Chemical structure, comparison antioxidant capacity and separation antioxidant of hen, duck and quail egg white protein hydrolysate

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Abstract. Amino acid linkages as proteins are nutritional substance which important for diet intake. Purification protein processing undergo heating procedure process followed by additional of proteolytic enzymes or acid had been resulting in protein hydrolysates. A protein hydrolysate describe as many free amino acids bound together through a complex mixture of peptides. Egg white protein hydrolysates is one of subject interested to study for human health or industry product. The objectives of the research are to determine and identification the antioxidant derived from egg white hydrolysate protein. Identification of chemical structure of albumen and albumen protein hydrolysate was examine using IR Spectrophotometry. While comparison of antioxidant capacity and antioxidant separation egg albumen was also investigate using FTIR method (Fourier Transform Infrared Spectroscopy). Hen, duck and quail albumen egg white and on hydrolisate form were used as research materials. The results were showing that different time and enzyme of hydrolisate of albumen protein were not influence at secondary structure of hydrolysate albumen protein. Phytochemical content such as alcohol and hydroxyl compound which have potential as functional group of antioxidant were detected in all of the samples. Their results of radical scavenging activities samples hydrolyzed by pepsin were respectively 89.40%, 50.25% and 85.13%. Whereas the radical scavenging activities of hydrolysates hydrolyzed by papain were 72.85%, 61% and 76.45% respectively.

Keywords: Eggs white hydrolisates, FTIR, antioxidant

1. Introduction
Consuming eggs may help child’s brain intelligence. As promised, egg consumption for adults could give physical strength. Eggs are very good protein source to consume for kids and adults as well. Egg is one of the food supply of animal protein source which has the most complete nutritional content such as protein, vitamins, lipids and other substances.

Recently many findings on egg research expose on the functional substances of eggs beyond the basic nutritional. Advance study were done to identify and characterize those functional substances as well for example antimicrobial, anticancer, antithrombotic, hypocholesterolemic, mineral binding and antihypertensive. Many food products based on egg has high nutritive value. Besides its important reserve of highly digestible proteins, lipids, vitamins and minerals, the egg contains molecules with
numerous health promoting and biotechnological properties.

Oxidative damage is one of the causes of human disease. Therefore, consumption of foods containing antioxidants or consumption of antioxidant supplements is important to prevent oxidative damage in humans [1]. Exogenous chemicals or endogenous metabolic processes will result in free radicals and other reactive oxygen species (ROS) in food system or human body [2]. A lot of researches reported that cancer, cardiovascular disease, hypertension, neurodegenerative disorders and aging were associated with accumulation of peroxides (such as Alzheimer’s disease).

The peptides derived from pork proteins [3], milk casein [4], soy proteins [5] and egg-yolk proteins [6] have been demonstrated to exhibit in vitro antioxidant activity. Consumers recognize these products are safe and healthy for consumption. Bioactive peptides could be produced by enzymatic hydrolysis of food proteins. Bioactive peptides resulted in antioxidant activities to against the peroxidation of lipids or fatty acids. Recently, foods development not only considers its nutritional sources but also its bioactive compounds. Thus, the research of food possessing bioactive functions has drawn attentions in recent time. The objectives of the research are to determine and identification the antioxidant derived from egg white hydrolysate protein.

2. Material and Method

2.1. Preparation of egg white isolate.

At the temperature and controlled pH, the preparation of egg white hydrolysate was prepared. Egg white was separate from yolk egg. The dried egg white was dissolved in distilled water at 30 mg/ml concentration and heated at 90°C for 10 min, then hydrolyzed using Pepsin at pH 2.00, 45°C and papain at pH 6.0, 50°C separately. During hydrolysis, the pH was maintained in order to remain at optimal value with 1mol/L NaOH or HCl. Samples hydrolysate were taken at different time hydrolysis 2, 3, and 4h intervals and the samples were heated using boiling water for 10 minutes to inactivate the enzyme. Hydrolysates after heating treatment were centrifuged at different speed from 4,000-16000 g for 10 min on 4°C temperature and the supernatants were collected and stored until use for the analysis. After each egg white into hidrolysate. hidrolysate each protein will be used for analysis SDS Page, antioxidant activity and other analyzes are required.

SDS PAGE was performed using 5% acrylamide in stacking gel and separating gels is 12.5% acrylamide. The first gel was prepared is 12.5% acrylamide and allowed to polymerize before with 4% stacking gel. Sample must be heated 3-5 min on boiling water. The migration process of electrophoretic was performed at 80 V in the second gel (stacking gel) and change to 120 v in first gel (separating gel, in 25 mM Tris, 192 mM Glycine and 0.1% SDS, pH 8.3. Sample was load to gel about 5-10µg. As comparison use protein marker to determine molecular size protein sample (170 kDa). After running gel were stained with comassie brilliant blue R250 for about 30 minutes. Staining solution composed of 25% ethanol, 0.1% Comassie Brilliant Blue R-250 and 8% acetic acid. Destaining steps were done by soaking gel into solution (25% ethanol and 8% acetic acid) [7].
2.2. **Determination on antioxidant activity of hen, duck and quail egg white protein hydrolisate.**

To evaluate the antioxidant activity of those kinds of egg white protein was done by DPPH method was prepare 2 mL of sample HEPHPH diluted with 2 mL of DPPH solution (0.15 mM DPPH in ethanol 95%) on 15 mL Volumetric Flasks, after that the solution mixed with vortex and incubated at 37°C for 30 min at dark room. After color change to yellow the absorbance of solution formed was measured at 517 nm with a spectrophotometer. BHT will use as control and standard sample respectively. Antioxidant capacity result will be analyzed using ANOVA analysis with 95% confidence level using SPSS Version 16.

2.3. **Comparison antioxidant capacity of protein hydrolisate.**

By selecting the highest average antioxidants from each sample consisting of different types of eggs, different kind of enzyme and different hydrolysis time, aimed to compare the antioxidant capacity of protein hydrolysate. Each sample will be selected, that have the highest antioxidant capacity and each other of samples will be compared.

2.4. **The identification of the chemical structure of the protein hydrolisate using Fouries Transform Infra Red Spectroscopy (FTIR).**

FTIR were used for analysis the chemical structures of different hydrolysates. Infrared spectrophotometer (Model–Nicolet Nexus-470, Thermo Nicolet Corporations, Madison, WI, USA) was used for the measurements in a transmission mode using a demountable thin layer IR-cell, with KBr windows in the frequency region 400–4000 cm⁻¹. Samples were placed at sample compartment with automatic accessory 46 recognition at the scanning speed of 2.8 mm/s. Before that sample was mix with KBr. Each spectrum was recorded in resolution of 4cm⁻¹.

2.5. **Separation antioxidant hydrolysates by ion exchange chromatography.**

Based on antioxidant activity results, the HEPHPH exhibited the highest antioxidant activity, and thus were employed for separation of antioxidant hydrolysate. The protein hydrolysate applied to a DEAE Toyopearl column (1.5×60cm) which was equilibrated with 0.1 mol/L Tris-HCl, pH 8.00.
The same buffer was used for washed out the unbound proteins. The absorb proteins were eluted stepwise with 0–30 mol/L NaCl in the Tris-HCl buffer. Fractions of 4mL were collected and the UV absorption was monitored at 280nm. Antioxidant activity was determined by each fraction. All peaks were collected analyze antioxidant activity by DPPH.

3. Result and Discussions

3.1. Preparation of egg white isolate.
The highest antioxidant activity was observed in the HEWPH 2H, HEWPH 3H and DEWPH at 3H hydrolysis, which presented 89.44%, 88.22% and 85.13%. DPPH scavenging activity of hydrolysate protein shows difference significant (P<0.05). High amount of peptide hydrophobicity or hydrophobic amino acids was associated with the protein hydrolysates with high level of DPPH free radical scavenging activity [8]. Therefore, the 3 kind of treatment was selected for further study. Because the protein hydrolysates have smaller molecular size that can be more beneficial for human health, so the protein hydrolysates are separated from the original protein generally.

![Figure 2. SDS PAGE analysis of HEPE2H, HEPE3H and DEWPH compare with kind of egg hydrolysate](image)

Figure above tell about comparative between kinds of egg as control with sample have high antioxidant activity from all treatment. The result SDS PAGE show two major of egg white protein ovalbumin and ovotransferrin at the 45 kDa and 75 kDa respectively for three kind of egg. Ovotransferrin is an egg white glycoprotein reported has antioxidative properties [9]. The high vicinity in that figure is less than 100 kDa and the lowest vicinity from sample without treatment is 10 kDa that protein with 10 kDa is lysozyme. Different sample show same of vicinity, all samples just show 10 kDa vicinity. The protein is lysozyme. That’s mean every treatment influence to protein content. This may cause by heating processing that can damage some protein in egg. But there also tell about lysozyme have antioxidant functionality. Another experiment also report about another function of lysozyme. Lysozyme also can be antimicrobial source. In addition, antioxidant activity was influenced by molecular weight of protein. In the previous research, the antioxidant activity of corn gluten meal hydrolysates was found related to molecular weight of hydrolysates and its concentration. MW 500–1500 Da peptides antioxidant activity was showed stronger than peptides above 1500 Da and peptides below 500 Da [10]. However, the overall antioxidative activity has been postulated that it has to be ascribed to the integrative effects of these actions rather than to the individual actions of peptides.

From the result it can be implied that the antioxidant activity was mainly determined influenced by the hydrolysates or peptides with low molecular wight, obtained from WPI [11], [12] were doing a
research that characterised the peptides which have antioxidant effect derived from the hydrolysates of free-lecithin egg yolk and found that the molecular weight of proteins or peptides determined their efficacy in antioxidant activity, the strongest antioxidant activity was showed by 5 kDa permeate. The DPPH radical scavenging activity of the hydrolysed WPI increased probably due to the change of protein structure, leading to radical quenching ability that become greater.

3.2. **Determination on antioxidant activity of hen, duck and quail egg white protein hydrolisate.**

The hydrolysates with the highest hydrolysis time and high speed produced by pepsin had higher DPPH radical scavenging activity at 100 mg/ml. This indicates that the interaction time, enzyme and speed can strongly influence the antioxidant properties of the sample. The use of different types of enzymes and different time hydrolysis show significant effect on the antioxidant capacity (P<0.05) for all sample HEWPH. The pepsin enzyme presented an effective effect in DPPH radical scavenging activity, higher than the papain enzyme. The Highest one on HEWPH is 89.40% on HEWPH 2H hydrolysis by pepsin.

The highest antioxidant activity is DEWPH 3H by pepsin. DEWPH 3H by pepsin have 85.13% antioxidant capacity. On this study was shown that DEWPH available to inhibition 50% of DPPH radical scavenging higher than BHT. Egg white as antioxidant source has a great potential for consumers request to provide additive-free, fresher and more natural food. All treatment and interaction on DEWPH sample show significant difference (P<0.05). It is possible to obtain a natural antioxidant source from Egg product. The pepsin enzyme presented an effective effect in DPPH radical scavenging activity, higher than the enzymes papain and BHT as synthetics source antioxidant.

The hydrolysates with the highest hydrolysis time and high speed produced with pepsin had higher DPPH radical scavenging at 100 mg/mL but not high than BHT. The highest on QEWPH is hydrolysate QEWPH 4H by papain 61.01%. This indicates that the time and speed can influence the antioxidant properties of the sample. The pepsin enzyme again presented an effective effect in the DPPH radical scavenging activity, larger than the enzymes papain. Different time hydrolysis, interaction treatment enzyme and hydrolysis, interaction of enzyme type and speed, interaction time and speed hydrolysis and interaction third treatment showed a significant difference (P<0.05).

3.3. **Comparison antioxidant capacity of protein hydrolisate.**

From all sample the protein hydrolysate that have antioxidant activity is hydrolysate hydrolysis by pepsin. Protein hydrolysate from hen egg (HEWPH) is the highest one. It is able inhibit scavenging radical 89.40%. Egg white proteins are widely used as functional and nutritional ingredients in food products and their hydrolysates obtained by protease treatment are water soluble and wave high nutritional value. The efficient way to recover potent bioactive peptides is by enzymatic hydrolyzing of food proteins, including antioxidant activity.

3.4. **The identification of the chemical structure of the protein hydrolysate using Fouries Transform Infra Red Spectrocopy (FTIR).**

3.4.1. **FTIR of Egg White Protein.** All spectra were baseline corrected after background correction. Figure 1 shows the result FTIR spectra for hydrolysate without treatment enzyme hydrolysis, different time hydrolysis and centrifuge speed. FTIR analysis sample without sample will be positive standard to hydrolysate hydrolysis by enzyme.
Figure 3. FTIR analysis of hen egg white protein

Figure 4. FTIR analysis of duck egg white protein

Figure 5. FTIR analysis of quail egg white protein

Figure 5 explain the range of FTIR analysis from different kind of hydrolysates. From figure 5 HEWPH range is $1396 \text{ cm}^{-1}$ to $3299 \text{ cm}^{-1}$, DEWPH $1237 \text{ cm}^{-1}$ to $3302 \text{ cm}^{-1}$ and QEWPH range $1384 \text{ cm}^{-1}$ to $3304 \text{ cm}^{-1}$. We know that FTIR can explain chemical molecule structure in sample and chemical bonds in a molecule. Figure 5 was compared with sample which contain high antioxidant.

3.4.2. FTIR of HEWPH and DEWPH. The Fourier Transform Infrared (FTIR) spectroscopy is a valuable tool for characterization and identification compounds or functional groups (chemical
bonds). The spectroscopic analysis of polymeric molecules, including proteins, is complex due to the molecular vibrations arising from numerous atoms [13]. We can judge the origin of different extracts accurately and effectively, trace the constituents in the extracts, identify the medicinal materials and evaluate the qualities of medicinal materials by using the microscopes fingerprint characters of FT-IR spectrum. FTIR spectroscopy is an established timesaving method to characterize and identify functional groups [14]. The data concluded that most common compounds in the hydrolysates are alcohol and hydroxyl compound, aliphatic and aromatic nitro compounds. The presence of the above mentioned functional groups might attributed to the phytochemical composition and antioxidant activity of the hydrolysates. The results are confirmed with Infrared Spectra analysis interpretation, which seems to be a practical approach. Alcohol and hydroxyl compound are chemical bonds that have potential as functional group of antioxidant.

3.5. **Separation antioxidant hydrolysates by ion exchange chromatography.**

Ion Exchange chromatography of the protein hydrolysate was performed subsequently. The result is two kind of HEWPH sample have III peak (peak I, peak II and peak III)

Every peak was separated and the antioxidant capacity was tested by DPPH. Antioxidant capacity peak of HEWPH is 58.50% 60.56% and 54.97% respectively.

![Figure 6. Antioxidant capacity and peak of HEWPH separation by ion exchange chromatography](image)

![Figure 7. Antioxidant capacity and peak of DEWPH separation by ion exchange chromatography](image)

Dissimilar with HEWPH sample, after separation, DEWPH have 4 peaks. Peak I and II bound to each other and peak III was the more larger one than others. The capacities of antioxidant that came from DEWPH was lower than HEWPH. DEWPH property of antioxidant capacity only have 32.23% on peak III and the another peak was the lowest. The antioxidant capacity of peak I, II and IV respectively is 24.87%, 22.66% and 19.28%. Antioxidant capacity of sample will be affected by different time hydrolysis of hydrolysate. Protease enzyme will affects the antioxidant activity, beside...
of time hydrolysis enzyme and also affects amount, size, composition of free amino acid and peptides and their amino acid sequence which in turn influences the antioxidant activity of the hydrolysates [5,15, 16]. In their research, [17] reported that different type of enzymes will produce hydrolysates from native and heated soy protein isolates. They reported that by using different type of enzymes generated in the formation of a mixture of peptides with different degrees of hydrolysis and accordingly different ranges of antioxidant activity.

Egg white proteins are found in food products and usually used as ingredients because it’s functional nutrition and their hydrolysates that obtained by protease treatment are water soluble and have high nutritional value [18]. Some protein hydrolysates, such as egg white [19] potato (Solanum tuberosum) [4], fish muscle [20], and tuna liver [21] have been reported to take control of both ACE inhibitory capacities and antioxidant activity.

4. Conclusion
After separation of the antioxidant capacity from its hydrolysate showed different antioxidant activity and different peak. HEWPH showed unbound peak between three of peak while for DEWPH showed that two peak are bounded and one peak is unbounded. HEWPH remains the highest antioxidant capacity. HEWPH was able to inhibit free radical scavenge as 60.56% and the highest on DEWPH only 32.23%.

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