A Novel Collagen Extraction Method Based on Microwave Irradiation

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Abstract. Microwave was used as a thermal source to extract collagen from the cattle hide in the present work. The effects of microwave on collagen extraction yields were studied under different irradiation temperature, time and solid-liquid ratio. The optimal extraction process was obtained by an orthogonal experiment. The results showed that the extraction yeild of collagen was positively correlated with irradiation temperature. With the solid-liquid ratio decreased and the irradiation time, the extraction yeild increased within limits. Under the condition of 37 °C, 7 h and 1:45 of solid-liquid ratio, the extraction yeild was the highest (14.35 %). And the composition, structure and properties of the extracted collagen were characterized by amino acid analysis, FTIR, UV-Vis, and VP-DSC. Amino acid analysis confirmed that the composition of the products were similar to that of standard type I collagen. The UV absorption peak of the product conformed to the characteristics of collagen. Moreover, the absorption peak of the collagen products in the infrared spectrum did not migrate, indicating the triple helix structure of the collagen was stable. Furthermore, the VP-DSC results showed that the thermal denaturation temperature of collagen products was 38.82 °C. Therefore, these results proved that the natural structure of the product was still maintained, which provided a new choice for efficient extraction of collagen.

1 Introduction

Collagen is the most abundant protein in mammals, accounting for 25-30% of the protein weight. This fibrous structural protein consists of three left-handed spiral peptide chains with right-handed supercoil structures [1] Due to its excellent biophysical properties, it is widely used in medicine [Fehler! Verweisquelle konnte nicht gefunden werden.], food [Fehler! Verweisquelle konnte nicht gefunden werden.], cosmetics[Fehler! Verweisquelle konnte nicht gefunden werden.] and so on. And it has vast development potential and broad market application prospects. The development of extraction of collagen containing natural structure will bring huge commercial value and social benefits. At present, the methods for preparing collagen mainly include acid extraction [Fehler! Verweisquelle konnte nicht gefunden werden.], enzymatic extraction [Fehler! Verweisquelle konnte nicht gefunden werden.-Fehler! Verweisquelle konnte nicht gefunden werden.], alkali extraction [Fehler! Verweisquelle konnte nicht gefunden werden.], hot water extraction [Fehler! Verweisquelle konnte nicht gefunden werden.], neutral salt extraction [10]. Among these methods, the collagen extracted by acid method has complete structure and high purity, but the extraction rate is low[Fehler! Verweisquelle konnte nicht gefunden werden.].

Since microwaves was used to treat nuclear waste in Harwell Laboratory at 1970, it had been widely used in various chemical fields as a transmission medium or heating energy source [Fehler! Verweisquelle konnte nicht gefunden werden.], gradually formed microwave chemistry. People began to pay attention to the excellent performance of microwave in chemical reaction process. At present, microwave irradiation has become an important technology to accelerate chemical reaction. Moreover, previous studies[Fehler! Verweisquelle konnte nicht gefunden werden.-Fehler! Verweisquelle konnte nicht gefunden werden.] proved that microwave-assisted method can shorten the time and increase the extraction rate while ensuring product quality.

In order to prepare structurally intact collagen more effectively, a new method for efficient extraction without affecting the activity of collagen molecules was explored. In this work, microwave was used

as heat source in the extraction process of extracting collagen by acid method under water bath heating was used as control group. The influence of solid-liquid ratio, microwave irradiation time and temperature on the extraction rate was investigated by single factor method. Then the extraction process was optimized by orthogonal method. The structure and properties of the product were characterized by FTIR, VP-DSC, UV-Vis. These results may offer theoretical supports for a new collagen extraction method based on microwave irradiation.

2 Materials and methods

2.1 Materials

Collagen was prepared from cattle hide made in laboratory. All other chemicals were commercially available of analytical grade from Chengdu Cologne Chemical Co., Ltd.

2.2 Methods

2.2.1 Preperation

The raw material was prepared according to the method of ZHONGKAI et al. [14]. Green hides which came from cattles were processed by conventional tanning process to prepare limed stock. After spliting, the samples with the size of 100 cm × 80 cm were taken symmetrically along the back line of the split and weighed as the basis of the following materials. Then the samples were delimed with 2% ammonium chloride and 0.5% hydrochloric acid for 1 h, followed by neutralizing with 0.5% hydrochloric acid solution which aimed to adjust the pH to 6.0-7.0. After that, the samples were rinsed with distilled water for 30 min and cutted into fragments of 0.5 cm × 0.5 cm. Finally, they were put into a dryer and set aside after air drying.

2.2.2 Extraction of collagen by microwave irradiation

3 g of the raw material was weighed accurately in a 100 mL beaker to extract collagen under certain conditions. Next, the crude extract was obtained by filtering the extract with 200 mesh gauze, and then centrifuged at 8000 r/min for 10 min by using desktop high speed centrifuge (TG-20; Sichuan Shuke instrument Co., Ltd; China). Subsequently, sodium chloride was added to the supernatant until the concentration was 3 mol/L. Afterward, collagen precipitation was collected by centrifugation again and dissolved in acetic acid solution of 0.5 mol/L. After the collagen was dialyzed with distilled water for 3 days, dialysis fluid was freeze-dried and put into the dryer for use. The product obtained by microwave extraction is called the experimental product (EP), while the product obtained under water bath heating was the control product (CP).

In order to optimize the experimental conditions, the single factor experiment was conducted. With the extraction yeild of collagen as indicator, collagen was extracted respectively at different irradiation temperatures (irradiation time 6 h, solid-liquid ratio 1:30), different solid-liquid ratio (irradiation temperature 35 °C, irradiation time 6 h) and different irradiation time (irradiation temperature 35 °C, solid-liquid ratio 1:40) to explore the effect of extraction conditions on the extraction yield of collagen. On the basis of single factor experiments, orthogonal experiment with 3 factors and 3 levels were carried out. Details on the orthogonal optimization, including irradiation temperature, time and solid-liquid ratio were given in Table 1.

Table 1. Details on orthogonal design.

Levels	Factors		
	A (°C)	B	C (h)
1	33	1:35	6
2	35	1:40	7
3	37	1:45	8

A: irradiation temperature; B: solid-liquid ratio; C: irradiation time

2.3 Analysis

2.3.1 Composition determination

A small part of the raw material was used to determine the proximate composition, including ash, moisture, crude fat andcrude protein according to the method of AOAC [15].

2.3.2 Yield

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 acetic acid solution of 0.5 mol/L. With acetic acid solution of 0.5 mol/L as reference, the absorbance of collagen solution were recorded over the range of 200-400 nm at the scanning speed of 400 nm/min by ultraviolet spectrophotometer (UV1900; Shanghai Flying Art instrument Co., Ltd.; China) to determine the characteristic absorption peak of collagen. According to the absorbance of collagen solution at the characteristic absorption peak, the standard curve which was used to calculate the concentration of collagen in the crude extract was made. Then the yield of collagen was calculated according to formula 1:

$$Y = \frac{C*V*100}{M*F} \quad (1)$$

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 (%).

2.3.3 Amino acid analysis

25.0 mg of the collagen products were completely hydrolysed in the presence of hydrochloric acid of 6 mol/L at 120 °C for 24 h. The solutions were filtered and diluted. Thereafter, the amino acid composition of filtrate was determined by amino acid analyzer (L-8900; Hitachi Co., Ltd.; Japan).

2.3.4 Fourier transform spectroscopy (FTIR)

2 mg of the products were mixed with potassium bromide (1:100) respectively, grounded and pressed evenly for spectrum recording. Subsequently, they were scanned in the Fourier transform infrared spectrometer (Nicolet iS10; Seymour Technology Co., Ltd.; America) for 32 times with a wave number range of 400-4000 cm^{-1} at room temperature.

2.3.5 Differential scanning calorimetry (DSC)

0.5 mg/mL collagen solution were prepared with acetic acid solution of 0.5 mol/L. Acetic acid solvent of 0.5 mol/L was used as a reference control, and the solutions were degassed in the instrument

for 30 min followed by being tested in the temperature range of 20 °C to 60 °C by hypersensitive differential scanning calorimetry (VP-DSC; Microcal; USA) with the increasing rate of 1 °C/min.

3 Results and discussion

3.1 Composition of cattle hide

The composition of each component of the raw materials is shown in Table 2. It can be seen that the main components in the raw materials are protein and water, which together account for 89.3% of the tare weight, in addition to 7.26% fat and ash besides 3.44% other substances. Since the raw material is prepared from limed stock after degreasing, the fat contained only 6.4% of the tare weight. Followed on air drying, the moisture content of the raw material is reduced to 17.1%. After the pretreatment is completed, the content of protein in the material is as high as 72.2%, which is suitable for the extraction of collagen.

Table 2. Composition of material.

	Water	Ash	Protein	Fat
content g/100 g	17.1	0.86	72.2	6.4

3.2 Relationship between collagen concentration and absorbance

The standard curves of different concentrations of standard type I collagen solution were obtained at the characteristic absorption peak (234 nm) of collagen, as shown in Fig. 1. According to Lambert-Beer's law, there is a linear relationship between the UV absorption and the concentration in a certain concentration range **Fehler! Verweisquelle konnte nicht gefunden werden.**. It can be seen that the concentration and absorbance of collagen conform to the law, and the linear relationship is y=0.5799x+0.0307, besides, the linear relationship variance is 0.9955, which indicates that the concentration of collagen has a good linear relationship with absorbance and the obtained data are in good agreement with the fitting function.

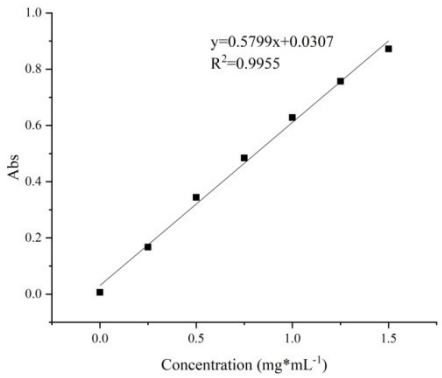

Fig. 1. Relationship between concentration and absorbance of standard type I collagen

3.3 Effect of microwave on collagen extraction yeild

3.3.1 Effect of irradiation temperature on extraction yeild

Collagen was extracted by microwave irradiation under the condition of solid-liquid ratio 1:30 for 6 h. The effect of irradiation temperature on the extraction of collagen was investigated by extracting collagen at different temperatures. Taking the temperature as the abscissa and the extraction yeild as the ordinate, the relationship between the extraction yeild and the irradiation temperature was obtained(figure 2).

It can be seen that the collagen extraction yeild is the lowest at 15 °C. With the increase of irradiation temperature, the extraction yeild increases rapidly, and the highest extraction yeild is exhibited while the irradiation temperature was 40 °C. When irradiation temperature is 15 °C, the temperature is cool, therefore, corresponding microwave power is low, resulting in small effect on collagen. Simultaneously, the acidic condition destroys the Schiff bond (-C=N-) and salt bond between the collagen molecules Fehler! Verweisquelle konnte nicht gefunden werden., causing its compact structure to become loose and collagen molecules to be extracted. With the temperature went up gradually, the solubility of collagen molecules increased and the thermal motion fortified, besides the augment of microwave power. The molecular motion is more intense under the action of high-frequency electric field, which makes the collagen structure looser and more soluble. Therefore, as the temperature rises, the extraction yeild increases. It is reported that the denaturation temperature of collagen is about 40 °C, and even lower when collagen is in an acidic environment, which is at 38-39 °C Fehler! Verweisquelle konnte nicht gefunden werden.. In order to extract collagen with natural structure, 35 °C was selected the optimum irradiation temperature.

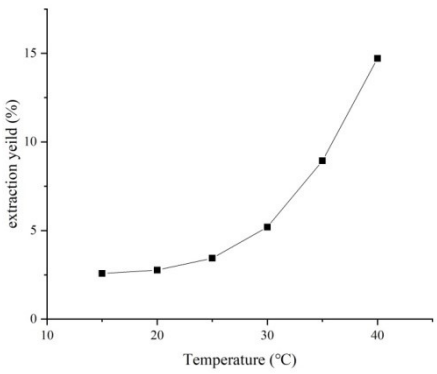

Fig. 2. Relationship between extraction temperature and extraction yeild of Collagen

3.3.2 Effect of solid-liquid ratio on extraction yeild

Under the conditions of irradiation temperature of 35 °C and irradiation time of 6 h, the collagen was extracted at different solid-liquid ratios. The relationship between the extraction yeild and solid-liquid ratio was shown in figure 3. It is known that when the solid-liquid ratio is large, as the solid-liquid ratio decreases, the extraction yeild rises, and with the solid-liquid ratio decreases gradually to 1:30, the extraction yeild increases sharply. Till the solid-liquid ratio reaches 1:40, the maximum is reached; while the solid-liquid ratio is less than 1:40, the solid-liquid ratio decreases leading to the decrease of extraction yeild, and finally reaches the relative equilibrium value. When the ratio is relatively large, there is little acetic acid solvent resulting in less amount of collagen. With the small solid-liquid ratio, more solvent exists since the probability of the material being irradiated becomes smaller, which is not conducive to collagen dissolution Fehler! Verweisquelle konnte nicht gefunden werden., and the increase of acetic acid content promotes the hydrolysis of collagen. Therefore, the solid-liquid ratio is the most suitable at 1:40.

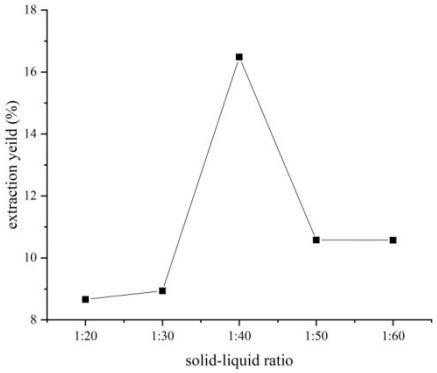

Fig. 3. Relationship between extraction yeild and solid-liquid ratio.

3.3.3 Effect of irradiation time on extraction yeild

On the basis of the above experiments, collagen was extracted at different times to explore the effect of irradiation time on collagen extraction under the solid-liquid ratio of 1:40 and the irradiation temperature of 35 °C. The relationship between the extraction yeild and the irradiation time is shown in Fig. 4. The extraction yeild of collagen increased with the prolongation of irradiation time, and the maximum value is reached between 6 and 9 hours. Afterwards, the extraction yeild of collagen decreased as the reduction of irradiation time. When the irradiation time is short, microwave can break the wall to a certain extent, and at the appropriate temperature, it can also loosen the triple helix structure of collagen to promote the dissolution of collagen **Fehler! Verweisquelle konnte nicht gefunden werden.**. If the time is too long, molecular exercise is intense, causing collagen to hydrolyze, resulting in a decrease in extraction yeild at higher temperatures **Fehler! Verweisquelle konnte nicht gefunden werden.**. Based on the results of single factor experiment, the experimental point with the highest extraction yeild was selected as the optimum extraction conditions, that is, the irradiation time was 6 h, the irradiation temperature was 35 ℃, and the solid-liquid ratio was 1:40.

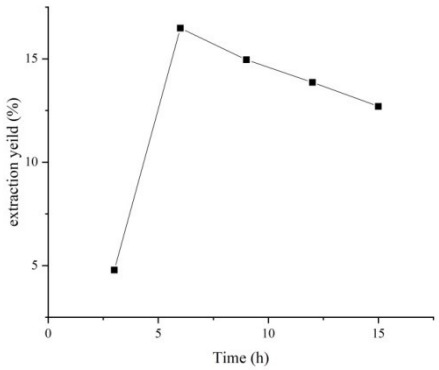

Fig. 4. Relationship between extraction yeild and solid-liquid ratio

3.3.4 Optimization of the collagen extraction based on microwave irradiation

According to the results of single factor experiment in which collagen was extracted by microwave irradiation, the extraction yeild was taken as the target parameter, and the orthogonal tests of three factors and three levels were carried out on the solid-liquid ratio (1:35, 1:40, 1:45), irradiation time (6, 7, 8 h) and irradiation temperature (33, 35, 37 °C). Wherein the extraction temperature

selected is no higher than the critical denaturation temperature which is 37 °C. The experimental results are shown in Table 3.

Table 3. Orthogonal test results of collagen extraction by microwave irradiation.

Number	A	B	C	Extraction yeild /%
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	

According to the analysis of the orthogonal test, the extraction yeild of No. 4 test was the highest (11.65%), and the corresponding extraction conditions were A_2=35 °C, B_1=35, and C_2=7 h. S and R are the variance and range of K_1, K_2 and K_3, respectively. The larger the variance and the range difference, the more significant the influence of this factor on the test results. The analysis results in $S_1>S_3>S_2$ and $R_1>R_3>R_2$, illustrating that the order of the influence on the extraction yeild was as follows: irradiation temperature > irradiation time > solid-liquid ratio. Optimal level obtained by the combination is $A_3B_3C_2$, that is, the irradiation temperature is 37 °C, the ratio of material to liquid is 45:1, and the irradiation time is 7 hours. Under this condition, the verification experiment showed that the extraction yeild of collagen was 14.35%, and the corresponding product was called experimental product; while heated under the same condition by traditional water bath, the extraction yeild of collagen was 9.30%, and the corresponding product was called control product. In contrast, microwave irradiation extraction technology can significantly improve the extraction yeild of collagen, and has a good application prospect.

3.4 Structure and properties of collagen extracted by microwave irradiation

3.4.1 Amino Acid Composition

The molar percentages of the respective amino acids in the EP and the CP are shown in Table 4. Glycine, proline and alanine were the main amino acids of the two products, and among them, the content of glycine was the highest. The molar percentage of glycine in the experimental product and the control product was 33.90% and 34.38% respectively, which accorded with the proportion of glycine in bovine collagen [21]. Besides, the contents of proline and alanine were about 10% of the total molar content. The ratio of hydroxyproline to proline of the experimental product was 0.59, which was similar to that of the control product (0.63), indicating that the stability of the two products was comparable[22]. In addition, trace amounts of tyrosine showed the presence of terminal peptide residues in the product [23]. The existence of a small amount of other amino acids (such as glutamic acid, arginine, aspartic acid, etc.) proved that the product was consistent with the primary structure of collagen, and its basic composition was collagen.

Table 4. Amino acid composition of experimental and control product.

Amino acid	Molar percentage /%	
	EP	CP
Asp	4.41	4.55
Thr	1.52	1.72
Ser	3.77	3.73
Glu	7.72	7.60
Gly	33.90	34.38
Ala	10.54	10.17
Val	1.32	0.22
Met	0.63	2.53
Ile	1.41	1.33
Leu	3.10	3.04
Tyr	0.22	0.43
Phe	1.89	1.96
Hylys	0.76	0.77
His	0.38	0.31
Lys	2.94	2.86
Arg	5.48	5.35
Hypro	7.40	7.42
Pro	12.60	11.64

3.4.3 UV-Vis Spectra

The absorbance of collagen solution in the range of 200-400 nm was measured by UV spectrophotometer with 0.5 mol/L acetic acid solution as the blank, and the EP, CP and standard type I collagen ultraviolet spectrum were obtained, shown in diagram 5. Collagen contains phenylalanine and tyrosine, which have sensitive chromogenic groups and characteristic absorption peaks at 258 nm and 278 nm, respectively. Their superposition of UV absorption gives collagen a maximum absorption peak below 300 nm [Fehler! Verweisquelle konnte nicht gefunden werden.]. The UV spectrum of the product showed that the standard type I collagen, experimental product and control product all had maximum absorption peak at 234 nm, which was accorded with the characteristics of collagen. At the same time, the results showed that microwave irradiation would not destroy the primary structure of collagen.

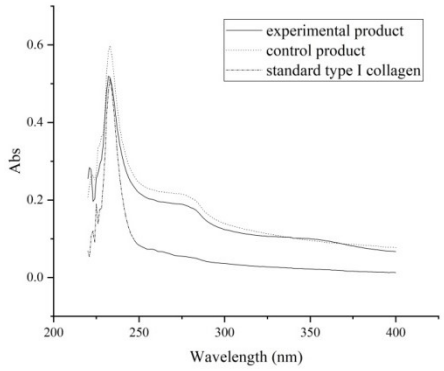

Fig. 5. Ultraviolet spectra of EP, CP and standard type I collagen.

3.4.4 FT-IR spectra

A scanning image by the Fourier transform infrared spectrometer of the EP, the CP and the standard type I collagen is shown in Fig. 7.

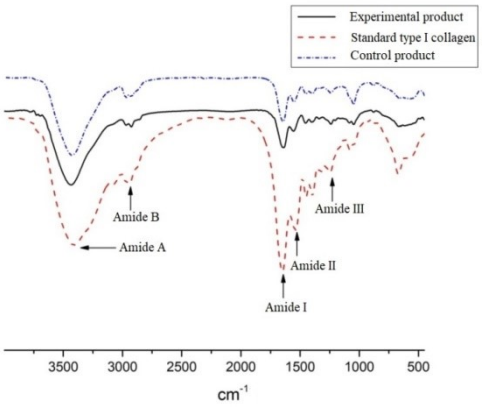

Fig. 6. Infrared spectra of EP, CP and standard type I collagen.

As can be seen from fig. 7, there is an absorption peak of the amide A band caused by the stretching vibration of NH at 3400-3440 cm^{-1}. Absorption peak at 2800-3000 cm^{-1} is a stretching vibration peak generated by the asymmetric CH_2 of the amide B band. The amide Iband absorption peak located between 1640-1660 cm^{-1} is the main characteristic absorption peak of collagen infrared spectrum, that is the vibration peak of the C=O group forming the hydrogen bond in the triple helix, which is often used for the secondary structure analysis of collagen **Fehler! Verweisquelle konnte nicht gefunden werden.**. Due to the C-N stretching vibration and N-H bending vibration of collagen, the absorption peak of the amide II band appeared at 1500-1600 cm^{-1}, and the absorption peak of the amide III band was produced between 1200-1360 cm^{-1}. Therefore, it can be speculated that the product possess a complete triple helix structure [26]. The absorption peaks of the experimental product, the control product and the standard type Icollagen were almost in the same position, which indicated that the microwave irradiation extraction technology would not destroy the structure of the product.

3.4.5 Denaturation temperature

There are two endothermic peaks of predenaturation transition and main denaturation transition on the typical collagen VP-DSC curve, corresponding to the transition temperatures Tm_1 and Tm_2, respectively. Among them, Tm_2 is an important indicator of the thermal stability of collagen, and

the greater the value, the higher the stability of collagen [27]. Table 5 shows the thermal denaturation temperature of collagen products obtained by different extraction methods. The thermal denaturation temperatures of the experimental product, the control product and the standard type I collagen were 38.82 °C, 38.91 °C and 39.09 °C, respectively, which were consistent with the thermal denaturation temperature of cowhide reported in the literature[Fehler! Verweisquelle konnte nicht gefunden werden.]. Compared with the control product, the thermal denaturation temperature of the experimental product did not change significantly, indicating that the extraction of collagen by microwave irradiation did not change its thermal stability. Based on the analysis of amino acid composition, and infrared spectrum, the collagen extracted by microwave irradiation can maintain the natural structure.

Table 5. Thermal denaturation temperature of the products and the standard.

Sample	EP	CP	Standard type I collagen
Denaturation temperature/℃	38.82	38.91	39.09

4 Conclusions

In this study, the collagen was extracted under the conditions of microwave irradiation by acetic acid with the traditional water bath heating as the control. The effect of microwave on the extraction yeild of collagen was explored, and the optimum extraction process was obtained by orthogonal experiment. A variety of methods, including FTIR, VP-DSC, UV-Vis and amino acid analysis, demonstrated that microwave irradiation did not change the natural structure of collagen. The results displayed:

(1) Irradiation temperature is positively correlated with extraction yeild. Within a certain range, the extraction yeild increases with the decrease of the solid-liquid ratio and the prolongation of irradiation time. And the optimum conditions for extracting collagen by microwave irradiation are 37 °C, 7 h and ratio of material to liquid 1:45. Moreover, extraction yeild significantly increased by microwave irradiation was 1.5 times higher than that of traditional water bath heating, which was 14.35%.

(3) Amino acid composition analysis confirmed that the basic composition of the product was collagen. Compared with the standard type I collagen, its UV absorption peak accorded with collagen characteristics and the absorption peak of infrared spectrum did not migrate. The thermal denaturation temperature was 38.82 °C. These results demonstrate that the product extracted by microwave irradiation was type I collagen with natural triple helix structure.

Acknowledgements

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