Original Research Article

EMULSIFYING AND LIPID PEROXIDATION INHIBITORY ACTIVITY OF CHICKEN HEAD PROTEIN HYDROLYSATE USING A COMBINATION OF PAPAIN AND BROMELAIN ENZYMES

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 that it can be used as raw material for protein hydrolysate with functional properties and as a lipid peroxidation inhibitor agent. The aim of this research was determination to determine the emulsifyingand 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 five replications, consisting of T1 (without hydrolysis), T2 (75% papain enzyme and 25% bromelain enzyme), T3 (papain enzyme 50% and bromelain enzyme 50%), and T4 (papain enzyme 25% and bromelain enzyme 75%). The data obtained were analyzed using analysis of variance (ANOVA), continue by Duncan's multiple range test (DMRT). The difference in the concentration ratio of papain enzyme and bromelain enzyme in chicken head protein hydrolysate showed 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 showed no significant difference (P>0,05) in emulsion stability. Chicken head protein hydrolysate shows potential as an emulsifier and lipid peroxidation inhibitor agent.

Keywords: Chicken Head, hydrolysate protein, functional properties, inhibition lipid peroxidation

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 (Fatimah, 2008) [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; antioxidants can prevent oxidation rancidity through many mechanisms [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]. Zinina et al. (2021) [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 being used for food for humans, pets, and animal feed, as well as fertilizer or thrown away without processing. Al Awwaly et al. (2020) [9] reported that the protein contained in chicken heads is around 12.29%, while Du et al. (2013) [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 hydrolysate from chicken heads 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. (2020) [11] stated that hydrolysing protein could be done using chemical methods (acids) and alkaline), bacterial fermentation, and enzymatic hydrolysis using protease enzymes. Andiana et al. (2023) [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 when performing hydrolysis 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 hydrolysis process 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]. In contrast, 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. MATERIAL 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, andammonium 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 four treatments and five. 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.*(2011) [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 weredetermine as describe by Selmane, *et al*(2008) [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 andadd 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

2.7Lipid Peroxidation Inhibition Assay

The peroxidation lipid inhibition and describe Estave et al (2015) [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 measure at For that reason, 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 capacity was calculated by using the following formula :

$$\text{Peroxidation lipid inhibition (\%)} = \left[1 - \frac{(Abs\ sample\ \ 144\ h - Abs\ sample\ \ 0h)}{(Abs\ blank\ \ 144h - abs\ blank\ \ 0h)}\right] \times 100$$

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 collected were analyzed using one-way analysis of variance (ANOVA) with significance of (P<0.01) and (P<0.05). The data were given as mean ± standard deviation. Duncan's multiple range test (DMRT) was used to compare means. Perform all analyses 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 enzyme papain and bromelain in chicken head hydrolysate protein gave a highly significant difference (P<0.01) in electrical conductivity. T2 and T1 showed the highest and the lowest electrical conductivity. High and low electrical conductivity in chicken head protein hydrolysate, when Low protein size will result in higher protein solubility; this is because protein have an open sulfide bond structure, creating electrostatic repulsion and high solubility [23]. The first time that the addition of a combination of enzymes to chicken head protein hydrolysate caused a significant increase in electrical conductivity compared to chicken head protein without hydrolysis.The result of the electrical conductivity of all hydrolysates can be seen in Table 1.

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]

The data is presented as mean ± SD. Duncan's multiple range test showed a high significant(P<0.01) in the mean values of the same column containing distinct letters.

The decrease in electrical conductivity is caused by the release of carboxylic groups during the incubation process. The hydrolysis process uses an enzymatic method; the protease enzyme will carry out the process of cutting the peptide bond, which affects the released carboxylic group, which will release several hydrogen ions [23]. 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 papain and bromelain 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 [37].

Kaewthong and Wattanachant (2018) [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. Cevik (2021) and Cho and Shang-Hoong (2021) [25,26] stated in research conducted that when the temperature used increases, the electrical conductivity value will increase due to the heating process due to the mobility of active ions due to a decrease in viscosity and loss of bubbles.Increasing electrical conductivity means the 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 [37].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 [27]. States that the higher the electrical conductivity value, the higher the ions in the liquid [28]. Protein hydrolysate will enzymatically cut peptide bonds based on the ability of the enzyme so that components in cells are more accessible to dissolve [29].

3.2 EmulsifyingProperties

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

Table 2. Emulsion Stability Chicken Head Hydrolysate Protein

Treatment*	Emulsion stability (%)	Emulsion activity
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]

The data is presented as mean ± SD. Duncan's multiple range test showed a significant(P<0.05) in the mean values of the same column containing distinct letters and no significant different (P>0.05).

The highest average value of emulsion stability was found in T1 without hydrolysis, with an enzyme value of 89.99%. Meanwhile, the lowest average of hydrolysate protein was found in T2 83.15%, while the combination enzyme in chicken heads hydrolysate protein and the highest average value of emulsion activity was found in T3 1.63 and the lowest average hyrolysate protein was found in T1 0.90. 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. Protein hydrolysate turtle grass with 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]. Proteins that are hydrolyzed from soybeans, casein, bonito, and chicken using the enzymes pepsin, trypsin, and α-chymotrypsin or using the enzyme bromelain. The results of the hydrolysate using the enzyme bromelain can isolate eleven peptides, including Glu Glu, Glu-Val, Ala-Asp-Glu, Ala-Glo-Asp, Asp-Glu, and Ser-Pro, which can increase the umami taste [30].The

by-product of chicken heads 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 [41]

The increase in hydrophobicity in chicken protein is that there is an increase in hydrophobic amino acids such as tryptophan, phenylalanine, and tyrosine. [30]. 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 [31].The emulsion-activity index (EAI) and emulsion-stability index (ESI) are widely used metrics for assessing hydrolysate performance emulsifying properties 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, length of hydrolysis time, and etc [38].According Al Awwaly, *et al* [39] 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 are amino acids that affect emulsions; besides that, the properties of emulsions are influenced by the nature of globular proteins, which easily interact with water..

3.3 Peroxidation Lipid Inhibition

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

Table 3. Peroxidation Lipid Inhibition Chicken Head Hydrolysate Protein

Treatment*	Peroxidation lipid 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}

The data is presented as mean ± SD. Duncan's multiple range test showed a significant(P<0.05) in the mean values of the same column containing distinct letters.

The highest average was in inhibition of lipid peroxidation was at T4 66.25% and the lowest average inhibition of lipid peroxidation was at T3 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 [32]. Oxidation is a complex process involving the formation and spread of free radicals; free radicals will bind lipid electrons in cell membranes, directly resulting in cell damage. This is in agreement with [33] that unsaturated fatty acid.

The research Sonklin, et al [34] reported that measuring the inhibition of lipid peroxidation where 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. Antioksidan merupakan substrat yang mampu menekan peroksidase lipid. Substrates with the ability to inhibit lipid peroxidation are called antioxidants. 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 [35].

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 95 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 [36]. Antioxidant properties can have an effect due to chain breaks in proteins; protein hydrolysate chains can ward off radicals, such as 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]. Less than 50 amino acids are found in bioactive peptides. The sequence of hydrophobic groups in arginine, lysine, and proline makes up bioactive peptides [43]. Antioxidants that are PAO inhibitors are found in hydrolyzed chicken heads. Cholic and deoxycholic acid percentages can rise with increased collagen peptide content from turkey heads. [16,42]. Composition, structure, and hydrophobicity all affect antioxidant action. The protease enzyme that is employed affects antioxidants; papain has a cutting site at the hydrophobic amino acid residues Ala, Val, Leu, Ile, Phe, Trp, and Tyr.

4. CONCLUSION

Differences in the percentage combination of papain and bromelain can affect electrical conductivity, emulsion activity, and inhibition of lipid peroxidation. It does not affect the stability of the chicken head protein hydrolysate emulsion. Emulsion activity and emulsion stability at T1 and T3 with values of 89.99% and 1.63. in comparison, the electrical conductivity value has the highest value at T2 of 1609.6 µm/Cm. The hydrolysis process of chicken heads with a combination of papain and bromelain can inhibit lipid peroxidation. Papain 25% and bromelain 75% have the best lipid peroxidation inhibitory activity, namely 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.

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