Extraction and Characterization of Local Duck Feet Collagen with Bromelain Enzyme Treatment

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Manuscript received April 27, 2026; accepted July 7, 2026; published September 30, 2026

***Abstract*—The purpose of this study is to use the bromelain enzyme to hydrolyze collagen from local duck feet and identify its properties. The enzyme concentrations used were 0, 0.3, 0.6, and 0.9%. The collagen characteristics tested included yield, pH, viscosity, molecular weight profile analysis using Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE), functional group profile analysis using Fourier Transform Infrared (FTIR), and thermal stability analysis using Differential Scanning Calorimetry (DSC). The results showed significant differences ($p < 0.05$) between treatments, with values of 1.36%, 7.86%, 10.76%, and 15.23% for wet yield and 0.07%, 0.56%, 0.82%, and 1.17% for dry yield. The measured pH values did not show significant differences ($p < 0.05$) with values of 4.17, 4.22, 4.44, and 4.52, respectively. The viscosity results showed significant differences ($p < 0.05$), namely 2.2, 2.3, 2.5, and 2.4. The SDS-PAGE molecular weight profile analysis results showed an α_2 band in the range of 100–140 kDa. The FTIR functional group profile analysis results showed clear amide absorption regions at A, B, I, II, and III. The thermal stability analysis results showed two melting peaks in the range of 154.24 °C to 211.04 °C. Based on the research conducted, it can be concluded that enzyme concentrations of 0.6% and 0.9% produce the best collagen characteristics.**

***Keywords*—collagen, local duck feet, bromelain, characterization**

I. Introduction

Animal connective tissues like ligaments, fascia, bones, blood vessels, and skin contain collagen, which is the primary structural protein [1]. This protein has unique characteristics because it is capable of forming insoluble fibers with high tensile strength and is composed of a triple helix structure of three polypeptide chains that coil to form a superhelix [2, 3]. Collagen extracted from natural sources has many uses in a variety of industries, especially food, cosmetics, and biomedicine, because of their mechanical and structural characteristics.

Collagen's exceptional biocompatibility, biodegradability, and low antigenicity have made it a popular natural biomaterial in biomedical applications. Until now, the main sources of commercial collagen have been bovine and porcine skin. However, the emergence of various diseases in ruminant livestock, such as Bovine Spongiform Encephalopathy (BSE), has prompted the development of safer and more sustainable alternative sources of collagen [3, 4]. One such potential source is duck feet, which have so far been classified as poultry by-products and have not been optimally utilized.

Duck by-products, particularly feet, possess notable nutritional value, characterized by high protein content and the presence of essential lipids. As the primary component of connective tissue, muscles, gums, and skin, collagen is the most prevalent protein in animal bodies [5]. To obtain collagen from animal tissue, various isolation methods are used, both chemical and enzymatic. Enzymatic methods are increasingly used because they minimize collagen structure damage and produce better collagen yield and quality [6].

One protease enzyme that has the potential to be used in the collagen isolation process is bromelain, an enzyme derived from pineapple extract [7]. Bromelain works by hydrolyzing peptide bonds, thereby breaking down proteins into smaller, more easily digestible fragments [8]. The use of bromelain in various concentrations (0.2% to 0.8%) has been proven effective in extracting collagen from duck feet, with the highest results observed at a concentration of 0.6%, which produced a yield of 7.77% [9]. This enzyme has also been reported to produce higher amino acid groups than other proteases such as alkalase, neutrase, and papain [10]. However, the use of the bromelain enzyme to isolate collagen from duck feet has not received much attention up to this point.

Therefore, research on collagen extraction from local duck feet using bromelain enzymes is very important to study. This research is expected to provide a new alternative source of collagen that is safe, economical, and has the potential to be applied in various industrial fields.

II. Materials and Methods

A. Materials

The material used in this study was duck feet obtained from a poultry slaughterhouse in the Sleman region. The chemicals used in this study were Sigma-Aldrich bromelain enzyme, NaOH, NaCl, ethanol, CH_3COOH, bromelain enzyme, Coomassie blue, SDS PAGE loading buffer, potassium bromide, and distilled water.

B. Sample Preparation

The collagen isolation process was carried out using a method that involved preparing the material by cleaning the duck feet of dirt using running water, then drying them [11]. After the duck feet were cleaned, they were cut into small pieces measuring 1–2 cm. The duck feet were then stored in a refrigerator for 24 h. The duck feet were thawed from a temperature of 4 ºC and ground until smooth using a chopper. Next, the fat removal process was carried out using 20% ethanol (1:10 w/v) for 24 h. The duck feet were then washed with distilled water and soaked in a 0.2 M NaOH solution with a ratio of 1:10 (w/v) for 24 h.

C. Collagen Extraction

Duck feet were then soaked in a 0.5 M CH_3COOH solution with the addition of bromelain enzyme. There were four treatments for the addition of bromelain enzyme, namely 0%, 0.3%, 0.6%, and 0.9% of the total sample with a soaking time of 24 h. The enzyme soaking was modified by adding NaOH solution until the pH reached 4.5–5. After that, the samples were centrifuged at 10.000 rpm in order to separate the supernatant, and solids. After that, 1.25 M NaCl was added to the supernatant, and it was agitated for a full day. To gather the precipitate, the mixture was centrifuged once more. The resulting precipitate was dialyzed into distilled water for 72 h after being dissolved in 0.02 M acetic acid at a weight/volume ratio of 1:10. The freeze-drying process, which may be used to examine collagen properties, was then employed to dry the collagen.

D. Yield Analysis

The dry weight of collagen generated (g) and the weight of cleansed fresh material (g) are compared to determine the yield [12]. Yield is obtained using the following formula:

$$\text{Wet collagen yield (\%)} = \frac{wet\ collagen\ weight}{weight\ of\ duck\ feet} \times 100\%$$

E. pH Analysis

pH testing was conducted by dissolving 1 g of collagen sample in 50 mL of distilled water and homogenizing it. The pH meter was turned on and left until it stabilized. The pH meter electrode was then dipped into the sample for a few moments until the pH value obtained stabilized.

F. Viscosity Analysis

Viscosity testing was performed by dissolving collagen samples in distilled water at a concentration of 0.3% (w/v). The viscosity of 100 mL of solution was measured using a Brookfield viscometer at a speed of 100 rpm and spindle no. 1 [13].

G. Functional Group Profile with FTIR

Collagen's functional group analysis was ascertained utilizing PerkinElmer Spectrum Fourier Transform Infrared Spectroscopy (FTIR). There are two in the 4000–400 cm^{-1} infrared spectrum area that is utilized. Collagen to be tested for functional group values was homogenized with potassium bromide (KBr) using a 1:10 ratio. The homogenized mixture was then pressed into a palette with a hydraulic press for reading on the FTIR device [14].

H. Molecular Weight Analysis of Collagen Was Performed Using SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

A gel electrophoresis comprising a 4% stacking gel and a 15% resolving gel was constructed. In 20 mm of NaOH and 0.1 M phosphate buffer (pH 8.3) at a 1:5 ratio, a sample of 0.01 g of duck feet collagen was dissolved. A vortex was then used to homogenize the solution. Four microliters of loading SDS buffer were combined with ten microliters of collagen solution, heated for five minutes at 85 degrees celcius in a water bath, and then chilled. The sample was centrifuged for 30 s at 3000 g. The material was electrophoresed for 150 min at 110 V. After that, the gel stained with 0.25% Coomassie blue, steeped for six hours, and then stained once again for fifteen minutes. After dissolving the gel in 10% CH_3COOH, it was examined [15].

I. Thermal Stability Analysis with Differential Scanning Calorimetry (DSC)

Duck feet collagen samples were rehydrated in acid or ionized water solutions. Thermal stability analysis was measured using Differential Scanning Calorimetry (DSC). The rehydrated collagen solution was placed in an aluminium container, covered, and heated at a rate of 10 °C per minute between 20 °C and 45 °C. The isothermal peak of collagen that appeared on the graph was observed [16].

J. Data Analysis

Data analysis used to test yield, viscosity, and pH characteristics used a one-way Complete Randomized Design (CRD) pattern, and if there were significant differences, it was followed by Duncan's New Multiple Range Test (DMRT). Statistical analysis was performed using Statistical Product and Service Solution (SPSS) software. Data analysis for functional group characteristics, thermal stability, and molecular weight used qualitative descriptive analysis.

III. Result and Discussion

A. Yield

Crude yield is the result obtained from collagen extraction with acid and enzyme addition. The yield results obtained can be seen in Table 1.

Table 1. Effect of enzyme concentration on collagen yield

Enzyme Concentration (%)	Wet	Dry
0	1.36 ± 0.52a	0.07 ± 0.00a
0.3	7.86 ± 0.90a	0.56 ± 0.21b
0.6	10.76 ± 1.04c	0.82 ± 0.34bc
0.9	15.23 ± 1.25d	1.17 ± 0.11c

[a,b,c] Different superscripts in the same column indicate significant differences ($p < 0.05$).

Based on the table above, it can be seen that the addition of the bromelain enzyme was 0%, 0.3%, 0.6%, and 0.9%. The calculations yielded average results for three repetitions in each sample from the addition of enzymes at the lowest concentrations, respectively, of 1.36%, 7.86%, 10.76%, and 15.23%. The calculation results for the extracted yield for dried collagen were 0.07%, 0.56%, 0.82%, and 1.17%, respectively. The yield results obtained showed a significant difference ($p < 0.05$). Based on these results, it can be seen that the higher the enzyme concentration added during the collagen extraction process, the higher the crude collagen yield. Based on the results, it can be seen that the addition of 0.9% bromelain enzyme produced the highest crude yield, while the lowest crude yield was obtained with the addition of 0% bromelain enzyme or without the addition of bromelain enzyme. The increase in bromelain enzyme concentration increases the crude collagen yield due to several key factors related to the proteolytic activity of the enzyme and its ability to break down collagen structures more effectively. Collagen extracted with the bromelain enzyme yielded 82.20%, which was significantly higher than the 66.94% yield from acetic acid extraction. This further

reinforces the effectiveness of bromelain in increasing collagen yield [17]. The amino-terminal and carboxyl-terminal ends of collagen's telopeptide region contain non helical regions that create a cross-linked structure [18]. Collagen fibers are bound by cross-links at this telopeptide region, which reduces their solubility in acidic solutions like 0.5 M acetic acid. These cross-links can be broken down by protease enzymes during the extraction process, improving collagen's solubility and extraction yield. Collagen solubility can be increased by increasing the amount of bromelain enzyme used during the extraction process, which can cleave peptide bonds. Increased collagen solubility will result in higher crude yield [19].

B. Collagen pH

pH testing aims to determine the degree of acidity or alkalinity of a solution. pH testing on collagen is carried out by dipping a pH meter directly into the wet yield. The collagen pH values can be seen in Table 2.

Table 2. Collagen pH based on differences in bromelain enzyme concentration

Enzyme Concentration (%)	pH
0	4.17 ± 0.01a
0.3	4.22 ± 0.60a
0.6	4.44 ± 0.52a
0.9	4.52 ± 1.01a

[a,b,c] Different superscripts in the same column indicate significant differences ($p < 0.05$).

Based on the table, it can be seen that collagen produced by adding bromelain enzyme at a concentration of 0% has a pH of 4.17 ± 0.01, collagen with an enzyme concentration of 0.3% has a pH of 4.22 ± 0.60, collagen with an enzyme concentration of 0.6% has a pH of 4.44 ± 0.52, and collagen with an enzyme concentration of 0.9% has a pH of 4.52 ± 1.01. The addition of enzymes in collagen isolation shows no significant difference ($p < 0.05$). Collagen extracted using bromelain enzymes at various concentrations (0.2% to 0.8%) show pH values ranging from 4.68 to 4.89 [9]. Various acids, such as citric acid, acetic acid, and their mixtures, have been shown to affect the yield, gel strength, viscosity, melting point, and gel point of gelatin extracted from collagen. For example, a 9% citric acid concentration has been shown to produce the best gelatin characteristics [20]. In addition, the difference in pH values obtained was influenced by the less than optimal penetration of the solution. The low pH was also caused by residual acetic acid, which increased the concentration of hydrogen ions in the sample. The decrease in pH is reinforced by the characteristics of acetic acid itself, which has a low pH of around 2.3 [21]. The low pH value produced has the advantage of being relatively more resistant to contaminating microorganisms.

C. Viscosity

Table 3. Collagen viscosity based on differences in bromelain enzyme concentration

Enzyme Concentration (%)	Viscosity
0	2.2 ± 0.10a
0.3	2.3 ± 0.15ab
0.6	2.5 ± 0.00b
0.9	2.4 ± 0.00b

[a,b,c] Significant differences ($p < 0.05$) are indicated by different superscripts in the same column.

The results of viscosity testing on local duck feet collagen with the addition of bromelain enzyme can be seen in Table 3.

The study's findings demonstrate that the addition of enzymes produced significant differences ($p < 0.05$) when compared to the control. In comparison to the control, the viscosity increased with the addition of enzymes. Based on the table, collagen without enzyme addition had a viscosity value of 2.2 ± 0.10, collagen with 0.3% enzyme addition had a viscosity value of 2.3 ± 0.15, collagen with 0.6% enzyme addition had a viscosity value of 2.5 ± 0.00, and collagen with 0.9% enzyme addition had a viscosity value of 2.4 ± 0.00. Stingray skin collagen has viscosity values ranging from 1.8 to 3.4 cP [22]. Collagen's primary physicochemical characteristic is its viscosity, which results from the existence of large protein chains that raise its molecular weight [23]. High viscosity values are caused by the presence of β and γ chains, while low viscosity values are caused by collagen denaturation at high temperatures. Collagen viscosity values are influenced by the temperature used, intermolecular forces, and the number of dissolved molecules [24]. The higher the viscosity value, the greater the electrostatic force generated by collagen molecules. Viscosity value is influenced by the temperature used during the collagen isolation process [25]. High viscosity value is due to the high proportion of inter- and intramolecular cross-links that can stabilize the collagen structure [26]. Analysis of molecular weight profiles using SDS-PAGE one technique used to describe collagen is collagen molecular weight profile analysis. The goal of molecular weight profile analysis is to determine the collagen protein's molecular weight. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used to analyze molecular weight profiles. Fig. 1 shows that molecular weight profile.

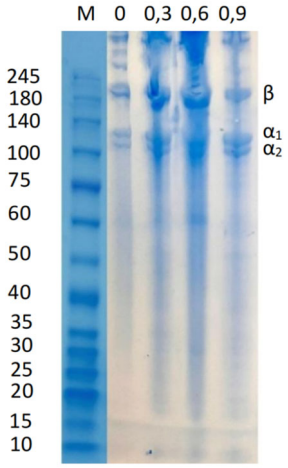


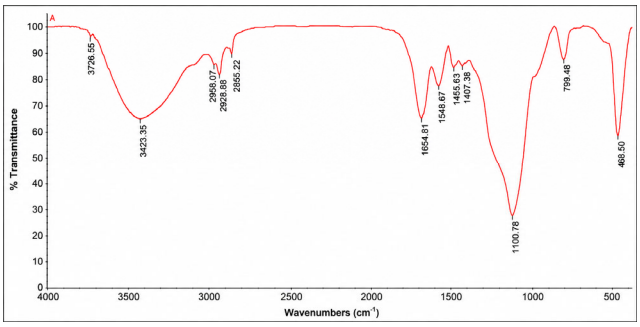
Fig. 1. Molecular weight profile of collagen isolated with different bromelain enzyme concentrations.

The figure shows that the local duck feet collagen has three primary bands, β, α_1, and α_2, when the bromelain enzyme is added [27]. The basic structure of alpha-1 and alpha-2 monomers, as well as the common theme of the regular collagen secondary structure in the beta and gamma bands, are typically detected in three bands in SDS-PAGE for type I collagen [28]. The beta band of type I collagen is roughly 250 kDa in size, whereas the alpha band is roughly 100–130 kDa. These findings lead to the conclusion that the collagen found in local duck feet is type I collagen due to the

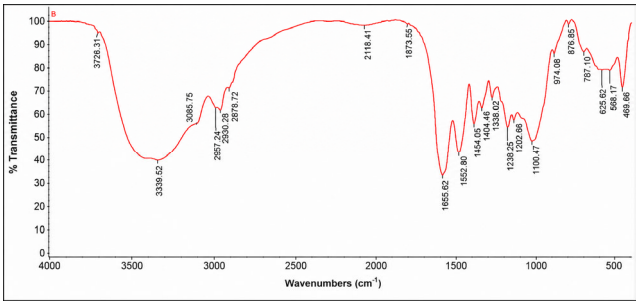
identification of the β, α_1, and α_2 chains. The basic structure of alpha-1 and alpha-2 monomers, as well as the common pattern of regular collagen secondary structure in beta and gamma bands, are typically detected in three bands for type I collagen in SDS-PAGE [29]. The beta band of type I collagen is roughly 250 kDa in size, whereas the alpha band is roughly 100–130 kDa. With the identification of β, α_1, and α_2 chains, the collagen found in local duck feet was determined to be type I collagen based on these findings. The size, charge, and shape of the protein, as well as the gel matrix's pore size, affect how quickly proteins migrate across a gel [30]. SDS-PAGE analysis can identify the presence of β-chain dimers, which indicates the presence of cross-links between molecules [31]. In addition, the identification of the β band also indicates that the triple helix structure of collagen is still intact and has not been completely degraded.

D. Functional Group Profile Analysis with FTIR

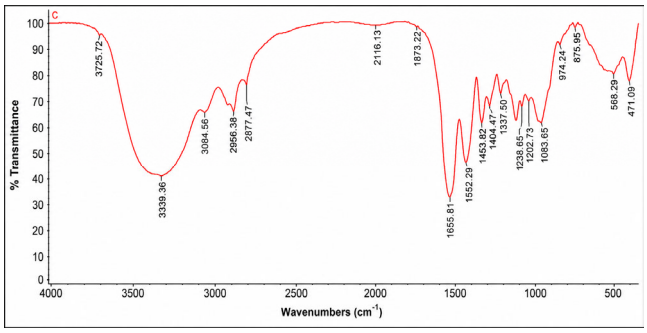
Functional group profile analysis was performed to determine the functional groups and secondary structure of collagen. Functional group values were determined using Fourier Transform Infrared Spectroscopy (FTIR). Functional group profiling with FTIR aims to confirm the compounds produced by collagen based on their functional groups [32]. Functional group values are determined based on the absorption peaks produced. The results of functional group profiling analysis can be seen in Fig. 2.



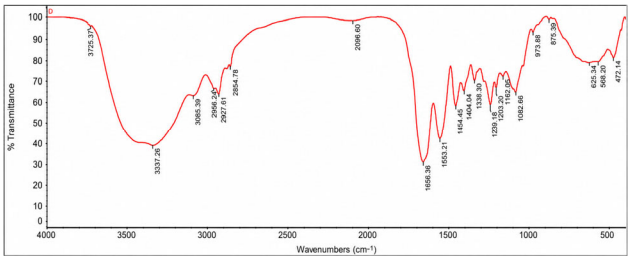
(a)



(b)



(c)



(d)

Fig. 2. FTIR spectra of local duck feet collagen. Spectra of local duck feet collagen: (a) 0%, (b) 0.3%, (c) 0.6%, (d) 0.9%.

Amide A, B, I, II, and III peaks are visible in the FTIR spectrum of collagen from local duck feet with varying bromelain enzyme concentrations. The sample's distinctive collagen structure is visible in the absorption peaks. The wavelength range of 3340–3400 cm^{-1} is generally associated with N–H stretching vibrations related to Amide A. Amide A band represents free N–H stretching vibrations and the presence of hydrogen bonds, and based on the literature, it usually appears in the range of 3300^{-1}–3400 cm^{-1} [33]. Amide B is related to asymmetric CH_2 stretching vibrations and is found in the range of 2925^{-1}–2935 cm^{-1}. The stretching vibration of the carbonyl group (C=O) in peptides, which is located between 1600 and 1700 cm^{-1}, is the representation of amide I. Amide II is located in the Amide II absorption range (1549 cm^{-1}), which is a mix of N–H bending and C–N stretching vibrations in type I collagen peptide bonds. It is characterized by the bending vibration of NH and the stretching of CH [34]. The amide III peak of collagen is at the 1200–1400 cm^{-1} wavelength peak [35]. The ratio of 1450 and the amide III group indicates the presence of a triple helix structure, which is a characteristic structure of collagen [36]. The absorption ratio of 1450 at amide III is 0.85, or close to 1.0. The peak positions of the collagen region spectra can be seen in the table below.

Table 4. Peak positions of collagen FTIR spectra

Region	Wave Number (cm^{-1})			
	0%	0.3%	0.6%	0.9%
Amida A	3423.25	3339.52	3339.36	3337.26
Amida B	2926.88	2930.28	2877.47	2971.61
Amida I	1654.81	1652.62	1655.81	1656.36
Amida II	1548.67	1552.80	1552.29	1553.21
Amida III	1100.78	1100.47	1238.65	1239.18

E. Thermal Stability Analysis with DSC

DSC analysis showed that all treatments produced two endothermic peaks representing the glass transition (Tg) and maximum melting point (Tmax). Collagen without enzyme showed Tg and Tmax at higher temperatures (149.83 °C and 211.04 °C), while the addition of bromelain caused varying temperature shifts. The 0.3% treatment significantly lowered the transition temperature, while the 0.6% and 0.9% concentrations again showed an increase in Tg and Tmax, indicating that the effect of bromelain on thermal stability is concentration-dependent.

This range of Tg and Tmax is still within the range reported in other species, such as sea cucumber collagen (87.8 °C; 162.4 °C) and snakehead fish skin collagen (70.07 °C; 222.16 °C). These variations can be attributed to differences in hydroxyproline and proline content, two amino acids that determine triple helix stability through hydrogen

bond formation. Higher thermal stability generally indicates a more preserved helical structure. In addition, differences in extraction methods also contribute, where acidic solvents are known to weaken collagen intramolecular bonds and lower the denaturation temperature. Overall, the 0.6% and 0.9% bromelain treatments produced more stable thermal profiles, indicating that the collagen triple helix structure remains intact and is potentially more resistant to heating processes in industrial applications.

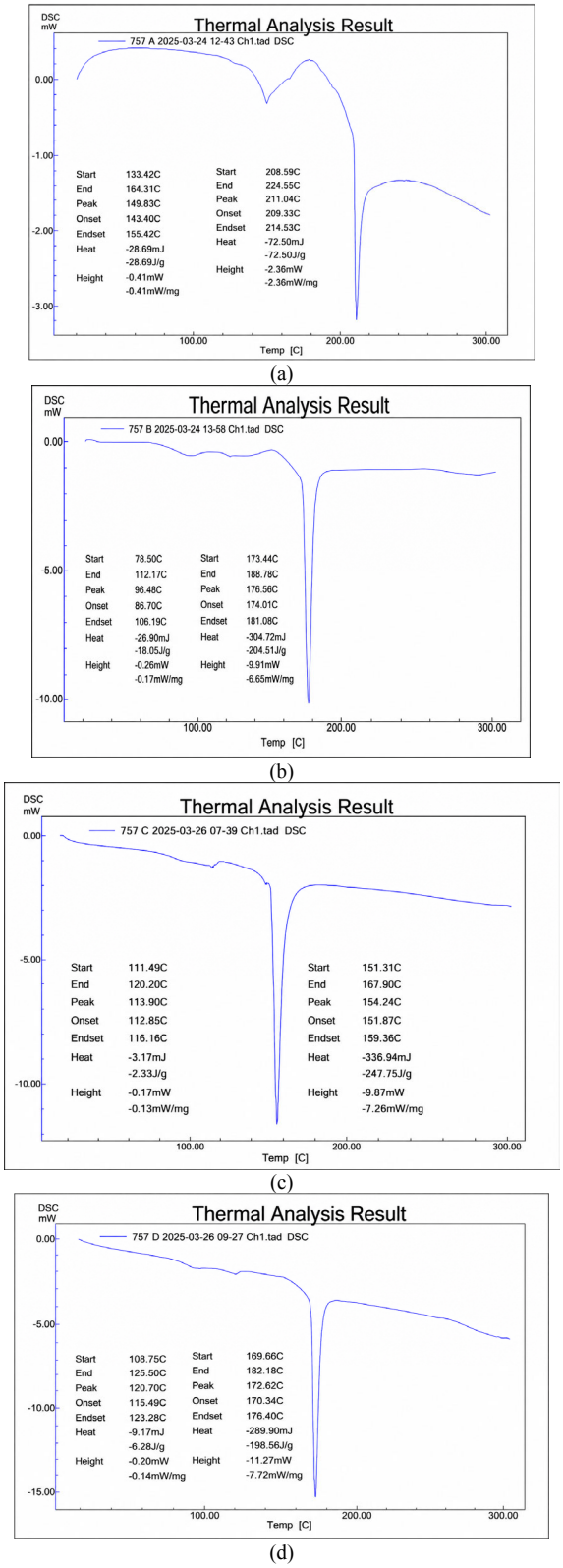


Fig. 3. Thermogram of local duck feet collagen. Thermogram of local duck feet collagen with bromelain enzyme samples (a) 0%, (b) 0.3%, (c) 0.6%, and (d) 0.9%.

Fig. 3 presents the DSC thermogram profiles of local duck feet collagen hydrolysates treated with different bromelain enzyme concentrations (0%, 0.3%, 0.6%, and 0.9%), indicating variations in their thermal characteristics and denaturation behavior. Based on the test results, collagen with 0% bromelain enzyme addition had two endothermic peaks at 149.83 °C and 211.04 °C with initial melting temperatures of 133.42 °C and 208.59 °C, respectively, and final melting temperatures of 164.31 °C and 224.55 °C. Collagen with 0.3% enzyme addition had two endothermic peaks at 96.48 °C and 176.56 °C with initial melting temperatures of 78.50 °C and 173.44 °C, respectively, and final melting temperatures of 112.17 °C and 188.78 °C. Collagen with 0.6% enzyme addition had two endothermic peaks at 113.90 °C and 154.24 °C, with initial melting temperatures of 111.49 °C and 151.31 °C, respectively, and final melting temperatures of 120.20 °C and 167.90 °C. Collagen with the addition of 0.9% enzyme had two endothermic peaks at 120.70 ºC and 172.62 ºC with initial melting temperatures of 108.75 ºC and 169.66 ºC, respectively, and final melting temperatures of 125.50 ºC and 182.18 ºC. The temperature on the thermogram consists of two peaks, which are the glass transition peak and the melting point peak (Tmax). The denaturation transition temperature of gamma sea cucumber collagen is 87.8 °C and the melting point peak is 162.4 °C [25]. The endothermic peak of snakehead fish skin collagen had two endothermic peaks at 70.07 ºC and 222.16 ºC, respectively, with an initial melting temperature of 213.34 ºC and a final melting temperature of 225.79 ºC [37]. A high denaturation temperature indicates that the collagen quality is better because the amino acids hydroxyproline and proline have pyrrolidine rings and hydrogen bonds that stabilize the polypeptide. The high thermal stability is caused by a higher amino acid content [38]. The use of extraction methods affects the denaturation temperature [25]. Extraction using acid produces lower results than extraction using water as a solvent. Acetic acid can break the triple helix structure of collagen by intramolecular bonds. A higher denaturation temperature indicates that the sample is more stable against heat and is not easily damaged in its application in products.

IV. Conclusion

Based on the research results, collagen isolation from locally discarded duck feet using bromelain enzyme demonstrated optimal performance at enzyme concentrations of 0.6% and 0.9%. Structural characterization of the obtained collagen confirmed that the product is type I collagen with a maintained triple helix configuration. The continuity of this helical structure confirms that the collagen has not undergone significant thermal or enzymatic denaturation, thus not being converted to gelatin.

Conflict of Interest

The authors state that there is no conflict of interest.

Author Contributions

The experiments were conducted by S. The manuscript was written by S, with assistance from M.Z.A. and Y.E. as supervisors. The project was supervised by M.Z.A. and Y.E.

The initial concept was developed by S., M.Z.A., and Y.E. All authors approved the final version of the manuscript.

FUNDING

Lembaga Pengelola Dana Pendidikan (LPDP) by Kementerian Keuangan Republik Indonesia

ACKNOWLEDGMENT

The author would like to thank to Lembaga Pengelola Dana Pendidikan (LPDP).

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