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Running title: Ovotransferrin peptides and Functional Properties

# Enzymatic Hydrolysis of Ovotransferrin and the Functional Properties of Its Hydrolysates

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**Abstract**

Bioactive peptides have great potentials as nutraceutical and pharmaceutical agents that can improve human health. The objectives of this research were to produce functional peptides from ovotransferrin, a major egg white protein, using single enzyme treatments, and to analyze the properties of the hydrolysates produced. Lyophilized ovotransferrin was dissolved in distilled water at 20 mg/mL, treated with protease, elastase, papain, trypsin, or α-chymotrypsin at 1% (w/v) level of substrate, and incubated for 0-24 hours at the optimal temperature of each enzyme (protease 55 ℃, papain 37 ℃, elastase 25 ℃, trypsin 37 ℃, α-chymotrypsin 37 ℃). The hydrolysates were tested for antioxidant, metal-chelating, and antimicrobial activities. Protease, papain, trypsin, and α-chymotrypsin hydrolyzed ovotransferrin relatively well after 3 hours of incubation, but it took 24 hours with elastase to reach a similar degree of hydrolysis. The hydrolysates obtained after 3 hours of incubation with protease, papain, trypsin, α-chymotrypsin, and after 24 hours with elastase were selected as the best products to analyze their functional properties. None of the hydrolysates exhibited antioxidant properties in the oil emulsion nor antimicrobial property at 20 mg/mL concentration. However, ovotransferrin with α-chymotrypsin and with elastase had higher $Fe^{3+}$-chelating activities (1.06±0.88%, 1.25±0.24%) than the native ovotransferrin (0.46±0.60%). Overall, the results indicated that the single-enzyme treatments of ovotransferrin were not effective to produce peptides with antioxidant, antimicrobial, or $Fe^{3+}$-chelating activity. Further research on the effects of enzyme combinations may be needed.

Keywords: bioactive peptides, enzyme hydrolysis, functional properties, hydrolysates, ovotransferrin

## Introduction

Chicken eggs are one of the best natural food products that are consumed all over the world (Abeyrathne et al., 2013a), and play a significant role in the human nutrition. Eggs are a rich source of protein and most of the egg proteins are present in the egg white and yolk, which account for 50% and 44%, respectively, of total egg proteins (Wu, 2014). Among the egg white proteins ovalbumin (54%), ovotransferrin (11%), ovomucoid (12%), globulins (8%), lysozyme (3.0%), and ovomucin (3.5%) are the major proteins (Abeyrathne et al., 2013a).

Ovotransferrin is a single-chain glycopeptide consists of 686 amino acids with a molecular weight of 78 kDa (Wu and Acero-Lopez, 2012). The native ovotransferrin is present in two forms: metal-free (apo) and metal-bound (holo), and the chemical and physical characteristics of the two differ significantly (Abeyrathne et al., 2013a). Apo-ovotransferrin is highly susceptible to chemical and physical treatments, whereas holo-ovotransferrin is resistant to chemical and thermal denaturation (Ko and Ahn, 2008). Furthermore, being a member of the transferrin family, ovotransferrin has an antimicrobial activity due to its iron-chelating capability (Wu and Acero-Lopez, 2012). Apo-ovotransferrin shows a high affinity to iron ions, and the antimicrobial activity of egg white is through the removal of iron from being used by microorganisms. The precipitation technique in the presence of ammonium sulfate (Warner and Weber, 1951), DEAE affinity-gel blue chromatography (Chung et al., 1991), Duolite C-476 (Guerin and Brule, 1992), and counter-current chromatography (Shibusawa et al., 1998) were commonly used to separate ovotransferrin, but the purity was low. To improve the purity of the ovotransferrin, chromatographic techniques such as immobilized-metal affinity chromatography (Al-Mashikhi and Nakai, 1987) and cation exchange chromatography (Guerin-Dubiard et al., 2005) were developed. Although the chromatographic methods improved the purity of ovotransferrin (over 89%), those methods were not practical for scale-up production. Ko and Ahn (2008) introduced an economic and

simple purification procedure for the large-scale production of ovotransferrin, and Abeyrathne et al. (2013b) introduced a new separation method without using ethanol. Recently, Ji et al. (2020) sequentially separated ovotransferrin along with 5 major egg white proteins with 90% purity and 77% yield.

The native ovotransferrin displays multiple bioactivities that include antimicrobial activity for a wide spectrum of bacteria, fungi, yeasts, and parasites (Valenti et al., 1982; Ibrahim et al., 1998; Ko et al., 2008a, b; Moon et al., 2012; Cooper et al., 2019), antioxidant activity (Ibrahim et al., 2007; Moon et al., 2014), anticancer activity against colon and breast cancer (Ibrahim and Kiyono, 2009), and immunomodulatory activity (Xie et al., 2002; Chiurciu et al., 2017; Lee et al., 2018; Zhu et al., 2019; Zhang et al., 2020). However, Moon et al. (2017) reported that native ovotransferrin does not contain any inhibitory activity against Angiotensin-Converting Enzyme (ACE). Furthermore, ovotransferrin was reported to have various food applications; use as a κ-carrageenan-based packing material once combined with Ethylenediaminetetraacetic acid (EDTA) (Seol et al., 2009), surfactant-free food-grade Pickering emulsion (Wei and Huang, 2019) and medical and pharmaceutical applications; metal supplement (Abdallah and Chahine, 1999), bone health promoter (Shang and Wu, 2019), heteroprotein complexes (e.g., ovotransferrin-lysozyme) for deliver hydrophobic nutraceutical such as curcumin (Wei at al., 2019), and therapeutic agent for the reproductive health of cows (Talukder et al., 2019).

Bioactive peptides are specific protein fragments that are inactive within the sequence of the native protein but have positive impacts on body functions or conditions once released by proteolysis or fermentation (Noh and Suh, 2015). Several peptides from egg proteins have been studied for their biological activities. The bioactive peptides in the native ovotransferrin were produced using various methods, including acid hydrolysis (Ibrahim et al., 2000; Lee et al., 2010), autocleaving under reduced conditions (Ibrahim and Kiyono, 2009; Moon et al.,

2014), and enzymatic hydrolysis (Huang et al., 2010; Shen et al., 2010; Moon et al., 2013; Wang et al., 2017; Ma et al., 2020). All the studies indicated that the functionality of ovotransferrin increased after the hydrolysis.

The application of natural bioactive compounds and peptides has received great attention as potential agents to improve human health in recent years (Wu, 2014). Extensive scientific evidence proved that the bioactive peptides derived from foods have beneficial effects in improving human health and preventing diseases (Möller et al., 2008). Ibrahim et al. (2000) reported that the peptide located within 109-200 sequences of the N-lobe of ovotransferrin showed a strong antimicrobial activity against *Escherichia coli* through a membrane damage mechanism. Furthermore, peptides of ovotransferrin possess antimicrobial activities against Gram-positive *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis,* and Gram-negative *E. coli*, *Pseudomonas aeruginosa* (Ma et al., 2020), and Gram-negative *Salmonella typhimurium* (Zohreh et al., 2014). Also, many scientific findings proved that peptides derived from ovotransferrin showed antioxidant activity.

Ovotransferrin hydrolysates showed stronger superoxide anion scavenging activity, oxygen radical absorbance capacity (ORAC), and 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity than native ovotransferrin (Kim et al., 2012). Two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) derived from ovotransferrin showed significant antioxidant activities when sonicated and hydrolyzed with thermolysin (Shen et al., 2010). An ovotransferrin peptide with the sequence of Ile-Arg-Trp is reported to have a significant oxygen radical-scavenging effect (Huang et al., 2010). Also, ovotransferrin hydrolysates obtained from promod 278P, thermolysin, and their combination had strong DPPH radical scavenging activities (Lee et al., 2017). Two ovotransferrin peptides with the amino acid sequence of Asp-Gln-Lys-Asp-Glu-Tyr-Glu-Leu-Leu and Lys-Asp-Leu-Leu-Phe-Lys showed the antiviral activity against Marek's disease infection (Giansanti et al., 2005). Especially,

ovotransferrin hydrolyzed with a food-grade promod 278P enzyme had a strong ACE-inhibitory activity (Moon et al., 2017). Majumder et al. (2015) reported that a peptide (Ile-Arg-Trp) derived from ovotransferrin contributed to antihypertensive activity by increasing $ACE_2$ and decreasing pro-inflammatory genes expression. Lee et al. (2006) found that one peptide from ovotransferrin with the peptide sequence of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr has an ACE-inhibitory activity that can be used as a pro-drug to reduce blood pressure. Recently, Yu et al., (2019) exhibited potent ACE-inhibitory of ovotransferrin hydrolysates of 19 enzymes and one combination obtained from *in silico* hydrolysis. In addition, the peptides of ovotransferrin possess various immunomodulatory activities (Liu et al., 2017; Jiao et al., 2019), anti-inflammatory (Wang et al., 2017), and cytotoxic (Yi et al., 2017) activities. Ovotransferrin-derived peptides were also reported to have potentials for many medical and pharmaceutical applications: the use of Ile-Arg-Trp, Ile-Gln-Trp, and Lys-Val-Arg-Glu-Gly-Thr to treat cardiovascular diseases (Chen et al., 2017), and Ile-Arg-Trp to decrease insulin-stimulated glucose uptake (Son et al., 2018).

Hence, the functional peptides derived from ovotransferrin may have great potentials to be used as antimicrobial and antioxidative agents in the food industry (Giansanti et al., 2015). Thus, the production of bioactive peptides from egg proteins will increase the values and potential applications of eggs. Recently, functional peptides derived from ovalbumin, ovomucoid, and ovomucin using single and two enzymes combined hydrolysis with strong bioactivities such as antioxidants, metal chelating, and ACE inhibitory activities were reported (Abeyrathne et al., 2014; Abeyrathne et al., 2015; Abeyrathne et al., 2016). There are many previous studies on ovotransferrin hydrolysates and their activities, but little work on analyzing multi-functionality of ovotransferrin hydrolysates in a study is available. This study determined the multiple functions of ovotransferrin hydrolysates, including antimicrobial, antioxidant and metal-chelating activities. Also, the functionalities of the hydrolysates

156 produced depend on the amino acid composition of peptides in the hydrolysates and their
157 physical natures such as solubility. However, enzyme hydrolysates will lead to either
158 improving or destroying existing activities. In this study we showed that enzymatic hydrolysis
159 can also destroy the activities of ovotransferrin under the investigated conditions. The
160 objectives of this research were to produce functional peptides from ovotransferrin using the
161 single-enzyme treatments and to analyze the functional properties of the hydrolysates
162 produced from ovotransferrin.
163
164 **Materials and Methods**
165 **Materials**
166 Lyophilized apo-ovotransferrin (over 85% purity and over 83% yield), which was
167 prepared according to the method described by Abeyrathne et al. (2013b), was obtained from
168 Iowa State University, Ames, USA. Standard enzymes; protease (from *Bacillus licheniformis*;
169 Alcalase® 2.4L; ≥ 2.4 U/g solution; EC 3.4.21.64), papain (from papaya latex; ≥ 10 U/mg
170 protein; EC 3.4.22.2), elastase (from the porcine pancreas; ≥ 4.0 U/mg protein, EC 3.4.21.70),
171 trypsin (from the bovine pancreas; ≥ 7500 BAEE U/mg solid; T9201) and α-chymotrypsin
172 (from the bovine pancreas; ≥ 40 U/mg protein; EC 3.4.21.1) were purchased from Sigma-
173 Aldrich (St. Louis, MO, USA). Other chemicals were purchased either from Sigma-Aldrich
174 (St. Louis, MO, USA), Daejung Chemical and Materials (Gyeonggi-do, Korea), HiMedia
175 Laboratories (Mumbai, India), or Research Lab Fine Chem Industries (Mumbai, India).
176
177 **Enzymatic Hydrolysis of Ovotransferrin**
178 The enzymatic hydrolysis of ovotransferrin was performed according to the method
179 described by Abeyrathne et al. (2014) with some modifications. Ovotransferrin (20 mg/mL)
180 solution was prepared by dissolving lyophilized ovotransferrin in distilled water and then the

pH of the ovotransferrin solution was adjusted for the optimal condition for each enzyme (protease pH 6.5, papain pH 6.5, elastase pH 8.0, trypsin pH 7.8, α-chymotrypsin pH 7.6) using 1 N HCl, 1 N NaOH, 0.1 N HCl and 0.1 N NaOH under room temperature. Standard enzymes were separately added to the ovotransferrin solutions with the enzyme: substrate ratio of 1:100 (w/w) and then incubated at the optimal temperature of each enzyme (protease 55 ℃, papain 37 ℃, elastase 25 ℃, trypsin 37 ℃, α-chymotrypsin 37 ℃) for 0 (immediately after addition of enzymes), 3, 6, 9, 12 and 24 hours. After incubation, the samples were heated at 100 ℃ for 15 minutes to inactive the added enzymes and the resulting solutions were freeze-dried and considered as the enzyme hydrolysates of ovotransferrin.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**

The degree of hydrolysis was analyzed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) described by Green and Sambrook (2014) under reduced conditions using Mini-Protean® Tetra System (Bio-Rad; Bio-Rad Laboratories, Inc., United States) and stained with Coomassie Brilliant Blue R-250 (Ameresco, Solon, OH).

**Analysis of the Functional Properties of Hydrolysates**

**Analysis of Antimicrobial Activity**

The antimicrobial activity of the hydrolyzed ovotransferrin was determined according to the agar well diffusion method described by Moon et al. (2012) with some modifications. Initially, all the bacteria were suspended in 0.1% w/v sterile peptone water and enriched for 2-3 hours before culture. In the antimicrobial test, 20-25 mL of plate count agar (HiMedia, India) for total plate bacteria was poured into sterilized Petri plates. After solidification, the agar surface was streaked with a sterilized cotton swab containing bacteria. After 30 minutes, 6 mm-diameter wells were aseptically punched on the agar surface using a sterilized cork-

borer, and 100 µL aliquots (20,000 ppm, 10,000 ppm, 5,000 ppm, 2,500 ppm, 1,250 ppm, and 625 ppm) of hydrolysate was pipetted into it. Finally, the Petri dishes were kept for 30 minutes to complete diffusion and incubated at 37 ℃ for 48 hours. The bacterial inhibition zones of the hydrolysates were observed against the positive control containing Augmentin® (SmithKline Beecham Ltd., West Sussex, United Kingdom) and the negative control containing sterilized distilled water. The antimicrobial activity of the hydrolysates was calculated as the antimicrobial index using the following formula (Patra et al., 2009):

Antimicrobial index = (inhibition zone of sample/inhibition zone of standard) × 100

**Analysis of Antioxidant Activity**

The 2-thiobarbituric acid reactive substance (TBARS) values of the hydrolysates were measured according to the TBARS assay described by Abeyrathne et al. (2014) with some modifications. One gram of olive oil (Aceites Agro Sevilla, Spain), 100 µL of Tween-20 (Research Lab Fine Chem, India), and 100 mL of distilled water were homogenized for 2 minutes in an ice bath using a homogenizer (D-500, Scilogex, Rocky Hill) at the maximum speed to prepare an oil-in-water emulsion. Samples for the TBARS assay were prepared by mixing 8 mL of an oil-in-water emulsion, 1 mL of distilled water, and 1 mL of ovotransferrin hydrolysates (20 mg/mL) followed by incubating at 37 ℃ for 16 hrs. After incubation, 1 mL of sample was transferred to a 15 mL Falcon tube followed by adding 2 mL of thiobarbituric acid (TBA)/ trichloroacetic acid (TCA) solution (20 mM TBA/ 15% TCA w/v) and 50 µL of 10% w/v butylated hydroxyanisole (BHA) (Research Lab Fine Chem, India) in 90% v/v ethanol. Then the solution was vortex-mixed and incubated at 90 ℃ for 15 minutes to develop color. At the end of the incubation, the sample was cooled in ice water for 10 minutes and centrifuged at 3,000 × g for 15 minutes at 5 ℃. Finally, the absorbance of resulting

supernatant was measured at 532 nm against a blank (1 mL of distilled water and 2 mL of TBA/TCA solution). The TBARS value of the hydrolysates was expressed as milligrams of malondialdehyde (MDA) per liter of emulsion.

**Analysis of $Fe^{3+}$-Chelating Activity**

The $Fe^{3+}$-chelating activity of the ovotransferrin hydrolysates was analyzed using the Ferrozine method (Carter, 1971) with slight modifications. In the experiment, 100 µl of ovotransferrin hydrolysates (20 mg/mL), 900 µl of deionized water, and 1 ml of 10 mg/kg $FeCl_3$ (Research Lab Fine Chem, India) were vortex-mixed in a 15 ml Falcon tube followed by incubating at room temperature for 5 minutes. After proteins and peptides in the sample were precipitated by adding 900 µl of 11.3% w/v trichloroacetic acid (Sigma-Aldrich, USA), the samples were centrifuged at 2,500 × g for 10 minutes at 5 ℃. Then, 1 ml of supernatant from the sample was transferred to a culture tube followed by adding 1 ml of distilled water, 800 µl of 10% w/v ammonium acetate (HiMedia, India), and 200 µl of ferroin color indicator (75 mg of ferrozine, 75 mg of neocuprion, and 1 drop of 6 N HCl in 25 mL of distilled water) and vortex-mixed. Finally, the samples were incubated at room temperature for 5 minutes and the absorbance was measured at 562 nm. The $Fe^{3+}$ chelating activity was calculated using the following formula:

$$Fe^{3+} \text{ chelating activity (\%)} = [1 - (\text{sample absorbance/blank absorbance})] \times 100$$

**Statistical Analysis**

All the tests were performed in triplicate and all the data were analyzed using Minitab® 17.1.0 statistical software (Minitab Ltd, Co., UK). One-way ANOVA in a completely randomized design was used and Tukey's test was performed for the significant

differences ($P < 0.05$) among mean values.

## Results and Discussion

### Enzymatic Hydrolysis of Ovotransferrin

Proteolytic enzymes hydrolyze proteins into amino acid monomers and peptides at the target-specific peptide bonds under optimal temperature and pH (Tapal and Tiku, 2019). Over the decades, various studies have been carried out to produce functional peptides from egg-derived proteins. Ko and Ahn (2008) reported that apo-ovotransferrin can be easily hydrolyzed using proteolytic enzymes. In the previous studies, ovotransferrin was hydrolyzed using thermolysin, pepsin (Shen et al., 2010), promod 278P (Moon et al., 2017), chymotrypsin (Kim et al., 2012), protamex, alcalase, trypsin, neutrase, flavorzyme, maxazyme, collupulin and Protex (Kim et al., 2012).

In this study, ovotransferrin was hydrolyzed using protease, papain, elastase, trypsin, and α-chymotrypsin under optimal temperature and pH for 0, 3, 6, 9, 12, and 24 hrs. According to the visual observations, the hydrolysates with protease showed the lowest precipitation and turbidity compared with other enzymes. Among the treatments, the papain and elastase hydrolysates had the highest turbidity and precipitation. Although visual observations showed significant differences among the 5 treatments, it was very difficult to differentiate the best incubation time because they were very similar at all combination times. Therefore, visual observations were not effective in selecting the best hydrolysates with the best incubation time.

The peptides with very small molecular weight were not retained in the 15% SDS-PAGE gel (Abeyrathne et al., 2014). Fig. 1 showed that protease almost completely hydrolyzed ovotransferrin after 3 hrs of incubation. However, elastase produced partially hydrolyzed ovotransferrin products even after 24 hrs of incubation. Treating ovotransferrin

with papain produced peptides with MW < 10 kDa, but another clear band appears around 25 kDa and smear showed between 25 – 10 kDa area (shown by the box in Fig. 2). Therefore, it cannot be considered as a completely hydrolyzed product. Trypsin also hydrolyzed the ovotransferrin, but it was not as effective as α-chymotrypsin (data not shown).

Elastase did not hydrolyze ovotransferrin well even after 24 hours of incubation (shown by the box in Fig. 3) and a significant number of bands were found in 15% SDS-PAGE (Figure 3). Therefore, elastase was not effective to produce ovotransferrin hydrolysates with low molecular weights. Kim et al. (2012) also discussed that acids, alcalase, and maxazyme treatments were more effective than neutrase and flavozyme in producing low-molecular-weight peptides from ovotransferrin.

All the single enzyme treatments with the best hydrolysates from ovotransferrin [protease 3 hrs at 55 ℃ (OTPro), papain 3 hours at 37 ℃ (OTPap), elastase 24 hrs at 25 ℃ (OTEla), trypsin 3 hrs at 37 ℃ (OTTrp) and α-chymotrypsin 3 hours at 37 ℃ (OTChy)] were selected and used for the analysis of the functional properties.

**Functional Properties of the Ovotransferrin Hydrolysates**

**Antimicrobial Activity of the Ovotransferrin Hydrolysates:** Antimicrobial peptides are abundant in many tissues and cells of plants and animals. The amino acid composition, amphiphilicity, cationic charge, and the size of peptides allow them to attach and penetrate membrane bilayers (Brogden, 2005). The antimicrobial peptides, usually with the molecular weight below 10 kDa, inhibit cell growth and kill several microorganisms (Kim and Wijesekara, 2010). Ibrahim et al. (2000) reported that the antimicrobial peptides from ovotransferrin (Leu109-Asp200) prevented *E. coli* through a membrane damage mechanism. They also stated that $Zn^{2+}$-saturated ovotransferrin exhibited stronger antimicrobial activity

than apo-ovotransferrin and other metal complexes. Compared with the antimicrobial activity of other egg white-derived peptides, little information on the antimicrobial activity of ovotransferrin-derived peptides is available.

According to present findings, 20 mg/mL ovotransferrin hydrolyzed products did not yield any inhibition zone in total plate counts compared to that of the Augmentin® (26.33±1.52 mm) under the investigated conditions. Similarly, Kim et al. (2012) noted that there was no clear zone at 20 mg/mL ovotransferrin against *E. coli* 0157:H7 and had a slight inhibition at 80 mg/mL concertation. Hence, the absence of antimicrobial activity of the ovotransferrin hydrolysates may be due to the destruction of antimicrobial and metal-binding sites by the enzyme hydrolysis. Also, the resistance of the bacteria and the ineffective concentration of the hydrolysate may have caused the loss of antimicrobial property under the investigated conditions. Furthermore, in order to express antimicrobial activity, protein materials should be completely diffused in the media. However, as per the visual observation, there were undiffused ovotransferrin hydrolysates even after incubation period, which can be a possible reason for ineffectiveness of antimicrobial activity. However, Zohreh et al. (2017) reported that the hydrolysis of ovotransferrin with trypsin and ficin showed antimicrobial activity against *S. aureus* ($G^+$) and *S.* Typhimurium. Also, the peptides from lysozyme (Ibrahim et al., 2001; Pellergrini et al., 1997; You et al., 2010), ovalbumin (Pellegrini et al. 2004), and ovomucin (Kobayashi et al., 2004) exhibited strong antimicrobial activities. Further experiments are needed to analyze the antimicrobial activity of the hydrolysates with different enzyme treatments.

**Antioxidant Activity of Ovotransferrin Hydrolysates:** Manso et al. (2008) explained that some substances termed as antioxidants should retard or at least attenuate the organic impairment by excessive oxidative stress at very low concentrations. Since there are potential

health risks associated with synthetic antioxidants such as butylated hydroxytoluene (BHT) and BHA, natural antioxidants became an attractive choice for the food and pharmaceutical industries (You et al., 2010). Ovotransferrin is reported to have a strong antioxidant property when supplemented along with diets. Oxidative stress is related to the etiopathogenesis of several chronic diseases. Lipids are the most heavily involved class of biomolecules to oxidative stress and the oxidation of the lipids generates several secondary products. Malondialdehyde (MDA) is the principal product of polyunsaturated fatty acid peroxidation (Rio et al., 2005). TBARS is a commonly used method of measuring lipid peroxidation. MDA forms an adduct with 2-TBA molecules, which gives a pink color (Dasgupta and Klein, 2014). In the present study, oil emulsion prepared with olive oil had whitish pink color compared to that of other emulsions containing ovotransferrin hydrolysates. Overall results in Fig. 4 indicated that the oil emulsion with native ovotransferrin and its protease hydrolysate had lower TBARS values than the control; 0.029 mg MDA/L and 0.036±0.003 mg MDA/L, respectively, indicating that both ovotransferrin and its protease hydrolysates have some antioxidant activity. However, other hydrolysates had weak antioxidant properties. According to the visual observations, there were significant amount of undigested protein materials with low solubility. Hence, the solubility of the protein materials is an important factor to express functional properties *in vitro*.

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The antioxidant activity of protein hydrolysates depends on the amino acid composition (Alemán et al., 2011), which are affected by enzyme activity and the conditions of the hydrolysis process such as pH, temperature, enzyme-substrate ration, and incubation time (Shahidi and Zhong, 2008). Kim et al. (2012) obtained approximately 3.2 to 13.5 superoxide-anion-scavenging activity and oxygen-radical-scavenging activity for the ovotransferrin hydrolysates of protamex, alcalase, trypsin, neutrase, flavorzyme, maxazyme,

collupulin, Protex, Promod 278, and α-chymotrypsin. Shen et al. (2010) reported that two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) from Thermolysin hydrolysates of ovotransferrin had significantly higher oxygen radical absorbance capacity, which increased with sonication. The hydrolysates obtained from ovalbumin with alcalase, pepsin, papain, and α-chymotrypsin (Abeyrathne et al., 2014), from ovomucoid with alcalase, trypsin and papain (Abeyrathne et al., 2015), and from ovomucin with alcalase, pepsin, trypsin, and papain (Abeyrathne et al., 2016) had lower TBARS values than that of the controls. However, the hydrolysates obtained from ovotransferrin under the investigated conditions were not much effective in preventing the formation of malondialdehyde in foods which leads to lipid oxidation as well as the occurrence of oxidative stress in animal cells.

**$Fe^{3+}$ Chelating Activity of the Ovotransferrin Hydrolysates:** In this study, enzymatic hydrolysis did not significantly increase the ferric iron-binding ability of peptides in all treatments as shown in Fig. 5. However, the ovotransferrin hydrolysates obtained from elastase and α-chymotrypsin slightly increased the ferric iron-binding property (1.06±0.88%, 1.25±0.24%) compared to that of the native ovotransferrin (0.46±0.60%), which could be caused by the release of new iron-binding sites by the enzyme activity (Figure 5). Rajapakse et al. (2005) stated that acidic (Asp and Glu) and basic (Arg and Lys) amino acid residues play important roles in the metal-chelating activity of the protein hydrolysates. Since elastase and α-chymotrypsin hydrolysates of ovotransferrin maintained a lower degree of hydrolysis, the hydrolysates maintained their iron-chelating residues even after hydrolysis. However, Keung et al. (1981) reported that the hydrolysis of holo-ovotransferrin with subtilin did not produce significant changes in the iron-binding capacity or the conformation of the iron-binding domains.

The native ovotransferrin is a member of the transferrin family and well-known for

its iron ($Fe^{3+}$)-binding capability (Ibrahim et al., 2000; Ko and Ahn, 2008; Lin et al., 1994). As previously described, the metal-binding peptides derived from egg white can retard lipid oxidation (Guerin-Dubaiard et al., 2007) as well as microbial growth (Ko et al., 2008b). Abeyrathne et al. (2013a) suggested that egg white peptides with metal-binding properties have great potentials as an iron carrier. However, the ovotransferrin hydrolysates produced from the current study were not effective in reducing oxidation nor metal-binding and it can be due to the destruction of metal-binding sites due to enzyme activity. Also, the physical structure of ovotransferrin hydrolysates may obstruct the metal-binding sites to prevent binding.

## Conclusion

The ovotransferrin from chicken egg could be completely or partially hydrolyzed using protease, elastase, papain, trypsin, and α-chymotrypsin under the investigated conditions. However, all the hydrolysates of ovotransferrin had poor antimicrobial, antioxidant, and $Fe^{+}$-chelating activities at 20 mg/mL concentration. Hence, those hydrolysates cannot be utilized as antimicrobials, antioxidants, and iron carriers in the food and pharmaceutical industries. We also found that enzymatic hydrolysis destroyed some functionality of the native ovotransferrin under investigated conditions. Further research is needed to produce functional peptides from ovotransferrin using different enzymes or enzyme combinations or finding the functionalities other than tested here.

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**FIGURE 01**

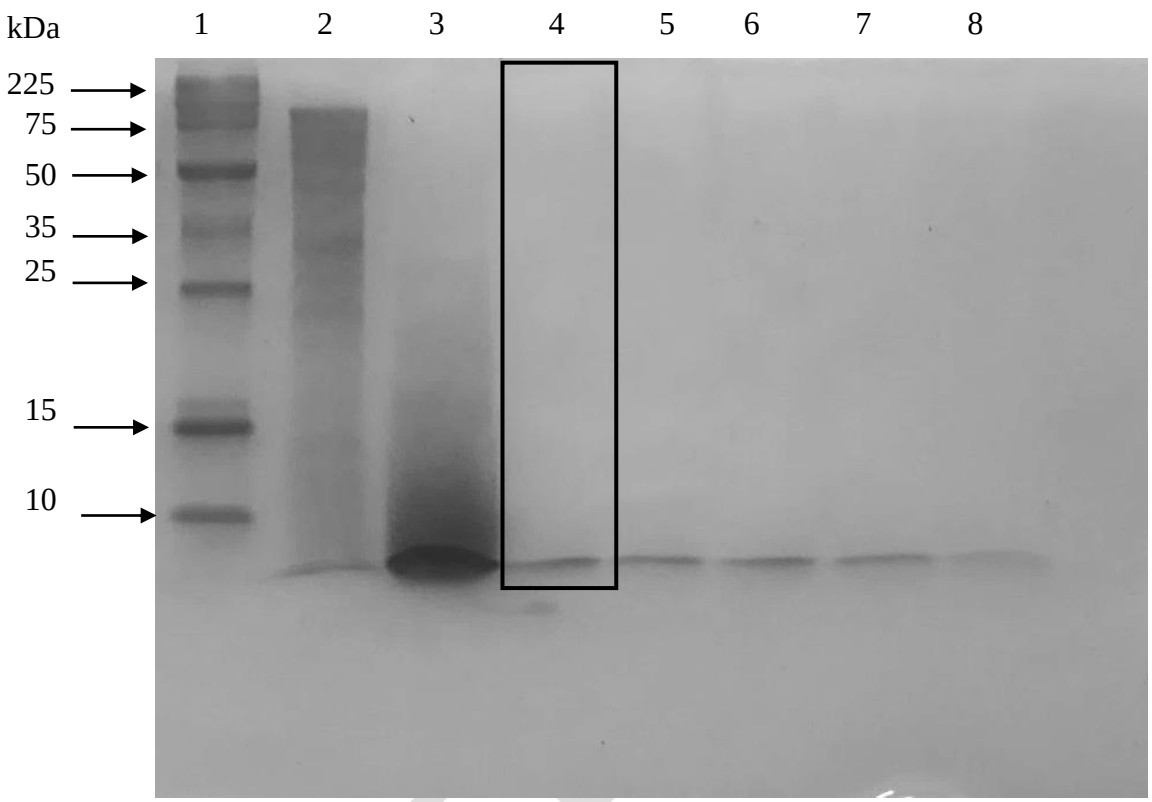


**Figure 1**. The SDS-PAGE image of ovotransferrin hydrolyzed with protease; Lane 1: marker, lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with protease 55 ℃ for 0, 3, 6, 9, 12 and 24 hours.

**FIGURE 02**

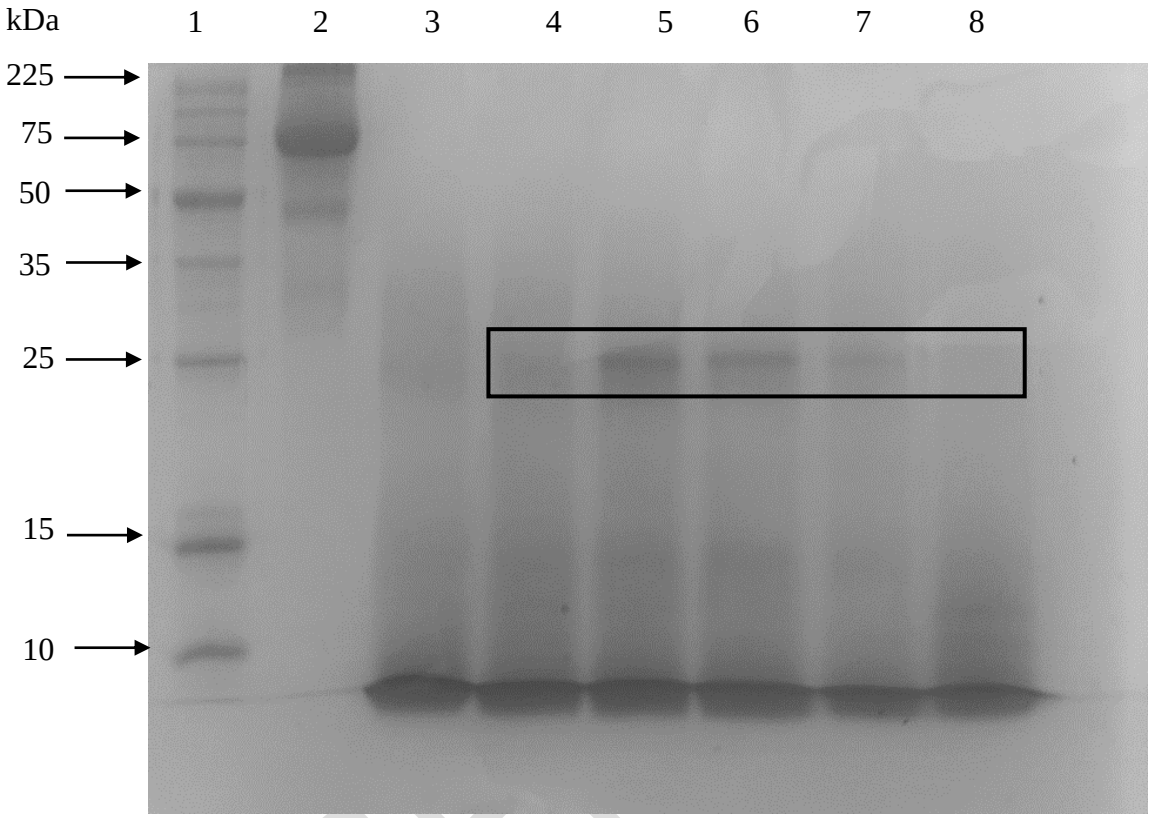


**Figure 02**. The SDS-PAGE image of ovotransferrin hydrolyzed with papain; Lane 1: marker, lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with papain 37 ℃ for 0, 3, 6, 9, 12 and 24 hours.

**FIGURE 03**

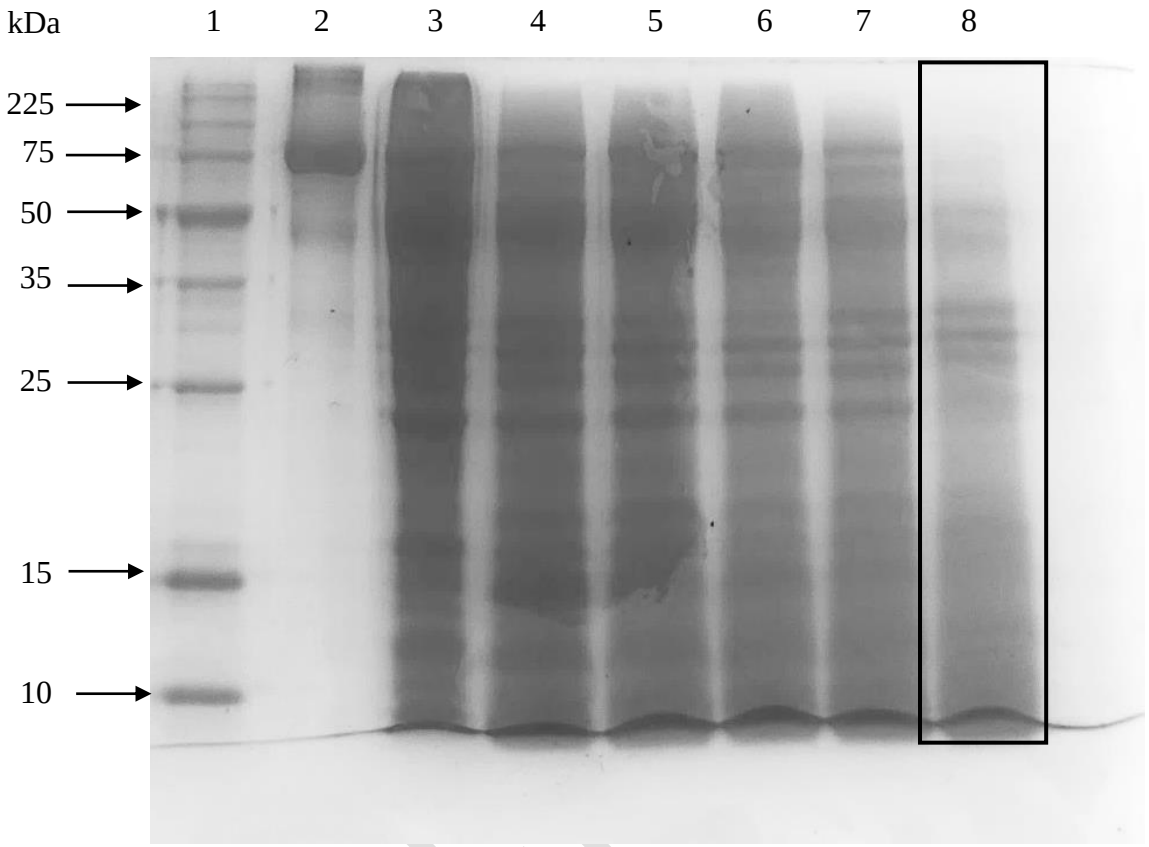


**Figure 3**. The SDS-PAGE image of ovotransferrin hydrolyzed with elastase; Lane 1: marker, lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with elastase 25 ℃ for 0, 3, 6, 9, 12 and 24 hours.

**FIGURE 04**

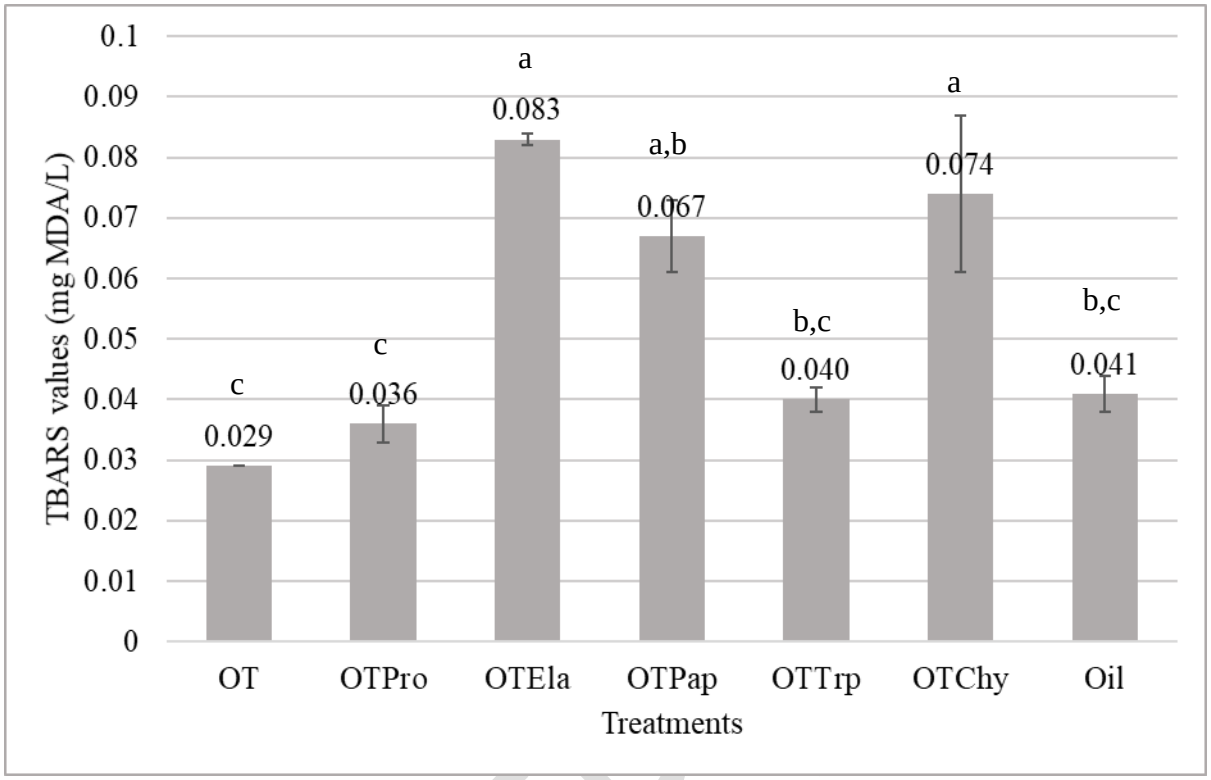


**Figure 04**. Graphical illustration of TBARS value of oil emulsion (mg of malondialdehyde/L) of the ovotransferrin hydrolysates; Values with different letters are significantly different ($P < 0.05$).

**FIGURE 05**

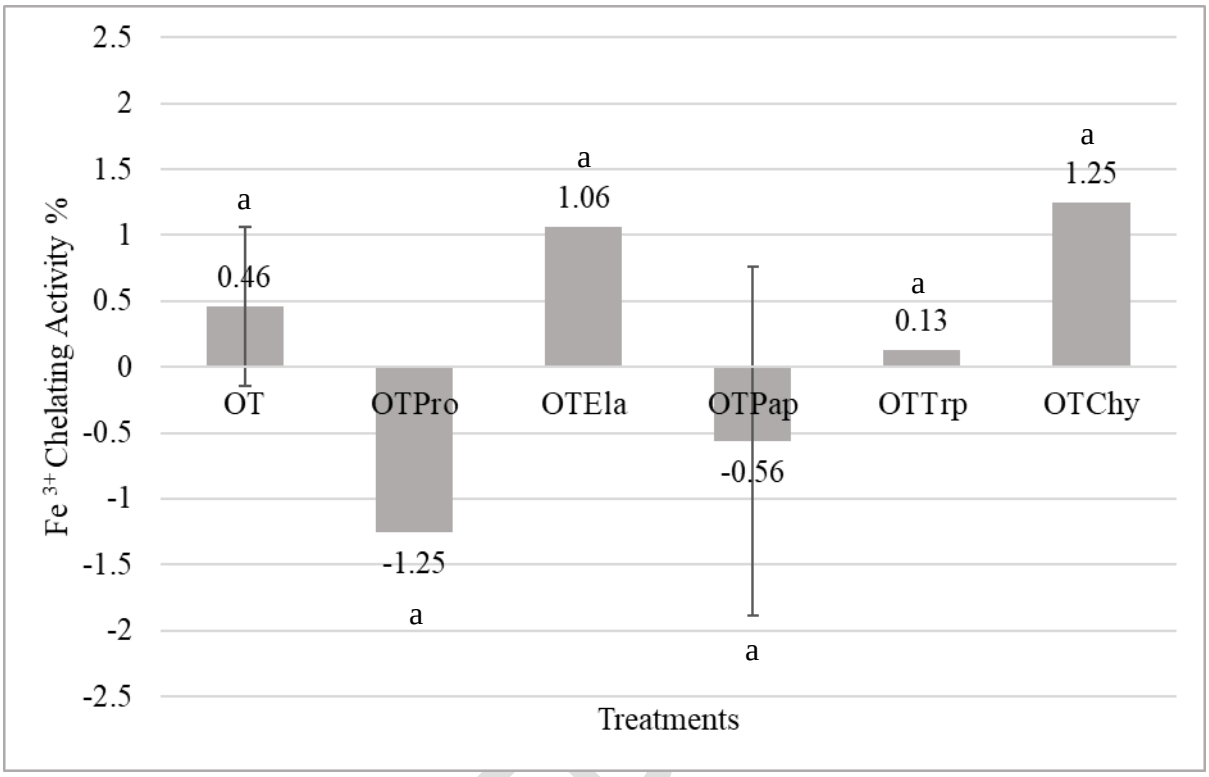


**Figure 05**. Graphical illustration of $Fe^{3+}$-chelating activity of the ovotransferrin hydrolysates; Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

718 **Figure 1**. The SDS-PAGE image of ovotransferrin hydrolyzed with protease; Lane 1: marker,
719 lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with protease 55 ℃ for
720 0, 3, 6, 9, 12 and 24 hours.
721 **Figure 02**. The SDS-PAGE image of ovotransferrin hydrolyzed with papain; Lane 1: marker,
722 lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with papain 37 ℃ for 0,
723 3, 6, 9, 12 and 24 hours.
724 **Figure 3**. The SDS-PAGE image of ovotransferrin hydrolyzed with elastase; Lane 1: marker,
725 lane 2: ovotransferrin (1 mg/ml), lanes 3-8: ovotransferrin hydrolyzed with elastase 25 ℃ for
726 0, 3, 6, 9, 12 and 24 hours.
727 **Figure 04**. Graphical illustration of TBARS value of oil emulsion (mg of malondialdehyde/L)
728 of the ovotransferrin hydrolysates; Values with different letters are significantly different ($P <$
729 0.05).
730 **Figure 05**. Graphical illustration of $Fe^{3+}$-chelating activity of the ovotransferrin hydrolysates;
731 Values are mean with standard error. Values with different letters are significantly different ($P$
732 $< 0.05$).
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