Isolation and Characterization of Collagen from Chicken Feet

P. Hashim, M. S. Mohd Ridzwan, J. Bakar

Abstract—Collagen was isolated from chicken feet by using papain and pepsin enzymes in acetic acid solution at 4°C for 24h with a yield of 18.16% and 22.94% by dry weight, respectively. Chemical composition and characteristics of chicken feet collagen such as amino acid composition, SDS-PAGE patterns, FTIR spectra and thermal properties were evaluated. The chicken feet collagen is rich in the amino acids glycine, glutamic acid, proline and hydroxyproline. Electrophoresis pattern demonstrated two distinct α-chains (α1 and α2) and β chain, indicating that type I collagen is a major component of chicken feet collagen. The thermal stability of collagen isolated by papain and pepsin revealed stable denaturation temperatures of 48.40 and 53.35°C, respectively. The FTIR spectra of both collagens were similar with amide regions in A, B, I, II and III. The study demonstrated that chicken feet collagen using papain isolation method is possible as commercial alternative ingredient.

Keywords—Chicken feet, collagen, papain, pepsin.

I. Introduction

COLLAGEN is a protein mainly present in animal connective tissues, comprising approximately 30% of total animal protein. Collagen is a polymer composed of the repetitious aggregation of tropocollagen monomers. Tropocollagen is a transitional molecular species introduced by procollagen, the soluble nonhelical peptide extensions at both N- and C-terminal ends. It then aggregates with increasing numbers of intramolecular and intermolecular crosslinks to synthesize collagen fibril [1].

Due to its unique characteristics, collagen has been used in various fields of industry such as leathers and films, beauty and cosmetics, biomedicals, pharmaceuticals, materials and foods [2]. Collagen can be extracted at low temperatures using different neutral salt or acid solutions from the connective tissues of animals including vertebrates and invertebrates [1].

Recently, most commercial collagens come from mammalian sources especially bovine and porcine. These porcine and other animal collagens are unsuitable for some religious and ethnic communities. Besides, they may be exposed to biological contaminants such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD) [3].

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Recently, acid extraction with pepsin hydrolysis was a common method to extract the collagen. Specifically, pepsin used was originally from porcine sources [4]. Therefore, it is of great interest to explore alternative collagen sources and isolation methods from chicken by-products such as feet and skin using plant based enzyme such as papain.

Malaysia has a high number of chicken livestock and in year 2012, its production was 231 million chickens and is increasing annually. In 2010, Malaysia ranked 7th production of chicken meat commodities among Asian countries with 1.3 million tons [5]. Hence, growing consumption of chicken especially in Malaysia and other countries in Asia such as China, India, Indonesia, Iran, Turkey and Japan are increasing. The chicken production has led to oversupply of byproducts such as head, skin and feet. These byproducts are mostly processed into animal feed. Study of the isolation and characteristic of collagen from chicken feet can increase the value-added potential of this byproduct and its utilization in medical, cosmetic as well as in food application. Therefore, the objective of this study was to isolate and characterize collagen from chicken feet using papain and pepsin enzymes. The characterization of collagen was carried out in terms of relative molecular weight distribution, amino acid composition, FTIR spectra and thermal characteristics.

II. Material And Procedure

A. Materials and Chemicals

Chicken feet were obtained from Ayamas Food Corporation Malaysia, Selangor. Ice storage was used during transportation of the raw materials to the laboratory to maintain the freshness. All chemicals and reagents used were OF analytical grade.

B. Sample Pretreatment

Samples were crushed using meat grinder and washed thoroughly to remove any contaminants. Samples were then packed in smaller containers and stored in a cold room at 21°C. The frozen samples were thawed overnight at 4°C prior to use.

C. Collagen Isolation

Papain and pepsin enzymes were used to isolate the collagen from chicken feet according to the method by Cheng et al. [4] with slight modifications. The ground samples were defatted under stirring with 10 volumes of ethanol for 6 h. The defatted samples were decalcified under stirring with 0.5 mol/L ethylenediaminetetraacetic acid disodium (EDTA-2Na) at 4°C for 6h. Later, to remove the non-collagenous protein

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materials, the treated samples were soaked in 0.1mol/L NaOH for 6h.

The residues were thoroughly rinsed with cold water and suspended in 0.5mol/L acetic acid containing 0.1% (w/v) enzyme at 4°C for 24h. The samples were centrifuged at 10,000 x *g* for 1h. The supernatant were salted out by adding NaCl with overnight stirring. Before the precipitate was obtained, the samples were centrifuged again at 10,000 x *g* for 1h. The final solution was dialyzed against distilled water by using dialysis bag for 24h. Finally, the product was lyophilized with freeze dryer. The collagen powder obtained was weighed and recorded.

D. Yield of Collagen

The yield of the collagen isolated was reported on dry weight basis according to the method of [6] as stated in (1).

% Yield (dry weight basis) =

$$\frac{dry\ weight_{collagen}}{(wet\ weight\ of\ sample - moisture\ content\ of\ sample)} x\ 100\% \quad (1)$$

E. Proximate Analysis

The chicken feet were analyzed for its moisture (oven drying method), ash, fats and crude protein (micro Kjedahl method) according to methods from [7].

F. Amino Acid Composition

The amino acids composition in collagen was determined using Amino Acid Analyzer High Performance Liquid Chromatography (Model: Waters 501 Millipore Corporation, USA). Each sample was hydrolyzed with 6 N HCl at 110 °C for 24 h. The hydrolyzed samples were then analyzed for the free amino acid content according to the method of [6].

G. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples were analyzed by SDS–PAGE using 12% gels with a constant current of 15mA [8]. After electrophoresis, the proteins were stained with 0.1% (w/v) of Coomassie Brilliant blue R-250 staining solution and destained with Destain Solution, Coomassie R-250.

H. Thermal Characterization

The denaturation temperatures of chicken feet collagen from different extraction method were measured by differential scanning calorimetry (DSC). The freeze dried collagen samples were placed into an aluminum lid and subjected to DSC platform. The reference pan was positioned on the opposite platform. The heat rate was 5°C/min and the temperature was increased from 27°C to 80°C. Three temperatures were measured as characteristics of the denaturation process of the chicken collagen produced by different isolation methods. The temperatures measured were the onset temperature (T_o), the peak temperature (T_p), and the endset temperature (T_f) [9].

I. Fourier Transforms Infrared (FTIR) Spectroscopy

The changes in the secondary structure were studied using FTIR spectroscopy [10]. IR spectroscopy of chicken feet collagen was taken using FTIR by preparing a KBr pellet containing 20 mg of the sample.

J. Statistical Analysis

All data were reported as mean ± standard deviation. The characteristics of collagen extracted from chicken feet were analyzed using t-test at 95% confidence level (p<0.05).

III. RESULT AND DISCUSSION

A. Proximate Analysis

TABLE I
PROXIMATE ANALYSIS OF CHICKEN FEET

Test	Composition (%)
Moisture	65.08 ± 0.90
Fat	3.90 ± 1.16
Protein	20.10± 0.98
Ash	8.16 ± 1.92

Chicken feet can be used as a protein substrate due to its high protein content of 20.10% (Table I). Pretreatment of the samples were necessary because of the slightly high amount of fat and ash content. Therefore, the samples need to undergo defatting and decalcifying process before extraction.

B. Collagen Yield

TABLE II
YIELD OF COLLAGEN FROM CHICKEN FEET

Treatment	Yield of collagen dry basis (%)
Papain, 0.1 % (w/w)	18.16 ±11.90[a]
Pepsin, 0.1 % (w/w)	22.94 ± 2.33[a]

The yield of chicken feet collagen using papain and pepsin enzymes are reported in Table II. The highest chicken feet collagen yield was isolated using pepsin aided in acetic acid (22.94%) and papain (18.16%). However, there is no significant difference of collagen yield between the two extraction methods. Our result was higher than collagen yield from silky fowl feet (7.31 ± 0.20%, dry basis) [4] and bird feet collagen (16.79 ± 4.79, dry basis) [11]. This suggested that papain can be a good alternative method for isolation of chicken feet collagen.

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C. Amino Acid Composition

TABLE III
AMINO ACID COMPOSITION IN CHICKEN FEET COLLAGEN AND OTHER COLLAGEN SOURCES

Compound	Papain (%)	Pepsin (%)	Chicken feet (%) [12]
Hydroxyproline	7.35±0.05	8.11±0.05	-
Aspartic Acid	7.66±0.19	7.63±0.19	9.10
Serine	3.37±0.01	3.02±0.01	3.23
Glutamic Acid	12.47±0.15	12.43±0.15	10.11
Glycine	16.97±0.05	18.91±0.05	32.66
Histidine	NA	NA	0.99
Arginine	7.66±0.04	7.34±0.04	8.50
Threonine	3.07±0.02	2.54±0.02	3.27
Alanine	7.71±0.09	8.88±0.09	12.96
Proline	9.62±0.02	10.75±0.02	12.08
Cysteine	0.39±0.07	NA	0.19
Tyrosine	1.19±0.11	1.01±0.11	0.79
Valine	3.62±0.02	3.07±0.02	4.72
Methionine	1.63±0.05	1.34±0.05	1.21
Lysine	5.83±0.15	5.37±0.15	4.19
Isoleucine	3.04±0.01	2.50±0.01	2.35
Leucine	5.44±0.01	4.56±0.01	4.64
Phenylalanine	2.99±0.11	2.54±0.11	2.12
Total	100	100	100
Imino acid	16.97	18.86	12.08

The amino acid composition plays a main role in the physical properties of collagen. Generally glycine, an abundant amino acid in collagen, constitutes around 30% of the total amino acid content. However, the value may vary according to species and body parts [13]. Amino acid compositions in chicken feet collagen extracted by papain and pepsin enzymes are shown in Table III. The chicken feet collagen had approximately 16-19% glycine as the major amino acid, followed by glutamic acid about 12%, proline in the range of 9-11%, hydroxyproline 7-8%, arginine 7% and low content of histidine and cysteine. The content of imino acid (proline and hydroxyproline) of chicken feet collagen was in the range of 17–19 % and this result is similar with imino acid of collagen from bovine (17.89%), porcine skin (17.74%) and bird feet (17.95%) [9]; which is higher than chicken feet studied by Liu et al. [12] which is 11.72%. When compared with our chicken feet collagen, proline content in bird feet was lower (4.29%) but high in hydroxyproline (13.66%) [9]; however, the hydroxyproline in chicken feet collagen [12] was not included. The high imino acid content, especially hydroxyproline is extremely important because it affects the functional properties of protein preparations made from collagen. It is suggested that collagen with high imino acid content may have wider applications in various fields [14].
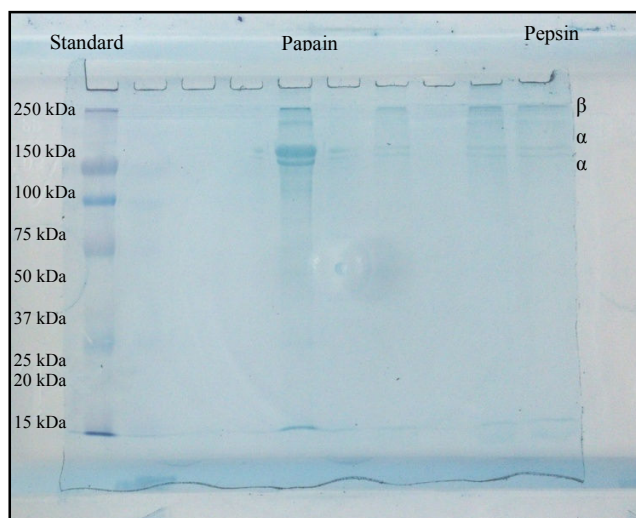
D. SDS-PAGE Pattern

Fig. 1 Protein molecular weight distribution of chicken feet collagen

The SDS–PAGE patterns of chicken feet collagen isolated by papain and pepsin are shown in Fig. 1. There is no difference in the protein patterns of sample extracted by different methods of extraction. Both papain and pepsin chicken collagen contained two distinct α-chains (α1 and α2) and β chain. Therefore, the results indicated that type I collagen is a major component of chicken feet collagen. Among subunits of the SDS–PAGE band, low molecular weight protein fragments could not be observed in all treatments. These results showed that contaminants such as hemoglobin or enzyme were successfully removed from chicken feet collagen. Furthermore, the results also proved that the collagen isolated still maintained their structural integrity throughout the purification processes [4], [15], [16], [17].

E. Thermal Characterization

TABLE IV
THERMAL TRANSITION TEMPERATURE OF CHICKEN FEET COLLAGEN AND OTHER COLLAGEN SOURCES

Collagen samples	Thermal transition temperature (°C)		
	$T_{onset}(T_o)$	$T_{peak}(T_p)$	$T_{final}(T_f)$
Chicken feet collagen (Pepsin)	48.60	53.35	57.68
Chicken feet collagen (Papain)	46.06	48.40	49.80
Pork collagen [18]	62.47	66.47	71.53
Calf skin [17]	56.84	67.84	78.38
Nile tilapia skin [17]	32.27	34.32	41.09

Thermal transitions of chicken feet collagen from pepsin and papain and other collagens are shown in Table IV. The thermal transition temperature of chicken feet collagen from pepsin and papain displayed a wide range (T_o: 46.06-48.60°C, T_p: 48.40-53.32°C, T_f: 49.80-57.68°C). The denaturation temperature of pepsin solubilized collagen is slightly higher (53.35°C) than the papain solubilized collagen (48.40°C). The thermal stability decreased in the order of pepsin > papain. However, the denaturation temperature of collagen produced from chicken feet was slightly lower than pork collagen,

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which is 66.4°C [18] and calf skin collagen (67.84°C) [17] but higher than tilapia skin collagen which is 34.32°C [17]. The higher transition temperature indicates that the collagen had higher stability in a high temperature environment [11].

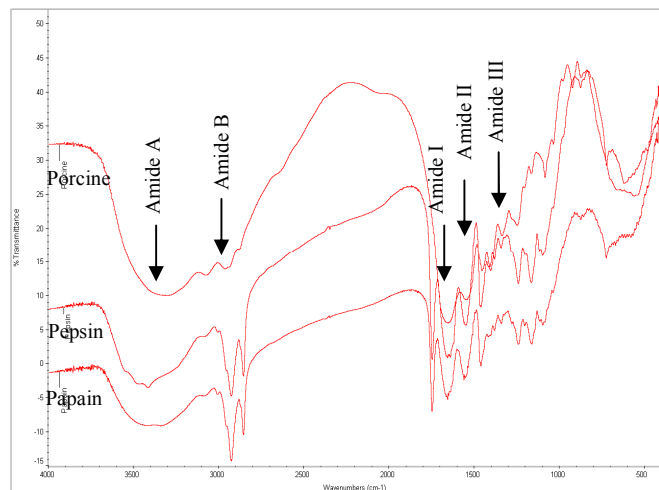*F. Fourier Transforms Infrared (FTIR) Spectroscopy*

Fig. 2 FTIR spectra of chicken feet collagen (pepsin and papain) and porcine collagen

FTIR spectra of chicken collagen exhibited the characteristic peaks of amides A, B, I, II and III as shown in Fig. 2. FTIR spectroscopy has been used to detect changes in the collagen structure. The absorption characteristics of amide A commonly associated with N-H stretching vibration occurs in the wave number range 3400-3440cm^{-1} [16], [19]. The absorption peaks of collagen isolated using papain and pepsin were found at 3412 and 3338cm^{-1} respectively, whereas porcine collagen was at 3293cm^{-1}. When N-H group is involved with H-bond in peptide chain, the position starts to shift to lower frequencies. Amide B peaks of chicken collagens were found at 2925cm^{-1}, representing asymmetrical stretch of CH$_2$. Similar absorption peaks between collagens suggested that all chicken collagens complex with hydrogen bonding between free N-H stretches attached with hydrogen in polypeptide chain [19], [20].

Amide I with characteristic wavenumber in the range of 1600-1700cm^{-1} is mainly associated with the C- or O-stretching vibration along the polypeptide backbone. The absorption peaks of chicken collagens were found at 1745cm^{-1}. This observation confirmed that the formation of hydrogen bond between N-H stretch (X position) and C- or O- (Gly) of the fourth residue is responsible for introducing triple helix [16], [19], [20].

Amide II is generally responsible for the combination of the N-H bending vibration coupled with the C-N stretching vibration and normally occurs at 1550-1600cm^{-1}. The shifting to lower wave numbers (cm^{-1}) of amide I and amide II, suggests the existence of more and stronger hydrogen bonds in the collagen structure. The higher hydrogen bonding in triple helical structure would result in higher structure order of collagen. Differences of band absorptions were possibly attributed to the different molecular structure between collagens [16], [19], [20]. According to Fig. 2, the absorption peak of amide A, I and II bands were similar to commercial porcine collagen. Moreover, the N–H stretching band (3300cm^{-1}) of denatured collagen (gelatin) was not detected. These results implied the collagen still preserved native conformation during purification processes [15].

The amide III band (1220–1320cm^{-1}) is associated with N-H deformation and C-N stretching vibrations. In addition, the absorption peaks around 1451–1450cm^{-1} were also found. This considerably corresponded to pyrrolidine ring vibration of proline and hydroxyproline as described by Muyonga et al. [20]. The band in the spectrum between 1200 and 1300 cm^{-1} are unique "fingerprint" of collagen molecular conformation attributed to particular tripeptide (Gly-Pro-Hyp)$_n$ [11].

IV. CONCLUSION

Chicken feet are an economically and technologically feasible substrate to extract collagen. The chicken feet collagen had similar pattern of amino acid composition, molecular weight pattern and structural properties compared to the commercial collagens. The thermal properties were also stable at high temperature. This study demonstrated that chicken feet collagen using papain isolation method is possible as commercial alternative ingredient.

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