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Study of Protein Hydrolysis and Peptide Antioxidants Activity of Chicken Slaughterhouse Waste and Its Potential for Feed Additives

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Abstract. Ensuring food safety in livestock requires specific feeding technology in agriculture by using feed additives in the form of antibiotics, prebiotics, probiotics, acidifiers, hormones and enzymes. Bioactive peptides improve the health status of humans and animals. Protein hydrolysis produce peptides that are safe, quickly metabolizable, less risky for livestock products to have contaminant residue. Bioactive peptides are still bound to the original protein, so they need to be released through an enzymatic process. This research explored the potential of chicken slaughterhouse waste to produce biopeptides by hydrolyzing proteins using various proteolytic enzymes. The slaughterhouse waste included chicken feet, intestines, filleting waste and blood plasma. The proteolytic enzymes used were papain, bromelain, protease by Rhizopus oligosphorus, probiotic protease. The observed variables were dissolved protein content with and without precipitation, protein hydrolyzate content, and the degree of enzyme hydrolysis. The research was conducted using exploratory methods. The results showed that the dissolved protein content in the chicken slaughterhouse waste protein concentrate was 1,585 mg/ml (feet), 2,361 mg/ml (intestines), 1,787 (filleting waste) and 2,372 mg/ml (blood plasma). Blood plasma protein concentrate showed the highest yield among other chicken slaughterhouse waste protein concentrates, namely 0.14 mg/ml (hydrolysis of papain), 0.18 mg/ml (hydrolysis of bromelain), 0.56 mg/ml (hydrolysis of R.oligosphorus protease) 0.68 mg/ml (hydrolysis of probiotic proteases). The highest degree of hydrolysis was shown in blood plasma hydrolyzates using probiotic protease enzymes, namely 28.72%. The highest antioxidant activity was 92.92% as observed in chicken feet protein hydrolyzate which was hydrolyzed using papain. Therefore, chicken feet, intestines and fillet waste can produce protein concentrates through precipitation using ammonium sulfate, and plasma using acetone. The highest protein concentration was in blood plasma protein which also produced the highest hydrolysis from hydrolyzing blood plasma proteins with hydrolyzed probiotic protease. The highest antioxidant activity was observed in chicken feet protein hydrolyzate which was hydrolyzed using papain enzyme and incubated for 6h.

Keywords: chicken slaughterhouse, protein hydrolyzate, antioxidant, feed additive

Abstrak. Penelitian bertujuan mengeksplorasi potensi limbah rumah pemotongan ayam (RPA) untuk produksi bioeptida menggunakan berbagai beberapa protease. Materi penelitian adalah limbah RPA yaitu ceker, usus, sisa fillet dan plasma darah. Papain, bromelin, ekstrak kasar *R. oligosporus*, ekstrak kasar probiotik adalah sumber protease untuk hidrolisis protein limbah RPA. Peubah yang diamati adalah: kadar protein terlarut dan protein hasil pengendapan, kadar protein hidrolisat dan derajat hidrolisis enzim. Penelitian dilakukan dengan metode eksploratif dengan ulangan tiga kali. Hasil penelitian menunjukkan kandungan protein terlarut konsentrat protein limbah RPA berturut-turut sebesar 1,585 mg/ml (ceker), 2,361 mg/ml (Usus), 1,787 (sisa fillet) dan 2,372 mg/ml (plasma darah). Kandungan protein hidrolisat plasma darah menunjukkan hasil tertinggi diantara konsentrat protein limbah RPA lain yaitu 0,14 mg/ml (papain), 0,18 mg/ml (bromelin), 0,56 mg/ml (protease *R.oligosphorus*), 0,68 mg/ml (protease probiotik). Derajat hidrolisis tertinggi ditunjukkan pada hidrolisat plasma darah menggunakan enzim protease probiotik yaitu 28,72%. Aktivitas antioksidan tertinggi sebesar 92,92%. ditunjukkan oleh hidrolisat protein ceker yang dihidrolisis selama enam jam menggunakan papain. Berdasarkan hasil penelitian dapat disimpulkan bahwa Konsentrat protein dari ceker, usus dan limbah fillet dapat diperoleh melalui pengendapan menggunakan amonium sulfat, sedangkan plasma menggunakan aseton. Konsentrat protein plasma darah menunjukkan konsentrasi protein tertinggi, hasil hidrolisis tertinggi ditunjukkan oleh hidrolisat protein plasma darah dengan hidrolisis enzim protease probiotik, aktivitas antioksidan tertinggi ditunjukkan oleh hidrolisat protein ceker yang dihidrolisis menggunakan enzim papain dengan waktu inkubasi 6 jam.

Kata kunci: Limbah-ayam, hidrolisat-protein, antioksidan, aditif pakan

Introduction

Ensuring the availability of safe food is an urgency amid the COVID-19 pandemic, especially the provision of animal protein containing essential amino acids as a buffer for the body's immune system. Technology is needed to ensure the safety of food from livestock through on-farm technology, namely technology in feeding, which is carried out using feed additives in the form of antibiotics, prebiotics, probiotics, acidifiers, hormones, and enzymes. Since January 1, 2018, AGP (Antibiotic Growth Promoter) antibiotics are prohibited because they cause residues in livestock products, making them unsafe for consumption. It opens opportunities for alternative additives that have almost the same level of effectiveness, such as by utilizing the biological function of proteins in the form of peptides. Bioactive peptides can improve the health status of humans and animals. Peptides are derived from protein so they are safe, quickly metabolizable, and less risky of residue contamination in livestock products. Bioactive peptides are still bound in the original protein so that they are released through an enzymatic process. Shahi et al. (2020) stated that antioxidant peptides are regarded healthy and safe compounds with low molecular weight, low cost, high activity, and high absorbability. In contrast to the synthetic antioxidants, these antioxidants can also be ascribed to the higher stability in different conditions, safety, nutritional value, and high functionality. Elias et al. (2008) and Nimse et al. (2015) stated that except for some well-known natural antioxidants (such as vitamins, bioflavonoids, carotenoids, proteins, amino acids, etc.), peptides also have the same antioxidant mechanism.

Several enzymes can be used to break protein polypeptide chains, including papain, bromelain, and microbial protease enzymes. This enzyme is widely available and is now more easily available in the market. Previous studies mention that the papain enzyme is superior in producing antioxidant peptides. The most important factor in the production of bioactive peptides is the molecular weight of the peptide, and the common method to produce specific molecular weight peptides is the ultrafiltration membrane system. A multilevel hydrolysis system (utilizing several enzymes simultaneously) can produce peptides of the hydrolysis process that produces bioactive peptides. Enzymatic hydrolysis can increase the functional ability of peptides to donate electrons to free radicals compared to inactivated peptides. The functional properties of bioactive peptides are largely determined by the composition and arrangement of amino acids in order to function as antioxidants. The addition of enzymes will be in line with the increase in the number of peptides and free amino acids produced in the hydrolyzate product so that the percentage of inhibition of free radical activity will also increase along with the presence of hydrolyzing enzymes. Waste is a source of animal protein that has not been exploited other than as a cheap alternative animal protein food. Therefore, waste can be explored for its potential as a source of peptides that have bioactive characteristics as feed additive agents. This research examines the hydrolyzate potential of waste and its potential as a source of feed additives for livestock, especially antioxidants.

Materials and Methods

We used chicken slaughterhouse waste including chicken feet, intestines, filleting waste and blood plasma. The proteolytic enzymes were papain, bromelain, crude extract of R. oligosphorus and probiotic *Raja Kaya*. The research was conducted using an exploratory method to obtain protein hydrolysates which has the potential as a feed additive. The observed variables were protein concentration in crude extract and ammonium sulphate precipitation of chicken slaughterhouse waste, levels of protein hydrolysates, degree of enzyme hydrolysis and antioxidant activity.

Production of Protein Concentrates (PC) of Chicken Slaughterhouse Waste

The concentrate of chicken slaughterhouse waste was made by weighing 100 g each of chicken feet, filleting waste, and intestine, and 100 ml of chicken blood plasma. Chicken feet, filleting waste and intestines were each incorporated into 400 ml of water and autoclaved for 30 minutes at 1 atm pressure. Then, it was filtered and stored overnight in the refrigerator to separate the filtrate from the fat.

The filtrate (except chicken feet) was then precipitated with 40, 50, 60 and 70% of ammonium sulfate saturation. Blood plasma was precipitated using cold acetone at concentrations of 40, 50 and 60% (v/v). The best results based on the protein content were selected to be applied in the manufacture of protein concentrate feet, filleting waste, intestine and blood as well as to test the degree of hydrolysis and antioxidant activity.

Production of Crude Extract of Proteolytic Enzymes

Proteolytic enzymes (proteases) are enzymes that break down protein. This research is using crude extracts of fruit bromelain, papaya latex, *R.oligosphorus* and probiotic. It were obtained from the centrifugation at 3500 rpm for 15 min. Crude extract of *R.oligosphorus* was obtained from fermenting soybeans for 3 days at room temperature. Ammonium sulphate precipitation was carried out by mixing the crude extract at 40% (bromelain), 55% (papain) and 50% (*R. oligosporus* and probiotic) saturation. After centrifugation at 10,000 rpm for 15 minutes, the yield was mixed with 5 ml buffer phosphate 0.05M pH 7.2.

The protease activity was measured according to the method of Bergmeyer and Grassl (1983) using 2% hammersten casein as a substrate. The absorbances of sample and blank were carried out at 578 nm.

Protein Hydrolysis

Protein hydrolysate was obtained through the hydrolysis process of chicken slaughterhouse waste protein extract (feet, intestine, fillet waste and blood plasma) using crude enzymes of papain, bromelain, Rhizopus oligosporus and probiotics. The hydrolysis reaction used the dissolved protein concentration of each protein extract. The specific activity of the enzymes used was 0.602 U/mg (bromelain), 0.270 U/mg (papain) and 1.703 U/mg (R. oligosporus) and 0.783 U/mg (probiotics). hydrolysis was carried out at the same protein concentration of chicken slaughterhouse waste extract. The hydrolysate was obtained after centrifuged at 10,000 rpm for 30 minutes. The protein content of hydrolyzate was measured by Bradford solution using a spectrophotometer at 595 nm. The degree of hydrolysis (DH) is calculated by the formula (Rutherfurd, 2010) below.

$$\text{DH}\ (\%) = \frac{\text{Protein after hydrolysis}}{\text{Protein before hydrolysis}}\ x\ 100$$

Antioxidant activity assay

The test of antioxidant activity used the DPPH (2.2-diphenyl-1-picrylhydrazil) according to Lu and Foo (2000). The absorbance of the sample was measured at a wavelength of 517 nm. Antioxidant activity was calculated based on the equation of the percentage of the difference between the control absorbance and the hydrolysate against the inhibitory power. Tests were carried out to measure the antioxidant ability of protein hydrolysate by measuring the ability to neutralize DPPH as free radicals. The color of the DPPH will be reduced in the presence of antioxidants that donate hydrogen ions. Antioxidant activity (AA) is calculated by the formula:

$$\%\ \text{A.A} = \frac{\text{Abs. DPPH} - \text{Abs. Sampel}}{\text{Abs. DPPH}}\ x\ 100$$

Results and Discussion

Dissolved Protein

The results showed that the protein content of the crude extract and precipitation of slaughterhouse waste ranged from 0.502 mg/ml to 1.219 mg/ml, with the highest concentration found in blood plasma, followed by chicken feet, intestines, and fillet waste (Table 1).

Plasma contains 7% protein, 0.9% inorganic ions, and 0.8% small organic molecules. Total protein in plasma is 6-8 g/dL, and the highest concentration of plasma protein is albumin, which is 3.2-5.6 g/dL (Meisenberg and Simmons, 2012). The high inorganic ions (Na, K, Cl, Ca, Mg) in plasma interfere with ammonium and sulfate ions binding to the water coat on protein molecules, so proteins cannot be precipitated. In this study, plasma proteins were successfully precipitated using 40% (v/v) cold acetone. The protein of filleting waste, chicken feet, and intestine can be precipitated using ammonium sulfate salt at different levels of saturation, namely 40% (foot and intestine) and 70% (filleting waste). Filleting waste mostly consists of bone and a little bit of meat attached to it. The organic components of bone are mainly protein, and 90% of bone protein is collagen (Cansu and Boran, 2015). Chicken feet are mainly composed of skin and bone tissue that contains a large amount of collagen. Native collagen can be extracted from various animal body tissues like bones, tendons, lung tissue, or even connective tissue (Ferraro et al., 2017; Santos et al., 2013; Paschou et al., 2018).

Dissolved protein content in the intestine comes from the intestinal mucosa which contains intestinal membranes, mucosa, intestinal microorganisms and digestive enzymes. Different amount and types of protein in the chicken slaughtered house waste causes differences in the level of ammonium sulfate salt used to precipitate the protein. Meanwhile, Table 2 shows varying concentrations of dissolved protein extracted from chicken slaughterhouse waste by precipitation using ammonium sulfate at saturation levels of 40%, 50%, 60%, and 70%. The highest concentration of dissolved protein levels of chicken feet was at 40% ammonium sulfate saturation level, namely 1.005 mg/ml, which decreased as the level of ammonium sulfate saturation increased, until salting-out at 70% saturation level. Intestinal protein extracts showed the highest concentrations at 40% and 50% saturation levels of ammonium sulfate, namely 0.818 mg/ml and 0.817 mg/ml, respectively. For further testing, protein extract with 40% ammonium sulfate was used. Meanwhile, the protein extract of the rest of the fillet showed no precipitation at the saturation level of 40% and 50%, but the highest concentration of dissolved protein (0.381 mg/ml) was at the 70% ammonium sulfate deposition level. As for the blood plasma protein extract, precipitation was not carried out using ammonium sulfate because the precipitation results between the pellet and the filtrate showed the same concentration of soluble protein. However, precipitation using acetone at 40% acetone precipitationproduced a high concentration of dissolved protein in the blood plasma extract, namely 4.963 mg/ml.

Hydrolysates Protein and Degree of Hydrolysis

The advantage of hydrolysis is the possibility of producing different profiles of peptide mixtures from the same raw material.

Table 1. Protein content of crude extracts and precipitation of slaughterhouse waste

No	Slaughterhouse waste	Crude extract (mg/ml)	Precipitation (mg/ml)
1	Chicken feet[1]	0.662	1.005
2	Intestine[1]	0.565	0.818
3	Fillet waste[2]	0.502	0.381
4	Blood Plasma[3]	1.219	4.963

[1)]40% ammonium sulphate saturation; [2)]70% ammonium sulphate saturation; [3)]40% (v/v) acetone

These deviations of peptide profiles are highly dependent on the processing parameters (enzyme specifications, hydrolysis temperature, and duration, raw material/water ratio, etc.) and may result in finished products with very highly diverse functional properties (Leduc et al., 2020).

The protein concentrate used in the enzymatic hydrolysis test was protein deposited by ammonium sulfate (chicken feet, intestine, fillet waste) and ethanol (plasma). The results showed that the concentration of hydrolysate protein ranged from 0.01 mg/ml to 0.68 mg/ml (Table 3). The highest protein concentration in protein extracts of chicken slaughter waste was indicated by the results of hydrolysis using crude enzymes from probiotic. The highest protein concentration was shown by the blood plasma protein extract which was hydrolyzed using crude enzymes from microbial origin, namely 0.56 mg/ml (hydrolysis using R oligosphorus enzymes) and 0.68 mg/ml (hydrolysis using probiotic enzymes). Different hydraulic results using different enzymes has caused different product sizes because the ability to cut amino acid chains is random depending on the characteristics of the enzyme. The results showed that the hydrolysis of protease enzymes was the highes because probiotics contained 13 types of Bacillus sp and Sacharomyces cereviseae, thus a stronger hydrolysis ability. Blood plasma is also a substrate rich in dissolved protein.

Table 3 shows that the highest degree of hydrolysis (28.72%) was shown by plasma hydrolysate with protease probiotic, and the lowest (0.41%) was shown by intestinal hydrolysate with protease *R. oligosporus*. The degree of hydrolysis shows the ability of enzymes to break down protein molecules into peptides. In this study, the highest hydrolysis ability was shown by the crude enzymes of probiotics in breaking down proteins. Probiotic proteases have high hydrolysis ability, presumably because they contain several types of proteases, so that more protein can be degraded into peptides in the protein extract of chicken slaughterhouse waste. Sufficient concentration and curing time will be able to break hydrogen and covalent bonds of a protein that is difficult to dissolve. As stated by Kezwoń et al. (2016), hydrolysis depends not only the concentration of the enzyme, but also the length of incubation of the protein with the enzyme. The longer the incubation time, the greater the opportunity for enzymes to cut protein molecules into peptides.

Table 2. Hydrolysates protein after hydrolysis using various protease

Protein source	Protein hydrolysates (mg/ml)			
	Papain	Bromelin	*R. oligosphorus*	Probiotic[1]
Chicken feet	0.05	0.01	0.01	0.10
Intestine	0.08	0.01	0.01	0.17
Fillet waste	0.19	0.17	0.07	0.18
Blood Plasma	0.14	0.18	0.56	0.68

[1]Commercial liquid probiotics contain 13 strains of *Bacillus* sp. and *S. cerevisiae*

Table 3. Degree of hydrolysis of concentrate protein from chicken slaughterhouse using various protease

Protein source	Degree of Hydrolysis (%)			
	Papain	Bromelin	*R. oligosphorus*	Probiotic
Chicken feet	2.90	0.50	0.47	6.36
Intestine	3.28	1.07	0.41	7.36
Fillet waste	10.75	9.71	8.95	9.97
Blood Plasma	5.96	7.63	23.75	28.72

Table 4. Antioxidant test results of protein hydrolyzate of chicken slaughterhouse waste hydrolyzed using various protease

Source of Hydrolysates	Antioxidant activity (%)			
	Bromelin	Papain	R. *oligosporus*	Probiotic
Chicken feet	48,62	92,92	38,77	86,77
Intestine	58,46	74,15	44,62	32,62
Fillet waste	5,54	9,23	44,00	29,85
Blood Plasma	65,54	68,31	39,08	65,23

Antioxidant activity

Table 4 shows the antioxidant activity of each protein hydrolysates with different enzyme hydrolysis. All hydrolysates show the highest antioxidant activity with papain hydrolysis, except blood plasma hydrolysate. The highest antioxidant activity of all hydrolysates was observed in chicken feet (92.92%), and the lowest was in the filleting waste protein with bromelain (5.54%). This result showed a higher antioxidant activity than that of Susanto et al. (2018), i.e., 55.10% obtained from the treatment of 3% papain enzyme concentration with 36 hours of curing time. The difference in antioxidant activity is due to different test samples, namely protein concentrate that no longer contained fat and carbohydrates. It indicates a very high antioxidant potential of chicken feet protein hydrolysate. Liu et al (2012) stated that more than 40% of chicken feet protein is composed of poorly soluble protein and has a low level of digestibility when consumed by humans. Chicken feet protein can be explored to produce functional micronutrients. These proteins have the potential to produce hydrophobic amino acids capable of donating hydrogen ions in reducing free radicals, such as DPPH (Lin et al., 2010). The hydrolysis process is expected to provide natural antioxidant peptides and exert higher antioxidant properties. Enzymatic hydrolysis can be chosen because it is more effective at targeting broken proteins and is safe for manufacture of food, cosmetic and pharmaceutical fields. Papain enzyme is a commonly used exopeptidase enzyme group because it can avoid substrate damage, very easy procurement, and is relatively inexpensive. Enzymatic hydrolysis is the use of enzymes in hydrolyzing proteins to produce hydrolysate products that are protected from changes and product damage. Several enzymes can be used to break the protein polypeptide chain, including the papain enzyme. This enzyme is widely available in the market. Several studies also mention that the papain enzyme is superior in producing antioxidant peptides. Harnedy et al. (2017) stated that compared to endogenous enzymes, exogenous proteases are more efficient in hydrolysis and producing controlled hydrolysis products.

The types of food proteins and enzymes directly affect the structure of peptides antioxidants. amino acid residues such as tyrosine, methionine, lysine, tryptophan, and cysteine are often found in polypeptides that have strong antioxidant activity. Cysteine contains a thiol group that can react directly with free radicals, which is important for peptide oxidant activity (Eftekharzadeh et al, 2010; Najafian and Babji, 2015).

Conclusions

Protein concentrates from chicken feet, intestines, and filleting waste can be obtained through precipitation using ammonium sulfate, while plasma using acetone. Blood plasma protein concentrate shows the highest protein concentration. The highest hydrolysis results were demonstrtaed by hydrolyzing blood plasma proteins with hydrolyzed probiotic protease enzymes. The highest antioxidant activity was shown by the chicken feet protein hydrolysate

which was hydrolyzed using papain enzyme and incubated for 6h.

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