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Hen Egg White Lysozyme Extraction Using SiO_2: Effect of pH and Mineral on Antimicrobial Activity

Khothibul Umam Al Awwaly[1]*, Abdul Manab[1], Manik Eirry Sawitri[1], Eny Sri Widyastuti[1] and Yuny Susanti Haniyah[2]

[1]Animal Product Technology Study Program of Animal Husbandry Faculty Brawijaya University, Malang, East Java Indonesia
[2]Agriculture and Animal Husbandry Polytechnic MAPENA Tuban, East Java, Indonesia
**Corresponding author*

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A B S T R A C T

The purpose of this research was lysozyme extraction from hen egg white using SiO_2. The treatment at first research was pH, at second research was the combination of mineral and ionic strength. At first research albumen from hen eggs white was diluted with buffer phosphate solution at pH 3.0; 5.0; 7.0 and 9.0 was further treated with SiO_2, at second research albumen from hen eggs white was diluted with mineral (NaCl, KCl and NH_4Cl) and ionic strength (0.1, 0.3 and 0.5) was further treated with SiO2. The Results showed that buffer phosphate solution at pH 3.0; 5.0; 7.0 and 9.0 produced the lysozyme extract with lysozyme activity 24578107.48, 23100847.76, 20820046.85 and 21732916.25 (U/minutes) respectively, Specific activity 1412531.5, 1271037.25, 511862.38 and 521482.71 (U/mg) respectively, Lysozyme recovery 52.20, 49.06, 44.22 and 46.16 (%) respectively, and protein content 22.32, 29.14, 40.67 and 42.15 (mg/ml) and SDS–gel electrophoresis shown several bands indicated lysozyme, ovalbumin and conalbumin. NH_4Cl 0.5 M produced highest lysozyme activity (74682875.98 U/minute), specific activity (6379289.65 U/mg) and lysozyme recovery (340.86 (%)), but KCl 0.5M gave lowest lysozyme activity (30810358.01 U/minute), specific activity (2600055.60 U/mg) and lysozyme recovery (141.77 (%)), SDS–gel electrophoresis shown several bands indicated lysozyme, ovalbumin and conalbumin. Lysozyme was fairly adsorbed on the SiO_2 surface at both pH 3, 5, 7 and 9, lysozyme extraction using adsoption method on SiO_2 gave lysozyme with highest antibacterial activity at pH 3. Lysozyme extraction using adsoption method on SiO_2 gave lysozyme with highest antibacterial activity at NH_4Cl 0,05M treatment. These results strongly suggested an electrostatic nature of the adsorption behavior. Results of this study revealed that electrostatic interaction between SiO_2 and lysozyme and hydrogen bonds led to binding lysozyme on SiO_2.

Introduction

Egg white is a natural source of proteins, three major proteins, i.e. lysozyme, ovotransferrin and ovalbumin. (11). Lysozyme (Mucopeptide N-

Acetylmuramoylhydrolase, EC 3.2.1. 17) is a enzyme that catalyzes the hydrolysis of β-1,4-glycosidic linkage between the C-1 of N-acetylmuramic acid (NAMA) and the C-4 of N-acetylglucoseamine (NAGA) of the peptidoglycan in the bacterial cell wall causing cell lysis. Lysozyme have isoelectric point between 10.5 and 11.5 and is stable under acidic pH conditions, especially when thermally treated (Chang *et al*., 2000). Lysozyme shows inhibitory activity towards a broad spectrum of Gram positive bacteria including Staphylococcus and Streptococcus, the activities spectrum of lysozyme can be extended to Gram-negative bacteria after a slight modification (Lesnierowski *et al*., 2013). Nowadays, lysozyme is widely used as food preservative and antimicrobial agent (Cunningham *et al*., 1991). Lysozyme has become increasingly important in the processing of foods due to its function as a natural food preservative.

Therefore, developing a simple efficient methodology for lysozyme production is needed. Various lysozyme-isolating methods have been investigated. Proteins, including lysozyme, horseradish peroxidase, catalase, and trypsin, adsorb strongly to SiO2 particles (sizes ranging from 9 to 40 nm). In the process, lysozyme undergo a partial loss of structure and generally a significant loss in enzyme activity.

Lysozyme is a small spheroidal hydrolase responsible for breaking down the polysaccharide walls of Gram-positive bacteria and those of some Gram-negative bacteria. It is a natural antibacterial protein found in saliva as well as in egg white and mucus. Lysozyme is a compact globular protein with 129 residues, consisting of five α helices, a three stranded antiparallel β sheet, and a large amount of random coil and β turns. Also its structure is stabilized by four disulfide bonds (Murzin *et al*., 1995), with most of the cysteins located in the *α* helices. The enzyme has an approximately ellipsoidal shape, with a large cleft in one side forming the active site which can bind six carbohydrates to execute its effective catalyst function (Wu and Narsimhan, 2008).

The SiO_2 particles have similar surface chemistry (hydrophilic OH terminated surface), which ensures that any difference in interaction with protein molecules (Vertegel *et al*., 2004). In this work, SiO2 was selected as being representative material and lysozyme as representative for enzyme extraction.

Materials and Methods

Materials

The materials used in this study were Hen Egg White, acetic acid (Merck), NaCl (Merck), KCL (Merck), NH_4Cl (Merck), Phosphate Buffer (Na_2HPO_4 0.1 M) (Merck), Silica (SiO_2) (PT. Panadia Corporation Indonesia), distilled water, 30 % bis-acrylamide (Merck), 1M HCl pH 6.8 (Merck), 1M tris HCL pH 8.8 (Merck), aquabidest, 10 % APS (Merck), 10% SDS (Merck), TEMED (Merck), R-250 Coomasie blue (Merck), methanol (Merck), glacial acetic acid (Merck), 50 % glycerol (Merck), 1% Bromophenol blue (Merck), *Micrococcus lysodeikticus* (Sigma), phosphate buffer (pH 6.24) of NaH_2PO_4 (Merck) and Na_2HPO_4 (Merck), Peptone (Oxoid), Lysozyme (Sigma Chemical), 95 % ethanol (Merck) and 85 % phosphoric acid (H_3PO_4) (Merck). Fresh hen eggs were purchased from a local market. Albumen was collected by screening it through a nylon net (2 mm network) to remove the chalazae.

Method

The absorption spectra of all

liquids/solutions were recorded with a Model Ultrospec 4000, UV-Visible spectrophotometer equipped with a thermostatic cell holder. SiO_2 was suspended in deionized water and mixed before use. Hen Egg white was dissolved in deionized water and stored at 4°C. Different pH was prepared to adjust the acidity of the solution. Electrolyte solution was prepared to adjust the ionic strength of the solution.

The first treatment were suspension pH (Daly *et al*., 2003; Jiang *et al*., 2001; Lesnierowski *et al*., 2013; Nezu *et al*., 2008) using Group Randomized Design, at second treatment using salt (NaCl, KCl and NH4Cl) and ionic strength (0,1; 0,3 and 0,5M) was factorial with Group Randomized Design, the variables were Lysozyme activity, Spesific activity, Lysozyme Recovery and Protein content and electroforesis.

Determination of Lysozyme Activity

Micrococcus lysodeikticus cells (Sigma) were suspended in 0.067 M phosphate buffer (pH 6.24) at room temperature and the absorbency at 450 nm (A 450) between 0.6 and 0.7 was measured. Thereafter, 2.98 mL of this suspension, 20 mL of enzyme solution containing between 1000 to 2000U/mL activity, was added at 0 time (the time hydrolysis was initiated); then thoroughly mixed with a vortex mixer. The absorbency at 450 nm as compared with the reference buffer solution was recorded every 30 s for 2 min, using a spectrophotometer (Jasco 7800; Spectroscopic Co. Ltd., Tokyo, Japan). A decrease in the absorbency at 450 nm (DA 450) of 0.001/min was taken as 1 unit of enzyme activity (U) and the results were expressed as units/mL calculated as: Activity (U/min) = (DA 450/min) / (0.001/min 3 0.02 mL) Specific activity (U/mg) = (U/mL) / (protein mg/mL). (Jiang *et al*., 2001).

Determination of Lysozyme Recovery

To compute recovery (%), total lysozyme activity of the collected centrifuged supernatant and of the recovered eluate (AI-CLPPS chromatography) was divided by the total lysozyme activity of the corresponding starting albumen sample. In other words; lysozyme recovery (%) = (total lysozyme activity of the supernatant or of the pooled eluate) / (total lysozyme activity of the corresponding starting albumen sample) x 100% (Jiang *et al*., 2001).

Determination of Lysozyme Specific Activity

Lysozyme Specific activity was calculated by dividing the protein content of the sample*:* Lysozyme Specific Activity (U / mg) = (U / ml) / (mg protein / ml) (Jiang *et al*., 2001).

Determination of Protein Content

Protein content was determined by the Bradford method (Bradford, 1976) with Bio-Rad protein assay dye reagent at 595 nm. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) (0.2 - 1.4 mg/mL) was used as a standard curve to calculate the protein content.

Protein Molecular Weight

Molecular weight fraction of lysozyme protein extracts was determined by SDS-PAGE (6).

Results and Discussion

pH Treatment

Buffer phosphate solution at pH 3.0; 5.0; 7.0 and 9.0 did not gave significant effect (P>0,05) on lysozyme activity, specific activity, lysozyme recovery and protein

content of lysozyme. The results showed that buffer phosphate solution at pH 3.0; 5.0; 7.0 and 9.0 produced the lysozyme with lysozyme activity 24578107.48, 23100847.76, 20820046.85 and 21732916.25 (U/minutes) respectively, Specific activity 1412531.5, 1271037.25, 511862.38 and 521482.71 (U/mg) respectively, Lysozyme recovery (%) 52.20, 49.06, 44.22 and 46.16 (%) respectively, and protein content 22.32, 29.14, 40.67 and 42.15 (mg/ml) as shown at Table 1.

The results for the separation of lysozyme from hen egg white mixture with four pH of suspension were manifested with SDS–gel electrophoresis as shown in Fig. 1. Fig. 1 shown SDS-PAGE pattern, presenting several bands on the gel. SDS-PAGE revealed a 14.4 kDa protein shown to be lysozyme, the impurities indicating conalbumin and ovalbumin, the natural protein of hen egg white, the impurities was absorbed by SiO_2 particle at pH 3, 5, 7 and 9.

The results indicated that there is an interaction between SiO_2 and lysozyme, when lysozyme is mixed into SiO_2 particle suspension, the adsorption of lysozyme occurred by the electrostatic attraction. When the distance between lysozyme and SiO_2 is short enough, the hydrogen bond will form between SiO_2 and lysozyme at polar side chains of amino acid residues. Thus, the interaction between SiO_2 and lysozyme were the combination of non-covalent electrostatic interactions and hydrogen bonds led to binding of lysozyme on SiO_2 particle.

The activity of lysozyme depends on its specific conformation, the covalent and non-covalent interactions among its amino acid residues affect to lysozyme activity. When a certain compound is added to a protein solution, the internal non-covalent interactions of the peptide chain may be altered or even destroyed. The unfolding kinetics of lysozyme when adsorbed onto silica particles shows that upon adsorption, the proteins show a rapid conformational change at both secondary and tertiary structure levels (Shang *et al*., 2007; Fei and Perrett, 2009). The adsorption of lysozyme on SiO_2 particles decreases as the pH decreases. This agrees well with previous observations of lysozyme adsorption onto negatively charged silica, where it was proposed that lysozyme adsorption was strongly influenced by increased protein-protein electrostatic repulsion at a lower pH, electrostatic interaction is the main mechanism controlling the adsorption of lysozyme to SiO_2 (Nezu *et al*., 2008).

The active site of lysozyme is located at the opposite side of the positively charged patch (Vertegel *et al*., 2004; Daly *et al*., 2003). Thus, we may speculate that the first structural changes upon adsorption result is only moderate loss in activity because the structural perturbations are somewhat distant to the active site. More significant perturbation, however, occurs closer to the active site as the native α-helix content is further lost (Vertegel *et al*., 2004). Lysozyme structure and function upon adsorption onto silica particles is strongly dependent upon the size of the particles. Less significant perturbation of lysozyme secondary structure is observed when the protein is adsorbed onto smaller particles under otherwise similar conditions (Vertegel *et al*., 2004).

Minerals and Ionic Strength

Mineral (NaCl, KCl, NH_4Cl) and ionic strength (0,1, 0,3 and 0,5M) did not gave significant effect (P>0,05) on lysozyme activity, specific activity, lysozyme recovery and protein content. The results showed that NH_4Cl 0.5M gave highest lysozyme activity

(74682875.98 U/minute), specific activity (6379289.65 U/mg) and lysozyme recovery (340.86 (%)) but KCl 0.5M gave lowest lysozyme activity (30810358.01 U/minute), specific activity (2600055.60 U/mg) and lysozyme recovery (141.77 (%)) as shown at Table 2.

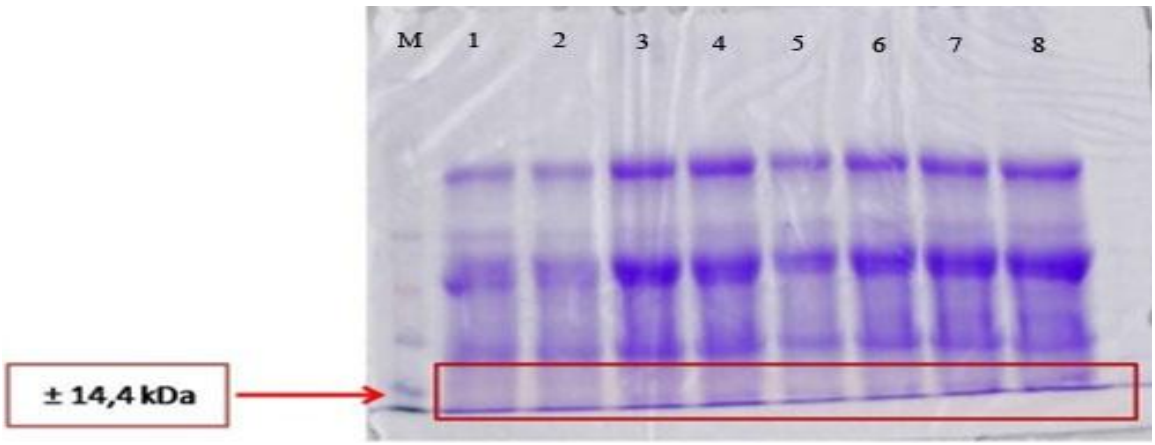
Table.1 Extraction of Hen Egg White Lysozyme Using Sio2 by Ph Treatment

pH	Lysozyme activity (U/minutes)	Specific activity (U/mg)	Lysozyme recovery (%)	Protein content (mg/mL)
3	24578107.48	1412531.51	52.20	22.32
5	23100847.76	1271037.25	49.06	29.14
7	20820046.85	511862.38	44.22	40.67
9	21732916.25	521482.71	46.16	42.15

Table.2 Extraction of Hen Egg White Lysozyme Using Sio2 with Mineral and Ionic Strength Treatment

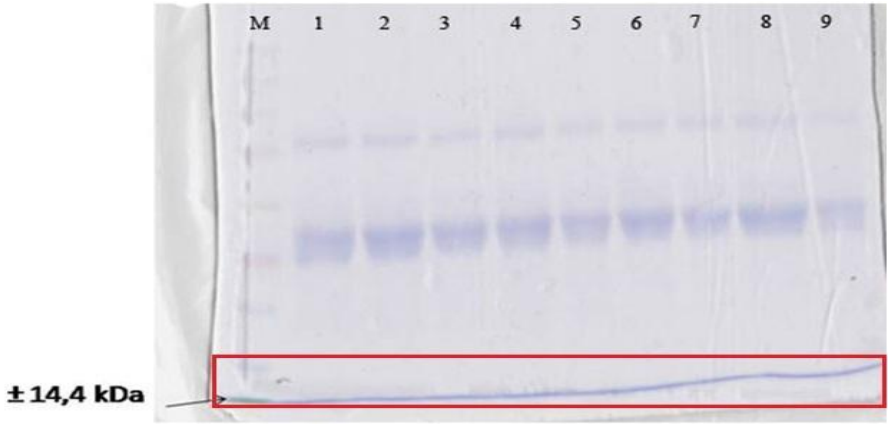
Kind of Mineral	Ionic strength (M)	Lysozyme activity (U/minute)	Specific activity (U/mg)	Lysozyme recovery (%)	Protein content (mg/mL)
NaCl	0,1	53275406.04	4223372.15	276.41	12.64
	0,3	41592155.79	3462220.66	213.70	12.03
	0,5	33942924.55	2872862.91	173.50	11.93
KCl	0,1	63560284.90	5401226.56	343.67	11.68
	0,3	35490657.77	3111599.75	185.25	11.49
	0,5	30810358.01	2600055.60	141.77	11.81
NH4Cl	0,1	62355677.51	5506474.61	255.23	11.02
	0,3	36702170.33	3234062.94	166.62	11.30
	0,5	74682875.98	6379289.65	340.86	11.65

Fig.1 SDS–Gel Electrophoresis of Lysozyme Extract



M, molecular mass markers; Lane 1 and 2, pH 3; Lane 3 and 4, pH 5; Lane 5 and 6, pH 7; Lane 7 and 8, pH 9

Fig.2 SDS–Gel Electrophoresis of Lysozyme Extract



M, molecular mass markers; Lane 1, NaCl 0.1 M; Lane 2, NaCl 0.3 M; Lane 3 NaCl 0.5 M; Lane 4, KCl 0.1 M; Lane 5, KCl 0.3 M; Lane 6 KCl 0.5 M; Lane 7, NH_4Cl 0.1 M; Lane 8, NH_4Cl NH_4Cl 0.3 M; Lane 9 NH_4Cl 0.5 M

The results for the separation of lysozyme from hen egg white using Mineral (NaCl, KCl, NH_4Cl) and ionic strength (0,1, 0,3 and 0,5M) of suspension were manifested with SDS–gel electrophoresis as shown in Fig. 2. Fig. 2 shown SDS-PAGE pattern, presenting several bands on the gel. SDS-PAGE revealed a 14.4 kDa protein shown to be lysozyme, the impurities indicating conalbumin and ovalbumin, the natural protein of hen egg white, the impurities was absorbed by SiO_2 particle mineral solution (NaCl, KCl and NH_4Cl) and ionic strength (0.1, 0.3 and 0.5M).

Increase of ionic strength of NaCl and KCl decreased lysozyme activity, specific activity and lysozyme recovery, otherwise, NH_4Cl with ionic strength at 0.5 M produced higher lysozyme activity, specific activity and lysozyme recovery.

There is a speculation that NaCl and KCl disturb lysozyme adsorption to SiO_2, NaCl potential to disturb electrostatic properties between lysozyme and SiO_2 (10), higher ionic strength of NaCl and KCl decrease lysozyme extract. Contrary, NH_4Cl did not disturb electrostatic properties of lysozyme and SiO_2, higher ionic strength of NH_4Cl increased lysozyme extract produced and higher lysozyme activity, specific activity and lysozyme recovery.

In conclusion, the adsorption of lysozyme on SiO2 were investigated at different pH Conditions, mineral and ionic strength. Higher adsoption of lysozyme on SiO2 at pH 3, NH_4Cl and ionic strength at 0.05 M increased lysozyme activity, specific activity and lysozyme recovery. These results strongly suggested electrostatic interaction between SiO2 and lysozyme and hydrogen bonds led to binding lysozyme on SiO2.

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