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Characterization of Functional Groups of Bioactive Peptides from Chicken Feet as Antioxidant

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***Abstract*—This study conducted to characterization of functional groups of bioactive peptides from chicken feet as antioxidant. The research method was experimental and laboratory exploration. Optimization the bioactive peptides from chicken feet as antioxidant through pH and temperature, hydrolysis of papain and ultrafiltration. The variables observed included proximat analysis of chicken feet powder, colour of dissolve protein, antioxidant activity and functional group characteristics of bioactive peptides. The results of this study indicate an increase in antioxidant activity by bioactive peptides of pH treatment, temperature, hydrolysis of papain enzyme and ultrafiltration process. The functional group characteristics also exhibit diversity with each other.**

***Keywords*— Chicken feet extract; Antioxidant Activity; FTIR; Functional Groups; Ultrafiltration**

I. INTRODUCTION

Shank is part of chicken feet that have been used only as a variety of processed foods. More than 40% of chicken feet protein was composed of a protein that is difficult to dissolve and has a low level of digestibility when consumed by humans. Chicken feet protein potentially produces hydrophobic amino acids couses capable of donating hydrogen ions in reducing free radicals such as DPPH [1].

The process of extraction of the bioactive peptide from chicken feet is influenced by several factors such as pH and temperature. Optimization of bioactive peptide function as a source of antioxidants can be done with several methods such as through hydrolysis. The hydrolysis process is expected to provide natural antioxidant peptide and exert a higher antioxidant properties. Enzymatic hydrolysis may be chosen because it is more effective at the target protein that is broken and safe for the utilization of its products in the fields of food, cosmetics and pharmaceuticals. Papain enzyme is a class of exopeptidase enzymes that are often used because it can avoid substrate damage and the procurement is very easy and relatively cheap. So it is necessary to optimize the antioxidant activity of peptides from chicken feet through hydrolysis of papain enzymes.

The ultrafiltration method is also potentially used in optimizing antioxidant activity. Hydrophobic amino acids are mostly obtained from peptides with molecular weight less than 10,000 daltons (BM <10 kDa). The use of ultrafiltration system or often called molecular weight cut off is very potential in producing active peptide (BM <10 kDa). The antioxidant activity of a peptide is also determined by the type of amino acid side chain of the constituent. The functional groups play a role in donating hydrogen ions in capture DPPH. This study aims to identified the characteristics of the functional groups with FTIR.

II. MATERIALS AND METHODS

A. Materials

The Chicken plug comes from broiler type Lohman 202 strain of PT.Panca Patriot which comes from UPT Agri Science Technopark Islamic University of Lamongan. $(NH_4)2CO_3$ (Merck), Ethanol 40% (Merck), Petroleum ether (Merck), Anhydrous $NaSO_4$ (Merck), $CuSO_4$ (Merck), H_2SO_4 (Merck), Zn (Merck), NaOH 40% (Merck), HCL 0.1 N (Merck), methyl red indicator 15 (Merck), 0.1 N NaOH (Merck), Buffer phosphate (0.2 mmol / L, pH 7.2) (Merck), Papain Dry Extract (Sigma), Aquaculture (Merck), Reagents Bradford (Sigma), Bovine Serum Albumine (BSA) (Sigma), Glacial Acetic Acid (Merck), and 0.1 mM DPPH (Sigma) solution.

B. Instruments

Analytical balance (Mettler PM 200 Switzerland), Vortex (Janke 43480), refrigerated (Panasonic), 3.5 cm magnetic stirrer (Labinco), Refrigerated Micro 22 R (Hettich) centrifuge, and CG.818T (Schott Gerate) pH meter, Water type J.26 digital bath, Shaker (Hettich), Blender (Panasonic), Oven (Hettich), eppendorf, micropippet 10µl - 1000 µl (Hamilton syringe), beaker glass (Pyrex), blue tip, yellow tip, micropippet 10µl - 1000 µl (Hamilton syringe), Kuvet, UV-2100 spectrophotometer (Unico), *amicon millipore* ultra-0.5. Infrared Fourier transform spectroscopy (FTIR).

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C. Sample Preparation

Extraction of bioctive peptides of chicken feet using a combination an modification [2] [3] [4]. Dry chicken feet extract taken as much as 20 grams and then added 80 ml of phosphate buffer (0.2 mmol / L, pH 7.2) and then in homogenizer / vortex at 6,000 rpm for 10 seconds 3 times [3]. The first treatment was used pH 6.8, pH 6 and pH 4 at temperature 25^0C, 50^0C, 65^0C. The second treatment wa used hydrolysis process was done with the addition of papain enzyme 2% concentration with incubation time 48 hours. The third treatment was used ultrafiltration with a micon millipore 0.5 [2],Then heated 50^0C for 10 minutes with hot magnetic stirrer [2]. Maceration uses the shaker with a speed of 100 rpm for 24 hours [4]. The dissolution was carried out using a 2M (NH_4) $2CO_3$ (1: 4) ammonium bicarbonate solvent using the maceration method [4]. Then centrifugation was done at 5000 x g at 4oC for 15 minutes. The extracted supernatant was dried by using a freeze dryer [4]. Drying using a freeze dryer aims to remove solvents still attached to the supernatant and to extract the obtained results in powder form to be stored for longer analysis purposes.

D. Proximat Analysis

Water content measured by AOAC methode (2005: 950.46), Fat content measured by AOAC methode (2005: 960.39), Total Protein content measured by AOAC methode (2005: 992.15), Ash Content measured by the AOAC methode (2005: 992.19), carbohydrates were measured by the AOAC methode (2005: 992.8).

E. Colour measurement

The Color Test was determined using Chromameter [5] with illuminant C for daylight using standard BaCl2. the tristimulus coordinates system L., a8, and b * (CIE Lab Color scale) are used in this measurement. The value of L means brightness and its value ranges from 0 = light and 100 = dark. The color at the center point (a * = 0, b * = 0) is achromatic (gray). On the flat axis, positive a * means red-purple, whereas negative a * means bluish green. As for the vertical axis, positive b * yellow and negative b * b * means blue. Furthermore, C8 is a chroma metric, which correlates with the saturation of the color calculated by the formula ($C8 = (a^{*2} + b^{*2})^{0.5}$).

F. Measurement of Antioxidant Activity

Analysis of antioxidant activity was performed by DPPH method [6]. The sample was taken as 0.5 mL, Added 2.7 mL DPPH 20 ppm then shaken, incubated in dark conditions for 30 min, Measured absorbance at 517 nm wavelength, DPPH as negative control measured its absorbance at 517 nm wavelength, Calculated% Antioxidant activity with

$$\% AA = \frac{\text{Abs. DPPH - Abs. Sample}}{\text{Abs. DPPH}} \times 100$$

G. Determination of Functional Groups

Samples were prepared by weighing 0.01 grams and homogenized with 0.1 grams of potassium bromide (KBr) anhydrous with an agate mortar. Then in a press with a vacuum hydraulic using a pressure of 1.2 psi to obtain a transparent pellet ready to be analyzed with FTIR.

3. RESULTS AND DISCUSSIONS

A. Chemical Composition of Chicken feet Powder

Chemical composition of chicken feet is presented in the table 1.

Table 1. The Proximat Content of Chicken Feet Powder

Parameter	Value
Protein (%)	42,68±2,61
Fat (%)	25,50±1,19
Water content (%)	3,90±0,04
Ash content (%)	11,29±1,02
Carbohydrate (%)	16,63±1,38

Based on table 1 it is known that the highest protein content value in chemical composition of chicken feet is 42,68%, followed by fat content, Carbohydrate and Ash. This shows that the protein content of broiler chicken feet is not reduced due to the drying process and smoothing of the sample. These protein levels are also comparable to previous research with a range of protein content values of 42-47% [4] [7].

Identification of chicken feet protein level is very important for the next stage is as early data to know the dissolved protein concentration and peptide extract that serves as an antioxidant. Other studies have shown that chicken feet protein levels are mostly composed of collagen with a rendement value of 18-20% by weight [8]. Hydrolysis of collagen and non collagenic proteins in chicken feet has the potential to produce a variety of peptides and free amino acids that function as antioxidants [9].

Water content of chicken feet powder achieved 3.90% indicated the optimum drying process was done. This aims to keep the sample maintained its quality when used in a long period of time. The presence of low water in a food will cause the structure of the protein more closed so that the potential denatured and the loss of bioactive peptide will be smaller [10].

B. Colour Analysis of Dissolve Protein

Colour measurement is important as an indicator of quality of protein extract obtained. The results of colour measurement are presented in Table 2.

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Table 2. Result of Colour Measurement

Sampel	L*	C	Hue (°H)
pH 6,8 (control) , Temperature 25^{0}C (Control)	83.10	17.07	-88.51
pH 6, Temperature 25^{0}C (Control)	83.88	16.94	-88.45
pH 4, Temperature 25^{0}C (Control)	85.08	16.94	-88.39
pH 6,8 (control), Temperature 50^{0}C	83.79	17.58	-88.68
pH 6, Temperature 50^{0}C	85.82	17.61	-88.69
pH 4, Temperature 50^{0}C	85.83	17.57	-88.65
pH 6,8 (control), Temperature 65^{0}C	85.82	18.46 ^{b*}	-88.86
pH 6, Temperature 65^{0}C	85.82	18.44	-88.89 ^{b*}
pH 4, Temperature 65^{0}C	86.72 ^{b*}	18.35	-88.82

Description: Superscript (b*) in the same column shows highest value but not significantly different (P>0.05)
The negative Hue (°H) indicates a tendency t ward a blue-green colour; L* value range 0 (black) to 100 (white); C Value indicates the intensity of the colour from low (faded) to high (concentrated).

Based on result of analysis of variance indicate that in general there is no difference of influence (P>0,05) variation of pH and temperature treatment to colour of protein solutions. Based on the colour observations confirm that no damage to protein extracts due to the treatment of pH and different temperatures. The use of weak acids such as acetic acid does not affect the form and swelling of collagen proteins that are mostly present in the skin of chicken legs [11]. The conformation or the form of triple-α-helix protein collagen will remain and there is no transition of helix-coil form ation on heating at temperature <60^{0}C [12].

The value of L * obtained ranges from 83.10 - 86.72 means the colour of the solution tends to be clear white. C values obtained ranging from 17.07 to 18.46 indicate very low colour intensity (faded) instead of concentrated. The value of L* ranges from 0 (black) to 100 (white); C values show the colour intensity from low (faded) to high (concentrated) [13].

However, C value there is a significant effect difference (P> 0,05) due to temperature factor. The effect of temperature on the mean of C value is presented in table 3.

Table 3. *Duncan* test of effect of temperature on C value

Value of C				
	Temperature	N	Subset	
			1	2
Duncan	Temperature 25^{0}C (control)	12	16,9825^a	
	Temperature 50^{0}C	12	17,5875^{ab}	17.5875^{ab}
	Temperature 65^{0}C	12		18.4142^b

Description: Superscript (b) in the same column shows the highest value with significance (P<0.05)

Table 3 shows that there was a change in color intensity (C value) (P <0.05) in solutions of chicken feet protein extract. The highest intensity is seen in the temperature 65^{0}C with C value of 18.4142. This means that the solution tends to be more concentrated than other temperature treatments. This condition confirms earlier variables that at the 65^{0}C temperature tends to cause a protein in gelatination process.

C. Functional Group Characteristics

In this research, FTIR testing is done on 3 selected samples in each stages of research. FTIR spectra obtained on measurements of protein and chicken peptide samples were presented in Fig. 1.

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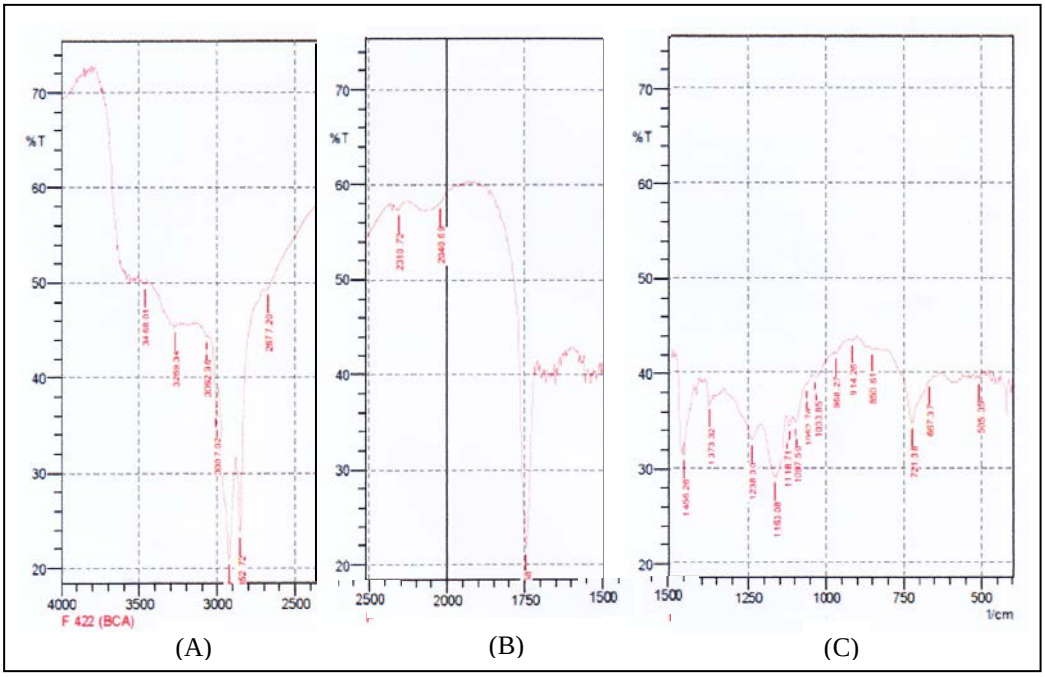

Fig. 1. FTIR spectra of Bioactive Peptide Chicken feet on Treatment: (A) pH 4 Temperature 50^0C, (B) Papain 3% at 36 hours, (C) Ultrafiltrate

Fig. 1 shows that there are differences in absorption of wave numbers in each sample. This indicates that the active groups present in each sample also differ from each other. Based on the existing FTIR spectra we can estimate the functional groups of each sample by looking at the wave number at the apex of the absorption then adjusted to the previous research source. These forecasts are presented in Table 4.

Table 4. Cluster Function Groups on Bioactive Peptides of Chicken Feet

Sample	Wave Numbers at the Absorption Peak (cm^{-1})	Forecast Cluster Function	Refferens
(A)	3466,01	N-H stretching (stretching) of a paired primary amine group or a secondary amide group of its own	[14]
	3269,34	N-H stretching of the secondary amide group	[14]
	3062,96 - 3007,02	NH2 simetric stretching in the primary amide group	[14]
	2677,20	N-CH_2 *stretching*	[15]
(B)	2310,72	*Overlapping (overlapping) C-H stretching on chains CH_2-, -CH_3*	[15]
	2040,69	*Aromatic isonitrile* –N=C *stretching*	[14]
(C)	1456,26	C = O stretch of hydrogen bond in pairs with COO-, NH_2 deformation, CH_2 bending (pembengkoan)	[16]
	1373,32	Aromatic C-N *stretching, C-O-C antisimetric stertching*	[14], [16]
	1238,30	Aliphatic C-N *stretching*	[15]
	1162,08 - 1033,85	The amino-free group is -NH_2	[16]
	966,27 - 850,61	NH_2 wagging and twisting, C-O-C bridge	[16]
	657,37 - 505,35	NH_2 wagging, cluster N-H wagging on secondary amide, -OH bending	[16]

Based on Table 4 it is known that the sample A which is the result of the treatment of pH 4 at a temperature of 50^0C is dominated by the active amide group (R-NH_3) in both primary and secondary forms. The number of amide activated groups also shows that in the treatment sample pH 4 and temperature 50^0C there are still many collagen proteins. NH_2 symmetric stretching in the primary amide group is an imide residue of the ß-sheet structure which is a typical collagen group [14].

The primary amide group one nitrogen atom is connected to one carbon atom, whereas in the secondary amide group a nitrogen atom is connected with two carbon atoms [17]. Amides are formed when the hydroxyl group of the carboxylic acid (C (OH)) is replaced by a compound such as ammonia (NH_3) or an amine. So the nature of hydrophobicity is low and less soluble in water. This is consistent with the result of the previous variable that the protein solubility rate of chicken chick protein extract from the treatment of variation of pH and different temperature is not as big as the result of enzymatic hydrolysis result.

In sample B which is the result of active peptide extract with 3% papain enzyme treatment with 36 hours of curing time indicates the presence of alkyl chains (CH) and nitrogen groups such as Aromatic isonitrile -N = C stretching. The activity of the papain enzyme is capable of breaking the covalent bond in the polypeptide to form a new group. Nitrogen is a very easy atom to bind with other atoms primarily carbon atoms. Isonitrile group -N = C is an isomer whose molecule has the same structure but different functions. Th group is very easy to interact and release the hydrogen ions that are bound. It is underlying that the extract samples of enzymatic treatment results have high antioxidant activity [18]. These results were consistent with the previous variables that the 3% papain enzyme treatment with 36 hour curing duration resulted in the highest antioxidant activity of 55.1% / mg protein.

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In sample C which is a result of ultrafiltration with low molecular weight indicates the existence of variations of functional groups. The presence of the NH_2-free amino group indicates that the peptide bond termination of the undetected enzymatic process in the previous sample is due to very little concentration. In this sample C has been through the concentration process so that the cluster appears. In this sample there is also a C = O stretch bond, a paired hydrogen bond with COO-, NH_2 deformation, and CH_2 bending indicating a possible peptide comprising a hydrophobic amino acid [19]. There are also NH_2 wagging bonds, N-H wagging groups in secondary amides, and -OH bending showing the same. The presence of the NH_2 wagging and twisting clusters and the C-O-C bridge makes this peptide rotatable and wobbly so that it reacts with other atoms [18].

The properties of the functional groups present in the ultrafiltrate sample indicate that protein is a very simple peptides and has a small shape, size and molecular weight. The conformation makes the peptide actively donate hydrogen ions so that it can reduce DPPH [20]. This is evidenced by the results of previous variables that the highest antioxidant activity is achieved from the ultrafiltration of 60.92% / mg protein.

4. CONCLUSIONS

The conclusions of this study indicate an increase in antioxidant activity by bioactive peptides of pH treatment, temperature, hydrolysis of papain enzyme and ultrafiltration process. The functional groups characteristics also exhibit diversity with each other.

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