Research Article

Antioxidant and α-Amylase Inhibitory Properties Generated from Chicken Head Proteins by Dual-Enzyme Hydrolysis

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Abstract | The aim of this work was to examine the effect of different concentrations of the combination of bromelain and papain enzymes in hydrolyzing chicken head protein in terms of the physicochemical characteristics and bioactivity of the hydrolysate. A laboratory experimental method using a completely randomized design (CRD) was used in this research. In this study, chicken head protein was hydrolyzed using different combinations of bromelain (b) and papain (p), namely CHP (without hydrolysis); CHP1 (0.25% b and 0.75% p); CHP2 (0.5% b and 0.5% p); and CHP3 (0.75% b and 0.25% p). The data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. The results showed that different concentrations of the combination of bromelain and papain had a significant effect ($P<0.01$) on pH, soluble protein concentration, peptide concentration, degree of hydrolysis, IC_{50} of DPPH, reducing power, iron and copper chelating ability, and α-amylase inhibitory activity. CHP1 showed the best bioactivity with a DPPH IC_{50} value of 4.62 mg/ml, iron chelating ability of 81.07%, copper chelating ability of 35.97%, and α-amylase inhibitory activity of 32.67%. While the best reducing power was at CHP2. As a result, it can be concluded that the use of a combination of bromelain and papain enzymes in chicken head protein hydrolysate has the potential to produce good bioactivity.

Keywords | Chicken head, Bromelain, Papain, Bioactive peptide, Antioxidant, Protein hydrolysate

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INTRODUCTION

Today's lifestyles associated with unhealthy diets, lack of physical activity, use of pesticides, food additives, and air pollution can result in oxidative stress in the body. Oxidative stress results from the inability of the biological system to balance the production and accumulation of reactive oxygen species (ROS) with its detoxification. The result of oxidative stress is chronic diseases, such as cancer, diabetes and stroke (Sharifi-Rad *et al.*, 2020). Free radicals and oxidants that are produced in excess will produce oxidative stress in the body which results in damage to cell membranes, DNA, proteins, lipoproteins, and lipids. Free radicals can trigger lipid peroxidation which can damage cell membranes and lipoproteins and generate malondialdehyde which is mutagenic and cytotoxic (Pham-Huy *et al.*, 2008). Natural antioxidants derived from phytochemical compounds such as flavonoids and polyphenols can be used to reduce the harmful effects of oxidative stress (Pizzino *et al.*, 2017). Apart from that, active peptides derived from meat muscle and by-products are also known to have the potential to be used as antioxidants,

due to their ability to scavenge free radicals and chelate metals (Liu *et al*., 2016).

Antioxidant peptides can come from vegetable proteins such as legumes, or from animal proteins from muscle tissue, milk, egg, and by-products. Bioactive peptides derived from poultry by-products, such as chicken skin (Onuh *et al*., 2014), chicken cartilage (Yang *et al*., 2019), chicken blood (Hamzeh *et al*., 2019) and chicken feathers (Alahyaribeik *et al*., 2021) showed the ability and potential as antioxidant. Bioactive peptides have good electron donor ability and hydrophobic character, so they can act as antioxidants by donating electrons to free radicals and converting them into stable compounds (Najafian and Babji, 2014). The use of poultry by-products as a source of bioactive peptides is based on the high protein content, especially collagen which can be a good precursor of bioactive peptides (Lafarga and Hayes, 2014). Chicken heads are also one of the poultry by-products that can be used to produce bioactive peptides because the protein content is high, namely around 10.58% (Du *et al*., 2013). The conversion of chicken heads into bioactive peptides is also expected to change its status to a high-value added product.

Bioactive peptides are protein fragments that generally have 2-20 amino acid residues. Some of the biological activities possessed by bioactive peptides include antioxidant, antimicrobial, antidiabetic and antihypertensive (Zaky *et al*., 2022). Bioactive peptides can only be biologically active in their simple form and are inactive in their parent protein (Gruppi *et al*., 2022). The generation of bioactive peptides from livestock by-products can be done through chemical and enzymatic hydrolysis. However, enzymatic hydrolysis is preferred because of the specific action of the enzyme and reduces the risk of amino acid damage (Andiana *et al*., 2023). Each protease has a different cleavage site during the protein hydrolysis process. Papain tends to cleave on residues Ala, Val, Leu, Ile, Phe, Trp, and Tyr which are hydrophobic amino acids (Zhao *et al*., 2022), while bromelain on alanine, glycine, and leucine (Colletti *et al*., 2021). Hydrolysis by combining more than one enzyme simultaneously or separately can be more effective in producing peptides that have certain biological activities compared to single enzyme treatment (Wickramasinghe *et al*., 2022). However, scarce information is available regarding the use of combined enzymes, especially bromelain and papain, to produce peptides with antioxidant and α-amylase inhibitory activity. Therefore, this study aims to evaluate the effect of using different combined concentrations of bromelain and papain on the hydrolysis of chicken head protein in terms of the physicochemical characteristics and bioactivity of the hydrolysate.

MATERIALS AND METHODS

Materials

The material used in this research was chicken head. The part of the chicken head used was the entire head to the neck, except the beak. Chicken head protein was extracted using the pH-shifting method as described by Darine *et al.* (2010) with slight modifications. Papain was provided by Hunan Insen Biotech Co., Ltd. (Hunan Province, China). Bromelain was purchased from Shaanxi Rainwood Biotech Co., Ltd. (Shaanxi, China). All other chemicals used in this work were analytical grade.

Method and experimental design

The research method used was a laboratory experiment with a completely randomized design with 4 treatments and 5 replications. The treatment was as follows: CHP (without hydrolysis); CHP 1 (hydrolysis using bromelain 0.25% and papain 0.75%); CHP2 (hydrolysis using 0.5% bromelain and 0.5% papain); and CHP3 (hydrolysis using bromelain 0.75% and papain 0.25%). The hydrolysis process was carried out for 3 hours for each enzyme, so the total duration of hydrolysis was 6 hours and was carried out at optimum conditions for both enzymes (pH 7 and 50 °C).

Protein extraction

Chicken head protein was extracted using the alkaline solubilization method and concentrated by acid precipitation as described by Darine *et al.* (2010) with slight modifications. Fresh ground chicken heads are dried for 6 hours using an oven at 40°C, after drying they are then ground into flour. Chicken head flours were made into a suspension with a concentration of 10% (w/v). The suspension was homogenized using magnetic stirrer for 5 min, then the pH was adjusted to 12 by adding 10 M NaOH. After the pH reached 12, the suspension was stirred continuously for 1 hour. The suspension was then centrifuged at 4,000 rpm for 15 min and the supernatant was obtained. The chicken head protein in the supernatant obtained was then precipitated by lowering the pH of the supernatant to 4 by adding 1 M HCl, then continued with centrifugation at 5,000 rpm for 15 min. The proteins concentrate obtained in paste form were dried with a microwave dryer on low mode (± 39 ^{0}C) for 5 min.

Protein hydrolysis

The hydrolysis process was carried out separately for both enzymes (bromelain and papain). Chicken head protein was first hydrolyzed using bromelain for 3 hours, then continued with hydrolysis using papain for 3 hours. The hydrolysis process was carried out at pH 7 and 50 ^{0}C for both enzymes. The total percentage of enzyme used for both enzymes is 1% (w/w) of the weight of the protein concentrate used. In the first stage of hydrolysis, the

protein concentrate was homogenized with distilled water at a concentration of 2% (w/v). The pH was adjusted to 7 by adding 2 M NaOH. The pre-incubation process was carried out at a temperature of 50 °C for 20 min followed by the addition of bromelain (0.25%; 0.5%; and 0.75%). The incubation process was carried out for 3 hours, then the enzymatic reaction was stopped by heating the mixture at 85 °C for 20 min. The same steps were carried out for papain hydrolysis. The percentage of papain added was 0.75%; 0.5% and 0.25%, so the total percentage of the two enzymes was 1%. After the hydrolysis process using papain was carried out, centrifugation was carried out at 4,000 rpm for 15 min, the supernatant obtained was then stored at -20 °C (Yuan *et al*., 2020).

Physicochemical analysis

The pH was measured using pH meter (Hanna Hi98107). The soluble protein concentration was determined spectrophotometrically at 280 nm using a Nano Drop/ ND-1000 UV/Vis (Thermo Fisher Scientific). The A280 protein program was used to quantify the soluble protein concentration. Peptide concentration and degree of hydrolysis were determined by the method described by Zhan *et al*.(2021). A series of tryptone casein concentrations of 0, 0.04, 0.08, 0.12, and 0.16 mg/ml were prepared to make a tryptone casein standard curve. The standard curve was used to determine the peptide concentration based on the linear regression equation y = ax + b. The degree of hydrolysis (DH) was calculated using the following equation:

$$DH\ (\%) = A/Bx\ 100$$

Where A (mg/ml) is the peptide concentration in the sample after hydrolysis and B (mg/ml) is the protein concentration in the sample before hydrolysis.

Bioactivities assay

Assay of DPPH scavenging activity was determined according to a method reported by Hu *et al.* (2018). Antioxidant activity against DPPH radicals was expressed in IC_{50} values obtained by plotting DPPH radical scavenging activity (%) against the concentration of hydrolysate (mg/ml). The reducing power of hydrolysates was evaluated according to the procedure described by Latorres. *et al* (2018). The method described by Najafian and Babji (2015) and Sellal *et al.* (2019) was used to evaluate the ability of samples to chelate iron and copper, respectively. Alpha amylase inhibitory activity assay was performed by following the previous description (Fadimu *et al*., 2022).

Statistical analysis

The data obtained were presented in the form of mean ± standard deviation and analyzed using one-way analysis of variance (ANOVA) based on significance levels of 1% and 5%. Duncan's multiple range test was performed to compare means. Statistical analysis was performed using SPSS Statistics 26.

RESULTS AND DISCUSSION

pH of chicken head protein hydrolysate

The difference in the percentage of the combination of bromelain and papain enzymes in the hydrolysis of chicken head protein showed a significant difference (P<0.01) on the pH value of the hydrolysate. The data in Table 1 showed the decrease on pH in chicken head protein before hydrolysis (CHP) and after hydrolysis using bromelain and papain (CHP1, CHP2, and CHP3). The highest value was found in CHP, while the lowest value was found in CHP3, namely protein hydrolysate using 0.75% bromelain and 0.25% papain. A decrease in pH after hydrolysis was also reported to occur in undersized crawfish minced meat hydrolysate (Bonilla *et al*., 2022) and whey protein hydrolysate (Kleekayai *et al*., 2022). The decrease in pH after hydrolysis may be caused by the release of carboxyl groups from protein chains during the hydrolysis process by proteases (Banjongsinsiri *et al*., 2016). The decrease in pH in the hydrolysate can also be caused by the specific cleavage site of an enzyme. It is known that papain has specific cleavage sites at Arg, Lys, and Phe residues (Hou *et al*., 2017). Arginine and lysine are known to contain base groups in the side chains that are attached to the core of the two amino acid molecules. These two amino acids have pKa values of 12-13.7 for arginine and ~10.5 for lysine (Li *et al*., 2013). This reason may also explain why CHP1 which used a higher papain concentration (0.75%) had a higher pH value compared with CHP2 and CHP3.

Soluble protein concentration of chicken head protein hydrolysate

Different percentages of the combination of bromelain and papain enzymes gave a significant difference (P<0.01) on the average soluble protein concentration. The data in Table 1 showed that the average soluble protein concentration is in the range of 1.47-6.02 mg/ml. The highest value was at CHP1 and the lowest was at CHP. Susanto *et al.* (2018) reported that chicken feet protein hydrolysate using the papain enzyme had a dissolved protein content of 0.76-1.38 mg/ml determined by the Bradford method. This showed that chicken head protein hydrolysate using a combination of bromelain and papain has a higher soluble protein concentration compared to previous study. The enzymatic hydrolysis process can increase protein solubility by increasing protein-water interactions (Noman *et al*., 2022). Protease can reduce the molecular mass of proteins and release ionisable groups, resulting in increased protein

solubility (Saengsuk *et al*., 2021). This can be seen in the data that chicken head protein that has been hydrolysed had a higher concentration of soluble protein compared to before hydrolysis. Good protein solubility is related to the efficiency of adding protein to a food ingredient. There is an indirect correlation between the level of protein solubility and the degree of hydrolysis (Nongonierma and FitzGerald, 2011).

Peptide concentration of chicken head protein hydrolysate

The concentration of TCA-soluble peptide can be used as an indicator to measure the level of protein degradation. Analysis of variance showed that different concentrations of the enzyme combination bromelain and papain had a significant effect (P<0.01) on the peptide concentration of chicken head protein hydrolysate. The peptide concentrations in the samples can be seen in Table 1. A peptide concentration of 0.1550 mg/ml was found in CHP which is a chicken head protein that was not hydrolysed. When the chicken head protein was hydrolysed, the peptide concentration increased in CHP1, CHP2, and CHP3 with combined enzyme hydrolysis treatment compared to CHP. Similar results were also reported by Saengsuk *et al*. (2021) that the hydrolysis of restructuring pork steak using bromelain had a higher peptide concentration when compared to before hydrolysis. Enzymatic hydrolysis allows for an increase in peptide concentration in the hydrolysate sample, due to the simplification of the protein form into peptides (Ibrahim and Ghani, 2020).

Degree of hydrolysis of chicken head protein hydrolysate

The difference in concentration of the combination of bromelain and papain enzymes gave a significant difference (P<0.01) on the degree of hydrolysis. The degree of hydrolysis in chicken head protein hydrolysate is in the range of 10.54-55.99% as shown in Table 1. The lowest degree of hydrolysis was in CHP, while the highest was in CHP1. These findings were similar to that reported by Lee *et al*. (2012) who found that duck skin gelatin hydrolysate using a combination of collagenase+papain and collagenase+α-chymotyrpsin had a degree of hydrolysis of 51% and 55.21%, respectively. The degree of hydrolysis can be defined as the percentage of the number of peptide bonds cleaved to the total number of peptide bonds (Bao *et al*., 2017). The success of a hydrolysis process can also be assessed from the degree of hydrolysis, because antioxidant activity can be influenced by the degree of hydrolysis and amino acid compositions (Puspawati *et al*., 2021). A high degree of hydrolysis will also increase protein solubility (Taheri *et al*., 2013) as can be seen that CHP1 which had the highest degree of hydrolysis also had a highest concentration of soluble protein.

IC_{50} DPPH of chicken head protein hydrolysate

Analysis of variance showed that different concentrations of the enzyme combination bromelain and papain had a significant difference (P<0.01) on the IC_{50} of DPPH. The IC_{50} value of chicken head hydrolysate using bromelain and papain was in the range of 4.62-15.63 mg/ml. The lowest value was in CHP1, while the highest was in CHP as can be seen in Table 2. The IC_{50} value is the sample concentration required to scavenge 50% of DPPH free radicals. A lower IC_{50} value indicates better antioxidant activity in the sample (Fadimu *et al*., 2022). In this study, it was seen that CHP1 using 0.25% bromelain and 0.75% papain had the strongest antioxidant activity when compared with other samples. Yang *et al*. (2019) reported that chicken

Table 1: Physicochemical analysis of chicken head protein hydrolysate.

Treatments	pH	Soluble protein concentration (mg/ml)	Peptide concentration (mg/ml)	DH (%)
CHP	7.00 ± 0.00^{c}	1.47 ± 0.17^{a}	0.1550 ± 0.01^{a}	10.54 ± 0.92^{a}
CHP1	6.20 ± 0.10^{b}	6.02 ± 0.60^{d}	0.8230 ± 0.08^{c}	55.99 ± 5.19^{c}
CHP2	6.36 ± 0.11^{b}	5.00 ± 0.40^{c}	0.7040 ± 0.02^{b}	47.89 ± 1.31^{b}
CHP3	5.98 ± 0.13^{a}	4.02 ± 0.27^{b}	0.8010 ± 0.06^{c}	54.49 ± 3.86^{c}

Data expressed as mean ± SD. Mean values with different letters in the same column were significantly different (P<0.01) according to Duncan's multiple range test.

Table 2: Bioactivity assay of chicken head protein hydrolysate.

Treatments	IC_{50} of DPPH (mg/ml)	Reducing power (A_{700})	Iron chelating ability (%)	Copper chelating ability (%)	α-amylase inhibitory activity (%)
CHP	15.63 ± 1.64^{c}	0.1554 ± 0.02^{a}	42.15 ± 3.68^{a}	7.13 ± 1.24^{a}	4.87 ± 0.72^{a}
CHP1	4.62 ± 0.69^{a}	0.2452 ± 0.05^{b}	81.07 ± 4.07^{c}	35.97 ± 3.62^{c}	32.67 ± 1.74^{d}
CHP2	9.15 ± 1.58^{b}	0.2610 ± 0.04^{b}	70.97 ± 4.98^{b}	22.40 ± 2.83^{b}	20.46 ± 0.68^{c}
CHP3	7.43 ± 1.06^{b}	0.2272 ± 0.03^{ab}	75.70 ± 2.23^{bc}	31.86 ± 3.36^{c}	9.44 ± 0.92^{b}

Data expressed as mean ± SD. Mean values with different letters in the same column were significantly different (P<0.01) according to Duncan's multiple range test.

cartilage hydrolysate, fractions >10 kDa, and <10 kDa using trypsin for 3 hours had IC_{50} values of 9.80, 18.91, and 7.35 mg/ml, while Alahyaribeik *et al.* (2021) reported that the <3 and <10 kDa fractions from chicken feather hydrolysate using fermented by *Bacillus licheniformis* had IC_{50} values of 5.03 and 6.64 mg/ml, respectively. This showed that chicken head protein hydrolysate had antioxidant activity that was equivalent or even better than chicken cartilage and feather hydrolysate.

The antioxidant capability found in protein hydrolysate samples may originate from peptides that engage in hydrogen donation, thereby interrupting free radical reactions. These peptides react with free radicals to generate more stable products and prevent further oxidative reactions (Najafian and Babji, 2015). Generally bioactive peptides have less than 50 amino acid residues. Bioactive peptide sequences are usually composed of proline, arginine, and lysine which have hydrophobic groups (Akbarian *et al.*, 2022). The antioxidant activity of peptides is influenced by composition, structure and hydrophobicity. The antioxidant properties of a peptide are also influenced by the treatment given to the protein, such as the type of protease enzyme used, the degree of hydrolysis, and the structure of the peptide (Padaga and Aulanniam, 2017). Papain is known to have cleavage sites on residues Ala, Val, Leu, Ile, Phe, Trp, and Tyr which are hydrophobic amino acids (Zhao *et al.*, 2022), this may be the reason why CHP1 which used the highest concentration of papain had higher antioxidant activity than other hydrolysates. The degree of hydrolysis, peptide concentration, and soluble protein content in the samples may also influence its antioxidant activity.

Reducing power of chicken head protein hydrolysate

The difference in concentration of the combination of bromelain and papain enzymes had a significant difference ($P<0.01$) on the reducing power. The reducing power of chicken head protein hydrolysate using a combination of bromelain and papain was in the range of 0.1554-0.2610 as can be seen in Table 2. The highest value is in CHP2 and the lowest is in CHP, namely 0.2610 and 0.1554, respectively, showing a significant ($P<0.01$) difference. CHP was a chicken head protein that was not hydrolysed and had the lowest capability among the other samples, this can be caused by the large size of the peptide in CHP. Peptides that have a smaller size have a better ability to donate electrons and interact with free radicals (Onuh *et al.*, 2014). Reducing power can be indicated from the increase in absorbance at 700 nm in the sample. Acidic amino acids such as aspartic acid and glutamic acid can strengthen the reducing power. Grass turtle protein hydrolysate was reported to have a reducing power of 0.88 at a concentration of 15 mg/ml and DH of 19.52% (Islam *et al.*, 2021). That value was higher than the reducing power of chicken head protein hydrolysates. Reducing power assay is often used to evaluate the ability of antioxidants to provide an electron to free radicals. Samples with higher reducing power have better ability to donate electrons (Jemil *et al.*, 2014). Bioactive peptides in protein hydrolysate will donate an electron and perform a reaction with potassium ferricyanide (Fe^{3+}) to produce potassium ferrocyanide (Fe^{2+}), followed by reacting with ferric chloride to generate a ferric-ferrous complex (Chalamaiah *et al.*, 2015).

Metal chelating ability of chicken head protein hydrolysate

The difference in concentration of the combination of bromelain and papain enzymes had a significant difference ($P<0.01$) on iron and chopper chelating ability. As can be seen in Table 2, the iron chelating ability of chicken head protein hydrolysate was in the range of 42.15-81.07%, while the copper chelating ability was 7.13-35.97%. CHP1 showed the strongest iron and copper chelating ability compared to other samples, namely 81.07% and 35.97%, respectively. CHP showed the lowest iron and copper chelating activity when compared with all treatments and showed a significant ($P<0.01$) difference. This may be caused by the lowest degree of CHP hydrolysis compared to other treatments, because the degree of hydrolysis indicates the amount of simple peptides produced. Small peptides have a simple spatial structure and more exposed metal ion binding sites, resulting in higher ability of metal chelation (Zhang *et al.*, 2021). Thus, the smaller the amount of simple peptides in the protein hydrolysate, the lower the ability to chelate metals. Abeyrathne *et al.* (2016) reported negative results on the copper chelating ability of ovomucin hydrolysate, while Islam *et al.* (2021) reported that grass turtle protein hydrolysate had an iron chelating ability of 63.25% at a DH of 11.96% and a concentration of 20 mg/ml and a copper chelating ability of 66.90% at a DH of 11.96% and a concentration of 6 mg/ml. The ability to chelate metals by protein hydrolysates can be influenced by the type of substrate, type of enzyme, and enzyme concentration (Onuh *et al.*, 2014).

Ferrous ions are catalysts that play a role in the lipid oxidation process. Hydroxyl radicals can be formed from hydrogen peroxide through the Fenton reaction and metal-catalyzed Haber-Weiss reactions. Therefore, ferrous ion chelation can reduce the formation of hydroxyl radicals (Famuwagun *et al.*, 2021). Amino acids and peptides that contain sulphur, such as cysteine, methionine, and glutathione have the ability to chelate and remove metals from the body (Flora and Pachauri, 2010). The ability to chelate iron ions is also associated with the presence of the -NH group of the imidazole ring on the histidine amino acid residue, especially when the histidine is located at the

C-terminal (Canabady-Rochelle *et al*., 2015).

α-AMYLASE INHIBITORY ACTIVITY OF CHICKEN HEAD PROTEIN HYDROLYSATE

Analysis of variance showed that different concentrations of the enzyme combination bromelain and papain had a significant effect ($P<0.01$) on the α-amylase inhibitory activity. CHP showed the lowest α-amylase inhibitory activity, namely 4.87%, while the highest activity of 32.65% was in CHP1 as can be seen in Table 2. The inhibition value was relatively higher when compared with collagen hydrolysate as reported by Gaspardi *et al*. (2022). The inhibitory activity of the α-amylase enzyme by bioactive peptides may originate from the amino acid residues Tyr, Trp, Phe, Lys, and cationic residues that have a tendency to bind α-amylase (Islam *et al*., 2021). Protein hydrolysate can inhibit α-amylase through disrupting enzyme-substrate interactions at the active site of the enzyme. Peptides with certain amino acid sequences have the ability to block the active site of α-amylase and delay the hydrolysis of carbohydrates into glucose (Fadimu *et al*., 2022).

CONCLUSIONS AND RECOMMENDATIONS

Differences in the percentage combination of bromelain and papain enzymes could affect the physicochemical characteristics and bioactivity of chicken head protein hydrolysate. The highest soluble protein concentration, peptide concentration and degree of hydrolysis were in CHP1 with values 6.02 mg/ml, 0.8230 mg/ml, and 55.99%. Meanwhile, the pH value for all samples was in the range of 5.98-7.00. The hydrolysis process of chicken head protein using a combination of papain and bromelain can increase the bioactivity of the hydrolysate. This was shown in the non-hydrolyzed (CHP) sample which had the lowest bioactivity compared to other samples. This research briefly showed that CHP1 using 0.25% bromelain and 0.75% papain had the best antioxidant activity when assessed from its DPPH inhibitory activity with an IC_{50} value of 4.62 mg/ml, iron and copper chelating ability of 81.07 and 35.97%, respectively. CHP1 also showed the highest an α-amylase inhibitory activity, namely 32.67%. However, CHP2 using 0.5% bromelain and 0.5% papain showed the best reducing power among the others. Thus, chicken head protein hydrolysate using a combination of bromelain and papain has potential as an antioxidant and α-amylase inhibitor.

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NOVELTY STATEMENT

The novelty in this research is the use of a combination of bromelain and papain enzymes with different concentrations to produce chicken head protein hydrolysate which has antioxidant and amylase inhibitory activity.

AUTHOR'S CONTRIBUTION

PA and MGMS performed laboratory analysis, analyzed data, and wrote the manuscript. KUAA and AM designed the research, provided guidance, and revised the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

Abeyrathne EDNS, Lee HY, Jo C, Suh JW, Ahn DU (2016). Enzymatic hydrolysis of ovomucin and the functional and structural characteristics of peptides in the hydrolysates. Food Chem., 192: 107-113. https://doi.org/10.1016/j.foodchem.2015.06.055

Akbarian M, Khani A, Eghbalpour S, Uversky VN (2022). Bioactive peptides: Synthesis, sources, applications, and proposed mechanisms of action. Int. J. Mol. Sci., 23: 1-3. https://doi.org/10.3390/ijms23031445

Alahyaribeik S, Seyed DS, Tabandeh F, Honarbakhsh S, Ghazanfari S (2021). Stability and cytotoxicity of DPPH inhibitory peptides derived from biodegradation of chicken feather. Protein Express. Purif., 177: 1-8. https://doi.org/10.1016/j.pep.2020.105748

Andiana P, Umam K, AA, Manab A (2023). The potential of meat and slaughterhouse by- products as sources of bioactive peptides: A literature review. E3S Web Conf., 81: 1-9. https://doi.org/10.1051/bioconf/20238100010

Banjongsinsiri P, Pasakawee K, Noojuy N, Taksima T, Rodsuwan U (2016). Production of mushroom protein hydrolysates by enzymatic hydrolysis and their physicochemical properties. Food Appl. Biosci. J., 4(3): 161-170.

Bao Z, Zhao Y, Wang X, Chi YJ (2017). Effects of degree of hydrolysis (DH) on the functional properties of egg yolk hydrolysate with alcalase. J. Food Sci. Technol., 4(3): 669–678. https://doi.org/10.1007/s13197-017-2504-0

Bonilla F, Garcia A, Calumba K, Reyes V, Sathivel S (2022). Functional and morphological characterization of undersized crawfish minced meat hydrolysate powders produced with alkaline protease and bacterial protease. Food Struct., 31: 1-9. https://doi.org/10.1016/j.foostr.2022.100250

Canabady-Rochelle LLS, Harscoat-Schiavo C, Kessler V, Aymes A, Fournier F, Girardet JM (2015). Determination of reducing power and metal chelating ability of antioxidant peptides: Revisited methods. Food Chem., 183: 129-135. https://doi.org/10.1016/j.foodchem.2015.02.147

Chalamaiah M, Jyothirmayi T, Diwan PV, Dinesh KB (2015). Antioxidant activity and functional properties of enzymatic protein hydrolysates from common carp (*Cyprinus carpio*) roe (egg). J. Food Sci. Technol., 52(9): 5817–5825. https://doi.org/10.1007/s13197-015-1714-6

Colletti A, Li S, Marengo M, Adinolfi S, Cravotto G (2021). Recent advances and insights into bromelain processing, pharmacokinetics and therapeutic uses. Appl. Sci. (Switzerland). 11(18): 1-20. https://doi.org/10.3390/app11188428

Darine S, Christophe V, Gholamreza D (2010). Production and functional properties of beef lung protein concentrates. Meat Sci., 84(3): 315-322. https://doi.org/10.1016/j.meatsci.2009.03.007

Du L, Khiari Z, Pietrasik Z, Betti M (2013). Physicochemical and functional properties of gelatins extracted from turkey and chicken heads. Poult. Sci., 92(9): 2463-2474. https://doi.org/10.3382/ps.2013-03161

Fadimu GJ, Farahnaky A, Gill H, Olalere OA, Gan CY, Truong T (2022). In-silico analysis and antidiabetic effect of α-amylase and α-glucosidase inhibitory peptides from lupin protein hydrolysate: Enzyme-peptide interaction study using molecular docking approach. Foods, 11: 1-20. https://doi.org/10.3390/foods11213375

Famuwagun AA, Alashi AM, Gbadamosi OS, Taiwo KA, Oyedele D, Adebooye OC, Aluko RE (2021). Antioxidant and enzymes inhibitory properties of Amaranth leaf protein hydrolyzates and ultrafiltration peptide fractions. J. Food Biochem., 45(3): 1-13. https://doi.org/10.1111/jfbc.13396

Flora SJS, Pachauri V (2010). Chelation in metal intoxication. Int. J. Environ. Res. Publ. Health, 7: 2745-2788. https://doi.org/10.3390/ijerph7072745

Gaspardi ALA, da Silva DC, Ponte LGS, Galland F, da Silva VSN, Simabuco FM, Bezerra RMN, Pacheco MTB (2022). *In vitro* inhibition of glucose gastro-intestinal enzymes and antioxidant activity of hydrolyzed collagen peptides from different species. J. Food Biochem., 46(12): e14383. https://doi.org/10.1111/jfbc.14383

Gruppi A, Dermiki M, Spigno G, Fitzgerald RJ (2022). Impact of enzymatic hydrolysis and heat inactivation on the physicochemical properties of milk protein hydrolysates. Foods, 11(4): 1-16. https://doi.org/10.3390/foods11040516

Hamzeh A, Wongngam W, Kiatsongchai R, Yongsawatdigul J (2019). Cellular and chemical antioxidant activities of chicken blood hydrolysates as affected by *in vitro* gastrointestinal digestion. Poult. Sci., 98(11): 6138-6148. https://doi.org/10.3382/ps/pez283

Hou Y, Wu Z, Dai Z, Wang G, Wu G (2017). Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. J. Anim. Sci. Biotechnol., 8(24): 1-13. https://doi.org/10.1186/s40104-017-0153-9

Hu F, Ci AT, Wang H, Zhang YY, Zhang JG, Thakur K, Wei ZJ (2018). Identification and hydrolysis kinetic of a novel antioxidant peptide from pecan meal using Alcalase. Food Chem., 261: 301-310. https://doi.org/10.1016/j.foodchem.2018.04.025

Ibrahim ESK, Ghani MA (2020). The effect of enzymatic hydrolysis on the antioxidant activities and amino acid profiles of defatted chia (*Salvia hispanica* L.) flour. Food Res., 4: 38-50. https://doi.org/10.26656/fr.2017.4(S4).003

Islam MS, Hongxin W, Admassu H, Noman A, Ma C, Anwei F (2021). Degree of hydrolysis, functional and antioxidant properties of protein hydrolysates from Grass Turtle (*Chinemys reevesii*) as influenced by enzymatic hydrolysis conditions. Food Sci. Nutr., 9(8): 4031-4047. https://doi.org/10.1002/fsn3.1903

Islam MS, Hongxin W, Admassu H, Mahdi AA, Chaoyang M, Wei FA (2021). *In vitro* antioxidant, cytotoxic and antidiabetic activities of protein hydrolysates prepared from chinese pond turtle (*Chinemys reevesii*). Food Technol. Biotechnol., 59(3): 360-375. https://doi.org/10.17113/ftb.59.03.21.7087

Jemil I, Jridi M, Nasri R, Ktari N, Ben S-BSR, Mehiri M, Hajji M, Nasri M (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by *Bacillus subtilis* A26. Proc. Biochem., 49(6): 63–972. https://doi.org/10.1016/j.procbio.2014.03.004

Kleekayai T, O'neill A, Clarke S, Holmes N, O'sullivan B, Fitzgerald RJ (2022). Contribution of hydrolysis and drying conditions to whey protein hydrolysate characteristics and *in vitro* antioxidative properties. Antioxidants, 11: 1-15. https://doi.org/10.3390/antiox11020399

Lafarga T, Hayes M (2014). Bioactive peptides from meat muscle and by-products: Generation, functionality and application as functional ingredients. Meat Sci., 98(2): 227-239. https://doi.org/10.1016/j.meatsci.2014.05.036

Latorres JM, Rios DG, Saggiomo G, Wasielesky W, Prentice-Hernandez C (2018). Functional and antioxidant properties of protein hydrolysates obtained from white shrimp (*Litopenaeus vannamei*). J. Food Sci. Technol., 55(2): 721-729. https://doi.org/10.1007/s13197-017-2983-z

Lee SJ, Kim KH, Kim YS, Kim EK, Hwang JW, Lim BO, Moon SH, Jeon BT, Jeon YJ, Ahn CB, Park PJ (2012). Biological activity from the gelatin hydrolysates of duck skin by-products. Proc. Biochem., 47(7): 1150-1154. https://doi.org/10.1016/j.procbio.2012.04.009

Li L, Vorobyov I, Allen TW (2013). The different interactions of lysine and arginine side chains with lipid membranes. J. Phys. Chem. B, 117(40): 11906-11920. https://doi.org/10.1021/jp405418y

Liu R, Xing L, Fu Q, Zhou GH, Zhang WG (2016). A review of antioxidant peptides derived from meat muscle and by-products. Antioxidants, 5(32): 1-15. https://doi.org/10.3390/antiox5030032

Najafian L, Babji AS (2014). Production of bioactive peptides using enzymatic hydrolysis and identification antioxidative peptides from patin (*Pangasius sutchi*) sarcoplasmic protein hydolysate. J. Funct. Foods, 9(1): 280–289. https://doi.org/10.1016/j.jff.2014.05.003

Najafian L, Babji AS (2015). Isolation, purification and identification of three novel antioxidative peptides from patin (*Pangasius sutchi*) myofibrillar protein hydrolysates. LWT-Food Sci. Technol., 60: 452-461. https://doi.org/10.1016/j.lwt.2014.07.046

Noman A, Wang Y, Zhang C, Abed SM (2022). Antioxidant activity of hybrid sturgeon (*Huso dauricus* × *Acipenser schrenckii*) protein hydrolysate prepared using bromelain, its fractions and purified peptides. Pol. J. Food Nutr. Sci., 72(1): 79-89. https://doi.org/10.31883/pjfns/146317

Nongonierma AB, FitzGerald RJ (2011). Enzymes exogenous to milk in dairy technology: Proteinases. In: Encyclopedia of Dairy Sciences: Second Edition. https://doi.org/10.1016/B978-0-12-374407-4.00154-0

Onuh JO, Girgih AT, Aluko RE, Aliani M (2014). *In vitro* antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. Food Chem., 150: 366-373. https://doi.org/10.1016/j.foodchem.2013.10.107

Padaga MC, Aulanniam (2017). Susu sebagai nutrasetika untuk penyakit gangguan metabolik. Malang: UB Press.

Pham-Huy LA, He H, Pham-Huy C (2008). Free radicals,

antioxidants in disease and health. Int. J. Biomed. Sci., 4(2): 89-96. https://doi.org/10.59566/IJBS.2008.4089

Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, Squadrito F, Altavilla D, Bitto A (2017). Oxidative stress: Harms and benefits for human health. Oxid. Med. Cell. Long., pp. 1-13. https://doi.org/10.1155/2017/6341671

Puspawati NM, Wahjuni S, Ayu NKIK, Fudholi A (2021). Effect of different papain concentrations on the properties of chicken skin protein hydrolysates. Int. J. Design Nat. Ecodyn., 16(4): 445-450. https://doi.org/10.18280/ijdne.160411

Saengsuk N, Laohakunjit N, Sanporkha P, Kaisangsri N, Selamassakul O, Ratanakhanokchai K, Uthairatanakij A (2021). Physicochemical characteristics and textural parameters of restructured pork steaks hydrolysed with bromelain. Food Chem., 361: 1-10. https://doi.org/10.1016/j.foodchem.2021.130079

Sellal A, Belattar R, Bouzidi A (2019). Heavy metals chelating ability and antioxidant activity of Phragmites australis stems extracts. J. Ecol. Eng., 20(2): 116-123. https://doi.org/10.12911/22998993/96276

Sharifi-Rad M, Anil Kumar NV, Zucca P, Varoni EM, Dini L, Panzarini E, Rajkovic J, Fokou PVT, Azzini E, Peluso I, Mishra AP, Nigam M, Rayess YE, Beyrouthy ME, Polito L, Iriti M, Martins N, Martorell M, Docea AO, Setzer WN, Calina D, Cho WC, Sharifi-Rad J (2020). Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. Front. Physiol., 11: 1-20. https://doi.org/10.3389/fphys.2020.00694

Susanto E, Rosyidi D, Radiati LE, Subandi S (2018). Optimasi aktivitas antioksidan peptida aktif dari ceker ayam melalui hidrolisis enzim papain. J. Ilmu Dan Teknol. Hasil Ternak, 13(1): 14-26. https://doi.org/10.21776/ub.jitek.2018.013.01.2

Taheri A, Anvar SAA, Ahari H, Fogliano V (2013). Comparison the functional properties of protein Hydrolysates from poultry byproducts and rainbow trout (*Onchorhynchus mykiss*) viscera. Iran. J. Fish. Sci., 12(1): 154-169.

Wickramasinghe HS, Abeyrathne EDNS, Nam KC, Ahn DU (2022). Antioxidant and metal-chelating activities of bioactive peptides from ovotransferrin produced by enzyme combinations. Poultry, 1: 220-228. https://doi.org/10.3390/poultry1040019

Yang J, Sun-Waterhouse D, Xiao Y, He W, Zhao M, Su G (2019). Osteoarthritis-alleviating effects in papain-induced model rats of chicken cartilage hydrolysate and its peptide fractions. Int. J. Food Sci. Technol., 54(9): 2711-2717. https://doi.org/10.1111/ijfs.14182

Yuan J, Zheng Y, Wu Y, Chen H, Tong P, Gao J (2020). Double enzyme hydrolysis for producing antioxidant peptide from egg white: Optimization, evaluation, and potential allergenicity. J. Food Biochem., 44(2): 1-12. https://doi.org/10.1111/jfbc.13113

Zaky AA, Simal-Gandara J, Eun JB, Shim JH, Abd El-Aty AM (2022). Bioactivities, applications, safety, and health benefits of bioactive peptides from food and by-products: A review. Front. Nutr., 8: 1-18. https://doi.org/10.3389/fnut.2021.815640

Zhan J, Li G, Dang Y, Pan D (2021). Study on the antioxidant activity of peptide isolated from porcine plasma during in vitro digestion. Food Biosci., 42: 1-8. https://doi.org/10.1016/j.fbio.2021.101069

Zhang Y, Ding X, Li M (2021). Preparation, characterization and in vitro stability of iron-chelating peptides from mung beans. Food Chem., 349: 1-8. https://doi.org/10.1016/j.foodchem.2021.129101

Zhao F, Liu C, Bordoni L, Petracci I, Wu D, Fang L, Wang J, Wang X, Gabianelli R, Min W (2022). Advances on the antioxidant peptides from Nuts: A narrow review. Antioxidants, 11(10): 1-23. https://doi.org/10.3390/antiox11102020