

ISOLATION, PURIFICATION, AND CHARACTERIZATION OF BOVINE TENDON COLLAGEN AND ANALYSIS OF GLYCINE, PROLINE, AND HYDROXYPROLINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-FLUORESCENCE

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Received: 01 June 2018, Revised and Accepted: 11 November 2018

ABSTRACT

Objective: In this study, collagen isolated from bovine tendon was purified and characterized, and the optimum conditions for analysis of glycine, proline, and hydroxyproline were determined.

Methods: The collagen isolation process used 0.1 N NaOH as a pretreatment, 0.5 M acetic acid in the extraction, 0.9 M NaCl in the salting-out step, centrifugation and dialysis for purification, and freeze-drying as the final step. The characterization of the collagen included analysis of the organoleptic properties, pH, moisture content, viscosity, and ash content. A Fourier-transform infrared (FTIR) spectroscopy analysis and Casson's trichrome staining were also performed. The collagen was hydrolyzed in 6 N HCl for 24 h and derivatized using 9-fluorenylmethoxycarbonyl chloride. The optimum condition was conducted from the optimal wavelength, selection of mobile phase composition, and flow rate.

Results: The average content was 11.867±0.20% for glycine, 33.247±0.20% for proline, and 10.51±0.23% for hydroxyproline. The optimum condition analysis for collagen was achieved by high-performance liquid chromatography (HPLC) with a C18® column and a fluorescence detector (excitation: 265 nm and emission: 320 nm) with mobile phase acetate buffer (pH 4.2):acetonitrile (55:45), and the flow rate was 0.8 mL/min.

Conclusion: The collagen isolated from bovine tendon was obtained at a yield of 0.690%, and the identity was confirmed by FTIR functional group analysis and Casson's trichrome staining. The HPLC conditions using a fluorescence detector for analysis of glycine, proline, and hydroxyproline concentrations in the bovine tendon collagen were optimized. The analysis of amino acids gave the average levels of 33.247±0.20% for glycine, 11.867±0.20% for proline, and 10.51±0.23% for hydroxyproline.

Keywords: Amino acid, Bovine tendon collagen, Content, Derivatization, Fluorescence, Glycine, High-performance liquid chromatographic, Hydroxyproline, Optimization, Proline.

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INTRODUCTION

Collagen is a fibrous protein located in the extracellular space and in various connective tissues that account for nearly 30% of the total protein in vertebrate and invertebrate organ tissues [1]. The main sources of collagen are skin tissue, tendons, cartilage, and bones. Collagen is indispensable as a raw material in the food, cosmetics, and biomaterials industries, especially those in the health field [2]. With developments in technology, the collagen can be used to repair bone tissue because the protein is able to stimulate the growth of new bone cells. Judging from its benefits and uses, collagen has an important role for humans, especially in the health field, so the demand and interest in collagen use are expected to increase. The production of collagen in Indonesia is still not optimal. In 2003, Indonesia imported >6200 tons of collagen at approximately US \$1/g [3]. Collagen can be produced by isolation from bovine tendon, which is a particularly good source [2]. One-third of the protein mass of bovines is collagen, and the tendons have a fairly high content of 85%. In Indonesia, the need for beef is increasing; beef production from 2015 to 2016 was increased from 506.661 tons to 524.109 tons. Therefore, due to increasing cattle production, there have been several studies on collagen isolation from cow body parts, such as tendons. In this study, collagen isolated from bovine tendon was purified and characterized, and the optimum conditions for analysis of glycine, proline, and hydroxyproline were determined.

MATERIALS AND METHODS**Materials**

Brahman cross bovine tendon obtained from the Tapos Animal Slaughterhouse, Depok, undenatured type II collagen (Inter Health,

Nutraceuticals Incorporated), trans-4-hydroxy-L-proline (Sigma-Aldrich), standard amino acid L-proline (Sigma-Aldrich), standard glycine amino acid (Sigma-Aldrich), Masson's trichrome stain (Pusat Studi Satwa Primata IPB; Pathology Laboratory), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (Hangzhou Dingyan Chem. Co), boric acid (Merck), high-performance liquid chromatographic (HPLC) grade acetonitrile (Merck), HPLC grade methanol (Merck), sodium hydroxide (Merck), sodium Cl (Merck), hydrochloric acid (Merck), aquadest (Brataco), sodium acetate (Merck), glacial acetic acid (Merck), and KBr (Merck).

Overview of methods

The isolation and purification of collagen from bovine tendon were performed using the method of Lestari with modifications [4]. The process of isolation began with a pre-treatment step using 0.1 N NaOH, extraction with 0.5 N acetic acid, precipitation using 0.9 N NaCl, centrifugation and dialysis for purification, and lyophilization (freeze-drying) to obtain solid collagen. The following parameters of the isolated collagen were analyzed: Organoleptic properties, pH, moisture content, ash content, viscosity, and functional groups determined by Fourier-transform infrared (FTIR) spectroscopy. To confirm the presence of collagen in the isolated samples, Casson's trichrome staining was conducted.

After obtaining the collagen, a quantitative analysis was performed. An amino acid analysis was performed by HPLC with fluorescence detection. Beforehand, collagen needs to be derivatized with fluorogenic reagents to form fluorescent compounds because collagen does not have chromophores that absorb ultraviolet/visible light.

In this study, FMOC-Cl, a reagent that can react with primary and secondary amino acids was selected to form a fluorescent derivative required in the optimization of a selective and sensitive HPLC method for determination of the content of glycine, as a primary amino acid, and that of proline and hydroxyproline, as secondary amino acids, in isolated bovine tendon collagen.

Instrument

For HPLC, we used HPLC pumps (LC 20AT Solvent Delivery System; Shimadzu, Japan), a YMC-Triart® C18 column, a RF 20A fluorescence detector (Shimadzu), manual injectors, and data processors (LC Solution Software). We also used syringe HPLC (SGE, Australia), ultraviolet-visible (UV-Vis) spectrophotometer (UV-1601; Shimadzu), FTIR-8400S equipped with DRS-8000 (Shimadzu), LC 20/H ultrasonic cleaner (Elma), pH meters (pH 510; Eutech Instruments), freeze dryer (Eyla FDU 1200), centrifuges (NF 400R), vortex mixer (Wisemix VM-10), oven (Cole Parmer, Chemoscience), dialysis tubing cellulose membrane (12 kDa, diameter 49 mm; Sigma-Aldrich), Ostwald viscometer (Schott-Gerate), Millipore 0.45- μ m filter membranes, filter paper no. #40 (Whatman), and analytical balance.

Preparation of collagen standard solution

The collagen standard solution was prepared by weighing 50 mg of the standard collagen, which was then dissolved in 5 mL of 6 N HCl to be hydrolyzed in a 110°C oven 4 times (22, 23, 24, and 25 h). The solution was then dissolved in acetic buffer (pH 4.20) and diluted to a concentration of 10 μ g/mL.

Preparation of amino acid stock solutions

A 3.8-mM hydroxyproline stock solution was prepared by dissolving 50 mg of hydroxyproline in 100 mL of 0.1 N HCl. A proline stock solution of 4.34 mM was prepared by dissolving 50 mg of proline in 100 mL of 0.1 N HCl. A 6.66 mM glycine stock solution was prepared by dissolving 50 mg of glycine in 100 mL of 0.1 N HCl. Each solution was diluted to a concentration of 10 μ g/mL.

Preparation of FMOC-Cl solution

A solution of 15 mM FMOC-Cl was prepared by weighing 39 mg of FMOC-Cl (BM 258.7 g/mol) that was then diluted it with 10 mL of acetonitrile to a concentration of 1.5 mM.

Preparation of acetate buffer solution

A 15 mM acetate buffer was prepared by dissolving 1.6 g of anhydrous sodium acetate in 400 mL of aquadest. The pH was adjusted to 4.2 with glacial acetic acid P. The acetate buffer was then filtered through a Whatman no. 45 filter.

Preparation of boric buffer solution

A 100 mM boric buffer was prepared by dissolving 0.0618 g of boric acid (MW 61.85 g/mol), in 100 mL of Aquabidest. Then, the buffer was diluted to 10 mM and the pH was adjusted to pH 9 by dropwise addition of 6 N NaOH.

Isolation of collagen from bovine tendon

The bovine tendon was washed thoroughly, cut into 1-cm pieces, and then weighed to give approximately 1000 g portions. The bovine tendon slices (samples) were immersed in 0.1 N NaOH at a ratio of 1:5 for 3 days; the 0.1 N NaOH solvent was replaced every day.

The extraction process in 0.5 M acetic acid was performed over 3 days. After the extraction process was completed, the extracted filtrate was collected as filtrate 1. The residue from the sample was then extracted again using a new 0.5 M acetic acid solution for 3 days, and the extracted filtrate was collected as filtrate 2. Filtrates 1 and 2 were mixed for further purification by salting-out using NaCl until the final concentration reached 0.9 N from the extract volume. White clouds of collagen formed and floated in the acid extract. The acid extract was then centrifuged at 4000 rpm and 4°C for 20 min to precipitate the collagen.

Dialysis was performed by immersing a collagen-containing sac with 0.1 M acetic acid at a ratio of 1:10 of the volume of collagen solution for 1 day. The 0.1 M acetic acid was then replaced with aquadest. The pH of the new aquadest was adjusted for dialysis to ≥ 5 . The dialysis was performed at a temperature of <20°C or placed in a refrigerator. The collagen obtained from the dialysis process was placed in a container for lyophilization (freeze-drying) to collect it in the solid form. The whole process was performed at a low temperature (4°C).

Characterization of the bovine tendon collagen

Organoleptic properties

The organoleptic test was performed by looking at the physical appearance, color, and smell of the collagen isolated from the Brahman cross bovine tendon.

Functional group analysis by FTIR

First, 200 mg KBr and 2 mg standard collagen were mixed and crushed to give a homogeneous powder and then inserted into a DRS-8000 device (non-disc). The spectrum was acquired from 400 to 4000 cm^{-1} wave numbers. All collagen samples were treated equally to obtain the IR spectra.

Casson's trichrome/Masson's trichrome staining

An approximate 0.1- μ L sample of collagen from the bovine tendon isolation was placed in a glass object and allowed to air-dry. Then, the sample was soaked in Casson's trichrome solution for 5 min, washed with running water for 3–5 s, and dried by absorption with filter paper until dry. Fast dehydration was achieved by mixing the sample with 100% alcohol 3 times. The collagen tissue observation was performed using a light microscope with a 10 \times objective magnification. A positive result for the presence of collagen was characterized by the color of the sample turned blue.

pH values

A total of 1 g of sample was dissolved in 70 mL of aquadest until homogeneous. The pH meter was turned on and then calibrated by allowing the readings to stabilize at each pH. The electrode was dipped into the sample solution until a stable pH value was obtained [5].

Measurement of the water content

A glass weighing bottle was dried in an oven at 105°C for 1 h. Then, the dried glass weighing bottle was placed in a desiccator for 15 min and weighed. A 1 g sample was then added to the dry glass weighing bottle and weighed. The glass weighing bottle containing the sample was placed into an oven at 105°C for 5–6 min or until it reached a constant weight. The glass weighing bottle was placed into a desiccator for 30 min and then weighed [5].

Measurement of the ash content

A porcelain cup was cleaned and then dried in an oven at 105°C for 30 min. The cup was then placed in a desiccator for 30 min and weighed. A 1 g sample was added to the porcelain cup, and the cup with the sample was placed in a furnace at 600°C for 7 h. The cup containing the residual ash was placed in a desiccator for 30 min and then weighed to obtain the weight of the ash residue [5].

Viscosity test

The viscosity test was performed by preparing a solution of collagen sample at a concentration of 0.5% in aquadest, followed by measurement of the viscosity using an Ostwald viscometer. The solution was passed through the capillaries from the upper limit to the bottom, and the flow time was accurately recorded (stopwatch). The value of the viscosity was expressed in units of centipoises (cP).

Amino acid derivatization process

A 300- μ L test solution was taken for the derivatization process by adding 300 μ L of 10 mM boric buffer (pH 9) to 300 μ L of

1.5 mM FMOC-Cl (in acetonitrile). Then, 20 μ L of the sample was injected into the HPLC.

Optimization of the HPLC conditions for analysis of glycine, proline, and hydroxyproline

Determination of the optimal analysis wavelengths

In this step, the determination of the optimal emission wavelengths was performed. The determination of the excitation wavelength was performed using a UV-Vis spectrophotometer and standard solutions of hydroxyproline, glycine, and proline.

Selection of mobile phase composition

To select the mobile phase composition, several acetate buffer (pH 4.2):acetonitrile ratios were investigated as follows: 55:45, 60:40, and 65:35.

Selection of the mobile phase flow rate for analysis

The mobile phase flow rates (at the compositions described above) of 0.8, 1.0, and 1.2 mL/min were investigated.

Test system compatibility

A 300- μ L aliquot of collagen standard solution was added to 300 μ L of 10 mM boric buffer, pH 9, and then 300 μ L of 1.5 mM FMOC-Cl (in acetonitrile) was added. After the derivatization, 20 μ L of the sample was injected into the HPLC using the test mobile phases and selected flow rates. Six injections were made.

Calibration curve and linearity test

Each standard solution of glycine, proline, and hydroxyproline in different concentrations was injected into the HPLC at the selected conditions.

Determination of the limit of detection (LOD) and limit of quantitation (LOQ)

From the obtained calibration curve, the smallest detectable concentration (LOD) and smallest detectable quantitative concentration (LOQ) were calculated from the linear regression line of the calibration curve.

Determination of glycine, proline, and hydroxyproline concentrations in isolated collagen samples from bovine tendon

50 mg of collagen isolated from the bovine tendon was placed in a suitable container and mixed with 5 mL of HCl 6 N. The mixture was incubated in an oven at 110°C for 22, 23, 24, and 25 h, then cooled, and dissolved in acetate buffer to obtain a concentration of 10 μ g/mL.

The derivatization was then performed in the same manner as that used for the standard preparation. A 20- μ L aliquot of each sample solution was injected into the HPLC under the selected analysis condition. Three replicates of each sample were injected to obtain an average value.

RESULTS AND DISCUSSION

Isolation of collagen

The result of the bovine tendon that was immersed in the 0.1 N NaOH solution was turned a cloudy color on the 1st day, but on the 2nd and 3rd days, the solution became clearer. This finding indicates that the dirt on the bovine tendon on the 2nd and 3rd days was reduced.

After the extraction process, the weight of the bovine tendon increased as it absorbed the acetic acid solution, and its shape changed to a more developed and elastic-like jelly. Then, the separated acid filtrate was subjected to the salting-out process or to the addition of NaCl. NaCl binds the acetic acid, so the collagen contained in the filtrate precipitated. The salted-out collagen was separated by centrifugation at 4000 rpm and 4°C for 20 min, which allowed large collagen pieces to settle on the bottom of the centrifugation tubes.

The process of dialysis for purification began by passing 0.1 M acetic acid over the collagen, so the concentration of 0.5 M acetic acid dissolved in the collagen decreased. The ratio of the volume of liquid collagen and 0.1 M acetic acid was 1:5. The dialysis process was continued by passing it over the aquadest that served to dissolve the NaCl and raise the pH of the collagen to eliminate acid.

After the dialysis process was complete, the wet collagen was lyophilized using a freeze dryer so that we could obtain solid collagen in the form of fine fibers. The total weight of the dry collagen obtained was 7.273 g, so the yield of the bovine collagen sample was 0.690%.

Characterization of bovine tendon collagen

Organoleptic properties

The collagen obtained from the isolation was in a dry, filamentous, white, solid form (Fig. 1). The fishy odor produced by it was the result of the isolation from bovine tendon, and the acidic odor was caused by the isolation process using acetic acid.

Functional group analysis by FTIR

The FTIR spectroscopy for both the standard collagen and sample showed a typical absorption spectrum of collagen, i.e., absorption peaks in the amide uptake region of amides A, B, I, II, and III. The amide A in collagen shows asymmetrical stretching of NH with hydrogen bonds detected in the approximate range of 3200–3440 cm^{-1} (Sai and Babu, 2001). Amide B uptake shows the CH_2 asymmetric stretch of collagen in the range of 2935–2915 cm^{-1} . The amide I C=O stretch was observed at 1681.02 cm^{-1} and the absorption region was in the range of 1600–1690 cm^{-1} [6]. The amide II bands are for CN stretching and NH bending. The amide II spectra occurred in the absorption region of 1480–1575 cm^{-1} and the center was at 1553.71 cm^{-1} . The CH_2 bend region of amide II was found at 1450.52 cm^{-1} [6]. The amide III NH bending of the NH bound to the CN group was located in the region of 1229–1301 cm^{-1} . The amide III in the collagen samples was observed at a wave number of 1234.48 cm^{-1} .

Staining with Casson's trichrome

The collagen isolation from the bovine tendon showed a positive result of collagen tissue with the presence of blue color (Fig. 2). The blue color indicates the presence of collagen tissue derived from aniline blue stain.

pH value

The pH of the collagen samples was 6.64. The collagen pH value in this study was in accordance with the collagen standard established by BSN (2014), which states that the standard pH value of collagen ranges from 6.5 to 8. The neutral pH of the collagen samples obtained is due to the effect of neutralization with aquadest after dialysis using acetic acid [7].



Fig. 1. Organoleptic isolated bovine collagen

Water content

The water content values obtained from two measurements were 1.80% and 1.70%, with an average moisture content of 1.75%, which met the collagen quality requirement based on BSN (2014) ($\leq 12\%$). Samples that have high water contents can be damaged if left in open air because of microbial activity [7].

Ash content

The ash content values obtained from two measurements were 0.20% and 0.30%, with an average of 0.25%, which met the quality requirement of collagen based on BSN (2014) (≤ 1.0) [7].

Viscosity test

The viscosity values of the collagen samples were 0.702, 0.731, and 0.726 cP, with an average of 0.720 cP, which was low because the collagen sample was dissolved in heated water, and the higher the temperature, the lower the viscosity value. Heating can also damage the hydrogen bonds in collagen, so the collagen solution becomes diluted.

Formation of derivative compounds

The derivatizing agent used was FMOC-Cl because it can react with both the primary and secondary amino acids to form a fluorescent compound product. The derivatization reaction between the amino acids and FMOC-Cl is a substitution reaction, i.e., the Cl group in FMOC-Cl is replaced by the primary and secondary amine groups. The derivatization process with FMOC-Cl also requires a buffer with an alkaline pH of ≥ 8.0 . The collagen standard itself has a pH ranging from 6.5 to 8, and the collagen assay solution is dissolved in acetate buffer (pH 4.2); therefore, before being analyzed by the KCKCT method, the standard collagen solution is supplemented with a pH 9 boric buffer [8].

To select the FMOC-Cl reagent volume used, a stoichiometric calculation was performed to determine how many volumes of reagent were required to produce the optimum compound derivative. In this study, 300 mL of collagen test solution was used with 300 mL of boric buffer and 300 mL of FMOC-Cl.

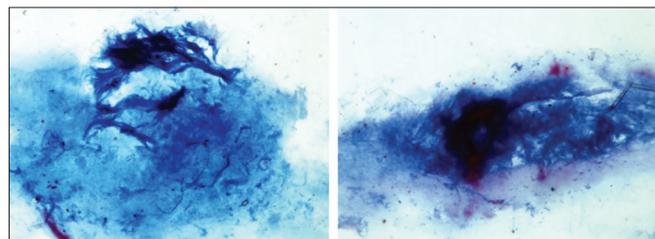


Fig. 2: Staining of bovine collagen samples with Casson's trichrome

Table 1: Hydrolysis time versus peak area of the derivatized compound

Hydrolysis time (h)	Peak area (mV/s)		
	Hydroxyproline	Glycine	Proline
22	94 073	203 781	365 141
23	57 014	156 464	201 178
24	63 202	238 078	141 636
25	24 087	155 995	80 549

Table 2: Peak areas of the derivatized compound versus wavelength

Wavelength	Peak area (mV/s)				
	Excitation (nm)	Emissions (nm)	Hydroxyproline	Glycine	Proline
265		320	63 202	238 078	141 636
		325	16 392	16 196	9343
		330	-	14 817	3902

Hydrolysis time optimization

The hydrolysis of the collagen was performed with deamination to remove amine groups from amino acid compounds to break the peptide bond using 6 N HCl at 110°C in an oven and was performed at the different hydrolysis times of 22, 23, 24, and 25 h. An hydrolysis of >24 h provided a larger glycine peak area than those of proline and hydroxyproline, as found by Schrieber and Gareis [9]. The peak areas for the hydrolysis times of 22, 23, and 25 h were not larger than that at the hydrolysis time of 24 h (Table 1). Thus, the optimal hydrolysis time was 24 h.

Wavelength analysis

The optimum wavelengths for the collagen compounds for fluorescence analysis were 265 nm for excitation and 320 nm for emission (Table 2). The result showed the uptake of 0.2018 A and the maximum wavelength was 265 nm.

Optimum analysis conditions

The mobile phase selected for the analysis of amino acids in the collagen samples consisted of acetate buffer: acetonitrile at a ratio of 55:45. At a mobile phase ratio of 60:40, glycine appeared at a retention time of 6.464 min, whereas hydroxyproline and proline were not detected. At a mobile phase ratio of 65:35, none of the amino acids were detected. This was because the mobile phases with ratios of 60:40 and 65:35 were more polar than that with a ratio of 55:45 because of the higher aqueous acetate buffer percentages; therefore, the amino acids had a greater affinity for the stationary phase (C18 column) and a lower affinity for the mobile phase; hence, glycine, proline, and hydroxyproline had longer retention times or may not have been eluted in the 60:40 and 65:35 mobile phase ratios (more polar than the 55:45 ratio).

After the optimum HPLC conditions were determined, the flow rate was optimized by evaluating three rates: 0.8, 1.0, and 1.2 mL/min. The flow rate of 0.8 mL/min was selected because it provided better column efficiency (small height equivalent to a theoretical plate [HETP]), a larger number of theoretical plates, and gave good separation because the resolution values between pairs of amino acids were >1.5 .

System conformity test

The data obtained from an average of six injections of the standard collagen solution were used. The following results for hydroxyproline were obtained: Average follow-up factor, 0.918; HETP, 2.27×10^2 ; resolution, 3.475; number of theoretical plates, 11,025.67; and coefficient of variation, 0.88%. The following results for glycine were obtained: Mean follow-up factor, 1.741; HETP, 7.91×10^3 ; resolution, 5213; number of theoretical plates, 31,660.617; and coefficient of variation, 0.49%. The following results for proline were obtained: Mean follow-up factor 0.888; HETP 2.29×10^2 ; resolution 4962; number of theoretical plates 10,951.5; and coefficient of variation, 1.40%. The results obtained from the system conformity test data satisfied the requirements because the coefficients of variation were $\leq 2\%$, the column efficiencies were good with small HETP values, the numbers of theoretical plates were high, and the resolution values were >1.5 .

Calibration curve and linearity test

The linear regression gave the following equations for the calibration curve lines: For glycine, $y=9351.2x+30.431$; for proline, $y=5824.4x+38.859$; and for hydroxyproline, $y=1655.4x+26.980$. The linearity tests gave a correlation coefficient (r)=0.9979 for glycine, $r=0.9985$ for proline, and $r=0.9981$ for hydroxyproline. The three

correlation coefficients (r) were not ≥ 0.999 possibly because the calibration curve solution contained all three amino acids or because of the effect of the derivatization process.

Determination of the LOD and LOQ

The LOD and LOQ values were, respectively, 0.990 and 3.301 $\mu\text{g/mL}$ for glycine, 0.998 and 3.327 $\mu\text{g/mL}$ for proline, and 0.900 and 3.001 $\mu\text{g/mL}$ for hydroxyproline. The LOQ values for the three amino acids were not within the concentration range of the calibration curve. It is possibly happen because, at the time, the calibration curve solutions were prepared they consisted of a mixture of the three amino acids; therefore, the LOD calculation and LOQ results were influenced by the linearity test and calibration curve.

Determination of glycine, proline, and hydroxyproline concentrations in collagen samples isolated from bovine tendons

The percentages were calculated according to the linear regression equation of each amino acid. The mean percentages of glycine, proline, and hydroxyproline were $33.247 \pm 0.20\%$; $11.867 \pm 0.20\%$; and $10.510 \pm 0.23\%$, which were similar to the levels of bovine collagen reported by Schrieber and Gareis, i.e., 33% glycine, 11% proline, and 10% hydroxyproline [9,10].

CONCLUSION

The collagen isolated from bovine tendon was obtained at a yield of 0.690%, and the identity was confirmed by FTIR functional group analysis and Casson's trichrome staining. The HPLC conditions using a fluorescence detector for analysis of glycine, proline, and hydroxyproline concentrations in the bovine tendon collagen were optimized. The analysis of amino acids gave average levels of $33.247 \pm 0.20\%$ for glycine, $11.867 \pm 0.20\%$ for proline, and $10.51 \pm 0.23\%$ for hydroxyproline.

CONFLICTS OF INTEREST

All authors have none to declare.

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