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Emulsifying and Lipid Peroxidation Inhibitory Activities of Chicken Head Protein Hydrolysate Using a Combination of Papain and Bromelain Enzymes

Moch. Geerhan Miraja Syahdan [a], Pramudya Andiana [a] and Lilik Eka Radiati [a*]

[a] Department of Animal Product Technology, Faculty of Animal Science, Universitas Brawijaya, JL. Veteran, Malang, East Java 65145, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Protein hydrolysis is a method used to produce protein hydrolysates containing simple peptides and free amino acids. The chicken head is one of the by-products that contain a high protein concentration so it can be use as a raw material for protein hydrolysate with functional properties and as a lipid peroxidation inhibitor. The aim of this research was determination to determine the emulsifying and lipid peroxidation inhibitory properties in chicken head protein hydrolysate using a combination of papain and bromelain enzymes. The method used in this research was a laboratory experiment using a completely randomized design (CRD) consisting of 4 treatments and 5 replications, consisting of T1 (without hydrolysis), T2 (75% papain enzyme and 25% bromelain

**Corresponding author: Email: lilik.eka@ub.ac.id;*

enzyme), T3 (papain enzyme 50% and bromelain enzyme 50%), and T4 (papain enzyme 25% and bromelain enzyme 75%). The difference in the concentration ratio of papain enzyme and bromelain enzyme in chicken head protein hydrolysate gave a very significant difference (P<0.01) in Electrical conductivity (EC), a significant difference (P<0,05) in lipid peroxidation inhibitory activity and emulsion activity, and no significant difference (P>0,05) in emulsion stability. Chicken head protein hydrolysate shows potential as an emulsifier and lipid peroxidation inhibitor.

Keywords: Chicken head; hydrolysate protein; functional properties; lipid peroxidation inhibition.

1. INTRODUCTION

Emulsion colloid systems have been found in many foodstuffs, cosmetics, and medicines. Oil-water emulsion systems can undergo lipid oxidation reactions. There is evidence that states that emulsion systems undergo oxidation more easily [1]. Lipid oxidation in food is the most crucial thing from a commercial point of view. This is because most oils are not consumed in bulk but dispersed into food, such as processed milk, meat, and sauces [2]. Lipid oxidation usually greatly influences a product's shelf life. However, this can be prevented with antioxidants, so that rancidity due to oxidation can be inhibited [3]. Emulsifier are substances that help to maintain stability and identify two opposing emulsion components, such as oil and water or water and oil. Emulsifier usually stabilize food products because they have functional properties, including foaming power, emulsion stability, emulsion activity, water-holding capacity, oil-holding capacity, and gel formation. After all, they can form fat and air globules [4]. Proteins originating from plants and animals can usually be used as food additives because they can be used as emulsifiers and stabilizers, which are effective in forming, stabilizing, and providing physicochemical properties to emulsifier systems in the food industry [5].

Protein is an essential nutrient in developing the body. Apart from well-established sources of protein, such as meat, poultry, and fish, and can even be obtained from plants, many sources of protein are still unexplored, one of which is the unutilized protein that comes from animal by-products, both non-ruminant and ruminant [6]. The results of slaughtering chickens produce several products besides carcasses, namely by-products from the chicken itself, including feathers, feet, skin, innards, chicken heads, and many more. Apart from that, the weight proportion of chicken heads is only 2.5 – 3.0% [7]. [8] reported that poultry slaughterhouses produce slaughter waste from chickens, which continues to increase every year. On the other hand, poultry by-products have not been utilized, which contain different amounts of protein. Utilization of by-products from poultry can increase the selling value of meat production and as an added value to maximize profits.

The use of chicken heads in Indonesia, limited to be used to humans food, pets, and animal feed, as well as fertilizer or thrown away without processing. Awwaly et al. [9] reported that the protein contained in chicken heads is around 12.29%, while Du et al. [10] reported that chicken head protein contains around 10.58%. It shows that the protein in chicken heads can be used as raw materials for making protein hydrolysate that has bioactive compounds and functional properties.

Protein hydrolysis is capable of producing polypeptide chains through a cutting process into amino acids and peptides with low molecular weight so that they can dissolve in water. Siddik et al. [11] stated that protein hydrolysis can be done using chemical methods (acids and alkaline), bacterial fermentation, and enzymatic hydrolysis using protease enzymes. Andiana et al. [12] stated that the chemical hydrolysis process (acid and base) can create extreme pH conditions. The use of acid and alkaline methods in hydrolysis is considered less effective because the performance of acids and bases is not specific. The hydrolysis process using enzymes has the advantage because the hydrolysis process is particular and enzymatic hydrolysis uses a pH that is not too extreme when hydrolysis takes place. The disadvantage of the enzymatic hydrolysis is that the price of the enzyme is relatively expensive. Protease enzymes can come from animals, plants, and microbes. Some examples are the protease enzymes alcalase, bromelain, papain, flavoryzme, and durazym [13,14].

Protease enzymes in hydrolyzing proteins have more specific cleavage sites. Therefore, hydrolyses proteins combining more than one enzyme can produce antioxidant compounds and

exert functional properties, compared to using a single enzyme to produce peptides with certain bioactivity [15,16]. Turtle hydrolysate using the papain enzyme can produce high emulsion activity [17]. While, using the bromelain enzyme in the protein hydrolysate can inhibit fat oxidation in the sesame seed protein hydrolysate [18].

The related use of papain enzymes and bromelain enzymes to produce protein hydrolysates that have functional properties and inhibit lipid peroxidation. This research uses a combination of papain enzymes and bromelain enzymes to produce chicken head protein hydrolysate, which contains functional properties that can be used as an alternative food additive and inhibits lipid peroxidation.

This research aims to produce chicken head protein hydrolysate using a combination of papain enzymes and bromelain enzymes, which have functional properties and inhibit lipid peroxidation.

2. MATERIALS AND METHODS

2.1 Materials

The material used in this research were chicken heads, HCl (Merck), NaOH (Makmur Sejati), papain (Hunan Insen Biotech), Bromelain (Shaanxi Rainwood Biotech), $FeCl_2.2H_2O$ (Pudak), linoleate acid (Sigma), olive oil, SDS, EtOH, and ammonium thiocyanate (MERCK). Tools used in the research were waterbath shaker (Jisico), centrifuge (corona 80-2), Spectrophotometer- Vis (Faithful 721/722), pH meterr (Hanna), and TDS-EC.

2.2 Methods

The method used in this research completely randomized design (CRD) with 4 treatments and 5. In this study, the different ratios of the combination of papain and bromelain enzymes were used to treat the hydrolysis process of chicken head protein. The research design can be seen as follows T1: Without Hydrolysis; T2: 75% papain + 25% bromelain; T3 50% papain + 50% Bromelain; T4 25% papain + 75% bromelain.

2.3 Preparation of Chicken Head Protein Concentrate

The fresh chicken head were cut into three parts, the beak removed, and pounded with a meat mallet. After that, they were dried for 6 hours in an oven at 40°C. Chicken head meat is mixed with distilled water (10% w/v). The initial pH of the homogenized sample was changed to pH 12 with 10 M NaOH, and stirring was carried out for 1 hour with a magnetic stirrer. After the mixture was taken, the mixture was put into a centrifuge tube and centrifuged for 15 minutes at a speed of 4,000 rpm. Then, using 1 M HCl, the supernatant's pH was adjusted to pH 4 and then centrifuged for 15 minutes at a speed of 5,000 rpm. The pellet was stored at -20°C overnight and then dried using a microwave dryer on low mode (± 39°C) for 5 minutes [9].

2.4 Preparation of Chicken Head Protein Hydrolysate

The hydrolysis procedure was carried out at an ideal pH of 7 and a temperature of 55°C for papain and bromelain. The amount of each enzyme was modified to suit the percentage in each treatment, and the ratio of the two enzymes to chicken head protein concentrate was 1:100 (w/w). Chicken head protein concentrate is combined with distilled water at a ratio of 2% (w/v) for the bromelain hydrolysis stage. The pH was then adjusted to 7 using 2 M NaOH, and the mixture was first incubated at 55°C for 20 minutes. According to the protocol, bromelain enzyme was added to the mixture and incubated under ideal conditions for 3 hours. The incubation process was stopped at 85°C for 10 minutes by heating the bromelain. After that, the mixture was adjusted to pH 7 using 2 M NaOH, and pre-hydrolysis was carried out for 20 minutes at 55°C, after adding the papain enzyme and incubating under ideal conditions for 3 hours. The incubation process was stopped at 85°C for 10 minutes. The hydrolysate solution was cooled to room temperature and centrifuged at 4,000 rpm for 15 minutes. The supernatant was collected and stored at −20°C [19].

2.5 Electrical Conductivity

The electrical conductivity of hydrolysate protein chicken head was measured using the method described by Yao et al. [20] with modifications. Hydrolysate protein chicken head taken 20 ml sample and put in beaker glass 50 ml. The electrical conductivity of the sample was determined using a TDS/EC meter.

2.6 Emulsifying Properties

Emulsion activity index (EAI) and Emulsion stabilization index were determine as describe by

Selmane et al. [21], Xue et al. [6], respectively. Olive oil 4 ml and sampel hydrolysate 1 ml were homogenizer using hand mixer for 1 min. Emulsion were pipetted out 0 and 10 min 100µL and add 14 ml SDS 0.1% into the test tube. The mixture was mixed thoroughly for 10 s using vortex mixer. The resulting dispersion was measured using a spectrophotometer 500 nm. EAI and ESI and EAI were calculated by the following formula :

$$EAI = 2.33 \times A_0$$

$$ESI (\%) = A_{10}/A_0 \times 100$$

Where

$A_0 = A_{500}$ at time of 0 minutes,
$A_{10} = A_{500}$ at time of 10 minute

2.7 Lipid Peroxidation Inhibition Assay

The lipid peroxidation inhibitory activity assay procedure described by Estave et al. [22]. A mixture of 2 mL sample, 2 mL linoleic acid in EtOH solution (0,13% (v/v)), and 1 mL water was combined. Ferric thiocyanate absorbance was used to measure the mixture's degree of oxidation at various points during its six days of incubation at 40°C in the dark. The absorbance was measured at 500 nm. Absorbance was measured after mixing 100 µL of the reaction solution with 7 mL of 75% (v/v) EtOH and 100 µL of 30% (w/v) ammonium thiocyanate. Blank was prepared using distilled water instead of sampel. Lipid peroxidation inhibition activity was calculated by using the following formula:

$$\text{Lipid peroxidation inhibition activity (\%)} = \left[1 - \frac{(Abs\ sampel\ 144\ h - Abs\ sampel\ 0\ h)}{Abs\ blank\ 144\ h - Abs\ blank\ 0\ h}\right] \times 100$$

Where Abs sample 144 h is the absorbance of sample after 144 h incubation; Abs sample 0 h is the absorbance of sample at 0 h; Abs blank 144 h is the absorbance of blank after 144 h incubation; and Abs blank 0 h is the absorbance of blank at 0 h.

2.8 Statistical Analysis

The data were presented as mean ± standard deviation. The data collected were analyzed using one-way analysis of variance (ANOVA) with significance of (P<0.01) and (P<0.05). Duncan multiple range test (DMRT) was used to compare means [23]. All analyses were carried out using the Microsoft Excel software.

3. RESULTS AND DISCUSSION

3.1 Electrical Conductivity

The analysis of variance showed that the treatment using different combinations of papain and bromelain in chicken head hydrolysate protein gave a highly significant difference (P<0.01) in electrical conductivity. The data in Table 1 showed that the electrical conductivity of T2 and T1 had the highest and the lowest electrical conductivity, respectively. High and low electrical conductivity in chicken head protein hydrolysate is caused by differences in protein molecular weight. Low protein molecular weight will increase the protein solubility. This is because protein have an open sulfide bond structure, creating electrostatic repulsion and high solubility [24]. The combination of bromelain and papain enzymes in chicken head protein hydrolysis increased significantly electrical conductivity compared to chicken head protein without hydrolysis.

Tabel 1. Electrical conductivity of chicken head protein hydrolysate

Treatment*	Electrical Conductivity (µs/cm)
T1	1322.8±19.32[a]
T2	1609.6±109.96[b]
T3	1582.8±63.10[b]
T4	1366.4±76.88[a]

All values are mean ± SD. Values with different letters are significantly different (P<0.01) according to Duncan's multiple range test

The decrease in electrical conductivity is caused by the release of carboxylic groups during the hydrolysis process. The hydrolysis process uses an enzymatic method will cleave the peptide bond, which affects the released carboxylic group and will release several hydrogen ions [24,25]. Electrical conductivity in protein hydrolysis is influenced by two factors, namely ion concentration and molecular size. The higher the ion concentration in the protein hydrolysate, the higher the electrical conductivity. Using bromelain and papain enzyme on chicken heads will open the protein structure during the hydrolysis process, potentially releasing calcium ions. In samples that received enzymatic treatment, this release will increase the conductivity value. Chelation between specific cations and the product's anionic groups is the reason for the drop in electrical conductivity level. Ionic interactions between these groups in

opposite charges result in the chelation process [26,27].

Nissen et al. [24] reported that chicken breast marinated using NaCl showed a more excellent electrical conductivity value with an electrical conductivity value of 16.49 – 85.03 µs/cm. Cho et al. [28], Akabari et al. [29] stated that the increase in temperature will increase the electrical conductivity. It's due to the heating process causes the mobility of active ions. Increasing electrical conductivity means that ions contained in it will increase with high temperatures, then hydronium and hydroxide ions will increase and will break peptide bonds in dissolved proteins and will be degraded into carboxylic acids, then increasing temperature will affect amino acids [26]. An increase in the electrical conductivity value causes an increase in amino acid yield, generally due to an increase in temperature. The papain and bromelain enzymes can transfer the matrix into cells, accelerating protein degradation and producing much soluble protein [29]. The higher the electrical conductivity value, the higher the ions in the liquid [30]. Protein hydrolysate will enzymatically cut peptide bonds based on the ability of the enzyme so that components in cells are more accessible to dissolve [31].

3.2 Emulsifying Properties

The analysis of variance showed that the treatment using different combinations of bromelain and papain in chicken head hydrolysate protein didn't give a significant difference (P>0.05) in emulsion stability and gave a significant difference (P<0.05) in emulsion activity. The emulsion stability and emulsion activity results can be seen in Table 2.

Table 2. Emulsifying Properties of Chicken Head Protein Hydrolysate

Treatment*	Emulsion stability (%)	Emulsion activity (mg/m^2)
T1	89.99±4.92	0,90±0,42^{a}
T2	83.15±4.06	1,61±0,39^{b}
T3	84.82±3.67	1,63±0,54^{b}
T4	83.27±10.11	1,28±0,33^{ab}

All values are mean ± SD. Values with different letters are significantly different (P<0.05) according to Duncan's multiple range test

The highest average value of emulsion stability was found in T1 with a value of 89.99%. Meanwhile, the lowest average of hydrolysate protein was found in T2 with a value of 83.15%. While the highest average value of emulsion activity was found in T3 with a value of 1.63 mg/m^2 and the lowest average value was found in T1 with a value of 0.90 mg/m^2. Protein-stabilized emulsions are usually formed by homogenizing the oil and water phases. This research uses a mechanical device using a homogenizer, which aims to break up and mix the oil and water phases and reduce the size of the oil droplets. Proteins are usually dissolved or dispersed in a water phase beforehand to homogenize because their outer surface is partly hydrophilic and needs some hydrophobic groups. Turtle grass protein hydrolysate using the papain enzyme had an emulsion stability of 72-90%. this is because the papain enzyme has a relationship with hydrophobic amino acids such as isoleucine, leucine, valine, alanine, and glycine [17]. The by-products of chicken that contain skin can have an impact on the amino acids generated because collagen contains hydroxyproline. This amino acid is created when protein hydrolyzation results in hydrophobic peptides [32].

The increase in hydrophobicity in chicken protein hydrolysate is cause by an increase in hydrophobic amino acids such as tryptophan, phenylalanine, and tyrosine [33]. The droplet surface is mostly an emulsion of oil and air. It is hydrophilic because it is coated with an amphiphilic emulsifier, which orients the non-polar groups connecting the oil phase. In contrast, the polar groups contact the oil and air phases. This applies to emulsions stabilized by surfactants with hydrophobic properties that differ from hydrophilic properties [34]. The emulsion activity index (EAI) and emulsion stability index (ESI) are widely used as metrics for assessing emulsifying properties of hydrolysate performance for stabilizing oil in water emulsions because of their high concentration of active amino acid. The functional properties of protein hydrolysates depend on several factors, including the protein source, type of proteolytic enzyme used, temperature, pH, and length of hydrolysis time. [35]. According to [36] the emulsion is influenced by the amino acid components that make up the protein. A balanced ratio of hydrophilic-lipophilic amino acids will determine the ability of the protein to form an emulsion. Non-polar amino acids can affect emulsions. Besides that, the properties of emulsions are influenced by the nature of globular proteins, which easily interact with water.

3.3 Lipid Peroxidation Inhibition

The analysis of variance showed that the treatment using different combinations of bromelain and papain in chicken head protein hydrolysate gave a significant difference (P<0.05) in lipid peroxidation inhibition. The lipid peroxidation inhibition of chicken head protein hyrolysate results can be seen in Table 3.

Table 3. Lipid Peroxidation Inhibition of Chicken Head Hydrolysate Protein

Treatment*	Lipid Peroxidation Inhibition (%)
T1	64,49±8,65 ^{ab}
T2	55,24±10,20^{ab}
T3	47,08±6,23^{a}
T4	66,25±10.79^{b}

All values are mean ± SD. Values with different letters are significantly different (P<0.05) according to Duncan's multiple range test

The highest average of lipid peroxidation inhibition activity was in T4 with a value of 66.25% and the lowest of lipid peroxidation inhibition activity was in T3 with a value of 47.08%. The oxidation process is the most important thing in foods rich in lipids. Fat oxidation can shorten the product's shelf life and cause an unpleasant odor. This phenomenon is known as rancidity [37]. Oxidation is a complex process involving the formation and spread of free radicals that will bind lipid electrons in cell membranes and directly result in cell damage. Josef et al. [38] stated that during long-term storage long time,quality and freshness of fish will decrease. Fatty acid content saturation in the body of skipjack tuna is more dominated by unsaturated fatty acids compound (PUFA) so fish oil is susceptible suffer from oxidative rancidity

Sonklin et al. [39] reported that in the lipid peroxidation inhibition assay, the sample will react with the ferrous ion (Fe^{2+}) formed in linoleic acid so that when the sample encounters $Fe^{2+,}$, it will react and become a ferric ion (Fe^{3+}) and form a brownish color. Antioxidants are agents that can suppress lipid peroxidation. Particularly, the long-term safety and anti-synthetic antioxidant properties of naturally occurring food-based antioxidants have recently been discovered since people need to live high-quality lives [40].

Physical interactions can influence the antioxidant activity of peptides in emulsion systems. The partitioning of peptides to the water–oil interface allows them to form a barrier that prevents free radicals from interacting with lipids. The influence of hydrophobic amino acids such as methionine, alanine, lysine, leucine, tyrosine, valine, and proline substantially affect antioxidant peptides. Inhibition of lipid peroxidation will prevent oxidized lipids [41]. Antioxidant properties from protein hydrolysate can prevent oxidation of unsaturated fats [16]. Protein hydrolysate can be antioxidants because they contain peptides that can donate hydrogen so that they can ward off free radicals [42,43].

4. CONCLUSION

Differences in the percentage combination of papain and bromelain can affect electrical conductivity, emulsion activity, and inhibition of lipid peroxidation, but it does not affect the emulsion stability of the chicken head protein hydrolysate. The highest the Emulsion activity and emulsion stability is in T1 and T3 with values of 89.99% and 1.63 mg/m^2, respectively. While the highest electrical conductivity value is in T2 with value of 1609.6 µm/cm.T4 show a highest lipid peroxidation inhibitory activity with the value of 66.25%. Thus, chicken head protein hydrolysates using a combination of papain and bromelain enzymes has potential as an emulsifier and inhibitor of lipid peroxidation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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