

A Novel and Efficient Collagen Extraction Method Assisted by Microwave Irradiation

by

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Abstract

A new and efficient microwave heating method was developed to extract intact Type I collagen from bovine limed splits. Orthogonal experiment was conducted to optimize the parameters of extraction, and the extraction yield was measured by ultraviolet spectra (UV). The hierarchical structures of the obtained collagen were determined by amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), UV, Fourier transform infrared spectra (FT-IR), circular dichroism spectra (CD), fluorescence spectra (FL) and ultra-sensitive differential scanning calorimetry (US-DSC). The results indicated the optimal conditions for this microwave assisted extraction were 37°C for 7 h by using solid to liquid ratio of 1:45. The resultant yield of collagen was 14.35% under the optimal conditions, which is 1.5 times higher than that obtained by the control water bath-heating method. Amino acid analysis, UV and SDS-PAGE revealed that the primary structure of collagen extracted with microwave assistance was type I collagen. Although the extraction yield was promoted drastically with microwave assisting, the secondary structure of the collagen involving triple helix still maintained according to FT-IR, CD, FL and VP-DSC results. In brief, microwave irradiation could be a new routine for collagen extraction with advantages of fast and effective. Moreover, this study might offer a potential choice for utilizing hide or skin limed split wastes.

Introduction

Statistically, more than 6.9 million tons of wet salted hides and skins are converted into leather worldwide annually.¹ Solid waste generated simultaneously from tannery processes is estimated at approximately 0.8 kg per kg of raw hides/ skin,²⁻⁴ of which about one-third is limed split wastes. These wastes can be utilized by microbiological methods as material for biodiesel or for composting.⁵⁻⁸ Particularly, limed split wastes have been used for the production of high value-added collagen as they are less contaminated by chemicals.^{9,10}

Type I collagen is the dominant protein in mammals, accounting for 25-30% of the total protein weight.¹¹ This fibrous structural protein consists of three left-handed spiral peptide chains with a right-handed supercoil structure named triple helix.¹² It is widely

used in biomedical, food, cosmetics and so on.^{13,14} Therefore, the development of collagen extraction remaining natural structure will bring momentous scientific significance and application value. At present, hides and skins are important resources for collagen extraction. The methods for preparing collagen mainly include acid extraction, enzymatic extraction, alkali extraction and neutral salt extraction.¹⁵⁻¹⁷ Among these methods, the collagen extracted by acid method sustains complete structure with high purity, but the extraction yield is lower.¹⁸

Since microwaves were used to treat nuclear waste in Harwell Laboratory at 1970, they have been widely used in various chemical fields as a transmission medium or heating energy source.^{19,20} Now, microwave irradiation has become an important technology to accelerate chemical reactions.^{21, 22} Moreover, previous studies proved that microwave-assisted methods could shorten the time and increase the extraction yield of collagen, gelatin and collagen hydrolysates.²³⁻²⁶ Unfortunately, the application of microwave in collagen extraction was limited to short-time pretreatment, and especially remaining collagen triple helix structure, has not been reported.

This study explores a new extraction method by microwave irradiation assistance, so as to prepare structurally intact Type I collagen more effectively. In this work, collagen was extracted by an acid method, and microwave irradiation was used as a heating source in the whole extraction process. A conventional heating process was used as a control, and the extraction yield was optimized by an orthogonal method and calculated based on UV testing. Structure and properties of the obtained collagen were characterized from its primary and secondary structures. This work would be a novel method for more efficient collagen extraction and provide scientific guidance for utilizing bovine limed split wastes effectively.

Experimental

Materials

Salted bovine hide was bought from local slaughterhouse in Chengdu, Sichuan province, China. Standard type I collagen was purchased from Sigma (USA) and without any further purification. Acetic acid was provided by Chengdu Kelong Chemical Co. LTD.

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All chemicals used for preparing limed pelt were industrial reagents, and the chemicals for collagen extraction and determination were commercially available of analytical grade.

Sample preparation

Pre-treatment of pelt

Limed pelt from salted bovine hide was prepared by a conventional beamhouse process. After splitting, the samples with the size of 100 × 80 cm were taken symmetrically along the backbone and weighed. Then the samples were delimed with 2% ammonium chloride and 0.5% hydrochloric acid for 1 h to adjust pH between 6.0 and 7.0, and the cross section of pelt was colorless by phenolphthalein indicator. The samples, afterwards, were rinsed with distilled water for 30 min and cut into fragments of 0.5 × 0.5 cm. Component analysis of the fragments was carried out according to the method of AOAC.²⁷ The main constituents of the raw material were proteins and water accounting for 17.1% and 72.2% respectively. The raw material was lyophilized and stored in sealing bag at 4°C.

Collagen extraction process

The 10 g dried limed pelt fragments were extracted with 0.5 mol/L acetic acid solution under 35°C at a solid to liquid ratio of 1:40 (w/v) for 8 h. The extraction with microwave-assistance was carried out in MCR-3C microwave reactor (Xi'an Yuhui instrument Ltd.), and the control was heated by using DF-101S water bath heater (Wuhan Ke'er instrument Company) at corresponding conditions. After extraction, the solution was filtered with 200 mesh nylon filter cloth to obtain crude extract. The crude extract was centrifuged at 8000 r/min for 10 min by using TG-20 high speed centrifuge (Sichuan Shuke instrument Co., Ltd; China) and supernatant was collected. Sodium chloride was added to the supernatant and until the concentration was 3 mol/L and precipitation appeared. Subsequently, the precipitation was collected by centrifugation again and dissolved in 0.5 mol/L acetic acid solution. The solution was dialyzed with distilled water for 3 days and lyophilized. The prepared collagen was stored in a dryer.

Extraction optimization

On the basis of single factor experiment, orthogonal experiments of three factors and three levels were carried out on temperature (33, 35, 37 °C), solid to liquid ratio (1:35, 1:40, 1:45) and extraction time (6, 7, 8 h). The extraction temperature was settled below the critical denaturation temperature of collagen (37°C).

Testing methods

Collagen extraction yield

With acetic acid solution of 0.5 mol/L as reference, the absorbance of collagen solution was recorded over the range of 200-400 nm at the scanning speed of 400 nm/min by UV1900 (Shanghai Flying Art instrument Co., Ltd.; China). Standard type I collagen solutions

of 0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/L were prepared with 0.5 mol/L acetic acid solution. According to absorbance of these solutions at the characteristic absorption peak, standard curve used to calculate collagen concentration of the crude extract was made. Confirmed collagen concentration and absorbance to Lambert-Beer's law, the linear relationship was $y=0.5799x+0.0307$, besides, the linear relationship variance was 0.9955. The yield of collagen was calculated according to following formula based on UV testing:

$$Y = (C \times V) / (M \times F) \times 100\%$$

Y – The extraction rate of collagen (%);

C – Collagen concentration in crude extract (mg/L);

V – Volume of crude extract (L);

M – the weight of raw material (mg);

F – percentage of protein in raw material skin (%).

Amino acid analysis

About 0.1 g collagen was completely hydrolyzed by 6 mol/L hydrochloric acid at 120°C for 24 h in the absence of oxygen. Hydrolyzed solution was filtered and diluted to 50 mL. An aliquot of 0.1 mL was applied to L-8900 analyzer (Hitachi Co., Ltd.; Japan).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Collagen was analyzed by SDS-PAGE gel electrophoresis with 8% separation gel and 5% concentrated gel system respectively. The collagen was dissolved in 0.5 mol/L acetic acid to obtain a final concentration 5 mg/mL collagen solution, then mixed with the sample buffer (0.0625 mol/L Tris-HCl, pH 6.8, containing 2.3% SDS, 10% glycerol and 5% mercaptoethanol) at a ratio of 1:1(v/v). The mixture was boiled for 5 min. Electrophoresis was performed by an electrophoresis instrument (Min-PROTENIN3, Bio-Rad Laboratories, Inc. USA) at a constant current of 60 V through the concentrated glue and then 120 V the separating glue. The protein markers with high molecular weight (Sigma Chemical Co., USA) were loaded alongside the collagen samples at a loading of 10 µL. The gels were stained by 0.25% Coomassie Brilliant Blue R-250 for 1h and eluted until the background color was colorless in 10% methanol and glacial acetic acid. Ultimately, the gray level of the gels was analyzed by software, Image J 1.48 v, to calculate the molecular weight distribution.

Fourier transform spectroscopy measurement (FT-IR)

Collagen was mixed with potassium bromide (1:100), ground and pressed evenly for spectrum recording. Subsequently, it was scanned by Nicolet iS10 Fourier transform infrared spectrometer (Seymour Technology Co., Ltd.; America) for 32 times with a wave number range of 400-4000 cm⁻¹ at room temperature.

Circular dichroism spectroscopy measurement (CD)

Collagen was dissolved in 0.5 mol/L acetic acid to prepare 0.05 mg/mL collagen solution, and the solution was monitored by CD using Jasco J-1500 spectrometer (Jasco Corporation, Japan). The container for the determination was a rectangular quartz cell with a path length of 1 cm. Spectrum was recorded from 250 to 190 nm with standard sensitivity under nitrogen atmosphere. The wavelength, scanning speed, band width, response and data pitch were set at 50 nm/min, 2 nm, 1 s and 0.5 nm, respectively.

Fluorescence Spectroscopy measurement (FL)

The fluorescence was measured under 25°C. Before the measurement, collagen concentration of 0.5 mg/mL in 0.5 mol/L acetic acid solution was equilibrated for 3 min in a fluorescence spectrophotometer (CaryEclipse, Agilent, America). The excitation wavelength was fixed at 275 nm, and the emission spectrum was recorded in the range of 280-400 nm under 25°C at scan rate of 120 nm/min. Fluorescence excitation and emission slits were 5 nm.

Synchronous fluorescence spectrum was scanned between wavelength 200 and 400 nm at the excitation wavelength of 275 nm. Both the excitation and the emission slits were 5 nm. Wavelength shift ($\Delta\lambda$) between excitation and emission was 15 nm. And the scanning temperature was 25°C with a scanning speed of 120nm/min.

Ultra-sensitive differential scanning calorimetry (US-DSC)

0.5 mg/mL collagen was prepared by dissolving collagen in 0.5 mol/L acetic acid and degassed in the instrument for 30 min. Then it was tested under the temperature range 20 to 60 °C by VP-DSC hypersensitive differential scanning calorimetry (Microcal; USA) with the increasing rate of 1 °C / min.

Results and discussion**Optimization of microwave-assisting collagen extraction**

According to the analysis of the orthogonal experiment (Table I), the highest extraction yield was 11.65%, and the corresponding extraction conditions were 35°C, solid to liquid ratio 1:35 and 7 h (Number 4). In the table, S and R were the variance and range of K_1 , K_2 and K_3 , respectively. The larger the variance and the range difference were, the more significant the influence of this factor was on the extraction yield, illustrating that the order of the influence on the extraction yield was as follows: irradiation temperature, irradiation time, solid to liquid ratio.

Under the optimal condition (extraction temperature 37°C, solid to liquid ratio 1:45 and irradiation time 7 h) from orthogonal experiment, the verification experiment showed that the collagen extraction yield could reach to 14.35%, while the yield under corresponding condition with water bath heating was only to 9.30%, showing microwave irradiation could improve the extraction yield significantly.

Table I
Orthogonal test results of collagen extraction by microwave irradiation

Number	Irradiation temperature /°C	solid-liquid ratio	Irradiation time /h	Extraction yield/%
1	33	1:35	6	5.98
2	33	1:40	7	10.77
3	33	1:45	8	8.59
4	35	1:35	7	11.65
5	35	1:40	8	7.78
6	35	1:45	6	10.38
7	37	1:35	8	11.48
8	37	1:40	6	10.80
9	37	1:45	7	11.49
K_1	25.34	29.11	27.16	
K_2	29.81	29.36	33.91	
K_3	33.77	30.46	27.85	
R	8.42	1.36	6.75	
S	11.84	0.35	9.19	

Table II
Amino acid composition of different kinds of collagen

Amino acid	Molar percentage /%		
	Experimental product	Control product	Type I collagen
Asp	4.41	4.55	4.68
Thr	1.52	1.72	1.81
Ser	3.77	3.73	3.66
Glu	7.72	7.60	7.52
Gly	33.90	34.38	33.95
Ala	10.54	10.17	10.48
Val	1.32	0.22	1.21
Met	0.63	2.53	1.47
Ile	1.41	1.33	1.24
Leu	3.10	3.04	2.94
Tyr	0.22	0.43	0.41
Phe	1.89	1.96	1.41
Hyls	0.76	0.77	0.86
His	0.38	0.31	0.52
Lys	2.94	2.86	3.17
Arg	5.48	5.35	5.65
Hypro	7.40	7.42	7.81
Pro	12.60	11.64	11.21

During acid extraction, the structure of salt bond and Schiff base between collagen molecules was destroyed by acid and resulted in collagen dissolving.²⁸ Acetic acid solution, as an extracting agent, was chemically simple containing molecules of water and acetic acid only. The electromagnetic field generated by microwave changed the ionization of acetic acid in water. Consequently, the extraction system contained more acid with microwave assisting compared with water bath heating, and the higher acidity weakened the intermolecule hydrogen bonds of collagen in pelt.²⁹ On the other hand, polar molecules in solution and pelt would have more complicated and extra oscillation with the change of microwave electromagnetic field, it benefit for molecular collision and chemical bond breaking. Just as microwave could accelerate and promote other chemical reactions, the more efficient collagen extraction was obtained under microwave irradiation with higher extraction yield.

Primary structure of collagen extracted by microwave assisted

Amino acid composition of collagen

The amino acid composition of collagen extracted under microwave irradiation (the experimental product) and water bath heating (control product) as well as standard type I collagen were shown in Table II. Compared with standard type I collagen and bovine collagen,³⁰ the two products contained about one-third glycine, 11% alanine and without tryptophan, meaning the extracted collagen had the characteristics amino acid of type I collagen. The ratio of hydroxyproline to proline of the experimental product was 0.59,

similar to that of the control product (0.63), indicating that the stability of the two products was comparable.³¹

Molecular weight of collagen

The electropherogram of three kinds of collagen was shown in Figure 1. It was clear that there were three distinct electrophoretic bands. In addition to the cross-linked β chain, the samples included at least

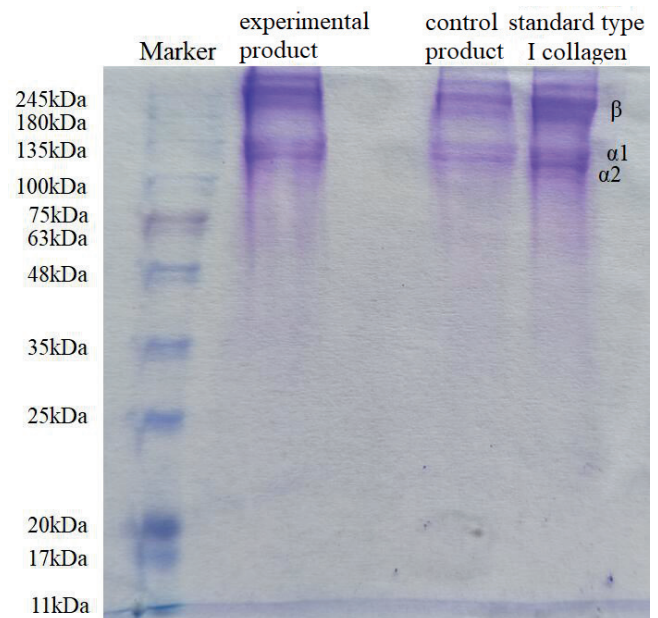


Figure 1. Electropherogram of different kinds of collagen

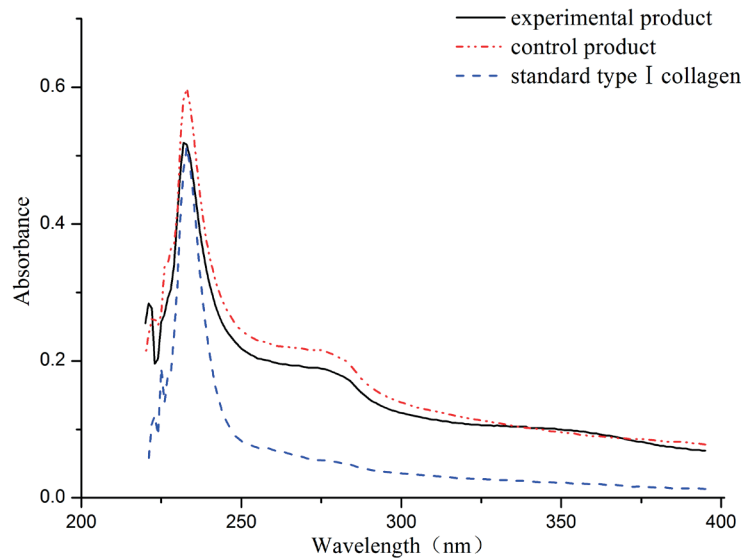


Figure 2. UV-Vis spectra of different kinds of collagen

two α chains (α_1 and α_2) with molecular weight larger than 100 kDa, conforming to the characteristic of type I collagen.^{32,33} According to the gray level measurement, the molecular weight proportion of experimental product above 100 kDa was 89.4%, indicating its higher purity. Furthermore, it was basically the same as that of the standard type I collagen and was not significantly different from that of control product.^{34,35}

UV-Vis Spectra of collagen

The ultraviolet spectra of standard type I collagen and the extracted products were shown in Figure 2. There was a maximum absorption peak of the products at 234 nm, and no significant difference between three samples could be observed, showing that microwave irradiation would not impair the primary structure of collagen.

Secondary structure of collagen extracted by microwave assisted FT-IR spectra of collagen

FT-IR spectrum of different kinds of collagen was shown in Figure 3. There were five amide bands. Amide A band was caused by the stretching vibration of N-H at 3400-3440 cm^{-1} . Amide B band at 2800-3000 cm^{-1} was a stretching vibration peak generated by the asymmetric CH_2 . Amide I band absorption peak between 1640 and 1660 cm^{-1} , the main characteristic absorption peak of collagen infrared spectrum, was attributed to the vibration peak of the C=O group, and it was often used for the analysis of secondary structure of collagen.^{36,37} Since the absorption peaks of the samples were almost at the same position, it inferred that the microwave irradiation extraction technique did not destroy the conformation of the product. Due to the C-N stretching vibration and N-H bending

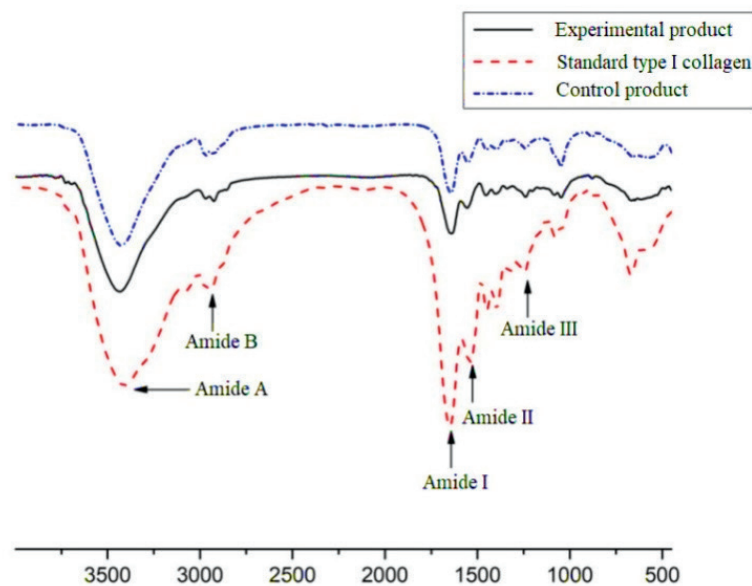


Figure 3. FT-IR spectra of different kinds of collagen

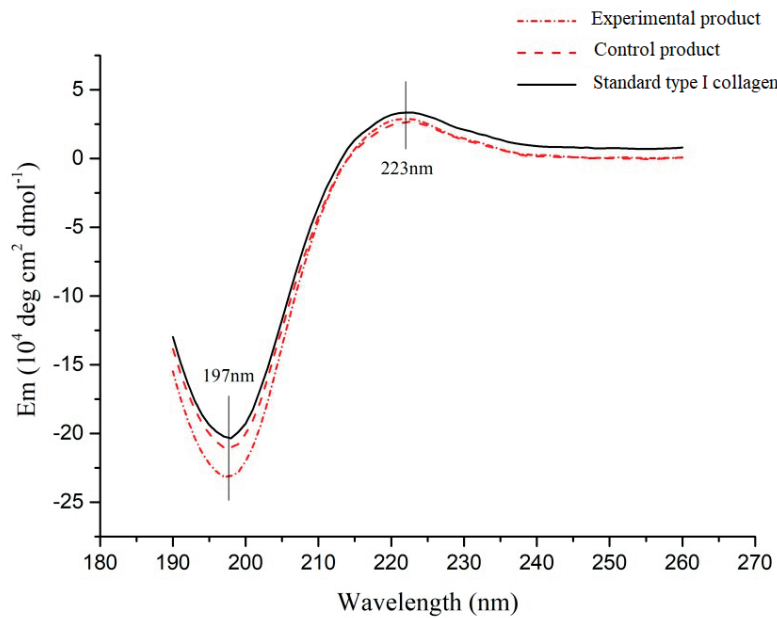


Figure 4. CD spectra of different kinds of collagen

vibration of collagen, the absorption peak of the amide II band appeared at $1500\text{--}1600\text{ cm}^{-1}$, and the absorption peak of the amide III band was produced between 1200 and 1360 cm^{-1} . Therefore, it could be speculated that the products possess a complete triple helix structure.³⁸

CD spectra of collagen

Circular dichroic spectrum of the samples was shown in Figure 4. The circular dichroic spectrum of collagen was characterized by a positive absorption peak around 225 nm and a negative absorption peak near 197 nm .³⁹ The collagen became denatured gradually, and the molar ellipticity of the absorption peak near 225 nm decreased while that near 197 nm increased.⁴⁰ There was a significant negative

absorption peak at 197 nm and a weak positive absorption peak at 223 nm in the circular dichroic spectrum of samples. These three kinds of collagen had almost same circular dichroic spectrum, indicating that the collagen under microwave irradiation still has a complete spatial configuration of the polypeptide chain.

The intrinsic fluorescence analysis of collagen

The fluorescence emission spectra of the samples is shown in Figure 5. Phenylalanine and tyrosine could be used as internal probes to measure intrinsic fluorescence.⁴¹ Collagen contained a high content of tyrosine, the main source of endogenous fluorescence. Tyrosine residues mainly exhibited the emission peak of 310 nm , and its fluorescence intensity increased with the rise of collagen

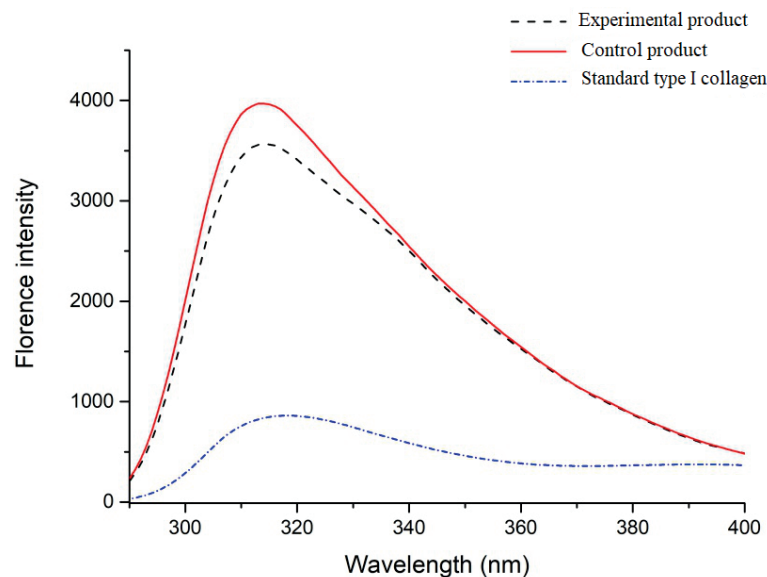


Figure 5. Fluorescence emission spectra of different kinds of collagen

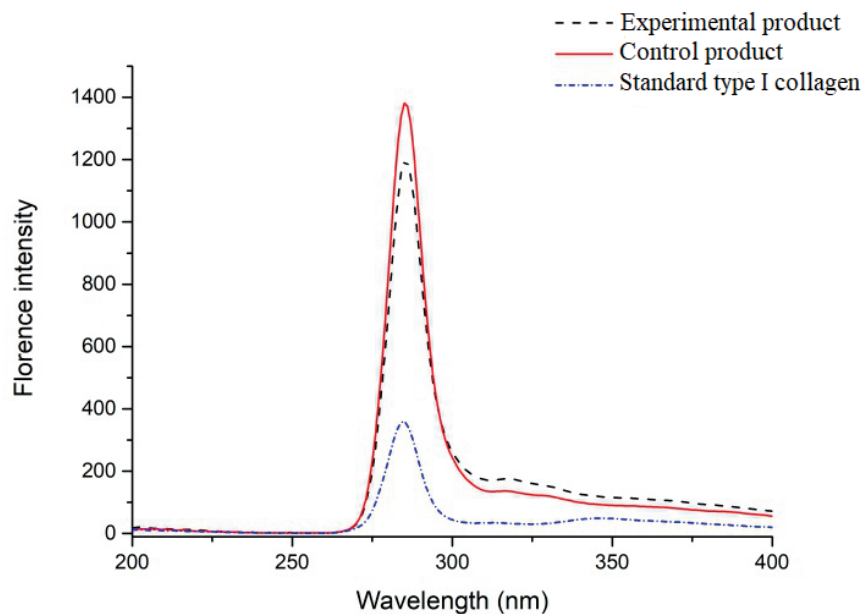


Figure 6. Synchronous fluorescence spectra of different kinds of collagen

concentration.⁴² Although the fluorescence intensities of the samples were different, there was a significant fluorescence emission peak in the range of 300-400 nm and the fluorescence spectrum curve didn't change. The same peak meant that the chemical condition of tyrosine in the experimental sample and the control was identical. It was evidence that there was no negative effect on collagen structure during microwave extraction process.

The synchronous fluorescence analysis of collagen

Figure 6 is a synchronous fluorescence spectrum of the products and the standard type I collagen. The spectrum not only maintained the sensitivity of general fluorescence correlation, but also possessed the characteristics of narrowing the spectral band, simplifying the emission spectrum and narrowing the spectral range.⁴³ It could reflect the effect of extraction methods on the endogenous fluorescence of collagen. The picture revealed the larger fluorescence intensity of the control product. This was because a conjugated rigid structure could increase the fluorescence intensity,⁴⁴ but under the action of microwave field, the movement of collagen molecules was more intense, and it wasn't conducive to molecular aggregation and conjugate formation. In addition, the characteristic peaks of synchronous fluorescence of the samples

were mainly absorbed by tyrosine residues, thus, a significant absorption peak appeared at 284 nm, indicating that the process of microwave irradiation extraction of collagen didn't damage the structure of collagen.

Denaturation temperature of collagen

Table III shows the thermal denaturation temperature of collagen obtained with different extraction methods. There were two endothermic peaks on the typical collagen VP-DSC curve, corresponding to the transition temperatures Tm_1 and Tm_2 , respectively. Among them, Tm_2 was an important indicator of the thermal stability of collagen, and the value and the stability of collagen were positively correlated.⁴¹ The thermal denaturation temperature of experimental product was 38.82°C, which was consistent with the literature.¹⁸ Compared with the control product, the extraction under microwave irradiation had little effect on thermal stability of collagen.

To sum up, the collagen extracted under microwave irradiation still had a complete spatial configuration of the polypeptide chain. This extraction method could prepare structurally intact collagen more effectively.

Table III
Thermal denaturation temperatures of different kinds of collagen

Sample	Experimental product	Control product	Standard type I collagen
Denaturation temperature/°C	38.82	38.91	39.09

Conclusions

Type I collagen was extracted from limed splits by using microwave irradiation as heating source. The yield of structurally intact Type I collagen was 14.35% at an optimum process temperature of 37°C, a solid to liquid ratio 1:45 and an irradiation time of 7 h. By comparison, the extraction yield was 9.30% by using the conventional water bath heating under corresponding conditions. The new microwave assisted extraction method was effective in preparing structurally intact Type I collagen, and the extracted collagen retained a complete primary structure and secondary structure of the collagen involving triple helix. This new method increased the extraction yield of collagen and thus would be potential way for limed splits utilization.

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References

- Puhazhselvan, P., Pandi, A., Sujiritha, P. B., *et al.*; Recycling of tannery fleshing waste by a two step process for preparation of retanning agent. *Process Safety and Environmental Protection* **157**, DOI10.1016/j.psep.2021.11.003, 2021.
- Hu, J., Xiao Z. B., Zhou R. J., *et al.*; Ecological utilization of leather tannery waste with circular economy model. *Journal of Cleaner Production* **19**, 221-228, 2011.
- Zhang, Z. K., Li, G. Y., Shi, B.; Physicochemical properties of collagen gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather technologists and Chemists* **90**, 23-28, 2006.
- Kanagaraj, J., Panda, R. C., Vinodh, K. M.; Trends and advancements in sustainable leather processing: Future directions and challenges—A review. *Journal of Environmental Chemical Engineering* **8**, doi.org/10.1016/j.jece.2020.104379, 2020.
- Colak, S., Zengin G., Oezguenay H., *et al.*; Utilization of leather industry pre-fleshings in biodiesel production. *JALCA* **100**, 137-141, 2005.
- Collivignarelli, C., Barducci G.; Waste recovery from the tanning industry. *Waste Management & Research* **2**, 265-278, 1984.
- Sandhya, K.V., Abinandan S., Vedaraman N., *et al.*; Extraction of fleshing oil from waste limed fleshings and biodiesel production. *Waste Management* **48**, 638-643, 2016.
- Vasudevan, N., Ravindran A.D.; Biotechnological process for the treatment of fleshing from tannery industries for methane generation. *Current Science* **93**, 1492-1494, 2007.
- Chen, Y. H., Li G. Y., Jia Y.; Rheological behaviour of alkali solubilized collagen from limed bovine split wastes. *Journal of the Society of Leather technologists and Chemists* **97**, 195-199, 2013.
- Li, D., Li G.Y., Jiang D. Y., *et al.*; Extracting of native collagen from bovine limed split wastes: pretreatment and extraction. *China Leather*, **37**, 8-11 2008.
- Zhang, X. X., Xu, S. C., Shen L. R., *et al.*; Factors affecting thermal stability of collagen from the aspects of extraction, processing and modification. *Journal of Leather Science and Engineering* **2**, <https://doi.org/10.1186/s42825-020-00033-0>, 2020.
- Shoulders, M.D., Raines R.T.; Collagen structure and stability. *Annual Review of Biochemistry* **78**, 929-958, 2010.
- Adamiak, K., Sionkowska A.; Current methods of collagen cross-linking: review. *International Journal of Biological Macromolecules* **161**, 550-560, 2020.
- Nagaraj, A., Etxeberria, A. E., Naffa, R., *et al.*; 3D-Printed Hybrid Collagen/GelMA Hydrogels for Tissue Engineering Applications. *Biology-Basel* **11**, DOI10.3390/biology11111561, 2022.
- Schmidt, M.M., Dornelles, R. C. P., Mello R.O., *et al.*; Collagen extraction process. *International Food Research Journal* **23**, 913-922, 2016.
- Hervas, F. F., Celma, P., Punti, I., *et al.*; The enzyme activity of trypsin on sheepskin trimmings in a two-step collagen extraction process. *JALCA* **102**, 1-9, 2007
- Matinong, A. M. E., Chisti, Y., Pickering, K. L., *et al.*; Collagen extraction from animal skin. *Biology-Basel* **11**, DOI10.3390/biology11060905, 2022.
- Shen, J. Q., Tang, J., Shen, Y. L., *et al.*; Study on extraction of bovine collagen by acid method and its physicochemical property and structure. *The Food Industry* **3**, 43-46, 2009.
- Jolly, J.; Microwave assisted reactions in organic chemistry: a review of recent advances. *International Journal of Chemistry* **4**, 29-43, 2012.
- Nozariasbmarz, A., Dsouza K., Vashae D.; Field induced decrystallization of silicon: evidence of a microwave non-thermal effect. *Applied Physics Letters* **112**, DOI10.1063/1.5020192, 2018.
- Diaz-Ortiz, A., Prieto P., de la Hoz A.; A Critical overview on the effect of microwave irradiation in organic synthesis. *Chemical Record* **19**, 85-97, 2019.
- Zhang, J. W., Chen W. Y.; A faster and more effective chrome tanning process assisted by microwave. *RSC Advances* **10**, 23503-23509, 2020.
- Mirzapour-Kouhdasht, A., Sabzipour F., Taghizadeh M.S., *et al.*; Physicochemical, rheological, and molecular characterization of colloidal gelatin produced from Common carp by-products using microwave and ultrasound-assisted extraction. *Journal of Texture Studies* **50**, 416-425, 2019.
- Park, J. H., Choe J. H., Kim H. W., *et al.*; Effects of various extraction methods on quality characteristics of duck feet gelatin. *Korean Journal for Food Science of Animal Resources* **33**, 162-169, 2013.
- Li, Y., Li J., Lin S.J., *et al.*; Preparation of antioxidant peptide by microwave- assisted hydrolysis of collagen and its protective effect against H₂O₂-induced damage of RAW264.7 Cells. *Marine Drugs* **17**, DOI10.3390/md17110642, 2019.
- Li, X, W., Li H. J.; Optimization of microwave-assisted extraction process for collagen from pigskin. *Food Science* **6**, 19-22, 2012.
- AOAC, Official methods of analysis. *Association of official analytical chemist Inc*, Arlington, 2000.
- Gaar, J., Naffa, R., Brimble, M.; Enzymatic and non-enzymatic crosslinks found in collagen and elastin and their chemical synthesis. *Organic Chemistry Frontiers*, **7**, 2789-2814, 2020

29. Yang, H., Xu S. C., Shen L. R., *et al.*; Changes in aggregation behavior of collagen molecules in solution with varying concentrations of acetic acid. *International Journal of Biological Macromolecules* **92**, 581-586, 2016.
 30. Li, H., Liu B.L., Gao L.Z., *et al.*; Studies on bullfrog skin collagen. *Food Chemistry* **84**, 65-69, 2004.
 31. Josse, J., Harrington W.F.; Role of pyrrolidine residues in the structure and stabilization of collagen. *Journal of Molecular Biology* **9**, 269-287, 1964
 32. Ilyas, K., Qureshi S.W., Afzal S., *et al.*; Microwave-assisted synthesis and evaluation of type I collagen-apatite composites for dental tissue regeneration. *Journal of Biomaterials Applications* **33**, 103-115, 2018.
 33. Ali, A. M. M., Benjakul, S., Prodpran, T., *et al.*; Extraction and characterisation of collagen from the skin of Golden Carp (*Probarbus Jullieni*), a processing by-product. *Waste and Biomass Valorization* **9**, 783-791, 2017.
 34. Wu, J. M., Li, Z. H., Yuan X. Y., *et al.*; Extraction and isolation of type I, III and V collagens and their SDS-PAGE analyses. *Transactions of Tianjin University* **17**, 111-117, 2011.
 35. Lin, Y. K., Liu, D. C.; Comparison of physical-chemical properties of type I collagen from different species. *Food Chemistry* **99**, 244-251, 2006.
 36. Kittiphattanabawon, P., Benjakul S., Visessanguan W., *et al.*; Isolation and Characterization of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chemistry* **119**, 1519-1526, 2010.
 37. Mehta, M., Naffa, R., Maidment, C., *et al.*; Raman and ATR-FTIR spectroscopy towards classification of wet blue bovine leather using ratiometric and chemometric analysis. *Journal of Leather Science and Engineering*, **2**, <https://doi.org/10.1186/s42825-019-0017-5>, 2020.
 38. Liu, S. C., Liu W. T., Li G. Y.; Effect of ionic liquids pretreatment on the extraction of collagen from calf skin. *JALCA* **114**, 391-399, 2019.
 39. Akita, M., Nishikawa, Y., Shigenobu, Y., *et al.*; Correlation of proline, hydroxyproline and serine content, denaturation temperature and circular dichroism analysis of type I collagen with the physiological temperature of marine teleosts. *Food Chemistry* **329**, DOI: 10.1016/j.foodchem.2020.126775, 2020.
 40. Ikoma, T., Kobayashi, H., Tanaka J., *et al.*; Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. *International Journal of Biological Macromolecules* **32**, 199-204, 2003.
 41. Ding, C. C., Zhang M., Wu K., *et al.*; The response of collagen molecules in acid solution to temperature. *Polymer* **55**, 5751-5759, 2014.
 42. Duan, L., Yuan, J. J., Yang, X., *et al.*; Interaction study of collagen and sericin in blending solution. *International Journal of Biological Macromolecules* **93**, 468-475, 2016.
 43. Cui, F.L., Wang J.L., Cui Y.R., *et al.*; Fluorescent investigation of the interactions between N-(p-chlorophenyl)-N'-(1-naphthyl) thiourea and serum albumin: Synchronous fluorescence determination of serum albumin. *Analytica Chimica Acta* **571**, 175-183, 2006.
 44. Ding, C. C., Zhang, M., Li G. Y.; Fluorescence study on the aggregation of collagen molecules in acid solution influenced by hydroxypropyl methylcellulose. *Carbohydrate Polymers* **136**, 224-231, 2016.
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