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Purification of Egg White Lysozyme from Indonesian Kampung Chicken and Ducks

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ABSTRACT

Egg white lysozyme (EWL) has considerably a wide functional protein exhibiting antibacterial activity mainly against Gram-positive bacteria. The EWL is widely applied in food industry and is considerably safe. Despite its high potency, EWL of Indonesian poultry has never been studied and exploited. This study was aimed to purify EWL from two Indonesian poultry: kampung chicken and Cihateup duck, and compared to egg of commercial laying hens. The eggs in this study were obtained from field laboratory of Faculty of Animal Science, Bogor Agricultural University (IPB) and classified in AA quality based on the interior quality. First attempt to purify the EWL was performed by using ethanol precipitation yielding purified EWL which was still contaminated by other proteins, hence designated as partially purified EWL. Final concentrations of partially purified EWL of kampung chicken, commercial laying hens, and Cihateup duck were about 5800, 5400, and 5500 µg/mL, respectively. To confirm whether the use of ethanol in the purification affecting EWL antibacterial activities, the activities were examined against *Staphylococcus aureus*. It demonstrated that the partially purified EWL exhibited ability to inhibit *S. aureus* at 6 and 26 h suggesting that the method was feasible as it did not interfere EWL antibacterial activities. Yet, based on SDS-Page, purity was the issue in ethanol precipitation method. Further attempt using ion exchange chromatography at pH 10 successfully purified lysozyme as indicated by a single band corresponding to lysozyme size (~14 kD) free from bands of other proteins. Altogether, a single step of ion exchange chromatography is sufficient and promising to isolate EWL from Indonesian poultry for various industrial purposes.

Key words: Indonesian poultry, lysozyme, egg, kampung chicken, Cihateup duck

ABSTRAK

Lisozim putih telur memiliki fungsi yang luas, salah satunya adalah memiliki aktivitas sebagai antibakteri terutama terhadap bakteri Gram positif. Lisozim dari putih telur juga banyak diaplikasikan di industri pangan dan dinyatakan aman untuk pangan. Sampai saat ini lisozim dari putih telur yang berasal dari unggas lokal (Indonesia) belum dipelajari dan dikembangkan. Tujuan penelitian ini adalah melakukan purifikasi putih telur dari telur unggas lokal, yaitu ayam kampung dan itik Cihateup dibandingkan dengan telur ayam ras. Sampel telur yang didapatkan dari Laboratorium Lapang Fakultas Peternakan diklasifikasikan pada grade AA berdasarkan kualitas interior. Tahap pertama purifikasi lisozim putih telur dengan presipitasi etanol menghasilkan protein lain selain lisozim (purifikasi parsial). Konsentrasi lisozim putih telur hasil purifikasi parsial telur ayam kampung, ayam ras dan itik Cihateup masing-masing adalah 5800, 5400, dan 5500 ug/mL. Untuk melihat efek penggunaan etanol dalam proses purifikasi terhadap aktivitast antibakteri lisozim, kemampuan daya hambat lisozim terhadap *Staphylococcus aureus* telah dianalisis. Lisozim putih telur hasil purifikasi parsial dapat menghambat *S. aureus* pada inkubasi selama 6 dan 26 jam menunjukkan bahwa teknis presipitasi etanol mempertahankan aktivitas antibakteri lisozim telur-telur tersebut. Meskipun demikian, metode tersebut tidak menghasilkan lisozim dengan tingkat kemurnian yang tinggi sehingga diperlukan metode lainnya. Metode kromatografi penukar ion pH 10 berhasil memisahkan lisozim dari protein yang lain. Hal ini dapat terlihat dari adanya pita tunggal hasil elektroforesis yang sama dengan standar lisozim (~14 kD) tanpa adanya protein yang lain. Satu tahap dari kromatografi penukar ion telah dapat memisahkan lisozim putih telur unggas lokal.

Kata kunci: unggas lokal, lisozim, telur, ayam kampung , itik Cihateup

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INTRODUCTION

Egg white lysozyme (hereafter called EWL) is a relatively small enzyme consisting of 129 amino acids with about 14.3–14.6 kD in its size (Johnson & Larson, 2005; Stadelman & Coterril, 1984). Despite EWL represents only 3%-4% of the egg white dry weight or about 2500–3000 ppm fresh-weight bases (Liburdi *et al*., 2014; Stadelman & Coterril, 1984), it is being widely used mainly in food industry due to its antibacterial properties (Tirelli & De Noni, 2007; Benkerroum, 2008; Schneider *et al*., 2011). It is usually added directly into food products (Liburdi *et al*., 2005) including cheese (Davidson, 2001), vegetable, seafood, pasta, and salads (Davidson, 2001). Application of lysozyme, in combination with bacteriocin nisin, has also been applied in meat and meat products (Nattress *et al*., 2001; Gill & Holley, 2003; Cegielska *et al*., 2009; Abdou *et al*., 2007; Cegielska *et al*.,2008; Malicki *et al*., 2004). Even more, EWL is very frequent to be used as antimicrobial enzyme incorporated into food packaging materials (Babiroli *et al*., 2012; Duan *et al*., 2008; Edward *et al*., 2011; Gucbilmez *et al*., 2007; Mecitoflu *et al*., 2006; Min *et al*. 2005; Kandemir *et al*., 2005;). Further, EWL is considerably safe to be used in food system (Karkaet & De Meulanaer, 2007) as declared by World Health Organization (WHO) and Food and Agriculture Organization (FAO) joint committee in 1992.

The inhibition of EWL was reported not only against saprophytic bacteria, but also against important food pathogens, such as *Listeria monocytogenes* and *Clostridium botulinum* (Hughey & Johnson, 1987). Among the 15 examined bacteria species, *Clostridium tyrobutyricum*, *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* were completely inhibited by EWL. *Bacillus cereus*, *Campylobacter jejuni*, *C. botulinum* types A, B and E, *Yersinia enterocolitica* and *L. monocytogenes* were among the bacteria moderately inhibited, whereas *Clostridium perfringens*, *Escherichia coli* O 157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* were not inhibited (Murry *et al*., 2004; Hughey & Johnson, 1987).

Antibacterial activity of EWL is due to its ability to disrupt the bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) of the peptidgoglycan in bacterial cell walls (Callewaert *et al*., 2012; Lesnierowski & Kijowski, 2007). EWL is mostly active against Gram-positive bacteria, while Gram-negative bacteria are relatively resistant as the peptidoglycan layer of Gram-negative bacteria is protected by outer membrane compartment (Turner *et al*., 2013). The mechanism by which EWL hydrolyzes the β(1-4) glycosidic linkages from NAM to NAG is well studied (Wohlkonig *et al*., 2010; Held & Smaalen, 2014). The reaction is likely proceeds via a covalent intermediate mechanism, in which Glu35 and Asp52 act as acid and covalent catalysts, respectively. Crystal structure of lysozyme revealed that Asp52 site is surrounded by several conserved polar residues with which it forms a complex hydrogen bonded network. Asp52 is therefore should be unprotonated and hence negatively charged throughout the 3 to 8 pH range over which lysozyme is catalytically active.

In attempts to exploit EWL advantages as described above, purification of EWL is unavoidable. The classic purification method of EWL was introduced by Alderton & Fevolid (1946) employing crystallization technique, which takes a week or more (Olieric *et al*, 2007). Despite the purity is remarkably high, the method is not feasible due to time constrain. Further, other methods for EWL purification were developed, including ion-exchange and affinity membrane chromatography, and ultrafiltration (Grasselli *et al*., 1999; Ghosh & Cui, 2000; Jiang *et al*., 2001; Arca *et al*., 2004). In addition, partial purification techniques were also widely developed. These include partitioning of lysozyme by the polyethylene glycol/salt aqueous 2-phase system (Su & Chiang, 2006), selective precipitation and recovery of lysozyme with anionic surfactant di-(2-ethylhexyl) sodium sulfosuccinate (AOT) and acetone (Shin *et al*., 2003), selective precipitation of non-lysozyme proteins in the egg white by heat-induced denaturation and gelation applied at 70 °C (Chang *et al*., 2000), and by incubation in the presence of 30% ethanol (Jiang *et al*., 2001), to name but a few. In term of application purpose, the simplest and fastest method yielding the purest EWL is the most feasible for EWL purification.

Indonesian native poultry, both chicken and duck, are considered to be important genetic resources, particularly in relation with meat and egg productions (Nataamijaya, 2010). Kampung chicken and Cihateup duck are Indonesian poultry that are widely used both for meat and egg purposes. In respect to taking the advantage of their egg productions, the attempts have so far been limited only in processing the egg to other egg-based products. Neither study nor application, to our knowledge, has been performed in egg white functional proteins, including lysozyme, from kampong chicken and Cihateup duck.

This study was aimed to purify antibacterial activity of EWL from kampung chicken and Cihateup duck by using two approaches: ethanol precipitation and ion-exchange chromatography. In the first attempts, there is a risky of degradation of EWL by the use of ethanol during the purification. To address, authors have examined antibacterial activites of the purified EWL againts-*Staphylococcus aureus*. Among various combinations of previously reported purification methods for EWL, in this study we successfully isolated EWL through a single step of ion-exchange chromatography. Further, the purified EWL displays remarkable antibacterial activity against *S. aureus.*

MATERIALS AND METHODS

Collection of Egg White Fractions of Kampung Chicken and Cihateup Duck

Fresh eggs of kampung chicken and Cihateup duck were obtained from field laboratory of Faculty of Animal Science, Bogor Agriculture University (IPB). The collected eggs were based on exterior quality including shell cleanliness, the absence of embryo and high viscosity. While the cleanness was observed directly with naked eye, the presence of embryo and the viscosity

were observed through candling technique according to Stadelman & Cotteril (1984). Prior to further experiment, interior qualities of the eggs were also examined based on Stadelman & Cotteril (1984). The examined qualities included whole weight, egg white weight, the Haugh unit (HU) value, and yolk yellowness. Briefly, the eggs were weighed using 0.01 g analytical balance (HWH Corporation, USA) and then gently cracked and broken in glass table. HU value was calculated using the egg weight and egg white height (Doyon *et al*., 1986). The yolk yellowness was measured using yolk color fan (Roche, Germany). For comparison, eggs of non-Indonesian laying hens (hereafter called commercial laying hens) were also collected and treated as described above. Egg white was then separated and kept for further experiment.

Lysozyme Purification

Partial purification. The experiment was performed based on Gemili *et al*. (2012) with slight modifications. Briefly, egg white was diluted 3-4 fold with 50 mM NaCl solution. The precipitation of egg white proteins, other than lysozyme, the pH of the cocktail was adjusted to 4.0 by step wise dropping of 1 N acetic acid and it was then diluted with an equal volume of 40% (v/v) and followed by 8 h incubation at room temperature. The mixtures were then centrifuge at 15,000 g for 15 min at 4 ^{o}C to separate precipitated protein and soluble protein in supernatant. The precipitant was discarded and the lysozyme in the supernatant was secured for purity, sizing, and concentration analysis and antibacterial activity as well.

Purification using ion exchange chromatography. The experiment was performed based on Strang (1984) with slight modifications. Briefly, egg white from one egg was filtered by using 3 layer cheesecloths and followed by 5 times dilution with 100 mM glycine/NaoH buffer pH 10.0. Two gram of dry carboxymethyl cellulose (CMC) was then added to the bulk and stirred for 15 min to adsorb the EWL. Suspension was then centrifuged at 15,000 g for 5 min and CMC-containing pellet was collected. The pellet was then washed with 100 mM glycine/NaOH buffer pH 10.0, in an equal volume as egg white, followed by centrifugation as before to have washed pellet. The washed pellet was resuspended in glycine buffer as before and then poured into 1 cm diameter column. The first elution was performed by glycine buffer as before. The final elution was performed using glycine buffer containing 0.5 M NaCl. The eluates were collected in 10 mL Falcon tube, so-called fractions, and the presence of proteins in each fractions was monitored by using the Agilent 8453 UV-vis spectrophotometer (Agilent Technologies, USA) at 280 nm.

Purity, Sizing, and Final Concentration of EWL Determination

The purity and size of eluted protein were confirmed by using 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) stained with Coomassie brillian blue R-250. For sizing in SDS-page, molecular weight protein markers of PageRuler Unstaind Protein Ladder (Thermo Scientific, USA) were used as size standard. The markers contain a mixture of 14 recombinant, highly purified, unstained proteins with the size ranging from 10 to 200 kD. The final concentration of partially purified EWL was determined by using Lowry method (Lowry *et al*., 1951; Goldring, 2012). Meanwhile, the final concentration of purified EWL from ion exchange chromatography was determined by using the Agilent 8453 UV-vis spectrophotometer (Agilent Technologies, USA) at 280 nm on the basis that the absorbance at this wavelength of a 0.1% (1 mg mL^{-1}) solution is 24.6 for lysozyme. This value was calculated by using extinction coefficient (ε) of Tyr and Trp were 1576 and 5225 M^{-1} cm^{-1}, respectively, at 280 nm. The absorbance method is not feasible for the partially purified EWL since some contaminant proteins (with difference extinction coefficient) were present.

Antibacterial Activity Against *Staphylococcus aureus*

The experiment was performed based on combined methods of Jenzano *et al*. (1986) and Kumar *et al*. (2001). Stock cells of *S. aureus* ATCC 25923 was cultured in Lauria-Bertani broth media, pH 7 at 37 ^{o}C in the absence or in the presence of purified lysozyme. The culture of *S. aureus* incubated in the absence of purified lysozymes was considered as a control. To monitor the growth of *S. aureus,* optical density (Abs) at 600 nm was observed at initial incubation (0 h) and at 7 and 26 h incubation time. Antibacterial activity of purified lysozyme to inhibit *S. areus* was determined as percent of inhibition referring the ratio of turbidity of *S. aureus* in the presence of lysozyme to that of the control. The inhibition was calculated by the following formula:

Inhibition (%)= [(A control - A sample treated with *S. aureus*)/ A control] x 100%

Data Analysis

Quantitative data were statistically analyzed by using analysis of variance (Anova) with Tukey as post-hoc test used to determine the differences among the means (Steel & Torrie, 1995). Unless stated otherwise, all experiments were performed triplicate in which 3 egg were used for each replication.

RESULTS AND DISCUSSION

None of eggs used in this study showed the presence of embryo and shape abnormalities (data not shown). The average weight of eggs from kampung chicken, Cihateup duck, and commercial laying hens were 38.11±3.52, 58.93±3.27, and 59.86±0.15 g, respectively. The weight of eggs from commercial laying hens was considerably normal according to Roland *et al*. (1984) and Tugiyanti & Iriyanti (2012). Despite weight of eggs of kampung chicken was significantly lighter ($P<0.01$) to that of eggs of commercial laying hens, the value was close to the range of average weight of egg sof kampung chicken, which is ranging from 39 to 48

g (Sulandari *et al*., 2007). The weight discrepancies between kampung chicken and commercial laying hens are acceptable due to the differences in their genetic and feeding management. The discrepancies were also been considerably common as reported by Sulandari *et al*. (2007). The weight of Cihateup duck was considerably similar to that of eggs of commercial laying hens but significantly higher to that of eggs of kampung chicken (P<0.01). The similarity of the weight of egg of Cihateup and commercial laying hens in this study is intriguing, since duck egg is commonly heavier to that of chicken egg. Noteworthy, the average weight of Cihateup duck egg in this study is about 10 g lighter than that of previously reported by Dudi (2007). The differences in production management and age of the duck used in these experiments might account for the difference between the current study and previous reports. Hence, exterior analysis of the egg used in this study showed the acceptable quality for further experiments.

Interior analysis of the egg is shown in Table 1. It was clear that the egg white heights among the egg were considerably similar. There is a conflicting assumption on the relation between egg weight and egg white height. Wilgus & VanWagenen (1936) report that there is no relation between egg weight and egg white height, while Silversides & Scott (2001), as supported by Siyar *et al.* (2007), strongly suggest for the bias of strain and age on the egg white height. As strain and age might affect the egg weight, Silversides & Scoot (2001) and Menezes *et al.* (2012) also implied the bias of egg weight on the egg white height. Our result, however, supported Wilgus & VanWagenen (1936) proposal as it indicated that there was no bias of egg weight on the egg white height as indicated by the similarity of the height across the egg with difference weight (Table 1).

Table 1 also showed the similarity of HU values among the eggs. While the similarity is acceptable, HU value of egg of kampung chicken is considerably intriguing as Eisen *et al*. (1962) reports that egg weight affects HU value. Given weight of eggs of kampung chicken is significantly lower to that of Cihateup duck egg and commercial laying hens, the HU value of kampung chicken egg, according to Eisen *et al*. (1962) assumption, is supposed to be different from the others. Yet, Table 1 displayed variance of HU value of kampung chicken egg was considerably high (> 20% of the mean) which might indicate high variety of the value among the eggs tested in this study. This high variance might account for the intriguing HU value of kampung chicken. Nevertheless, according to HU values, the eggs used in this study were classified as AA quality, as the HU values were higher than 72 (Brown, 2000).

Table1. Exterior and interior quality of egg

Measured variables	Cihateup duck	Commercial laying hen	Kampung chicken
Egg white height (mm)	8.10±0.86	7.23±0.76	5.26± 1.94
HU value	90.06±5.26	84.71±5.76	77.88±16.95
Yolk yellowness	9.00±0.00	7.22±1.29	7.67± 2.05

The yolk yellowness scores, as shown in Table 1, were similar for all eggs tested in this study (P>0.05). The yellowness was measured by using a yolk color fan which ranged from 1 to 14 scales, so-called yellowness score. The higher score indicated the brighter color of the yolk. The color is mainly modulated by pigment contained in consumed feed. Yolk coloring compound is *xanthophyll*, a carotenoid pigment found in corn, alfalfa crops and corn gluten meal. Egg yolk color is influenced largely by xanthophyll in feed. Carotenoid, a precursor of vitamin A, is a pigment found in plants and animals (Kljak *et al*., 2012; Stadelman & Cotterill, 1984). Altogether, external and interior qualities of the eggs used in this study were considerably good sources for lysozyme.

Purification Lysozyme

First attempt to isolate lysozyme from egg white of the above-mentioned egg was performed using partial purification technique as reported by Gemili *et al*. (2007). The method is basically based on the ability of miscible solvent to specifically precipitate contaminant proteins due to desolvation effect by the solvent. Further, the precipitate and soluble proteins were separated based on their gravity. Desolvation effect of the miscible solvent may differ to each protein according to their surface charges. Thus, in our study, concentration of ethanol, as a miscible solvent used in the experiment, was adjusted to precipitate egg white proteins other than lysozyme (see experimental method section for the detail). The use of ethanol to purify proteins based on this mechanism was also reported by Tschaeliessnig *et al.* (2014) in attempt to obtain high purity recombinant antibodies. The use of 40% (v/v) ethanol in our experiment is based on Gemili *et al*. (2007) reporting that this concentration yielded best balance of purity and activity of EWL. Thus, it was expected final supernatant only contain lysozyme.

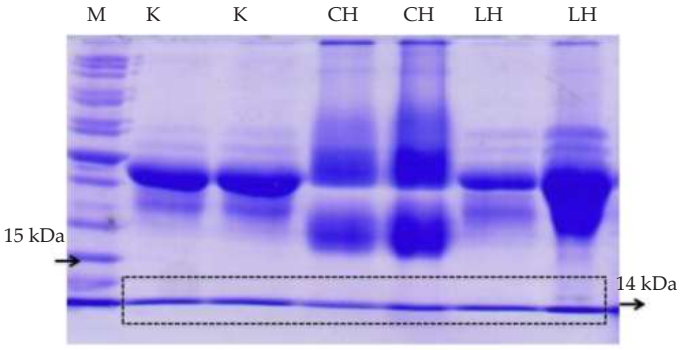

Figure 1. Electrophoresis results of lysozyme partial purification. 15% SDS-Page of partially purified egg white lysozyme of kampung chicken (K), Cihateup duck (CH) and commercial laying hens (LH). Two identic samples from each egg were loaded in two adjacent wells. The bands correspond to lysozyme size are indicated in the dashed box. M lane corresponds to low molecular weight protein markers (Termo Scientific, USA). The marker band corresponds to 15 kD shown in the figure, as it is closed to lysozyme`s size. Size label for other bands were omitted for clarity.

The result showed in Figure 1 displays the band of reactivated EWL after ethanol precipitation. The bands correspond to EWL size (~14 kD) is smear and mixed with other bands suggesting the following possibilities: (1) Amount of EWL that was recovered in precipitation is considerably low; and (2) EWL precipitated together with other egg white proteins at the concentration used in the experiment. However, in term of purity, ethanol precipitation indeed partially isolated EWL from the other proteins. Despite non-lysozyme proteins were also present, however the amount and number, as indicated by the band thickness and number, are remarkably less than the whole egg before precipitation.

Final concentrations of partially purified EWL of kampung chicken, commercial laying hens, and Cihateup duck were about 5800, 5400, and 5500 μg/mL, respectively. These values were statistically similar and considerably acceptable, as the values did not imply the total amount of EWL, which was certainly biased by the egg weight. In compare to Lesierowski & Kijowski (2007), these values were considerably higher by about 2 and 4-folds. The possible explanation for this evidence was the presence of contaminant protein in our purified EWL that contributed to the final concentration of the solution.

The use of ethanol in this technique is considerably risky as it may affect the activity of final EWL (Miyawaki & Tatsuno, 2011). To confirm whether or not the partially purifed EWL retained its bacterial activity, the inhibition assay against *S. aureus* was performed and the result is shown in Figure 2. It is interesting that the reactivated EWL retains its antibacterial activity at 7 h incubation at 37 °C. It confirmed that partially purified EWL in this study was indeed active. Alternatively, the use of ethanol at the concentration used in this study did not seriously affect folding of EWL to be active as an antibacterial protein. The inhibition of *S. aureus* at 7 h incubation time was ranging from about 15%-25%, in which the highest inhibition was shown in partially purified EWL of Cihateup duck egg. The inhibition of

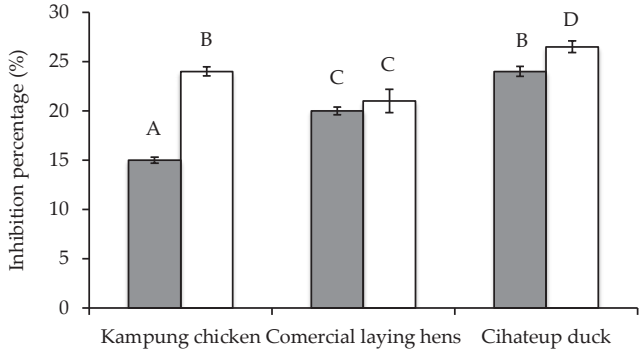

Figure 2. Inhibition of partially purified egg white lysozyme against *Staphylococcus aureus* during incubation at 7 (■) and 26 (□) hours at 37 °C. The error bars corresponds to standard error of the means from two independent experiments in which 3 eggs were used in each experiment. Means with different letters are significantly different (P<0.01).

EWL of egg of commercial laying hens was significantly (P<0.01) lower than that of EWL of the duck, but higher than that of the kampung chicken. The average of the inhibition effect of the EWL is increased (about 22%-27%) at longer incubation time (26 h). However, at this incubation time there was no significant difference (P>0.05) among the EWL. Given the role of the EWL to inhibit *S. aureus* at this incubation was considerably insignificant, the reducing of *S. aureus* population is mainly due to the death of aureus cells caused by the unbalance of cells population and available nutrient in the growth medium at longer incubation time.

Despite it remained active, the presence of remarkable contaminant in the partially purified EWL was the concern to argue that the method was considerably not feasible to isolate pure EWL for further purposes. Hence, the development of alternative and better method to isolate EWL is unavoidable.

To address this issue, we attempted to purify lysozyme using another method: ion exchange chromatography as was initially proposed by Strang (1984) and further modified by Luding *et al.* (2011), Safarik *et al.* (2007), Arica *et al.* (2004) and Li & Chen (2002), just to name a few. The method is theoretically feasible to be used as a single step to purify EWL on the basis of isoelectric point (pI) of EWL is extremely higher (10.7) compared to that of other egg white proteins (< 6.5) (Anton *et al*., 2006; Machado *et al*., 2007; Luding *et al*., 2011). The only egg white protein with pI close to EWL`s pI is avidin, which is 10.0. However, this magnitude is considerably sufficient to discriminate EWL and lysozyme based on their charges when the environment pH is adjusted between 10 to10.7. This is the reason of the use of buffer with pH 10 in our experiment. At this pH, in exception of EWL and avidin, egg white proteins are supposed to be positively charged. While EWL will be considerably negatively charged at pH 10, avidin is supposed to be uncharged. Based on this condition, when carboxymethil-cellulose (-CH_2COOH; pKa 3.5-4.5) or sulfopropyl (-CH_2SO_4; pKa 2-2.25) is dissolved in pH10, these materials are negatively charged. When these materials used as beads upon the purification, the negatively charged of the beads bind to positively charged of EWL, while other proteins do not dissolve and elute as flow-through fractions. Further, complex of EWL-the beads is disrupted by stronger ionic strength solution (NaCl) to obtain beads-free EWL, so called purified EWL.

Elution profiles upon ion exchange chromatography for all eggs displayed several peaks (Figure 3A, 4A, and 5A**)**, in which the first peak (ranging from fraction 1 to 4 or 5) was the highest peak indicating that more protein eluted in this first peak. These proteins were eluted earlier (in the first peak) because of their binding affinities to negatively charged column were extremely weak. Later peaks were relatively smaller and broader which implied proteins amount were relatively smaller and ionic strength were remarkably more positively charged compared to those in earlier peaks. More positive charges may increase binding affinity to negatively charged column and thus requires more NaCl to be eluted.

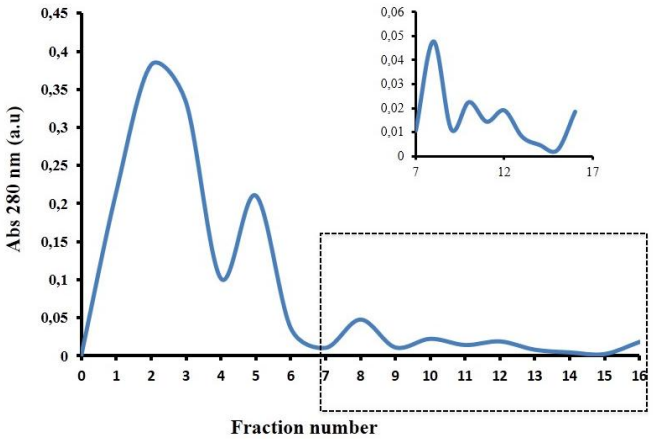

Figure 3A. Elution profile upon purification of egg white lysozyme of kampung chicken using ion exchange chromatography as monitored using UV-vis spectrophotometer at 280 nm. The peaks shown after 7th fraction (dashed box) were enlarged as shown in the right-top of this figure.

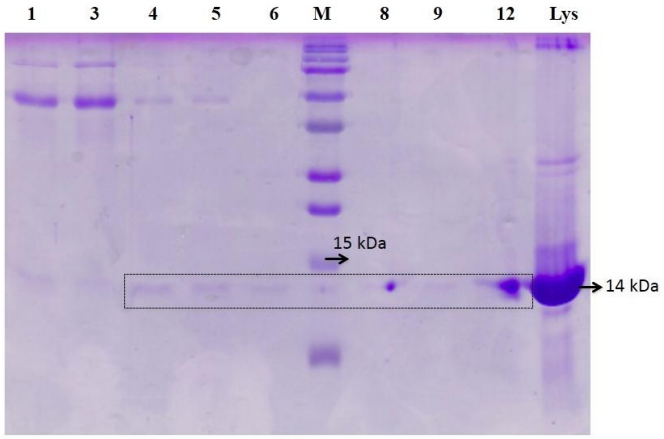

Figure 3B. 15% SDS-Page of purified egg white lysozyme of kampung chicken using ion exchange chromatography. The number shown in the top of gel corresponds to fraction number in elution profile (3A) in which 10-µL aliqoutes taken from each respected fraction was loaded into the well. The bands correspond to lysozyme size are indicated in the dashed box. M lane corresponds to low molecular weight protein markers (Termo Scientific, USA). The marker band corresponds to 15 kD shown in the figure, as it is closed to lysozyme`s size. Size label for other bands were omitted for clarity.

SDS-Page in Figure 3B, 4B, and 5B display proteins bands taken from representative fractions in each peaks a shown in Figure 3A, 4A, and 5A. First peaks from all egg gave many bands in SDS-Page, which, according to their sizes, are considerably non-lysozyme egg white proteins. This evidence is plausible since non-lysozyme egg white proteins are supposed to be negatively charged, due to their pIs, and therefore the binding affinities to negatively charged column are abolished due to repulsion effect. A single band corresponding to the size of lysozyme (~14 kD) was obtained after the purification from all egg were obtained in started from fraction of 6 (in kampung chicken and Cihateup duck, Figure 3B, 5B) or fraction of 8 in commercial laying hens (Figure 4B). Despite of a single band, the band is remarkable thinner compared to that of obtained from partial purification (Figure 1). It might be due to technical issue upon the purification. Elution volume in ion exchange chromatography was adjusted lower and broader than that of partial purification, thus total

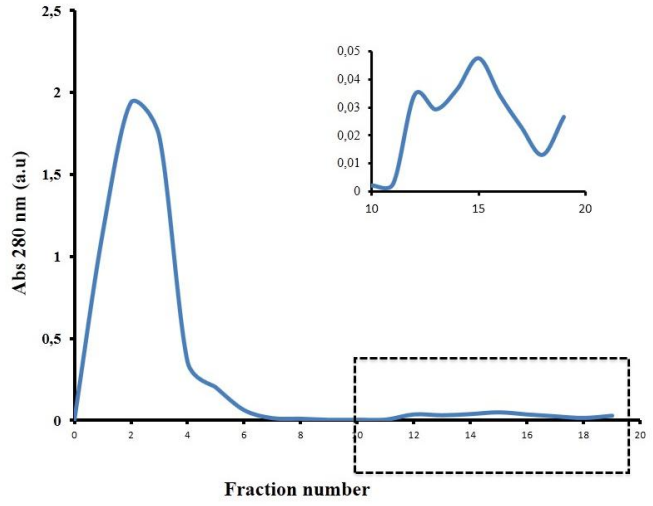

Figure 4A. Elution profile upon purification of egg white lysozyme of commercial laying hens using ion exchange chromatography as monitored using UV-vis spectrophotometer at 280 nm. The peaks shown after 10th fraction (dashed box) were enlarged as shown in the right-top of this figure.

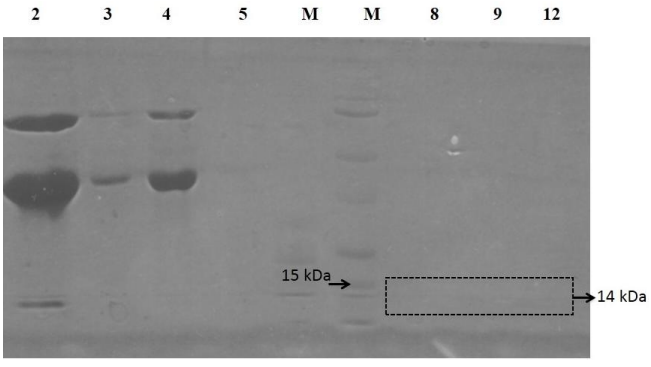

Figure 4B. 15% SDS-Page of purified egg white lysozyme of commercial laying hens using ion exchange chromatography. The number shown in the top of gel corresponds to fraction number in elution profile (4A) in which 10-µL aliqoutes taken from each respected fraction was loaded into the well.. The bands correspond to lysozyme size are indicated in the dashed box. M lane corresponds to low molecular weight protein markers (Termo Scientific, USA). The marker band corresponds to 15 kD shown in the figure, as it is closed to lysozyme`s size. Size label for other bands were omitted for clarity.

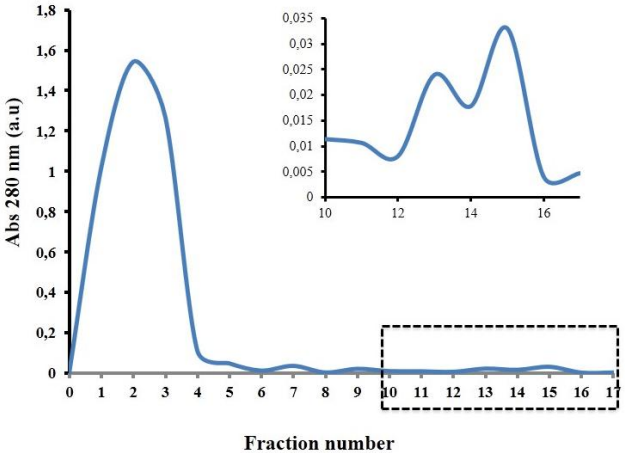

Figure 5A. Elution profile upon purification of egg white lysozyme of Cihateup duck using ion exchange chromatography as monitored using UV-vis spectrophotometer at 280 nm. The peaks shown after 10th fraction (dashed box) were enlarged as shown in the right-top of this figure.

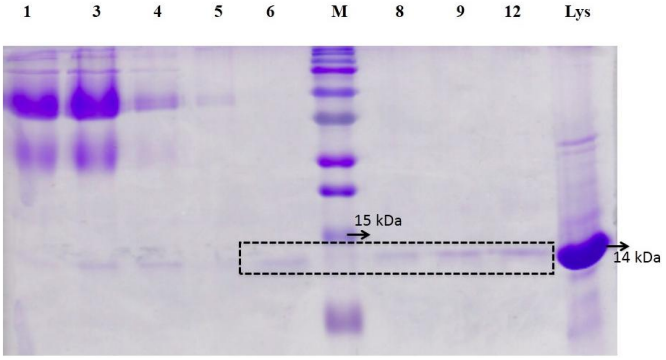

Figure 5B. 15% SDS-Page of purified egg white lysozyme of Cihateup duck using ion exchange chromatography. The number shown in the top of gel corresponds to fraction number in elution profile (5A) in which 10-µL aliqoutes taken from each respected fraction was loaded into the well. The bands correspond to lysozyme size are indicated in the dashed box. M lane corresponds to low molecular weight protein markers (Termo Scientific, USA). The marker band corresponds to 15 kD shown in the figure, as it is closed to lysozyme`s size. Size label for other bands were omitted for clarity.

EWL was more distributed, hence the concentration in each fraction was relatively lower. Total concentration of purified EWL under Abs280 nm was about 0.31, 0.02, and 0.43 mg from 20 mL of white egg of kampung chicken, commercial laying hens, and Cihateup duck, respectively. Noteworthy, amount of lysozyme secured from egg white of commercial laying hens was remarkably lower compared to that of kampung chicken and Cihateup duck. It is probably due to most part of lysozymes eluted earlier together with other egg white proteins (shown in fraction 2 of Figure 4A). Lyozyme from commercial laying hens might behave differently at pH used in this experiment, however this speculation need to be confirmed. In fractions of 4 to 5 of kampung chicken and Cihateup duck the band corresponding to lysozyme size is contaminated with another band with apparent size about 65 kD. This contaminant protein is probably avidin which has close isoelectric point to lysozyme theferore it may eluted almost at the same time with lysozyme.

CONCLUSION

The results in this current study clearly show a single step ion exchange chromatography is promising to be used to purify EWL from Cihateup duck, Kampung chicken, and as well as commercial laying hen eggs. The purity and amount of purified through this method is considerably better compared to ethanol precipitation. Indeed, the absence of ethanol in ion exchange chromatography promotes the purified EWL remains in its optimum active conformation. Purified EWL from ion exchange chromatography retains its bacterial activity as good as the partially purified EWL. The activity may also be better than that of the partially purified EWL as ethanol-treated enzymes (a step performed in ethanol precipitation) has never exhibited 100% activity of completely free-ethanol enzymes.

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