Isolation of collagen from chicken feet with hydro-extraction method and its physico-chemical characterisation

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Abstract. Chicken slaughterhouses generate a large volume of chicken feet as a waste or by product. Nowadays, the chicken feet have not been optimally utilised. One of the ways to increase added value of the chicken feet is to utilize collagen contained in it. The objectives of this study were to obtain the best treatments in pre-treatment and hydrolysis stages of collagen extraction from chicken feet using hydro-extraction method, and to characterise the physico-chemical properties of the isolated collagen. Collagen extraction consisted of three stages of pre-treatment, hydrolysis, and hydro-extraction. Pre-treatment stage used NaOH concentrations of 0.5, 1, and 2 M, and soaking times of 2, 4, 6, and 8 hours. The hydrolysis used CH3COOH concentrations of 0.1, 0.3, and 0.5 M, and soaking times of 1, 2, and 3 hours. The best condition of pre-treatment was 0.5 M NaOH concentration with 6 hours of soaking time, while the best condition of hydrolysis was 0.5 M CH3COOH concentration with 1 hour soaking time. FTIR spectroscopy showed that the isolated collagen amino acids consisted of amide A, amide B, amide I, amide II, and amide III groups. The dominant amino acid contents were glycine, L-serine, L-proline, L-glutamic acid, and L-alanine.

Key words: chicken feet, hydro-extraction, hydrolysis, collagen, pre-treatment.

1. Introduction
Collagen is an extracellular matrix of connective tissue which is abundant, about 30% of the total protein. Collagen consists of 26 types; one of the collagen types is type I collagen which is the main component in tendon, bone and skin tissue [1]. Type II collagen is present in the cartilage and the conjunctiva of the eye. Type III collagen is found in blood vessels. About 80-90% of collagen types that are in the human body are type I, II, and III. Differences in collagen types are characterized by bond complexity, structural uniformity, connection variants (slice), presence of non-helix domains, assembly and function. The collagen structure unit is a rod-shaped tropocollagen and consists of three polypeptide units that play a role in the formation of triple helix structure. Collagen consists of 3 large and repetitive polypeptide chains. Amino acid composition and physicochemical characteristics of collagen are varies and depend on tissues [2]. The amino acid composition of collagen found in chicken feet is dominated by glycine, glutamic acid, proline, and hydroxyproline [3]. The use of collagen is quite extensive both in the fields of biomedicine, cosmetics, and food. Collagen has been widely applied for biomedical purposes, pharmaceutical, food, drug, and cosmetics industries. Collagen of animal skin or hide can be converted to leather by a tanning process to produce a variety of products [4, 5, 6, 7, 8]. Applications of collagen in biomedicine are as a homeostatic agent, regeneration in bone tissue replacement, membrane oxygenator, contraception (barrier method), implant, and drug delivery system. Utilisations
in the field of cosmetics are as active ingredients to prevent the occurrence of premature aging (ant aging) either in the form of lotions or creams. The food industry utilises collagen as an emulsifier and foaming agent. Collagen has several advantages including easily absorbed in the body, low antigenicity, high water affinity, non-toxic, biocompatible, biodegradable, relatively stable, and can be prepared in various forms as needed [9].

Collagen found on the market today generally comes from cow hide and pig skin. The use of collagen from these materials has constraints from religious aspects [3]. An alternative source of collagen is collagen isolated by freezing temperature until the chicken feet water. Chicken feet are a waste from chicken slaughterhouse with a large volume of waste. Based on animal husbandry and animal health statistics data in 2017, there were 1.698 billion broilers in Indonesia [10]. During this time the potency of chicken feet has not been utilised optimally; chicken feet only became a waste of the chicken slaughterhouse. One way to increase the added value of chicken feet is to utilise their collagen.

The collagen isolation process can be carried out using the existed methods of acid-soluble collagen and pepsin-soluble collagen [11]. Collagen can be extracted chemically or with combination between chemical and enzymatic processes [12]. However, both extraction techniques have disadvantages, so other collagen isolation techniques are needed. Hydro-extraction is a method of isolating collagen using water as a solvent to extract collagen. Determination of NaOH and CH₃COOH concentrations, and also the duration in isolation of collagen is necessary to shorten the isolation time and to minimise the chemicals used in the collagen extraction. Physico-chemical characteristics of collagen isolated by hydro-extraction from chicken feet are also important to understand in product development from slaughterhouse by products.

The objectives of this study were to obtain the best treatments in pre-treatment and hydrolysis stages of collagen extraction from chicken feet using hydro-extraction method, and to characterise the physico-chemical properties of the isolated collagen.

2. Material and Method
2.1. Material and Equipment
Main material used in this study was chicken feet. The materials used to produce collagen were distilled water, NaOH, and acetic acid. The materials used in quantitative protein analysis were Bradford's solution (10 mg Coomassie Brilliant Blue (CBB G-250), 5 ml 95% ethanol, 10 ml of 85% orthophosphoric acid solution and distilled water) and bovine serum albumin (BSA) standards. Analysis for swelling and level of collagen solubility used filter paper and 5 M NaCl. Proximate analysis used distilled water, kjeltabs grains (catalyst tablets), H₂SO₄, NaOH, HCl, H₃BO₃ containing a mixture of 0.1% bromocresol green indicator and methyl red 0.1% with a ratio of 2:1, NaOH-Na₂S₂O₃ and hexane solvent 96%. Other ingredients included ingredients for characteristic analysis of collagen.

Equipment used in this study were cooler box, analytic scale, thermometer, UV-Vis spectrophotometer, water bath shaker, and freeze dryer, Erlenmeyer glass, measuring cup, and stirring rod. The equipment for the proximate, protein content, fat content, and ash content analyses. FTIR (Fourier transform infrared) type Bruker infrared spectrophotometer was used for the functional groups analysis and ultra-performance liquid chromatography (UPLC) was used for the amino acid test.

2.2. Method
2.2.1. Chemical preparation and characterisation of chicken feet. The chicken feet was transported to the laboratory under freezing conditions, then thawed, and cleaned all remaining dirt by washing with water. Chicken feet were cut to a size of approximately 0.5-1 cm using a knife or scissors and stored at freezing temperature until the chicken feet were used. The proximate analysis of the clean chicken were carried out including water, protein, ash and fat contents [13].
2.2.2. **Collagen isolation** (modification for Liu et al. [14], modification from Sukkwai et al. [15] and Nur’aenah [16]). Collagen isolation process with hydro-extraction method consists of three stages, namely pre-treatment with NaOH solution, hydrolysis with acetic acid solution, and extraction with distilled water. The first stage is the process of pre-treatment with NaOH solution aimed to eliminating non-collagen proteins and other impurities such as fats, minerals, pigments and odours. The NaOH concentrations used were 0.5, 1, and 2 M with a soaking times of 2, 4, 6 and 8 hours, then every 2 hours the NaOH solution was replaced with a new NaOH solution, but still used the same chicken feet. The ratio between chicken feet and NaOH solution was 1:10 (b/v). NaOH solution from the immersion process was tested quantitatively by the Bradford analysis to determine the best NaOH concentration and soaking time. The chosen chicken feet were neutralized by distilled water before were used for the next stage. The second stage was hydrolysis with a solution of acetic acid (CH₃COOH) to change the structure of collagen fibres to facilitate the extraction process. The concentrations of acetic acid used were 0.1, 0.3, and 0.5 M with soaking times of 1, 2, and 3 hours. The ratio between chicken feet and acetic acid solution was 1:10 (b/v). The samples were washed using distilled water until neutral. The third stage was extraction by hydro-extraction method. The extraction was carried out at 40°C for 2 hours using a water bath shaker with a speed of 150 rpm. The ratio between chicken feet and distilled water was 1:2 (b/v). Extraction result was in the form of collagen solution, then the solution was dried with a freeze dryer to obtain dry collagen and the yield was calculated.

2.2.3. **Characterisation of collagen.** Collagen characterization consisted of yield analysis [17], functional group analysis with FTIR [18], and amino acid analysis [19, 20].

2.2.4. **Proximate analysis** [13]. Chemical characterization of chicken feet raw material was carried out by proximate analysis consisting of water, protein, ash, and fat content analyses.

3. Results and Discussion

3.1. **Chemical characteristics of chicken feet**

The chemical composition of material needs to be analysed for the identification of chemical content contained in the material and as indicator of material quality. Chemical composition analysis used in this study was a proximate analysis. The proximate analysis results of chicken feet were presented in table 1. Potency of chicken feet can be seen from the collagen content of 5.64-36.38 % of total protein [21]. The presence of ash and fat in materials can interfere effectiveness of collagen extraction [22]. Extraction disturbances occur because fat and ash can block the penetration of water to extract collagen from chicken feet, so it needs to be removed at the pre-treatment stage.

| Proximate composition | Composition (% w/w) | Composition (% w/w) |
|-----------------------|---------------------|---------------------|
| Water                 | 64.22±0.66          | 65.08±0.90¹        |
| Ash                   | 6.95±0.44           | 5.98±0.37²         |
| Protein               | 18.09±0.07          | 17.42±0.73²        |
| Fat                   | 8.22±0.19           | 12.04±0.44²        |

¹Hashim et al. [3], ²Liu et al. [21]

3.2. **Pre-treatment of the chicken feet with NaOH solution**

Collagen extraction process begins with a pre-treatment process which aims to remove non-collagen protein. The alkaline solutions that can be used to remove non-collagen proteins are NaOH and Ca(OH)₂ [23]. The alkaline solution (NaOH) can break down most of the telopeptide regions of collagen molecules which cause slight swelling of materials [24]. The telopeptide regions are the ends of the open triple helix chain (short non-helix structures) [25]. The condition of swelling can cause easily released of non-collagen proteins and other impurity components such as fat and pigments in the collagen matrix [26]. This causes the NaOH solution used to soak the chicken feet to turn brownish. The concentration
of dissolved protein found in NaOH solution because of the influence of concentration and soaking time could be seen in Figure 1. Figure 1 showed the concentration of protein (mg/l) non soluble collagen measured every 2 hours for 8 hours. The samples used at a concentration of 2 M were damaged which were indicated by the materials that became soft, so that the non-collagen protein value increased sharply from the second to forth hours. This results in macro particles being extracted in the measurement of dissolved proteins.

![Figure 1](image_url)

**Figure 1.** The concentrations of protein in the NaOH solution residues; NaOH 0.5 M, NaOH 1 M, NaOH 2 M.

The results of the split plot analysis showed that the differences in soaking time and their interaction with soluble protein levels were significant (p < 0.05). NaOH concentration had no significant effect on soluble protein levels (p> 0.05). The data were then tested by Duncan's Multiple Range Test (DMRT). The use of 0.5 M NaOH concentration for 6 hours was the best treatment from the pre-treatment stage which showed the lowest soluble protein concentration of 0.046 mg/ml. The concentration of dissolved protein showed that non collagen protein has been maximally extracted. This concentration was chosen because lower concentrations can reduce the risk of collagen component damage [27]. Hydrolysis is the process of breaking chemical bonds with the addition of water, where water is broken down into the form of H⁺ and OH⁻, generally were used in breaking down certain polymers [28].

The efficiency of the pre-treatment stage in NaOH solution is influenced by several factors including time, temperature, NaOH concentration, and raw materials used [8]. The use of alkaline solutions in the pre-treatment process is more effective in the process of extracting non-collagen proteins and only causes low level of collagen loss [23]. The extraction of non-collagen compounds occurs due to the breakage of some of the bonds between fibres in the structure of collagen in alkaline conditions [29]. The duration of soaking and alkali ratio will cause depolymerisation and deacetylation. NaOH has a role in separating strands from collagen fibres [24].

### 3.3. Chicken feet hydrolysis with CH₃COOH
Neutral pH causes decrease of collagen solubility [30]. The hydrolysis stage is carried out with acids which cause changes in the structure of collagen fibres to facilitate the extraction process of collagen in the next stage. Hydrolysis using acetic acid solution causes the material expanding and collagen solubility will occurs [31]. Based on the results of the study, the swelling increased along with the increase in acetic acid concentration and soaking time (figure 2). The biggest swelling (27.39%) was found at 0.5 M acetic acid concentration with a 3 hour soaking time, whereas the lowest (2.61%) was found at 0.1 M acetic acid concentration with 1 hour soaking time. The variance analysis showed that the differences in acetic acid concentrations affected the swelling significantly (p<0.05), while the
differences in soaking times and their interactions did not significantly influence the swelling (p > 0.05). Nur’aenah [16] stated that the swelling increases when the higher concentration of acetic acid used and the longer of soaking time. Acetic acid serves as a catalyst that helps the performance of water in the process of hydrolysis, so that it has a large influence on the results of extraction [28].

During the soaking process with acetic acid, collagen fibres will have a swelling process, resulting in a decrease in the internal cohesion of the collagen fibres [32]. During swelling, the cross-linking structure of the collagen molecule undergoes an opening and acetic acid acts as a catalyst between the bonds [28], so that water can enter the space of the collagen fibrils which causes swelling of the chicken feet (figure 3). Swelling of collagen in acidic solution is influenced by the osmotic pressure difference between protein and solution to reach the point of balance of osmotic pressure [33]. Increased collagen swelling at low pH is caused by the breakdown of the reticular tissue and elastin tissue by breaking intermolecular interactions and hydrogen bonds. Breaking intermolecular interactions can reduce the cohesion of protein molecules found in chicken feet, to facilitate the extraction process. Collagen protein changes in the form by swelling, because of the interaction between collagen fibrils and acidic solutions. This interaction is indicated by an increase in the percentage of weight on chicken feet after soaking in a certain period. The magnitude of swelling indicates more space in collagen fibrils can be entered into the solution.

The results of variance analysis of collagen solubility showed that the differences in the soaking time of acetic acid affected significantly the level of collagen solubility (p < 0.05). Acetic acid concentration and the interaction of acetic acid concentration and soaking time did not significantly influence the level of collagen solubility (p > 0.05). The data was then tested further by DMRT. Figure 4 showed the level of solubility of chicken feet collagen. The highest level of collagen solubility (1.04 %) was found in 0.5 M acetic acid concentration with 3 hour soaking time, which is equivalent to losing 0.011 gram. The lowest level of collagen solubility (0.46 %) was at 0.5 M acetic acid concentration with 1 hour soaking time equivalent to 0.005 gram loss. Wulandari et al. [31] reported that the concentration of acetic acid determines the pH value of the solution, so that it regulates the level of charge of collagen which affects electrostatic interactions, the degree of solubility, and the extractability of collagen from the tissues.
Figure 4. The degree of collagen solubility on the chicken feet during soaking by acetic acid: ■ soaking time of 1 hour, □ soaking time of 2 hours, ◇ soaking time of 3 hours.

Collagen is a hydrogel so that increases in solubility causes an increase in the swelling [30]. Swelling of the skin structure affects the separation of collagen fibre structure and can interfere the non-covalent bond of collagen which ultimately can facilitate extraction [24]. Therefore, in the hydrolysis process, the swelling factor is more important than the level of collagen solubility. Concentration of 0.5 M is the concentration level which gave the highest swelling compared to the concentration of 0.1 M and 0.3 M, and had insignificant soaking times. The lowest collagen solubility was at a acetic acid concentration of 0.5 M with a 1 hour soaking time with a large swelling. Based on the variance analysis on swelling and the level of collagen solubility, the best treatment chosen at the hydrolysis stage was 0.5 M acetic acid concentration with 1 hour soaking time.

3.4. Hydro-extraction

Before extracting collagen, chicken feet washed first with distilled water. The aim was to decrease the pH until neutral. Chicken feet extraction was carried out with distilled water, with a ratio of 1:2 (b/v). Extraction was carried out at 40°C for 2 hours using a water bath shaker with a speed of 150 rpm.

3.4.1. Yield. The results (table 2) showed that the yield of collagen dry base from chicken feet extracted by hydro-extraction method was lower compared to collagen yield obtained using enzymatic methods, sodium chloride-soluble collagen (SSC), and acetic acid-soluble collagen (ASC). Collagen extraction is currently used by pepsin enzyme (PSC) and acid method (ASC). Both methods have several disadvantages such as requiring a long time, a lot of chemicals and high production cost. Hydro-extraction method is an extraction method that has the advantage of producing collagen with a relatively fast production, requires little equipment, can be produced continuously, small amount of waste, and lower production costs. The difference in yield caused by differences in the extraction method used, the concentration of the solution used to remove non-collagen protein, the type of raw material used, temperature differences, and the duration of extraction process [35]. The amount of collagen wasted during the pre-treatment and washing process can cause a decrease in yield [36]. Low yields occur due to collagen leaching during the washing process or denaturation by acid during the hydrolysis process [37]. The hydro-extraction process has a low reaction speed and low ability to penetrate into a tissue.

| Source of collagen and method of extraction | Yield(% w/w) |
|--------------------------------------------|--------------|
| Chicken feet, hydro-extraction             | 0.14         |
| Chicken feet skin, sodium chloride-soluble collagen (SSC) | 1.13¹      |
| Chicken feet skin, acetic acid-soluble collagen (ASC) | 14.49¹     |
| Chicken feet, papain enzyme                | 18.16²      |
| Chicken feet, pepsin enzyme                | 22.94²      |

¹Zhou et al. [38], ²Hashim et al. [3]
The chicken feet have a dense or less porous physical form which causes low level of swelling, so that absorption of water is low and the ionization reaction with water during the extraction process is reduced. The chicken feet have a thick structure that requires extracting material that is able to penetrate into the material aggressively. The small number of cross links opened during the swelling process can reduce yield [39]. Soluble collagen in acetic acid cause reduce of collagen yield [31].

3.4.2. Functional group. The functional group of a compound can be identified by the FTIR (Fourier Transform Infrared) spectroscopic analysis by detecting infrared light as a source of electromagnetic radiation. If certain infrared frequencies are absorbed by a sample of organic compounds, vibration will occur in the molecule of the compound [40]. Characteristics of chicken feet collagen functional groups detected by FTIR are shown in table 3.

The functional group analysis with FTIR can be used to confirm the existence of a triple helix structure, which is known from the value of the amide III absorption ratio (1241.01 cm\(^{-1}\)) with an absorption peak at 1450 cm\(^{-1}\) which is close to 1 [41]. The intensity ratio between the amide III band and the 1450 cm\(^{-1}\) band is 1.16. FTIR analysis results showed that the value in accordance with the statement, so this indicates that the chicken feet collagen had not been degraded into gelatine because of the presence of the triple helix structure. The results of functional group analysis with FTIR is shown in figure 5.

### Table 3. The functional groups of chicken feet collagen.

| Amide type | This research data | Collagen literature | Absorption area standards | Information                  |
|-----------|--------------------|---------------------|---------------------------|------------------------------|
| Amide A   | 3426.65            | 3308\(^1\)          | 3400-3440\(^2\)           | N-H stretch                  |
| Amide B   | 2924.37            | 2925\(^4\)          | 2915-2935\(^3\)           | Asymmetrical stretch of CH\(_2\) |
| Amide I   | 1637.13            | 1630\(^1\)          | 1600-1700\(^4\)           | C=O stretch                  |
| Amide II  | 1409.51            | 1548\(^1\)          | 1550-1600\(^4\)           | N-H bend and C-N stretch    |
| Amide III | 1241.01            | 1242\(^1\)          | 1229-1301\(^5\)           | N-H bend and C-N stretch    |

\(^1\)Zhou et al. [38], \(^2\)Liu et al. [42], \(^3\)Coates et al. [43], \(^4\)Hashim et al. [3], \(^5\)Kong et al. [44]

3.4.3. Amino acid. The amino acid composition determines the type and physical characteristics of collagen. The amino acid composition of collagen from chicken feet was expressed in mg/kg (table 4). The largest amino acids in collagen found in chicken feet extracted with pepsin soluble collagen method were glycine (18.91%), glutamic acid (12.43%), proline (10.75%), and alanine (8.88%) [3]. The difference in amino acid content can occur due to differences in the species of material used and the extraction method used. Glycine is the most dominant amino acid found in collagen, and all types of collagen are characterized by tripeptide (Gly-X-Y) repetition, where X is proline and Y is hydroxyproline responsible for forming triple helix structures [18]. Collagen with the amino acid glycine, alanine, and proline in sufficiently high amounts, and contains little amino acids histidine and tyrosine, and does not contain cysteine is a characteristic of type I collagen [45]. High levels of proline and hydroxyproline are rarely found in proteins other than collagen [46]. Hydroxyproline is an amino acid that has a function as a heat stabilizer from collagen [18].
Figure 5. The FTIR spectrum of chicken feet collagen.

Table 4. The amino acid composition of chicken feet collagen resulted by the hydro-extraction and enzymatic extraction.

| Amino acids       | Hydro-extraction (mg/kg) | Enzymatic extraction (%)<sup>1</sup> | Enzymatic extraction (%)<sup>2</sup> |
|-------------------|--------------------------|--------------------------------------|--------------------------------------|
| L-Glutamic acid   | 107.98                   | 10.07                                | 12.43                                |
| L-Phenylalanine   | 50.31                    | 4.69                                 | 2.54                                 |
| L-Isoleucine      | -                        | 0.00                                 | 2.50                                 |
| L-Valine          | 24.63                    | 2.30                                 | 3.07                                 |
| L-Alanine         | 96.10                    | 8.96                                 | 8.88                                 |
| L-Arginine        | 93.97                    | 8.76                                 | 7.34                                 |
| Glycine           | 253.36                   | 23.62                                | 18.91                                |
| L-Lysine          | 42.31                    | 3.95                                 | 5.37                                 |
| L-Aspartic acid   | 72.03                    | 6.72                                 | 7.63                                 |
| L-Leucine         | 22.80                    | 2.13                                 | 4.56                                 |
| L-Tyrosine        | 21.14                    | 1.97                                 | 1.01                                 |
| L-Proline         | 113.65                   | 10.60                                | 10.75                                |
| L-Threonine       | 50.04                    | 4.67                                 | 2.54                                 |
| L-Histidine       | -                        | 0.00                                 | -                                    |
| L-Serine          | 124.14                   | 11.58                                | 3.02                                 |
| L-Cysteine        | -                        | 0.00                                 | -                                    |
| L-Methionine      | -                        | 0.00                                 | 1.34                                 |

<sup>1</sup>Zhou et al. [38]; <sup>2</sup>Hashim et al. [3]

4. Conclusion
Based on this study, it can be concluded that the use of 0.5 M NaOH for 6 hours was the best condition for pre-treatment in chicken feet collagen extraction. The best treatment of hydrolysis was the concentration of 0.5 M acetic acid with 1 hour soaking time. The collagen yield was 0.14%. FTIR spectroscopy showed amide groups of collagen consisted of amide A, amide B, amide I, amide II, and amide III. The highest amino acid contents were glycine, L-serine, L-proline, L-glutamic acid, and L-alanine which are the composition of amino acids which build collagen structure.
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