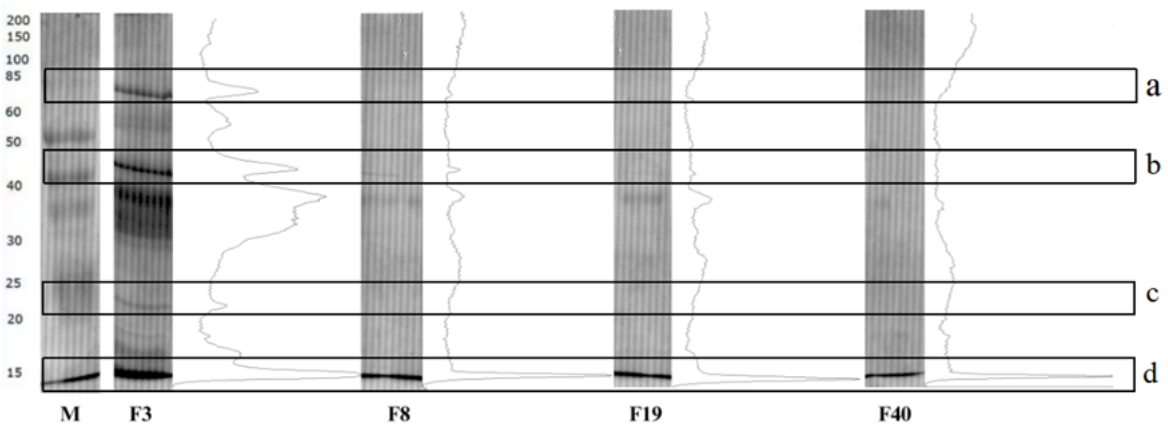

Figure 3. Electrophoresis pattern of egg white Mojosari duck purification using image 14.7 software. M: marker, F3: fraction-3, F8: fraction-8, F19: fraction-19, F40: fraction-40. (a) ovotransferin, (b) ovalbumin,(c) ovomucoid, (d) lysozyme

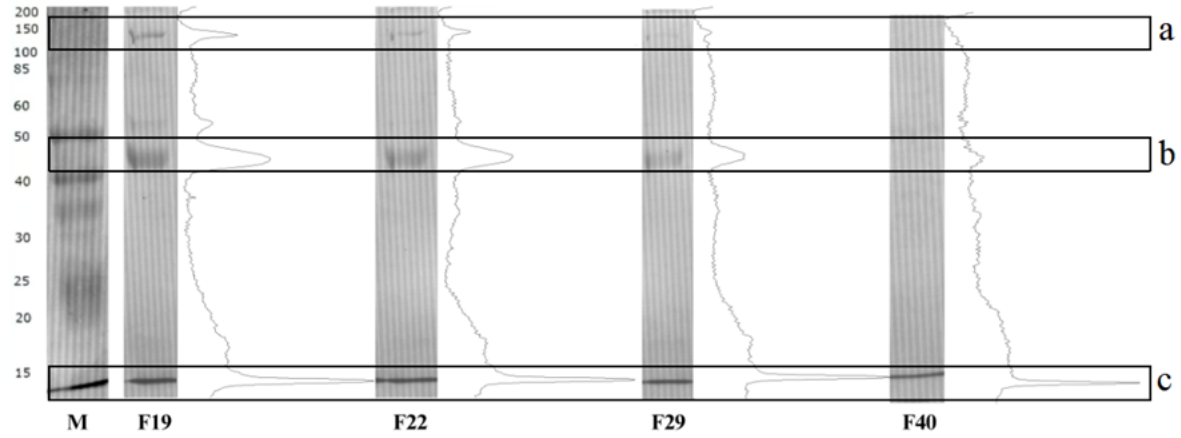

Figure 4. Electrophoresis pattern of egg white Alabio duck purification using image 14.7 software. M: marker, F19: fraction-19, F22: fraction-22, F29: fraction-29, F40: fraction-40, (a) ovomucin, (b) ovalbumin, (c) lysozyme

in various fractions, and (2) The bond between contaminant proteins (ovalbumin and ovomucin) from Alabio duck eggs with Amberlite FPC 3500 resin is stronger than that from Mojosari duck eggs. This is in accordance with Razali *et al.* (2021), who stated that proteins purified from sources with contaminant proteins at high concentrations tend to be distributed in various fractions. In addition, the difference in binding between ovalbumin and ovomucin from Alabio and Mojosari duck eggs is a common phenomenon of the absence of proteins with precisely the same physicochemical properties (Khorsidian *et al.* 2022).

An interesting fact was revealed that in all fractions, no avidin (68.30 kDa) band was found in the SDS-PAGE results of both Mojosari and Alabio duck egg whites. This indicates that the protein is not bound with the resin. This phenomenon is thought to be due to the relatively low concentration of avidin in eggs, which is only 0.05% of total egg white protein and much lower than lysozyme (3.5%) (Awade *et al*. 1999; Li *et al*. 2022). In addition, the distance between the pI of lysozyme and the pH of the mixture is higher than that of avidin so that the binding force of lysozyme to the resin is stronger, and avidin is blocked by lysozyme bound to the resin.

Another interesting fact was that ovomucin, ovalbumin, and ovotransferrin have a much lower pI than lysozyme (Awade *et al*. 1999). The three proteins are thought to be negatively charged at the pH used during the ion exchange chromatography process, so they should not bind to Amberlite FPC 3500 resin. However, the presence of these proteins along with lysozyme in SDS-PAGE (Figures 3 and 4) indicates that these proteins can still interact with the resin. This indicates that some molecules of the three proteins are not homogeneously negatively charged. Some molecules of the three proteins are positively charged and eventually able to bind to Amberlite resin. This unusual behavior can occur because the pI value of a protein is only theoretical without considering environmental conditions that can affect the real isoelectric point in the solution. Budiman *et al.* (2012) mentioned that some amino acids in extreme environments (e.g. too hydrophobic environment) will shift their isoelectric point away from their theoretical value. In addition, the bond between the resin and the three proteins occurs unspecifically (unspecific binding) due to physical entrapment/absorption with the resin. Mohamad *et*

al. (2015) mentioned that both can cause protein molecules to be physically immobilized in the resin even though they are chemically unbound.

Table 2. Purity measurement of duck egg white lysozyme

Lysozyme Fraction	Density	Lysozyme purity (%)
Mojosari duck		
F3	14550.9	47.67
F8	12318.28	75.44
F19	7357.51	82.54
F40	5609.84	94.92
Alabio duck		
F19	8457.71	38.46
F22	7464.69	48.2
F29	5295.54	50.44
F40	4119.01	96.09

In general, the purification method developed in this study successfully purified lysozyme from both Mojosari and Alabio duck eggs. This is characterized by the fraction of chromatography results containing lysozyme bands without the presence of contaminant proteins. The density and purity of lysozyme band electrophoresis results in each fraction are then calculated based on the percentage of the peak quantitatively (Table 2). Each of the results of isolation and purification of duck egg lysozyme showed a thickening of the lysozyme band starting from the absorbance peak, namely the 19th fraction with a density area calculated based on ImageJ 14.7 software at BM 14.3 kDa for Mojosari ducks 7357.51 and 8457.71 for Alabio ducks. At the peak of absorbance, the lysozyme band began to look more clearly until the 40th fraction of the band was mainly composed of lysozyme. Based on the measurement results, it was observed that the purity of lysozyme increased towards the final fraction. In Mojosari ducks, the purity was 94.92%, and Alabio ducks had a purity of 96.09%. This purity value is close to the research of Abeyrathne *et al.* (2014), with a purity level of 97%. However, it is still lower than the research of Wulandari (2018), which is 100% for Cihateup duck lysozyme.

Characteristics of Local DEWL

Lysozyme fractions from each collected duck egg white were dialyzed, and protein concentration, total lysozyme, yield, and lysozyme activity were measured. Table 1 shows that the concentration, total, yield, and activity of lysozyme purified from Mojosari and Alabio duck eggs are very different ($P<0.05$). The concentration and total lysozyme produced from Mojosari duck eggs were higher than those from Alabio duck eggs. Meanwhile, the yield of lysozyme produced from Alabio ducks was higher than that from Mojosari ducks. In the protein purification process, yield is the main parameter determining its effectiveness. This indicates that the purification of lysozyme from Alabio duck eggs is much more efficient than that from Mojosari ducks because it has a higher yield value. Meanwhile, differences in the concentration and total weight of lysozyme obtained from the purification process were caused by many factors, both technical and non-technical. Technical factors include the purification process carried out using a resin that has been inactivated for a long time, so it is thought to affect the molecular binding capacity of the resin. In addition, non-technical factors that affect the difference in the two parameters are genetic factors, feeding, and egg quality, which cause the initial content of lysozyme in eggs used in the purification process to be different.

Table 3. Characteristics of egg white lysozyme isolates of Alabio ducks and Mojosari ducks

Variable	Mojosari duck	Alabio duck
Protein concentration (mg/mL)	0.12 ± 0.001a	0.081 ± 0.005b
Lysozyme weight (mg)	37.63 ± 0.40a	18.27 ± 0.29b
Yield (%)	15.54 ± 0.22a	24.86 ± 2.92b
Enzymatic activity (u/mg)	12319.44 ± 457.1a	8189.39 ± 822b

Small letters written next to the numbers in the same row indicate that there are significant differences ($P<0.05$)

The protein content of contaminants in Alabio duck eggs tends to be higher than in Mojosari duck eggs, as shown in the SDS-PAGE results (Figures 1 and 2). The presence of high contaminants causes a lot of lysozyme to be wasted due to lack of purity. In addition, it should be noted that the eggs used in this study were fresh eggs aged <3 days. This affects the Haugh Unit (HU) of duck eggs, according to Purdiyanto (2018) fresh eggs with a storage age of 5-7 days have a high HU compared to eggs with an age of >7 days. Mojosari duck egg white has HU 91.78, while Alabio duck has HU 92.30 (Melisa 2023). This difference in HU value has implications for differences in egg white viscosity, which causes egg whites with higher viscosity levels to physically envelop the resin so that the lysozyme obtained cannot be eluted optimally. This was proven by Wulandari's research (2018), which used Cihateup ducks with HU 90.06 and produced lysozyme protein levels of 0.53 mg/mL. This result is lower than lysozyme isolation using purebred chicken egg white (HU 84.71) of 1.51 mg/mL and native chicken (HU 77.88) of 2.32 mg/mL.

The difference in concentration and total amount of lysozyme is also closely related to the total protein content of the eggs. Eggs with high total protein will also have high lysozyme levels. The difference in protein levels is influenced by the type of feed and its absorption ability (Sunarno *et al*. 2023). However, the two ducks used in this study were given the same feed treatment (commercial feed with a crude protein of 17-18%). Therefore, it is unlikely that the feed influenced the lysozyme levels in the eggs of the two ducks. The most likely factor affecting the difference in lysozyme levels is the ability of the two ducks to absorb the feed. Ferket and Gernat (2006) state that each type of poultry has a different absorption ability and feed conversion ratio. These differences can ultimately affect the chemical composition of the final product (meat or eggs). However, no study has specifically examined the direct

relationship between feed and the lysozyme concentration in poultry eggs.

Table 3 also shows that the total lysozyme of Alabio ducks and Mojosari ducks was 37.63 mg and 18.27 mg, respectively. The total lysozyme in this study was much lower than the research of Wulandari (2018), which produced lysozyme isolate with a lysozyme weight of 210.81 mg and a yield of 24% in Cihateup ducks. On the other hand, research by Abeyrathne *et al.* (2014) also showed a higher yield of 88.9% with a purity level of 97%, while, in this study, the most significant yield only reached 24.86% with a purity of 96.06%. In general, it is known that protein purity and yield have an unproportional relationship. The higher the protein purity, the lower the yield. This is due to the part of the protein that is wasted at various stages during the purification process. The difference in lysozyme weight and yield may also be due to the number of collected fractions.

Significant differences in the enzymatic ability of lysozyme from Alabio and Mojosari ducks are shown in Table 3. Lysozyme is an enzyme that can hydrolyze glycosidic bonds in the peptidoglycan of bacterial cell membranes. Therefore, the enzymatic activity referred to in this study is its ability to lyse the peptidoglycan of the bacterial target. Micrococcus luteus is ideally used as a bacterial target for lysozyme enzymatic activity testing based on its nature as a Gram-positive bacterium. Micrococcus luteus is susceptible to lysozyme activity because it has a peptidoglycan layer, a substrate for lysozyme. According to Carillo *et al*. (2014), the enzymatic activity of lysozyme is defined as the decrease in turbidity of the suspension of Micrococcus luteus observed with a 450 nm spectrophotometer. One unit of lysozyme is defined as a decrease in absorbance (450 nm) of 0.001 per minute.

Based on Table 3, Alabio duck lysozyme has lower enzymatic activity than Mojosari duck. This is thought to be because the two protein lysozymes have some structural differences in their enzymatic activity. According to Hegyi and Gerstein (1999), the function of a protein, including enzymes, is influenced by various factors, including the structure of the protein. The protein's amino acid sequence influences the protein structure's conformation. Although Alabio and Mojosari duck lysozyme have the same identity (lysozyme), the amino acid sequence details of the two proteins are believed to have differences. The difference in structure is believed to be not on the active side of lysozyme, namely Glu (glutamic acid) and Asp (aspartic acid). According to Tanaka (2021), the active sides of lysozyme (chicken) are Glu-35 and Asp-52. According to Langley *et al.* (2017), all active lysozymes have conserved active sides, namely Asp and Glu. Since Alabio and Mojosari duck lysozymes are enzymatically active, both lysozymes are believed to have Asp and Glu active sides. If the active side is different, the enzymatic activity of the two lysozymes will be lost. Therefore, the difference in activity between the two enzymes is not located on Asp and Glu but is thought to be on the amino acids around the two active sides. The arrangement of amino acids around the active side of Mojosari duck lysozyme is more suitable for supporting its activity, both from binding to the substrate, the catalysis process, and others. Previously, lysozyme from native chicken and Cihateup duck was also reported to have different activities (Wulandari 2018). In general, the two groups of lysozymes (Type C and Type G) do have differences in activity accompanied by differences in their amino acid sequences. Nonetheless, all lysozymes under type C and type G share similarities in the active sides of Glu and Asp (Langley *et al.* 2017). Interestingly, the two types differ in other amino acids, with amino acid similarity below 10% (Ye *et al.* 2010). This indicates that differences in activity in lysozyme from different sources are a common phenomenon reported in many other studies.

Structural differences due to amino acid variations in the lysozyme of Alabio and Mojosari ducks were confirmed due to differences in the genetic background of the two ducks. Alabio ducks and Mojosari ducks may have different sequences of lysozyme coding genes that affect the amino acids they translate (non-sense or missense mutations). The difference in amino acids then affects the structure of the protein, which results in changes in activity or stability (Teng *et al.* 2008). The findings of Myint *et al.* (2011) stated that the missense polymorphism in the gene encoding lysozyme in quail was shown to be a factor behind the variation in the antibacterial activity of lysozyme. However, until now, there has yet to be a study on this matter for the two local duck breeds studied.

Enzymatic Activity of Local DEWL on Temperature Treatment

Although the data in Table 4 clearly shows a difference in lysozyme activity from Alabio and Mojosari ducks ($P<0.05$), the stability of both lysozymes to heat treatment remains unknown. Interestingly, the findings of Hou *et al*. (2023) stated that, generally, there is a trade-off between stability and enzyme activity. This phenomenon states that to achieve high stability, enzymes often sacrifice their activity as compensation. Enzymes with high stability generally have a more rigid structure. This is unsuitable for enzymatic activity because it is less flexible in binding and adjusting the substrate during catalysis. Borrowing this theory, Mojosari duck lysozyme with higher enzymatic

Table 4. Enzymatic activity of local DEWL under temperature treatment

Temperature	Mojosari duck	Alabio duck
Control	11943.30±490.92a	8966.67±1452.44a
Pasteurization LTLT (63 °C, 30')*	4100.00±1231.61b	429.22±124.63b
PasteurizationHT-ST (72 °C, 15")**	7395.56±1671.66c	-
Sterilization (121 °C, 15')	-	-

LTLT (Long Temperature Long Time), HTST (High Temperature Short Time), (-) indicates no enzymatic activity. Lowercase letters behind the numbers on the same column indicate significant differences ($P<0.05$).

activity is expected to have lower stability than that of Alabio duck.

Table 4 shows that Alabio duck lysozyme is more sensitive to heat treatment. LTLT treatment of Alabio ducks significantly decreased the enzymatic activity from 8966.67 U/mg to 429.22 U/mg. This decrease is equivalent to a more than 95% loss of activity. Meanwhile, in Mojosari duck lysozyme, the decrease in activity due to LTLT treatment occurred from 11943.3 U/mg to 4100 U/mg or a loss of only about 65%. In the HTST treatment, the activity of Alabio duck lysozyme was not detected, indicating that the lysozyme became completely inactive. Meanwhile, lysozyme from Mojosari ducks still showed an activity of 7395.56 U/mg, equivalent to 62% of the control activity (a decrease of 38%). On the other hand, total sterilization rendered both lysozyme completely inactive. These results suggest that the relationship between activity and stability of Alabio and Mojosari duck lysozyme does not follow the general pattern of stability-activity trade-off previously reported by Hou *et al.* (2023). Alternatively, the relationship between the activity and stability of the two lysozymes is thought to follow the theory of Hofmeister effects, which explains that the contribution of dominant ionic bonds in proteins tends to cause their stability and activity to have a positive correlation (Riberio *et al*. 2021). Previously, this effect was reported to apply to glutaminase enzymes, as reported by Sakai *et al*. (2022). Meanwhile, the possibility of lysozyme following this effect was also previously reported by Garajova *et al.* (2017), who stated that the contribution of kosmotropic ions to lysozyme will increase its stability and activity. Conversely, an increase in chaotropic ions will have the opposite effect. This indicates that kosmotropic ions dominate the ionic bonds stabilizing lysozyme from both ducks. The main ions in kosmotropic proteins are hydrogen ions (H+), while chaotropic ones are ammonium (NH4+). This indicates that the amino acids that contribute to the stability of both lysozymes can produce hydrogen ions (acids) compared to amino acids with ammonium side groups.

This theory can also explain the difference in stability between Alabio ducks and Mojosari ducks. The more stable lysozyme from Mojosari ducks is thought to be due to more amino acids forming ionic bonds through kosmotropic cations than in Alabio ducks. This is also supported by previous assumptions that indicate differences in amino acid sequences in the two lysozymes. The sequence differences may cause changes in the amino acid profile of the two lysozymes. Hence, the ratio of amino acids with kosmotropic ionic forming side groups in Mojosari ducks is higher than that in Alabio ducks. However, this assumption still has to be proven through further research.

The difference in the effects of LTLT, HTST, and sterilization on Alabio and Mojosari duck lysozyme is believed to be due to the thermal energy generated by each heating method. Thermal energy can break intramolecular ionic bonds in lysozyme, which causes damage to the protein structure. In enzymatic reactions, an increase in temperature accelerates collisions between molecules by increasing the kinetic energy of the reacting molecules, which also breaks the bonds between molecules. On the other hand, lysozyme is a globular protein that is easily affected by temperature and quickly gets denatured. The heating process can reduce the activity value of lysozyme, and the most common reaction is structural denaturation. As per Chang and Li (2002), lysozyme is stable at 55 °C for 60 minutes, but at higher temperatures (65 °C), approximately 55% of the protein gets denatured. The denaturation rate of lysozyme increases almost 14 times over as the temperature increases from 65 °C to 75 °C, which is the denaturation temperature. Venkataramani *et al.* (2013) stated that there are changes in the secondary structure of lysozyme when increasing the temperature slowly until it reaches 65 °C, which is characterized by a decrease in α-helix and β-sheet structure. At 65 °C, the secondary structure becomes less stable, and the hydrogen bonds in the amide groups become weak. When lysozyme is at 70 °C, or the temperature is close to the denaturation temperature, there is a significant decrease in the α-helix structure. At higher temperatures, such as 90 °C, lysozyme undergoes conformational changes as indicated by the characteristic pattern of loss of protein aggregates and changes in helical conformation. Research by Carrillo et al. (2014) reported that heat treatments at 90 °C and 95 °C caused a decrease in lysozyme activity of up to 41.8%.

Table 4 indicates that the impact of low-temperature long-time (LTLT) treatment on lysozyme from Mojosari ducks is more harmful than that of high-temperature short-time (HTST) treatment. Although LTLT treatment is carried out at a lower temperature, it is conducted longer than HTST treatment. This causes the accumulation of more thermal energy, ultimately leading to the destabilization of lysozyme more severely. However, the effect differs for lysozyme from Alabio ducks, where HTST treatment was more extreme in destabilizing the protein than LTLT treatment. This difference is believed to be associated with the different amino acid profiles of the two lysozymes, as the thermal effects work differently on different amino acid compositions. This variation indicates that lysozyme from different duck eggs should be treated differently, particularly considering the risk of heat treatment stress.

CONCLUSION

Isolation and purification of Mojosari and Alabio duck egg white lysozyme had a purity level of 94.92-96.09%. The presence of lysozyme was confirmed by bands mainly composed of lysozyme in the SDS-PAGE. The type of egg used affects the characteristics of the lysozyme produced, such as protein content, lysozyme weight, yield, and lysozyme activity. In addition, thermal exposure such as LTLT, HTST, and sterilization, decreased lysozyme activity. Alabio duck lysozyme is thermosensitive, so it only survives at LTLT temperature. While Mojosari duck lysozyme can survive LTLT and HTST pasteurization temperatures. However, neither of them survived the sterilization temperature.

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