Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.101722

Characteristics of protein and amino acid in various poultry egg white ovomucoid

NURLIYANI[1]*, Yuny ERWANTO[1], RUMIYATI[2], Ari Surya SUKARNO[1]

Abstract

Ovomucoid (OVM) is one of the glycoproteins in egg white which is heat resistant protein and to have protease inhibitor activity, that has potential as an anti-cancer. The aim of this study was to purify and characterize ovomucoid from four types of poultry eggs (commercial chickens, native chickens, ducks and Muscovy ducks). We analyzed the protein profile on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the secondary structure on Fourier Transform Infra-Red (FTIR) Spectroscopy, amino acid profiles and protease inhibitor activity. The results showed that the ovomucoid profile on SDS-PAGE of most eggs was in the range of 35 kDa, except for commercial chicken egg which was 40 kDa. The ovomucoid spectra on FTIR of poultry eggs were similar, and indicate the presence of a specific band for carbohydrate contents at the wavelength of 894 to 1249 cm^{-1}. There was similar content in amino acid profile of ovomucoid, except duck ovomucoid showed the highest in methionine contents. The activity of protease inhibitors in duck ovomucoid was highest than other ovomucoid. In conclusion, among the ovomucoid of poultry eggs were found variations in SDS profile, specific band in FTIR spectra, amino acid content of methionine and also protease inhibitor activity.

Keywords: ovomucoid; electrophoretic profile; FTIR; amino acid; protease inhibitor.

Practical Application: Duck egg ovomucoid has the highest protease inhibitor activity, so that it has the potential to be used as an anticancer functional food or nutraceutical.

1 Introduction

Eggs consist of many functional proteins, with notable functional characteristics . However, the application of egg protein in industry is very limited (Abeyrathne et al., 2013). The complete balance and diversity of nutrients along with high digestibility and also affordable prices make eggs in a highlight. Recent studies have shown that egg protein mix weaning foods can be a cost-effective and possible means to address malnutrition in children in developing countries (Naeem et al., 2022). However, nutritionists suggestion limit egg consumption to decrease the incidence of cardiovascular disease. Whereas most experimental, clinical and epidemiological studies conclude zero correlation between dietary egg cholesterol and enhanced total plasma cholesterol. In fact, there is a strong evidence that eggs also contain many unobserved bioactive substances, which may be very useful in preventing or/curing diseases (Réhault-Godbert et al., 2019).

Egg whites or albumen, constitutes 60% of the total weight of eggs in which water and protein are the main components, followed by carbohydrate, ash, and trace amounts of lipids (1%). The contents of egg whites are water (88%) and protein (11%), with the remainder consisting of carbohydrates, ash, and trace amounts of lipids (1%). The main protein in egg white include ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%), and ovomucin (3.5%) closely related to albumen viscosity. The minor proteins are avidin (0.05%), cystatin (0.05%), ovomacroglobulin (ovostatin) (0.5%), ovoflavoprotein (0.8%), ovoglycoprotein (1.0%), and ovoinhibitor (1.5%) (Mine & Kovacs-Nolan, 2005; Abeyrathne et al., 2013).

Study on the use of egg white protein powder based edible film which has antimicrobial compounds from essential oils has proven potential as natural antimicrobials that can increase the safety of organic or natural foods (Kavas & Kavas, 2016). In addition, eighty-three peptides in egg white proteins were identified for ACE inhibitory activity. Among these peptides, Ile-Leu-Lys-Pro showed the highest activity with the residues Glu362 and Ala332 were the crucial binding sites in molecular docking (Li et al., 2022).

Ovomucoid is one of glycosylated proteins found in egg whites. The molecular weight of the ovomucoid is 28 kDa, but the band in SDS-PAGE which appears at 30 to 40 kDa, has made ovomucoid known as a trypsin inhibitor and is considered a major food allergen in egg white. Each ovomucoid molecule binds one trypsin molecule, and the 3-dimensional structure is secured with 3 disulfide bonds in it (Abeyrathne et al., 2013).

The secondary structure of chicken egg white ovomucoid (186 residues) is predicted from the amino acid sequence. The relative composition of the secondary structure obtained by the former method was: 27% α -helix (7 regions), 33% β-structure (10 regions), 23% β-turn (11 regions) and 7% random coil. Ovomucoid may be classified into the α+β type protein group and that the reactive site (Arg 89-Ala90) of trypsin inhibition is

Received 20 Sept., 2022
Accepted 06 Nov., 2022
[1]*Department of Animal Product Technology, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia*
*Corresponding author: nurliyani@ugm.ac.id

probably situated at the C-terminal β-structure end in domain II (Matsuda et al., 1981).

The possible use of proteinase inhibitors treat various diseases including cancer and AIDS, has led to extensive research on the structure, specificity and stability of inhibitors. Efforts to enhance protein activity and/or stability, requires comprehensive understanding of the association between molecular structure and stability. It has been found that globular proteins with a hydrophobic and compact interior are more stable than those with polar or charged amino acid residues in the interior (Quan & Benjakul, 2017).

Proteases in normal cells play a vital role in biological activity. In living systems, there is a balance between proteases and anti-proteases, and a disruption in this balance causes many diseases such as cancer. Tumor development phases (initiation, growth, metastasis and finally invasion to other sites) involve five groups of proteases, namely serine, cysteine, aspartate, threonine, and matrix metallo-proteases (Rakashanda et al., 2012).

Cancer is now the second most common cause of death globally according to estimates reported by the Global Burden of Disease (GBD) in 2013, the year 14.9 million cancer cases, and 8.2 million deaths by cancer were reported. While dietary habits are greatly associated with the development of cancer (Bisen, 2016), chemical drugs often have side effects. Therefore, it is important to formulate alternatives from natural materials such as plants or animals for cancer therapy.

Ovomucoid has been successfully separated from egg white by extraction using ethanol and citrate or with ethanol, saturated ammonium sulfate and citrate (Abeyrathne et al., 2013). Meanwhile, the secondary structure of chicken ovomucoid is already predicted from their amino acid sequence by Matsuda et al. (1981). However, there is limited the information about ovomucoid characteristics from various poultry eggs. Previous studies reported that eggs of different types of poultry have a very wide variety in the inhibitory residue in contact with the enzyme (Laskowski et al., 1987). It is suspected that among ovomucoid eggs from various types of poultry, the variation in the enzyme inhibitory site will also affect the activity of protease inhibitors. The purpose of this study was to isolate and purify egg white ovomucoid from various types of poultry and to determine the secondary structure, amino acid profile and its potential as a protease inhibitor.

2 Materials and methods

2.1 Isolation and purification of ovomucoid

Isolation and purification of ovomucoid were adapted from Abeyrathne et al. (2014), with slight modification in heating treatment after the addition of citric acid. In principle, egg whites (the eggs from native chicken, commercial chicken, duck and Muscovy duck) were separated from the yolks, dissolved with 1 volume of distilled water, added Fe^{3+} (0.8 mL/L of 500 mM $FeCl_3$) to saturate ovotransferrin then homogenized in high speed using a hand mixer for 2 min. Proteins in egg white solution, except for ovotransferrin and ovomucoid, were precipitated by the slow addition of 100% ethanol to make a final concentration of 43%, and centrifuged at 3,400 x g (20 min) at 4 °C. Furthermore, the ethanol extract was increased in concentration to 61% by slowly adding 100% ethanol, the solution was left overnight at 4 °C, centrifuged at 3,400 x g (20 min) at 4 °C. After that, the supernatant was taken and the ethanol was evaporated using a rotary evaporator (concentrated). Citric acid was added to lower the pH to 4.0, then heated at 80 °C for 30 min (modification from the original 65 °C for 20 min) and centrifuged 3,400 x g, then the supernatant containing ovomucoid was lyophilized.

Confirmation of purity was evaluated using SDS-PAGE, ovomucoid confirmation was performed using Western blott, the chemical structure of the purified ovomucoid protein was evaluated with FT-IR and its amino acid profile was analyzed by HPLC.

2.2 SDS-PAGE and immunoblotting

The results of the extraction and purification of ovomucoid protein were visualized using the SDS-PAGE method according to Laemmli (1970) on a discontinuous buffered system. SDS-PAGE analyzed the efficiency and yields of separations under reduced conditions using Mini-Protein II cell (Bio-Rad). Ten percent polyacrylamide gel, stacking gel 5% and Coomassie Brilliant Blue R-250 (Sigma) staining was used. Exactly 5 μL of 10 mg/mL ovomucoid sample was loaded into each well running. To check the purity of ovomucoid, gel pictures were taken after distaining.

Western Blott was used to confirm ovomucoid according to Xie et al. (2002) with modifications. After running the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (BioRad) at 90V for 2 h at controlled temperature. The transferred membrane was blocked with 1% bovine serum albumin dissolved in 0.5% Tris-buffered saline with Tween-20 (TBST). The transferred membrane was incubated at 4 °C for 2 h and shaken, then washed 3 times with 0.05% TBST for 10 min and shaken. To identify ovomucoid, the membrane was treated with anti-ovomucoid antibodies (after a 1: 5,000 dilution with buffer incubation at pH 7.2 as a primary antibody; Abcam) and kept for 2 h at 4 °C with shaking. The membrane was washed 3 times with 0.05% TBST solution at 10-min intervals. Rabbit antimouse IgG (H+L) conjugated alkaline phosphatase (AP) (Abcam) was used after diluting 1:5,000 in 1% BSA with buffer incubation at pH 7,2 as the secondary antibody, then incubated for 1 h at room temperature and washed with TBST 3 times with 10-min intervals. The membrane was washed one time for 5 min with TBS without Tween. Substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) were prepared 50 mg in 20 mL buffered substrate. Substrate solution was transferred into the well, incubated and shaken at a dark room until the color appeared then immediately washed with aquabidest to stop the reaction.

2.3 Evaluation of purified ovomucoid structure using FTIR

Infrared spectroscopy is one of the oldest and well established experimental techniques to analyze the secondary structure of polypeptides and proteins (Kong & Yu, 2007). The interactions between matter and electromagnetic radiation determined by FT-IR spectroscopy were visualized in the form of a spectrum.

Each molecule has a spectrum fingerprint that makes it unique and provide it to be differentiated from other molecules (Fadlelmoula et al., 2022).

Secondary structure of egg white ovomucoid protein from different sources (commercial chicken, native chicken, duck and Muscovy duck) was analyzed according to Abd-Elaziz et al. (2018) with slight modifications. Ovomucoid sample was prepared with potassium bromide (KBr), homogenized using vibrating mill, pressed and vacuumed to form a pellet. Infrared spectra were measured with a FTIR spectrometer (SHIMADZU PRESTIGE 21) at 25 °C. The pellet was scanned on FTIR at 300-4000 cm^{-1} region with a resolution of 16 cm^{-1} and 10 scans. The relative amounts of different secondary structures of ovomucoid protein in egg white were determined from the infrared second derivative amide spectra by manually computing the areas under the bands assigned to a particular substructure .

2.4 Amino acid analysis of ovomucoid protein

Amino acids were determined by HPLC (Thermo) according to Marino et al. (2010) with some modification. Firstly, 60 mg sample was weighed then put into a tube, and covered. Exactly 4 mL hydrochloric acid 6N was added and vortexed until homogenized. The sample was hydrolyzed at 110 °C for 24 h in an autoclave, cooled at room temperature and neutralized by NaOH 6 N. Solution of 40% *Pb*(CH3COO)4 and 15% oxalic acid were incorporated into the sample, put in vial and diluted with aquabidest until 10 mL. Exactly 3 mL of sample was taken and filtered with Whatman filter 0.20 µm. Then, 50 µL sample was added with 300 µL o-phthaldehyde (OPA), stirred for 5 min, then 10 µL of it was injected to HPLC.

HPLC condition was assessed in the following procedure using LiChrospher 100 RP-18 (5µm). Eluent: A) MeOH: 50 mM acetic-buffer: Tetrahydrofolic (THF) (80:15:5) pH 6.8, and B) 65% MeOH. Detector: Thermo Ultimate 3000 RS Fluorescence Detector. The elution of samples was performed at a flow rate of 1.5 mL/min by gradient elution for a total running time of 45 min, which started at 0.1 min (pump A = 100%, pump B = 0%), 15 min (pump A = 0%, pump B = 35%), 30 min (pump A = 0%, pump B = 100%), 40 min (pump A = 0%, pump B = 100%), and 45 min (Stop). Standard solution mix was prepared using 50 ppm of standard solution made from stock solution. This standard was diluted 2x with aquadest, put 50 µL and added 950 µL OPA. Solution was mixed, incubated for 5 min, and injected (10 µL) into HPLC.

2.5 Trypsin inhibitor activity of ovomucoid analysis

The analysis of trypsin inhibitor activity of ovomucoid was adapted from Quan & Benjakul (2018), with modifications. Briefly, ovomucoid sample (2.5 mg freeze dried) was dissolved into the phosphate buffer at pH 8 until the volume was 50 mL (the sample concentration was 0.05 mg/mL). Table 1 show treatments of samples, standards and blanks:

To terminate the reaction, 30% acetic acid (v/v) was added, and the release of ρ-nitroalaniline was monitored by measuring the absorbance at 410 nm using a spectrophotometer (DLAB SP-V1100) for each solution (sample, sample blank, standard, standard blank).

The trypsin unit (TU) is calculated based on the absorbance of the solution after being corrected with a blank solution, provided that each one trypsin unit (1TU) will increase the absorbance scale 0.01, it is formulated as: TU = Absorbance/0.01.

The trypsin inhibitor unit (TIU) is calculated as the difference between the standard TU (TU0, i.e. the reaction without sample solution), and the sample TU (TUx), it is formulated as (Equation 1):

$$TIU = TU0 - TUx. \quad (1)$$

One unit of trypsin inhibitory activity (TIA) was defined as the amount of inhibitor, which reduced trypsin activity by one unit. Trypsin inhibitory activity of ovomucoid was expressed as units/mg protein. The trypsin inhibitory activity (TIA) is calculated as the total unit of trypsin inhibition for each mg of the sample material, formulated as (Equation 2):

$$TIA = TIU / mg \; x \; Dilution \; factor. \quad (2)$$

The solutions to dissolve trypsin and Na-Benzoyl-DL-arginine-ρ-nitroanilide (BAPNA) were prepared as follow. Trypsin solution was obtained by diluting 2 mg of trypsin standard with 0.001N HCl to a volume of 40 mL. To make tris solution, 3.025 g of Tris plus 1.47 g of $CaCl_2$ was diluted with distilled water to a volume of 300 mL then adjusted to pH 8.2 by adding 0.1 N NaOH or 0.1 N HCl. The total volume was made up to 500 mL in volumetric flask (can be stored in the refrigerator for 2 months). BAPNA solution was made by incorporating 20 mg of BAPNA with 0.5 mL of concentrated dimethyl sulfoxide (DMSO), then heat the solutiom in a water bath at 37 °C until completely dissolved, and dilute it with tris solution to a volume of 50 mL.

Table 1. Treatments of sample, standard and blank.

Treatment	Sample	Sample blank	Standard	Standard blank
Sample	2.0 mL	2.0 mL	-	-
H_2O	-	-	2.0 mL	2.0 mL
Trypsin	2.0 mL	2.0 mL	2.0 mL	2.0 mL
BAPNA	5.0 mL	5.0 mL(*)	5.0 mL	5.0 mL(*)
Shake and incubate at 37 °C	10 min	10 min	10 min	10 min
30% acetic acid	1.0 mL	1.0 mL	1.0 mL	1.0 mL

(*) Added after the addition of 30% acetic acid.

2.6 Statistical analysis

The profil protein on SDS-PAGE and secondary structure on FTIR of egg white OVM were expressed in figure form. The OVM yield, amino acid profile and protease inhibitor activity of OVM in four types of poultry egg were analyzed statistically by ANOVA, and the mean difference between type of poultry OVM were compared by Duncan's multiple range test at $p < 0.05$ using SPSS software version 17.0.

3 Results and discussion

3.1 Ovomucoid isolation and purification

The results of isolation and purification of ovomucoid (OVM) from egg whites showed a clear protein band and no impurity in eggs of commercial and native chicken, duck and Muscovy duck. all type of poultry. However, impurity appeared in OVM of the standard chicken which also had a smaller band than the other (Figure 1).

Based on Figure 1, the molecular weight of OVM in SDS-PAGE is around of 35 kDa, but in commercial chicken OVM is about 40 kDa. This result is in accordance with Abeyrathne et al. (2015), that the molecular weight of OVM on SDS-PAGE ranges from 30 to 40 kDa, although the informed molecular weight of OVM is 20.1 kDa. Meanwhile, Kato et al. (1987) reported that OVM is a protein containing 186 amino acids with a molecular weight of 28 kDa.

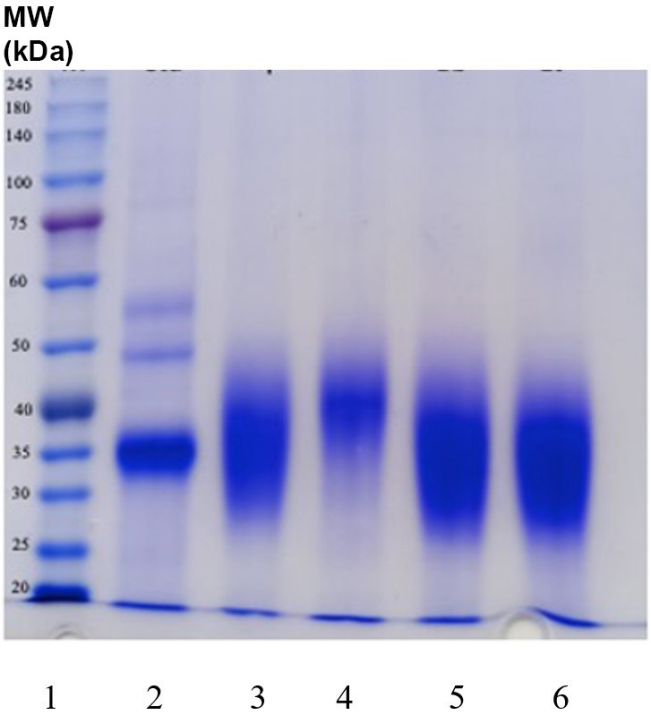

Figure 1. SDS-PAGE of OVM from different poultry types (Lane 1: protein marker, Lane 2: chicken OVM standard, Lane 3: Native Chicken OVM, Lane 4: Commercial chicken OVM, Lane 5: Duck OVM, Lane 6: Muscovy duck OVM).

3.2 Western Blott of ovomucoid

The results of Western Blott showed that OVM of native chicken, commercial chicken, duck and Muscovy duck and standard chicken OVM reacted with OVM antibodies (Anti-OVM) (Figure 2). This means that the results of the isolation and purification of OVM from 4 types of poultry eggs are indeed confirmed OVM proteins, and not other proteins.

3.3 Yield of purified ovomucoid

Based on Table 2, the average of OVM yield in native chicken was the lowest ($p < 0.01$) of all other types of poultry eggs. The present study indicate significant differences quantitatively in the content of protein type among poultry species, although the total protein contens in egg is stable between species (in around of 13%) (Réhault-Godbert et al., 2019). According to Réhault-Godbert et al. (2019) factors affecting egg quality include genetics, nutrition, rearing systems, physiological status, egg storage and heat treatment, and variability between avian domestic species. The composition of egg from traditional domestic species share common characteristics, but have possess some significantly different energy due to the change in relative proportion of yolk to egg white.

3.4 Protein structure of egg white ovomucoid on FTIR

Overall, the OVM of eggs of commercial chicken, native chicken, duck and Muscovy duck egg ovomucoid showed the same profile in FT-IR spectra. Compared to the standard, the OVM of chickens also showed the same profile, but the standard

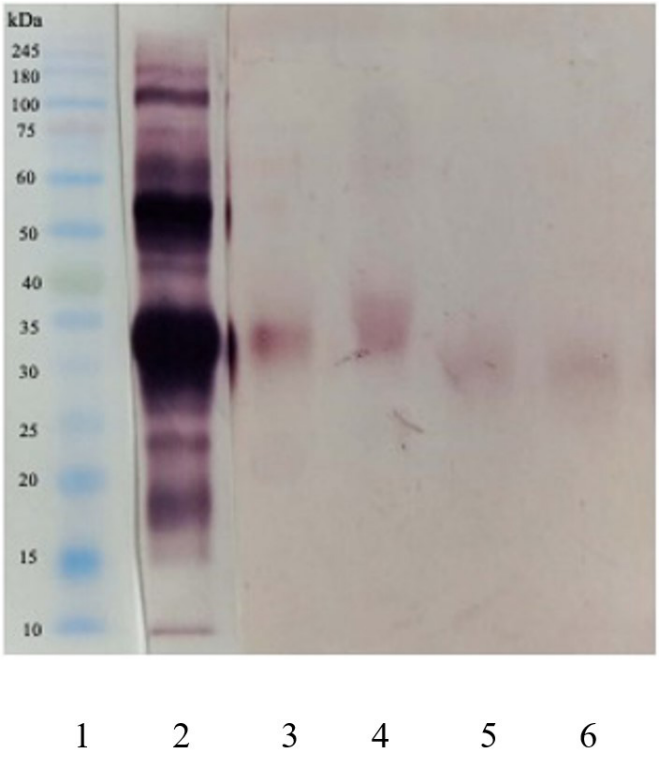

Figure 2. Western Blott of OVM from different poultry types (Lane 1): protein marker, Lane 2: standard chicken OVM, Lane 3: Native chicken OVM, Lane 4: Commercial chicken OVM, Lane 5: Duck OVM, Lane 6 (M): Muscovy duck OVM.

Table 2. Yield of purified ovomucoid from different poultry types.

Eggs	Sample No.	Egg numbers	Range of egg weight	Composite albumen weight (g)	OVM yield (g)	OVM yield (%)	Average OVM yield (%)
Commercial chicken	1	7	51.3-73.1	257.5	1.63	0.64	0.83 ± 0.18[a]
	2	3	62.7-71.7	126.5	1.06	0.85	
	3	3	57.8-63.5	112.4	1.12	1.00	
	Total			496.4	3.83	2.49	
Native chicken	1	8	43.6-54.8	357.4	0.52	0.145	0.41 ± 0.23[b]
	2	8	37.4-50.2	169.5	0.93	0.546	
	3	5	40.5-44.7	104.4	0.58	0.560	
	Total			631.3	2.03	1.26	
Duck	1	10	53.1-66.1	282.6	2.01	0.713	0.89 ± 0.15[a]
	2	4	61.4-64.9	112.4	1.08	0.959	
	3	3	59.6-68.9	112.7	1.13	0.999	
	Total			507.7	4.22	2.671	
Muscovy duck	1	7	56.2-67.1	188.8	2.09	1.11	1.19 ± 0.18[a]
	2	6	61-72	101.7	1.08	1.06	
	3	6	60.1-70.1	97.9	1.38	1.40	
	Total			388.4	4.55	3.57	
						p	0.008

Different letters within column indicate significant difference ($p < 0.008$).

did not show several peaks like the rest poultry in this study, which could probably due to different degree of purity and concentration of OVM standard as shown in the SDS-PAGE profile (Figure 1).

Some occurrences of poultry OVM bands and standard samples from Figure 3 can be seen on Table 3. Several bands showed similarity in wavelength between the poultry OVM in this study and the chicken OVM standard, such as in amide band I (1635-1651cm^{-1}), amide VI (532-601 cm^{-1}) and the carbohydrate-specific band appeared in all samples of poultry and alsoin the standard, namely band 864-894 and band 1072-1080 cm^{-1}. There was a difference in sharpness of the 1651 cm^{-1} band in the OVM of commercial chicken eggs which showed that it was less sharp than other poultry eggs, but in the standard OVM of chicken eggs it appear on 1635 cm^{-1}.

Previous studies reported different approximates of amide A band, namely 3500 (Yang et al., 2015), 3225-3280 (Krimm & Bandekar, 1986), 3310-3270 cm^{-1} (Barth, 2007), and 3300–3500 cm^{-1} (Ji et al., 2020). Therefore, OVM band of with band of 3394-3448 cm^{-1} in this study could be categorized as amide A. Amide A is with more than 95% due to the N-H stretching vibration. This mode of vibration does not depend on the backbone conformation but is very sensitive to the strength of a hydrogen bond (Krimm & Bandekar, 1986).

Commercial chicken OVM with band 1720 cm^{-1} was amide I because it was in the band range of 1600-1700 cm^{-1} similar to a finding by Krimm & Bandekar (1986). The amide I band arises principally from C=O stretching of the peptide group (Joshi & Soni, 2012).

The OVM band which appeared in all types of poultry in this study (1651) and in the standard (1635 cm^{-1}) are also the Amide I which according to Krimm & Bandekar (1986), ranges from

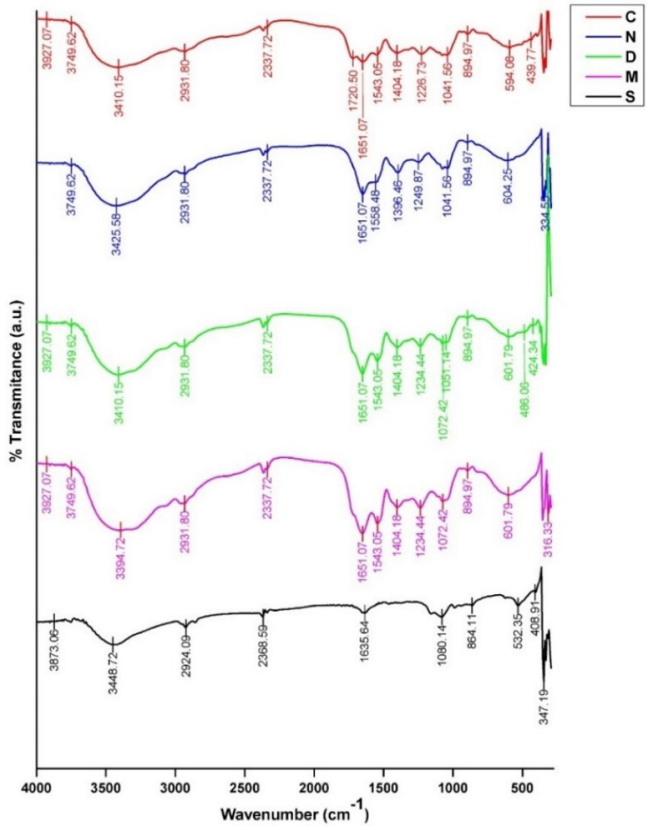

Figure 3. FTIR spectra of OVM from different poultry types (C: commercial chicken, N: native chicken, D: duck, M: Muscovy duck, S: standard).

1600 to 1700 cm^{-1}. Band 1651 is a helix structure (Zhang et al., 2003). Amide I band leads to stretching vibrations of the C=O

Table 3. Ovomucoid bands (cm^{-1}) from different types of poultry on FTIR.

No	Bands	Commercial chicken	Native chicken	Duck	Muscovy duck	Standard
1	Amide A	3410	3425	3410	3394	3448
2	Amide I	1720	-	-	-	-
3	Amide I	1651	1651	1651	1651	1635
4	Amide II	1543	1558	1543	1543	-
5	Amide II	1404	1396	1404	1404	-
6	Amide III	1226*	1249*	1234*	1234*	-
7	Carbohydrate	-	-	1072*	1072*	1080*
8	Carbohydrate	1041*	1041*	1049*	-	-
9	Carbohydrate	894*	894*	894*	894*	864*
10	Amide VI	594	601	601	601	532

*indicates the presence of carbohydrates bands (864-1249 cm^{-1}).

bond of the amide (Abd-Elaziz et al., 2018), and according to Kong & Yu (2007), 1600-1690 cm^{-1} band is a stretching CO. According to Joshi & Soni (2012)

amide I band mainly consists of helix (1650-1658 cm^{-1}) and β sheets (1620-1640 cm-1) structures (Joshi & Soni, 2012).

Ovomucoid of poultry that appeared in band 1543-1558 cm^{-1} was amide II which according to Kong & Yu (2007), ranges from 1480-1575 cm^{-1}. Amide II is a combination of N-H bending and stretching vibrations (Tarhan et al., 2020) or especially bending vibrations of the N-H bond (Abd-Elaziz et al., 2018). The band that appeared in OVM of 4 types of poultry in the present study was in a range of from 1396-1404 cm^{-1}, which is also amide II according to Joshi & Soni (2012) who reported that band in amide II ranged from of 1400 to 1600 cm^{-1}. The amide II band is primarily N-H bending with a contribution from C-N stretching vibration. This amide II band did not appear for the OVM standard.

The presence of the OVM band of 1226-1234 cm^{-1} in this study indicated the presence of amide III band, which did not appear in the OVM standard. According to Tarhan et al. (2020), the presence of amide III band between 1320 and 1220 cm^{-1} was due to combination of N-H bending and C-N stretching vibrations. Since amide III arises from N-H bending, its absorption is very weak in infrared (Joshi & Soni, 2012).

The specific carbohydrate band of poultry OVM in the present study (Table 3) appeared in band in a range of 864-1249 cm^{-1}. While OVM band of 894 cm^{-1} appeared in all poultry, the standard OVM was 864 cm^{-1}. Band of 1041 cm^{-1} appeared in commercial and native chicken OVM, band 1049 cm^{-1} in duck, Muscovy duck and standard sample did not show bands between 1041 and 1049 cm^{-1}. Furthermore, band of 1072 cm^{-1} appears on duck and Muscovy duck OVM, but on standard OVM appears in band of 1080 cm^{-1}. Band 1226 cm^{-1} appears on commercial chicken OVM, and band of 1249 cm^{-1} appears on native chicken OVM, while band 1234 cm^{-1} appears on duck and Muscovy duck OVM. However, OVM standard did not appear band in around of 1234-1249 cm^{-1}. Differences in the appearance and sharpness of bands in poultry and standard OVM may be due to different OVM concentration in the sample, in which the standard of chicken OVM contains many impurities (Figure 1). Ovomucoid is characterized by a band of 900-1200 cm^{-1} which indicates the presence of carbohydrate/glycan compounds in the form of N-acetylglucosamine compounds (Giosafto et al., 2016). As stated by Salahuddin et al. (1985), five carbohydrate moieties of the inhibitor in ovomucoid that are nonessential for its inhibitory activity (Laskowski et al., 1987) are linked to Asn residues in Asn-X-Thr/Ser sequences. The fifth Asn residue in domain III remains unglycosylated in some ovomucoid molecules. Although the three domains of OVM exhibit considerable sequence homology, domains I and II appear to be more closely related (Salahuddin et al., 1985).

The OVM band of 594-601 cm^{-1} appeared in 4 types of poultry in this study, except for standard chicken eggs. According to Kong & Yu (2007), the band of 537-606 cm^{-1} is an amide VI band, which is an out-of-plane C=O bending structure.

3.5 Amino acid profile of ovomucoid

Amino acid profile of OVM from various poultry types showed on Table 4. Based on Table 4, the concentration of individual amino acid in OVM of different poultry eggs is not significantly different, except for methionine ($p < 0.025$) which was the highest in duck egg among poultry. Methionine in duck eggs ovomucoid showed highest concentration among poultry type. According to Attia et al. (2020), the amino acid content of eggs is affected by poultry breeds and species, preparation method and egg components (whole, albumen, and yolk). A previous studies showed that methionine in duck egg whites is not different from that of chicken and guinea fowl, namely 2.36-2.49 g/100 g crude protein (Adeyeye et al., 2012). In this study, it was possible that the higher methionine levels in the OVM of duck eggs and Muscovy duck eggs than those of chicken eggs were due to different feed. Local ducks in Indonesia are usually grazing in paddy fields, so they may feed on snails, periwinkle (*Tympanotomus fuscatus)*, small crabs, rice grain, grass, and other. It is similar to Tumanggor et al. (2017) who reported the possibility of duck to eat grain, grass, snails, small crabs (*Cardiosoma armatum)*, sand, small rocks, and unknown materials when grazing. Higher methionine levels in OVM of egg white of ducks than chickens in this study were similar to findings by Quan & Benjakul (2019). Another study by Adeyeye (2013), indicated that the methionine level in duck whole egg

Table 4. Amino acid in egg white ovomucoid from different sources.

Amino acids	OVM Amino acids (ppm)				p
	Commercial chicken	Native chicken	Duck	Muscovy duck	
Aspartic acid	414.63 ± 228.44	436.28 ± 185.50	405.51 ± 115.32	390.38 ± 163.01	0.991
Glutamic acid	329.92 ± 191.26	325.78 ± 136.11	408.08 ± 158.77	365.40 ± 190.00	0.926
Serine	177.83 ± 102.62	167.42 ± 78.55	188.87 ± 76.07	182.52 ± 91.55	0.992
Histidine	123.16 ± 65.19	114.09 ± 52.69	138.90 ± 60.44	118.05 ± 63.71	0.960
Glycine	170.18 ± 97.92	152.12 ± 65.60	181.29 ± 64.50	165.89 ± 79.63	0.974
Threonine	210.08 ± 114.27	218.89 ± 78.38	261.94 ± 83.33	275.94 ± 122.69	0.824
Arginine	239.08 ± 150.14	188.99 ± 126.23	115.73 ± 67.23	120.03 ± 82.61	0.507
Alanine	171.37 ± 97.31	156.75 ± 81.36	169.32 ± 78.13	159.62 ± 89.73	0.996
Tyrosine	313.58 ± 187.04	320.31 ± 113.03	446.92 ± 135.30	400.49 ± 166.37	0.672
Methionine	47.75 ± 25.20[a]	60.65 ± 10.46 [a]	140.73 ± 41.79 [b]	103.56 ± 38.77 [a]	0.025
Valine	210.04 ± 117.79	220.04 ± 96.99	215.11 ± 69.35	200.79 ± 92.85	0.995
Phenylalanine	137.69 ± 78.70	131.03 ± 64.91	145.16 ± 63.48	139.65 ± 77.93	0.996
Isoleucine	96.11 ± 56.60	88.26 ± 48.31	81.84 ± 39.82	98.29 ± 56.94	0.977
Leucine	399.21 ± 224.06	395.22 ± 164.03	507.99 ± 209.93	439.65 ± 240.59	0.905
Lysine	411.75 ± 254.15	338.62 ± 163.36	468.08 ± 190.91	425.85 ± 220.93	0.895
Total AA	3452.40	3314.62	3875.79	3586.14	0.979
Total E AA	1629.13 (47.18%)	1566.82 (47.26%)	1959.58 (50.55%)	1801.80 (50.24%)	0.934
Total NEAA	1816.60 (52.82%)	1747.67 (52.74%)	1915.74 (49.45%)	1784.33 (49.76%)	0.995
Total acidic amino acid	744.55 (21.57%)	762.06 (22.99%)	813.59 (20.99%)	755.78 (21.08%)	0.995
Total basic amimo acid	773.99 (22.41%)	641,7 (19.36%)	722.71 (18.64%)	663.93 (18.51%)	0.972
Total neutral amino acid	871.67 (25.24%)	858.74 (25.90)	1099.02 (28.35)	1024.84 (28.57%)	0.889
EAA/NEAA	0.90[a]	0.90[a]	1.01[b]	0.99[b]	0.013

Different letters within the same row indicate significant difference ($p < 0.05$).

is higher than that of francolin and turkey. When methionine concentration in poultry diet increases, the eggshell thickness and strength decreases significantly, but albumen weight increases significantly (Fouad et al., 2016).

The percentage of essential amino acid (EAA) to the total amino acid (TAA) of egg white ovomucoid in this study was in a range of 47.18-50.5% and not significantly different across all four types of poultry (Table 4). This percentage was higher than the 43.50% egg whites of guinea fowl according to Adeyeye et al. (2012). However, the values 43.50% is well above the 39% considered be sufficient for ideal protein food for infants, 26% for children and 11% for adults.

The percentage of total ovomucoid non essential amino acid (NEAA) in this study ranged from 49.45-52.82% (Table 4). This result was lower than a previous NEAA study that reported the total NEAA in guinea fowl egg whites was 56.5% per 100 g of crude protein (Adeyeye et al., 2012). It has been reported that NEAAs are now considered as conditionally essential amino acids in diets and play pivotal roles in supporting chicken's health and maximum growth and egg-laying of chickens. Therefore, sufficient NEAAs in diets are important for increasing the efficiency of poultry and egg production (He et al., 2021).

There are no significant differences in the percentage of acidic, basic and neutral amino acids of ovomucoid in all poultry species in this study (Table 4). The total acidic amino acid (TAAA) of poultry ovomucoid in this study ranged from 20.99 to 22.99%, similar to that of guinea fowl egg whites, namely 22.7% (Adeyeye et al., 2012). The percentage of total basic amino acid (TBAA) and total neutral amino acid (TNAA) of ovomucoid in this study were 18.51-22.41% and 25.24 to 28.57%, respectively. The percentage of TBAA and TNAA in the guinea fowl albumen was 18.40 and 57.30%, respectively (Adeyeye et al., 2012). Table 4 shows that in this study, the EAA to NEAA ratio in the OVM of duck eggs (1.01) and Muscovy duck eggs (0.99) was higher ($p < 0.05$) than of chicken eggs (0.90). A previous studies reported a lower EAA:NEAA ratio in commercial chicken egg albumen, namely 0.800 - 0.829 (Attia et al., 2020). According to Romano et al. (2019), the EAA content varies greatly depending on the source. However, the best ratio of EAA:NEAA for any dietary protein is ≤ 0.90, which can be explained that a very large amount of NEAA to meet the requirement for EAA and the excess of NEAA must be eliminated through complex metabolic pathways. In addition, varying dietary EAA:NEAA ratios may affect cell metabolism. Therefore, the EAA:NEAA ratio of OVM in this study already in accordance with a good ratio. Based on the results of the present study and Adeyeye et al. (2012), feed factors and poultry genetic differences may have affected the percentage of TEAA, TBAA, and TNAA, and EAA:NEAA ratio to albumen ovomucoid.

In general, the dominant amino acids in poultry ovomucoid in this study were glutamic acid and aspartic acid, while the dominant essential amino acids were leucine and lysine (Table 4). These results were in agreement with the results of albumen amino acid profile analysis from four sources of commercial table egg in the retail market in Saudi Arabia (Attia et al., 2020). Glutamic acid is considered to be important as important for its vitaal roles in intestinal function and development, and its effects on intestinal morphology in poultry. Sufficient provision of lysine will affect the immune competence and digestive tract

functionality in poultry. Enhanching dietary lysine have been shown to increase egg production, egg weight, egg mass, and feed conversion efficiency in laying hens (Macelline et al., 2021).

3.6 Protease inhibitor activity of ovomucoid

Table 5 shows that there was no difference between the protease inhibitor activity of egg white ovomucoid in commercial and native chicken, and the highest activity was found in duck ovomucoid. This difference is due to structural differences between the ovomucoid of several types of poultry, which causes variations in the affinity of the ovomucoid to trypsin, thus affecting the degree of dissociation of the ovomucoid-trypsin complex. The higher the degree of ovomucoid-trypsin complex dissociation, the weaker the trypsin inhibitor activity, and vice versa. Nagata & Yoshida (1984) stated that association of a protease with protein protease inhibitor may be important in the disintegration of the inhibitor after it has acted in its original location. When the dissociation rate constants of the inhibitor-enzyme complex are much smaller than the association rate constants, then the complex once formed hardly dissociates during activity assay. Likewise, when the formation of a relatively unstable complex, which has greater rates of dissociation than those of association, then the protease inhibitor should be considered as a substrate rather than as an inhibitor.

The variation in the inhibition of trypsin may be due to the conformations diversity of the uniqueness and flexibility of the reactive site. This can be seen from the two kinds of inhibitor molecules that show structural variations in the reactive side loop. Inhibition of trypsin by inhibitors has been confirmed by structural studies of the inhibitor-trypsin complex which clearly demonstrated the binding of the inhibitor reactive site to the trypsin active site (Patil et al., 2012). The reactive site of chicken ovomucoid that interacts with trypsin is located in the Arg89-Ala90 sequence in the second domain (Matsuda et al., 1983). According to Shimazaki et al. (2018), interactions between enzymes and inhibitors depend on regulation of the enzyme activities. Reversible inhibition of enzyme activity is primarily caused by interactions such as association and dissociation between enzymes and inhibitors. Ovomucoid, from egg white, is a serine protease inhibitor that reversibly inhibits the activity of the serine protease trypsin. Reversible inhibitors can associate and dissociate to the targeted enzymes. The dominant force for binding serine protease and ovomucoid has been reported to be hydrophobic interactions.

Abnormally expressed proteases in the tumor microenvironment represent a main contributors to tumor growth and development, and also potentially a promising category of drug targets. However, a main challenge in the development of therapeutic protease inhibitors is the identification of inhibitors that are sufficiently selective, as drugs that are not sufficiently selective are related with toxicity induced by interference with a wide variety of physiological processes, and may be less effective as a result of simultaneous targeting of pro-and antitumorigenic proteases (Salameh et al., 2011).

Table 5. The average of trypsin inhibitor activity of egg white ovomucoid in various poultry.

No	Egg vomucoid	Trypsin Inhibitor (IU)
1	Commercial chicken	34.45 ± 2.51^{a}
2	Native chick	34.12 ± 0.29^{a}
3	Duck	56.52 ± 0.38^{b}
4	Muscovy duck	44.62 ± 3.47^{c}
	p	$p < 0.001$

Different letters within column indicate signif icant different ($p < 0.001$).

4 Conclusions

Ovomucoids in various types of poultry eggs showed variations in SDS-PAGE profile, secondary structure on FTIR spectra particularly in carbohydrtate band, amino acid content of methionine and protease inhibitor activity. Duck egg contained the highest methionine content and protease inhibitor activity, and showed a potential as anticancer.

Acknowledgements

We thank the Directorate General of Higher Education, the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for funding this research through "Penelitian Dasar" (No: 2197/UN1/DITLIT/DIT-LIT/PT/2021).

References

Abd-Elaziz, M. A., Osman, A. O., Ibraheim, W. M., & Zagazig, M. A. D. (2018). Comparative studies of egg albumin from different sources. *Journal of Agricultural Research (Lahore)*, 45(3), 1003-1010.

Abeyrathne, E. D. N. S., Lee, H. Y., & Ahn, D. U. (2013). Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents: a review. *Poultry Science*, 92(12), 3292-3299. http://dx.doi.org/10.3382/ps.2013-03391. PMid:24235241.

Abeyrathne, E. D. N. S., Lee, H. Y., & Ahn, D. U. (2014). Separation of ovotransferrin and ovomucoid from chicken egg white. *Poultry Science*, 93(4), 1010-1017. http://dx.doi.org/10.3382/ps.2013-03649. PMid:24706979.

Abeyrathne, E. D. N. S., Lee, H. Y., Jo, C., Suh, J. W., & Ahn, D. U. (2015). Enzymatic hydrolysis of ovomucoid and the functional properties of its hydrolysates. *Poultry Science*, 94(9), 2280-2287. http://dx.doi.org/10.3382/ps/pev196. PMid:26195809.

Adeyeye, E. I. (2013). The comparison of the amino acids profiles of whole eggs of duck, francolin and turkey consumed in Nigeria. *Global Journal of Science Frontier Research B*, 13(3), 10-20.

Adeyeye, E. I., Adebayo, W. B., & Ayejuyo, O. O. (2012). The amino acid profiles of the yolk and albumen of domestic duck (*Anas platyrhynchos*) egg consumed in Nigeria. *Elixir Food Science*, 52, 11350-11355.

Attia, Y. A., Al-Harthi, M. A., Korish, M. A., & Shiboob, M. H. (2020). Protein and amino acid content in four brands of commercial table eggs in retail markets in relation to human requirements. *Animals (Basel)*, 10(3), 406. http://dx.doi.org/10.3390/ani10030406. PMid:32121495.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta*, 1767(9), 1073-1101. http://dx.doi.org/10.1016/j.bbabio.2007.06.004. PMid:17692815.

Bisen, P. S. (2016). Therapeutic and preventive potential of functional food and cancer. *Cancer Medicine & Anti Cancer Drugs*, 1(1), 1-2.

Fadlelmoula, A., Pinho, D., Carvalho, V. H., Catarino, S. O., & Minas, G. (2022). Fourier transform infrared (FTIR) spectroscopy to analyse

human blood over the last 20 years: a review towards lab-on-a-chip devices. *Micromachines*, 13(2), 187. http://dx.doi.org/10.3390/mi13020187. PMid:35208311.

Fouad, A. M., Ruan, D., Lin, Y. C., Zheng, C. T., Zhang, H. X., Chen, W., Wang, S., Xia, W. G., & Li, Y. (2016). Effects of dietary methionine on performance, egg quality and glutathione redox system in egg-laying ducks. *British Poultry Science*, 57(6), 818-823. http://dx.doi.org/10.1080/00071668.2016.1222603. PMid:27684471.

Giosafto, C. V. L., Rigby, N., Sorrentino, A., Mulholland, F., Mills, C., & Mackie, A. R. (2016). Optimization of in vitro n-deglycosylation of ovomucoid protein. *MOJ Food Processing & Technology*, 2(6), 205-212. http://dx.doi.org/10.15406/mojfpt.2016.02.00058.

He, W., Li, P., & Wu, G. (2021). Amino acid nutrition and metabolism in chickens. *Advances in Experimental Medicine and Biology*, 1285, 109-131. http://dx.doi.org/10.1007/978-3-030-54462-1_7. PMid:33770405.

Ji, Y., Yang, X., Ji, Z., Zhu, L., Ma, N., Chen, D., Jia, X., Tang, J., & Cao, Y. (2020). DFT-calculated IR spectrum amide I, II, and III band contributions of N-methylacetamide fine components. *ACS Omega*, 5(15), 8572-8578. http://dx.doi.org/10.1021/acsomega.9b04421. PMid:32337419.

Joshi, D., & Soni, R. K. (2012). Laser induced gold nanoparticle egg-white protein conjugation and thermal denaturation. In International Conference on Fiber Optics and Photonics. Washington: Optica Publishing Group. http://dx.doi.org/10.1364/PHOTONICS.2012.MPo.3.

Kato, I., Schrode, J., Kohr, W. J., & Laskowski, M. Jr. (1987). Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all three of its domains. *Biochemistry*, 26(1), 193-201. http://dx.doi.org/10.1021/bi00375a027. PMid:3548816.

Kavas, N., & Kavas, G. (2016). Physical-chemical and antimicrobial properties of egg white protein powder films incorporated with orange essential oil on Kashar Cheese. *Food Science and Technology (Campinas)*, 36(4), 672-678. http://dx.doi.org/10.1590/1678-457x.12516.

Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica*, 39(8), 549-559. http://dx.doi.org/10.1111/j.1745-7270.2007.00320.x. PMid:17687489.

Krimm, S., & Bandekar, J. (1986). Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Advances in Protein Chemistry*, 38, 181-364. http://dx.doi.org/10.1016/S0065-3233(08)60528-8. PMid:3541539.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685. http://dx.doi.org/10.1038/227680a0. PMid:5432063.

Laskowski, M. Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., & Schatzley, B. L. (1987). Ovomucoid third domains from 100 avian species: isolation, sequences, and hypervariability of enzyme-inhibitor contact residues. *Biochemistry*, 26(1), 202-221. http://dx.doi.org/10.1021/bi00375a028. PMid:3828298.

Li, H., Chen, X., Guo, Y., Hou, T., & Hu, J. (2022). A pivotal peptide (Ile-Leu-Lys-Pro) with high ACE- inhibitory activity from duck egg white: identification and molecular docking. *Food Science and Technology (Campinas)*, 42, 1-11. http://dx.doi.org/10.1590/fst.66121.

Macelline, S. P., Toghyani, M., Chrystal, P. V., Selle, P. H., & Liu, S. Y. (2021). Amino acid requirements for laying hens: a comprehensive review. *Poultry Science*, 100(5), 101036. http://dx.doi.org/10.1016/j.psj.2021.101036. PMid:33770542.

Marino, R., Iammarino, M., Santillo, A., Muscarella, M., Caroprese, M., & Albenzio, M. (2010). Technical note: rapid method for determination of amino acids in milk. *Journal of Dairy Science*, 93(6), 2367-2370. http://dx.doi.org/10.3168/jds.2009-3017. PMid:20494144.

Matsuda, T., Watanabe, K., & Nakamura, R. (1983). Blocking effect of trypsin associated with ovomucoid on the antibody binding to ovomucoid. *Journal of Biochemistry*, 94(1), 43-49. http://dx.doi.org/10.1093/oxfordjournals.jbchem.a134352. PMid:6413500.

Matsuda, T., Watanabe, K., & Sato, Y. (1981). Secondary structure prediction of chicken egg white ovomucoid. *Agricultural and Biological Chemistry*, 45(2), 417-423.

Mine, Y., & Kovacs-Nolan, J. (2005). Biologically active peptides derived from egg proteins. In *European Symposium on the Quality of Eggs and Egg Products*. Netherlands: World's Poultry Science Association.

Naeem, M., Un-Nisa, M., Ahmad, N., Imran, M., Anwar, H., & Manzoor, M. F. (2022). Preparation of weaning foods by replacing plant proteins with egg protein. *Food Science and Technology (Campinas)*, 42, 1-9. http://dx.doi.org/10.1590/fst.44920.

Nagata, K., & Yoshida, N. (1984). Interaction between trypsin-like enzyme from *Streptomyces erythraeus* and chicken ovomucoid. *Journal of Biochemistry*, 96(4), 1041-1049. http://dx.doi.org/10.1093/oxfordjournals.jbchem.a134921. PMid:6394596.

Patil, D. N., Chaudhary, A., Sharma, A. K., Tomar, S., & Kumar, P. (2012). Structural basis for dual inhibitory role of tamarind Kunitz inhibitor (TKI) against factor Xa and trypsin. *The Federation of European Biochemical Societies Journal*, 279(24), 4547-4564. http://dx.doi.org/10.1111/febs.12042. PMid:23094997.

Quan, T. H., & Benjakul, S. (2017). Comparative study on the effect of duck and hen egg albumens on proteolysis and gel property of sardine surimi. *International Journal of Food Properties*, 20(3), 2786-2797. http://dx.doi.org/10.1080/10942912.2017.1374290.

Quan, T. H., & Benjakul, S. (2018). Compositions, protease inhibitor and gelling property of duck egg albumen as affected by salting. *Korean Journal for Food Science of Animal Resources*, 38(1), 14-25. PMid:29725221.

Quan, T. H., & Benjakul, S. (2019). Duck egg albumen: physicochemical and functional properties as affected by storage and processing. *Journal of Food Science and Technology*, 56(3), 1104-1115. http://dx.doi.org/10.1007/s13197-019-03669-x. PMid:30956290.

Rakashanda, S., Rana, F., Rafiq, S., Masood, A., & Amin, S. (2012). Role of proteases in cancer: a review. *Biotechnology and Molecular Biology Reviews*, 7(4), 90-101. http://dx.doi.org/10.5897/BMBR11.027.

Réhault-Godbert, S., Guyot, N., & Nys, Y. (2019). The golden egg: nutritional value, bioactivities, and emerging benefits for human health. *Nutrients*, 11(3), 1-26. http://dx.doi.org/10.3390/nu11030684. PMid:30909449.

Romano, C., Corsetti, G., Flati, V., Pasini, E., Picca, A., Calvani, R., Marzetti, E., & Dioguardi, F. S. (2019). Influence of diets with varying essential/nonessential amino acid ratios on mouse. *Nutrients*, 11(6), 1-19. http://dx.doi.org/10.3390/nu11061367. PMid:31216646.

Salahuddin, A., Sibghatullah, X., & Baig, M. A. (1985). Homologous structural domains in chicken egg-white ovomucoid: Characterization and acid denaturation. *International Symposium on Biomolecular Structure and Interactions, Suppl. Journal of Bioscience. Journal of Biosciences*, 8(1), 67-87. http://dx.doi.org/10.1007/BF02703968.

Salameh, M. A., Soares, A. S., Hockla, A., Radisky, D. C., & Radisky, E. S. (2011). The P(2)′ residue is a key determinant of mesotrypsin specificity: engineering a high affinity inhibitor with anticancer activity. *The Biochemical Journal*, 440(1), 95-105. http://dx.doi.org/10.1042/BJ20110788. PMid:21806544.

Shimazaki, Y., Tanaka, K., & Sakata, K. (2018). Enzyme inhibitory activity of ovomucoid extracted using a carboxypeptidase y-immobilized

membrane. *Chromatography (Basel)*, 39(1), 33-39. http://dx.doi.org/10.15583/jpchrom.2017.020.

Tarhan, O., Gözler, M., Yavuz, R. C., & Şimşek, M. (2020). Effect of heat treatment on protein fractions of edible poultry eggs. *Akademik Gıda*, 18(3), 233-240.

Tumanggor, B. G., Suci, D. M., & Suharti, S. (2017). Kajian pemberian pakan pada itik dengan sistem pemeliharaan intensif dan semi intensif di peternakan rakyat. *Buletin Makanan Ternak*, 104(1), 21-29.

Xie, H., Huff, G. R., Huff, W. E., Balog, J. M., Holt, P., & Rath, N. C. (2002). Identification of ovotransferrin as an acute phase protein in chickens. *Poultry Science*, 81(1), 112-120. http://dx.doi.org/10.1093/ps/81.1.112. PMid:11885890.

Yang, H., Yang, S., Kong, J., Dong, A., & Yu, S. (2015). Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nature Protocols*, 10(3), 382-396. http://dx.doi.org/10.1038/nprot.2015.024. PMid:25654756.

Zhang, X., Huang, L., Nie, S., Qi, X., & Zhang, Q. (2003). Secondary structure of insulin encapsulated within liposomes. *Yao Xue Xue Bao*, 38(11), 863-866. PMid:14992003.