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# Research Article
# Effect of pH and Temperature on Characteristics and Antioxidant Activity of Chicken Feet Protein

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## Abstract

**Background and Objective:** The micronutrients of Chicken feet can be explored and developed to improve its economical and functional value. The principles of degradation and dissolution of proteins depend by several factor as pH, temperature. The Study aimed to determine the effect of different pH and temperature on characteristic and antioxidant activity of chicken feet protein. Research conducted for 6 months at February 3, 2017 until August 15, 2017 and location of study in Animal Product Resources Laboratory of University of Brawijaya. **Materials and Methods:** The material chicken feet obtained from *Lohman 202* broiler strain of *UPT Agri Science Technopark* of Lamongan Islamic University of Indonesia The research method was experimental with Factorial Completely Randomized Design. Treatments included pH (control (6,8) , pH 6 and pH 4) and temperature (control (25°C), 50°C and 65°C) were repeated four times.. The variables observed were microstructure, dissolved protein concentration and antioxidant activity. **Results:** The results of this study indicated the interaction of pH 4 and temperature of 50°C resulted in the highest Dissolved protein concentration amount 1.15 mg $mL^{-1}$ and the highest antioxidant activity amount 46.55%. Antioxidant activity tends to increase as well as the decrease of pH. **Conclusion:** The treatment of pH and temperature variations in chicken feet protein extraction gave a difference of influence ($p<0.05$) to the concentration of solubility protein and antioxidant activity.

Key words: Chicken feet, DPPH, protein microstructure, peptides bioactive, micronutrients, antioxidant activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The micronutrients of chicken feet can be explored and developed to improve its economical and functional value. Currently, the environmental pollution causes exposure to very high free radicals, so it takes a good source of antioxidants, especially from natural ingredients as well as a source of amino acids. the consumer needs in the fields of food, pharmaceuticals, cosmetics and others. They were assumed that antioxidant compounds from natural ingredients are very important. Antioxidant peptides can be bind of metals and potensial hidrogen donor to stop the chain of free radical reactions in the body[1]. It has never been studied about the antioxidant activity associated with dissolved protein in chicken feet.

The principles of degradation and dissolution of proteins depend by several factor as pH, temperature, type and solvent concentration and extraction a. The optimization of protein solubility from chicken feet has been done using an ammonium bicarbonate ($(NH_4)_2CO_3$) solvent with different maceration durations[2]. Never has been studied the effect of pH and temperature on the optimal dissolved protein and its correlation with antioxidant activity obtained. This study aimed to determine the degradation of chicken feet at different pH and temperature to proximate content, colour, micro structure, dissolved protein concentration and the activity of antioxidant.

## MATERIALS AND METHODS

**Materials:** Chicken feet obtained from Lohman 202 broiler strain of UPT Agri Science Technopark of Lamongan Islamic University of Indonesia. ($(NH_4)_2CO_3$) (Merck), Ethanol 40% (Merck), petroleum ether (Merck), $NaSO_4$ anhidrat (Merck), $CuSO_4$ (Merck), $H_2SO_4$ (Merck), Zn (Merck), NaOH 40% (Merck), HCL 0.1 N (Merck), indicator of metyl red 15 (Merck), NaOH 0.1 N (Merck), phosphate buffer (0.2 mmol $L^{-1}$, pH 7.2) (Merck), aquadest (Merck), Bradford reagent (Sigma), Bovine Serum Albumin (BSA) (Sigma), Asam asetat glasial (Merck), DPPH 0.1 mM (Sigma) and scanning electron microscopic (SEM) type JSM-6360LA.

**Duration and location of research:** Research conducted for 6 months at February 3, 2017 until August 15, 2017. Location of study in Animal Product Resources Laboratory of University of Brawijaya, Indonesia.

**Sample preparation:** Preparation was done based on modification to the method ever done[2], sample was broiler chicken strain Lohman 202, age range 34-36 days and body weight 1.8-1.9 kg, than aging for 8 h at a temperature of 16°C. Further done the sorting and cleaning the chicken feet from the nails, outer shells and dirt attached on the claw with the aim of getting raw materials clean and good. Further more were pressure cooker for 5 min not longer than the method of Widyaningsih *et al.*[2] so as not to damage the components of bioactive compounds on chicken feet. The next step is wet milling done using a dried blender, chicken feet samples are ground in half-wet conditions. It aimed to expand the width of the surface and the uniformity of the sample to speed up the drying process. Then drying in the oven at 40°C for 24 h aimed to reduce the water content so that when the extraction process can be obtained optimum protein solubility. Then done dryed milling with dried blender and filtered to obtain a uniform checker powder (60 mess) and fine texture.

**Protein extraction:** The protein degradation and extraction used a combination of methods of Xing *et al.*[3] and Widyaningsih *et al.*[2] were modified. Sample of chicken feet powder was taken as much as 20 g and then added 80 mL of phosphate buffer (0.2 mmol $L^{-1}$, pH 7,2) then were homogenated with speed 22000 rpm for 10 sec counted 3 times[3]. Different pH level treatments were performed by adding acetic acid ($CH_3COOH$) to achieve the determined pH. Then a different temperature treatment was performed when stirring with a hot magnetic stirrer for 10 min. Further done maceration used shaker with a speed of 100 rpm for 24 h [2]. The dissolution was carried out using a 2M ammonium bicarbonat ((NH4) $2CO_3$) solvent with ratio (1:4) used the maceration methode[2]. Then centrifugation was done at 5000 rpm at 4°C for 15 min. The extracted supernatant was dried by using a freeze dryer. 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.

**Dissolved protein concentration measurement:** Measurement dissolved protein concentration done be Bradford Method because it is the fastest and most widely used. Bradford method was used to measure total protein concentration by colorimetry in solution using a Coomassie Brillant Blue (CBB) dye as an indicator. The CBB binds to proteins in an acidic solution giving a blue color, because the dye is protonated by the amino group of lysine and the tryptophan further binds to the hydrophobic area of the protein, thus changing its color to blue. The color change into the base of the solution can be measured its absorbance using

a visible spectrophotometer at a wavelength of 465-595 nm using standard Bovine Serum Albumin (BSA) solution[4].

**Microstructure analysis:** Microstructure analyzed with scanning electron microscopy (SEM) type JSM-6360LA . The sample is attached to set holder with double adhesive, then coated with gold metal in vacuum. Further more, the sample was taken in place in the SEM, then the topographic image was observed and magnification was 5000 times[5].

**Measurement of antioxidant activity:** Analysis of antioxidant activity was performed by DPPH method 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 (%)[6]:

$$\text{Antioxidant activity} = \frac{\text{Absorbance DPPH-Absorbance sample}}{\text{Absorbance DPPH}} \times 100$$

**Statistical analysis:** The resulted data will be analyzed with analysis of varians, if there is any difference of effect with significance ($p<0.05$) will be continued with duncan test using SPSS software application version[7] 16.0.

## RESULTS

In this research, the data of dissolved protein concentration of chicken feet powder were treated with several different pH and temperature variations and the top solution (A) and bottom solution (B) were taken. The average treatment outcome was presented in Table 1. Based on the results of analysis of variance shows that in the sample of the upper fraction solution (A) there is a significant difference of ($p<0.05$) pH and temperature factor either partially or its interaction to dissolved protein concentration. The results were not different in the samples taken from the fractional solution at the bottom (B). However, interaction factor of pH and temperature factor did not give significant effect ($p> 0.05$) to the same variable. This was due to the low concentration of dissolved protein obtained from the solution at the bottom, so the statistical calculation is not enough to give a real difference.

The highest concentration of 1.11 mg $mL^{-1}$ was obtained from the treatment of pH 4. This showed the optimum pH of the occurrence of protein precipitation as well as the isoelectric conditions achieved at the pH 4. The effect of temperature on the dissolved protein concentration has a nonlinear pattern, it is known that at room temperature (control) at 25°C the value of protein concentration obtained by 1.08 mg $mL^{-1}$ of equilibrium rose significantly in the treatment of 50°C temperature of 1.12 mg $mL^{-1}$ and decreased again significantly at the 65°C temperature treatment of 1.06 mg $mL^{-1}$.

Results Scanning Electron Microscopy (SEM) Dissolved protein of chicken feet that has been freeze dried was presented in Fig. 1. Based on Fig. 1 it was known that the shape and size of the obtained protein still looks large and complex. The size of its diameter ranging from 385-495 µm was shown in Fig. 1a. This indicated the number of collagen proteins more than other proteins.

A cross-sectional cross-section in which there is considerable fiber (fibrillary) collagen between the other components was shown in Fig. 1b. The magnification of SEM as shown in Fig. 1d which shows the presence of a smooth surface indicating the presence of non-collagen proteins present in chicken feet.

The mean value of antioxidant activity testing by DPPH method on chicken feet protein extract treated with different pH and temperature was presented in Table 2. Based on Table 2 and the results of the analysis of variance it is known that in the sample of the solution taken at the top (A) there was a significant effect difference ($p<0.05$) pH factor and the

Table 1: Effect of pH and temperature on dissolved protein concentration

| | Temperature (°C) | | | |
|---|---|---|---|---|
| pH | Control (25) | 50 | 65 | Average±SD (mg $mL^{-1}$) |
| Control 6,8 (A) | 1.07±0.01 | 1.08±0.05 | 1.08±0.08 | 1.07±0.01$^{a}$ |
| Control 6,8 (B) | 0.95±0.01 | 0.93±0.02 | 0.98±0.03 | 0.95±0.02$^{B}$ |
| 6 (A) | 1.08±0.07 | 1.14±0.05 | 1.03±0.09 | 1.09±0.06$^{a}$ |
| 6 (B) | 0.94±0.02 | 0.93±0.01 | 0.97±0.03 | 0.94±0.02$^{A}$ |
| 4 (A) | 1.10±0.08 | 1.15±0.06 | 1.08±0.00 | 1.11±0.03$^{b}$ |
| 4 (B) | 0.93±0.02 | 0.93±0.02 | 0.93±0.02 | 0.93±0.00$^{A}$ |
| Average±SD | 1.08±0.02$^{a}$ | 1.12±0.04$^{b}$ | 1.06±0.03$^{a}$ | |
| (mg/ml) | 0.94±0.01$^{A}$ | 0.93±0.00$^{A}$ | 0.96±0.03$^{B}$ | |

$^{a,b}$Different superscript (a,b) on the same row showed significant differences ($p<0.05$), $^{a,b,A,B}$Different superscript on the same column showed significant differences ($p<0.05$), A: Upper fraction solution, B: Lower fraction solution

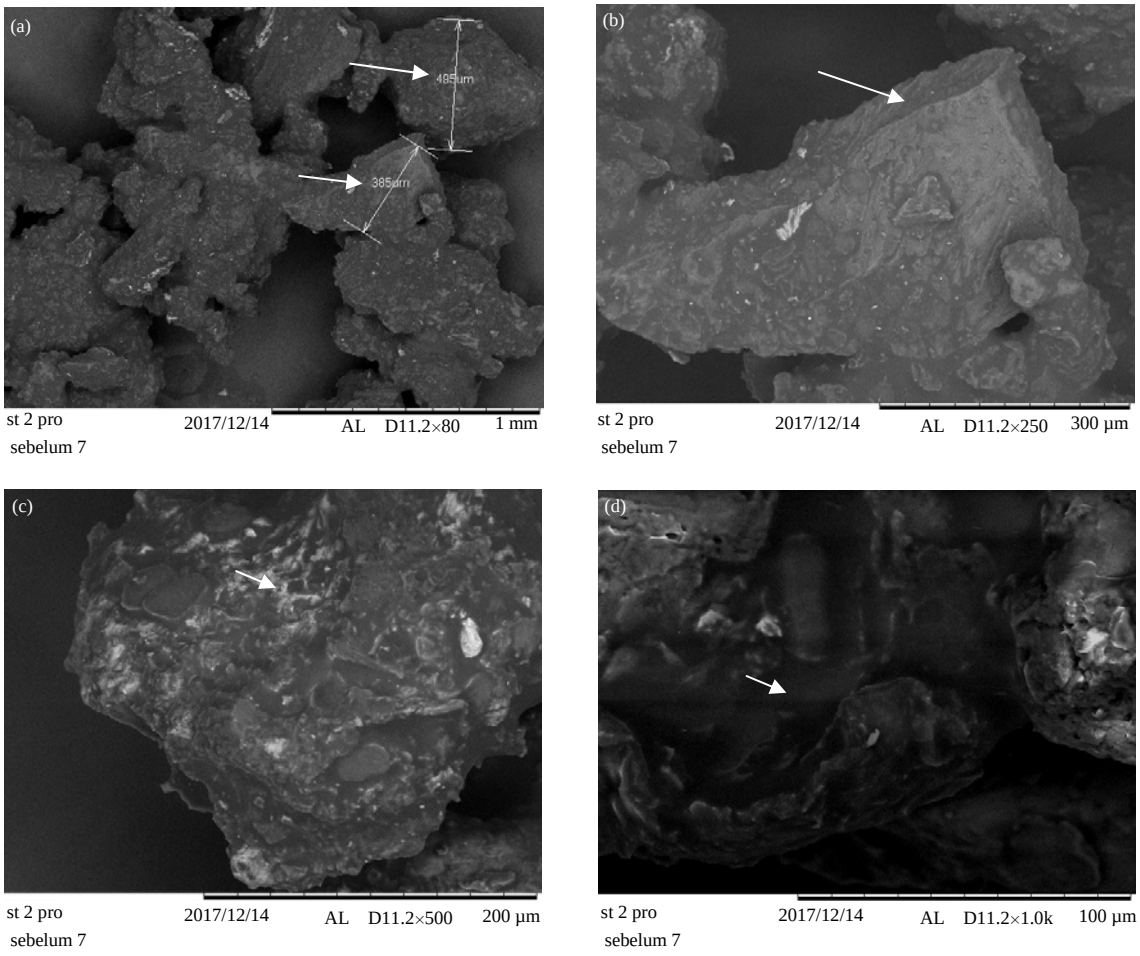


Fig. 1(a-d): Scanning electron microscope graph of chicken feet protein extract at variation of pH and temperature

Table 2: Variation of pH and temperature on antioxidant activity

| Samples | Dissolved protein concentration (A) (mg mL$^{-1}$) | Antioxidant activity (A) (%) | Dissolved protein concentration (A (B) (mg mL$^{-1}$) | Antioxidant activity (B) (%) |
|---|---|---|---|---|
| pH 6,8 , Temperature 25°C | 1.07±0.03 | 30.53±6.51 | 0.95±0.01 | 9.02±1.23 |
| pH 6, Temperature 25°C | 1.08±0.02 | 30.23±8.57 | 0.93±0.02 | 10.58±1.53 |
| pH 4, Temperature 25°C | 1.10±0.01 | 40.31±4.18 | 0.98±0.03 | 11.05±1.76 |
| pH 6,8, Temperature 50°C | 1.08±0.02 | 40.83±4.85 | 0.94±0.02 | 5.06±1.35 |
| pH 6, Temperature 50°C | 1.14±0.04 | 39.92±3.25 | 0.93±0.01 | 13.07±1.78 |
| pH 4, Temperature 50°C | 1.15±0.05[b] | 46.55±2.66[b] | 0.97±0.03 | 13.58±1.75 |
| pH 6,8, Temperature 65°C | 1.08±0.02 | 40.34±3.68 | 0.93±0.02 | 14.09±0.87 |
| pH 6, Temperature 65°C | 1.03±0.01 | 36.47±1.29 | 0.93±0.02 | 10.76±1.21 |
| pH 4, Temperature 65°C | 1.08±0.02 | 39.78±3.50 | 0.93±0.02 | 7.68±1.86 |

[b]Superscript shows the highest value significantly ($p<0.05$), A: Upper fraction solution, B: Lower fraction solution

temperature factor partially on the antioxidant activity, but not so with the interaction of these two factors. The interaction of pH and temperature factor did not give significant effect ($p> 0.05$) to antioxidant activity. The value of antioxidant activity obtained ranged from 30.23-46.55%. It was proportional to the value of protein concentration obtained.

Antioxidant activity tends to increase as well as the decrease of pH. The highest antioxidant activity of 46.55% was obtained from interaction of pH 4 and 50°C. The highest antioxidant activity was obtained at 50°C.

## DISCUSSION

The results and the above analysis were in accordance with the hypothesis that the dissolved protein concentration was strongly influenced by the pH and

temperature factors when the extraction is done. The pH conditions will affect the ionic strength and hydrogen bond in the protein[8]. While the temperature factor causes changes in primary protein structure both reversible and non reversible[9]. So the dissolution of chicken feet protein occurs due to differences in pH and temperature that cause changes in ions, bonds and protein structure.

The solubility of protein is strongly influenced by the interaction between isoelectric pH and ion of the solvent of salt. The highest absorption intensity of collagen proteins is obtained from the pH range of 4-5.5 in the addition of acetic acid and sodium phosphate solvent[10]. This confirmed current research, which to achieve the prescribed pH treatment is to use acetic acid and solvent sodium bicarbonate. The results were not different, the highest protein concentrations achieved at pH 4. The conditions of the isoelectric pH, the protein is in neutral charged, so that it will be easily withdrawn by the solvent and settling salt ions[11].

The highest concentration is obtained from the temperature treatment of 50°C because optimally process of denaturation of protein primer structure. Warming can lead to changes in the primary protein structure and even breakdown of the protein covalent bonds making it more soluble by the presence of salt[12].

At the temperature of 65°C there is a decrease in the concentration of dissolved proteins because begins the gelatinating process. Warming above 65°C causes some collagen proteins in chicken feet to have melting point[9], so that there is an irreversible shortening of the bonds and chains of tropocollagens called gelatinations[13].

The size is already smaller than the diameter of skin collagen and animal bones ever investigated range from[14] 500-880 μm. This condition indicated that the use of pH and partial temperature has broken the chain of chicken feet protein polypeptide until the size and shape is smaller.

A cross-section in which there is considerable fiber (fibrillary) collagen between the other components was shown in Fig. 1b. Data shows that collagen is formed from fibrils and tropocollagen to form a triple α helix structure to form a strong enough network matrix[15], while Fig. 1c showed that the compact form and protein density obtained are likely to be due to binding another major component of carbohydrates. The interactions of proteins and carbohydrates in animal tissues will form peptidoglycan bonds that produce solid and hard particles[16].

The magnification of SEM as shown in Fig. 1d showed the presence of a smooth surface indicating the presence of non-collagen proteins present in chicken feet. Non-collagen proteins are more easily dissolved at the isoelectric point conditions of pH 5-6, whereas collagen proteins begin to dissolve at 45°C due to the release of hydrogen and covalent bonds causing changes in the helical structure toward the coil transition[17]. The amount of dissolved protein is still low because the pH and temperature factors have not been able to completely break the polypeptide chain. These results underlie the enzymatic hydrolysis process carried out and discussed in subsequent chapters to allow more dissolved peptides to be obtained.

The higher the concentration of protein the greater the antioxidant activity. Proteins and peptides can function as antioxidants if they have hydrophobic active groups in them. Proteins containing peptides and amino acids rich in hydrophobic active groups are capable of donating hydrogen ions to reduce 2.2-diphenyl-1-picrylhydrazyl (DPPH) so that the free radical compounds can be inhibited[18]. Differences in the value of antioxidant activity obtained in this study indicated that different pH and temperature factors affect the hydrogen bonds and covalent bonds in chicken feet protein extracts so that the hydrophobic group optimization obtained also different between treatments with each other.

The highest antioxidant activity of 46.55% was obtained from interaction of pH 4 and 50°C. This was due to the protein obtained at the isoelectric point conditions, where the negative and positive charge attraction is in the same position. As a result, hydrogen ions in the hydrophobic group were easily released and become donors in the DPPH reduction process. pH <5 the protein condition is neutral charged so it is readily hydrolyzed by other compounds[19]. Which further described increased antioxidant activity is determined by the ability of proton donation and metal binding of a peptide to DPPH[20].

The highest antioxidant activity is obtained from the temperature 50°C as shown in Fig. 1a. This is due to the heating in temperature 50°C being able to break down the complex collagen structure into a simple polypeptide. The warming can make the hydrogen bonds of a protein to become unstable and thus more easily become protons and bind to other compounds in the presence of DPPH free radicals[21].

Antioxidant activity back down at the temperature treatment of 65°C. Decrease in antioxidant activity due to

the occurrence of gelatinations on chicken feet protein. Temperature 65°C couses the transformation of collagen structure into gelatin takes place, where the heating causes the fibrils chain to be hydrolyzed into tropocolagen[22]. Gelatinating proteins can occur when the temperature reaches the melting point[12]. The process of gelatinating collagen proteins causes the peptide component and amino acids present to lose hydrophobic groups, consequently the decrease antioxidant activity[23].

## CONCLUSION

The treatment of pH and temperature variations in chicken feet protein extraction gave a difference of influence ($p<0.05$) to the concentration of dissolved protein concentration and antioxidant activity. Antioxidant activity can still be increased through collagen hydrolysis in chicken feet, it is necessary to further study the use of enzymes in enhancing antioxidant activity.

## SIGNIFICANCE STATEMENT

The study discovered the effect of different pH and temperature on characteristic and antioxidant activity of chicken feet protein. The variables observed were microstructure, dissolved protein concentration and antioxidant activity. The treatment of pH and temperature variations in chicken feet protein extraction gave a difference of influence ($p<0.05$) to the concentration of solubility protein and antioxidant activity. This study will help the researcher to uncover the critical areas of chicken feet protein that many researchers were not able to explore. Thus a new theory on antioxidant activity may be arrived at.

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