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Extraction optimization and characterization of collagen from the lung of soft-shelled turtle *Pelodiscus sinensis*

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Abstract: The soft-shelled turtle (*Pelodiscus sinensis*) is a commercially important aquatic species with abundant collagen content and precious nutritious high quality goods. The present study was to optimize the collagen extraction from lung of *Pelodiscus sinensis*. Single-factor test was employed to investigate the effects of different extraction methods and the major factors that influence the collagen production in enzymatic extraction method on the collagen yield of lung. Optimization of the papain enzymatic extraction parameters was then examined using an orthogonal test design L9 (3^4). The optimum extraction conditions were obtained when the extraction temperature, papain enzyme dose, the ratio of solid to solution, and extraction time were 32°C, 4.0mg/ml, 1:35, and 12h, respectively. Under the optimized conditions, the collagen yield was up to 79.29%. The properties of turtle lung collagen were characterized by SDS-PAGE, UV scanning, and amino acid analysis. The results showed that the extracted lung collagen had high imino acid content at 21.8% and contained two α chains, β, and γ-components, belonging to the typical type I collagen. The amphibious aquatic turtle collagen is thought to be a viable novel source for replacement of terrestrial mammals and could provide scientific reference for the development of collagen polypeptide for terrapin animals.

Keywords: Soft-shelled turtle *Pelodiscus sinensis*, Collagen extraction, Optimization, Characterization

1. Introduction

Collagen is the main component of the extracellular matrix and constitutes approximately 30% of the total protein in vertebrates. It is widely distributed in all kinds of connective tissues of vertebrates to support and protect the body and organs [1]. Normally, the glycine content of collagen is as high as 30% and the proline and hydroxyproline account for about 25%, making the collagen has strong ability of holding water. Besides, collagen has properties of gel strength, emulsification, low viscosity, biological compatibility, water absorption and moisturizing effect as natural biological resources, which can be widely used in food, medicine, cosmetics and biomedical materials [2-4].

Collagen has been traditionally obtained from skins and bones of land-based mammalian animals, such as pig and cow. However, the outbreaks of domestic animal epidemic diseases, and some religious and customs reasons have resulted in restricted use of these animals [5, 6]. As a result, alternative sources, such as fish processing waste in aquaculture industry, including skin, bone, or swim bladders, have received increasing attention for collagen extraction. In recent years, many papers focused on the practical utilization of the by-products of fishes to produce collagen [5, 7-10]. The soft-shelled turtle (*Pelodiscus sinensis*) is a commercially important aquatic species in Asian countries including China, Japan and Korea etc., due to its high nutritional and medicinal values. In China, it is considered to be a rich delicious food with medical benefit. In 2010, more than 260,000 tons of this species were produced [11]. Compared to other coldwater fishes, the soft-shelled turtle is an aquatic animal living in relatively higher ambient environment at around 30 °C [12]. The turtle collagens have higher denaturation temperature (Td) and may have the advantage of higher thermal stability in biomedical applications. Additionally, as a large food and medicine source, people try to exploit the every parts of this turtle. Our colleagues and other researchers have previously reported the extraction and characterization of collagen from turtle calipash [13] and skin [12, 14] whereas no information regarding the characteristics of lung from

amphibian freshwater fish is available. Like the skin, the turtle lung (waste in the turtle processing operations) also contains large amount of collagen. If high-quality collagen can be extracted from lung, it would be a potential viable source of collagen.

Therefore, the objectives of this study were to compare the collagen yield of collagen from the soft-shelled turtle *Pelodiscus sinensis* lung (designated as TC-L) using acid solubilization with different proteolytic enzymes. It was also attempted to determine the optimum conditions for lung collagen extracting by employing the orthogonal L9 (3^4) test design, and further to investigate the chemical properties of the extracted collagen by SDS-PAGE electrophoresis, UV scanning and amino acid analysis.

2. Materials and Methods

2.1. Materials and Chemical Reagents

The soft-shelled turtles were obtained from the turtle farm in Zhejiang, China (body weight of 500 ± 50 g). All experimental procedures were approved by the Animal Ethics Committee of Zhejiang Wanli University. Tissues including lung, calipash and muscle were dissected quickly and put into the sealing bag under -20°C for further use. The chemical reagents including pepsin (3000U/g), papain (3500U/g), L-hydroxyproline standard and dimethyl amine benzaldehyde were purchased from Sangon Biotech (Shanghai) Co. Ltd. All regents used in this study were analytical grade.

2.2. Proximate Analysis of Turtle Tissue

The crude protein, crude fat, moisture content and ash content of turtle tissues were estimated by the AOAC official method. The crude protein was calculated by multiplying nitrogen content with a factor 6.25. The crude fat was determined by Soxhlet method using petroleum ether as solvent. Moisture content was determined by the hot air oven method. The ash content was determined by using muffle furnace at 550-600°C. The analyses were replicated three times.

2.3. Extraction of Lung Collagen

The collagen was extracted from soft-shelled turtle (*Pelodiscus sinensis*) lung (TC-L) according to the method described previously [13, 16] with minor modification. Briefly, the tissues of turtle lung were isolated, cut into small pieces, pretreated by 6% NaCl, the solid/solution ratio 1:20 (w: v) to soak 10 h at room temperature to remove non-collagenous proteins, and then with the same amount of 10% isopropyl with NaCl solution to soak 20 h at room temperature to remove fat. Then, the pretreated samples were used in enzymatic extracting process. Afterwards, collagen was precipitated by salting out, dialysising and purificating. Dry collagen was lyophilized at low temperature by using a vacuum freeze drier (FD-1A-50, Beijing, China).

2.4. Determination of Collagen Content

Hydroxyproline is the characteristic amino acids in collagen. We evaluated the extraction effect of collagen indirectly by measuring the content of hydroxyproline according to the method described in ISO (1978) [17] by using spectrophotometric analyzer (NanoDrop2000, Thermo Fisher Scientific, USA), with slight modifications. To calculate the hydroxyproline content, a standard curve was built in Excel soft ware with absorbance value as the ordinate and L-hydroxyproline concentration as the abscissa. The regression equation was y = 0.0394x–0.0053, the variation coefficient R2 was 0.9964, which showed that it can be used as a standard for the determination of collagen content. The extraction yield of collagen was calculated and expressed as collagen content in final extraction solution divided by the collagen content in initial tissue.

2.5. Optimization of Collagen Extraction

2.5.1. Single-Factor Test of the Extracting Technology

Table 1. *Single-factor test design for collagen extraction*

	Factors															
	Acid type	Acid concentration (mol· L^{-1})		pH		Temperature (°C)		Enzyme dose		Solid/solution ratio (w:v)		Time (h)		Enzyme type	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II
1	Acetic acid (I)	0.1	0.05	2.0	2.0	10	10	0.5	1.5	1:15	1:15	6	6	Pepsin	Pepsin
2	Citric acid (II)	0.5	0.1	2.5	3.0	15	15	1.0	2.5	1:20	1:20	10	10	Papain	Papain
3		1.0	0.5	3.0	3.5	20	20	1.5	3.5	1:25	1:25	15	15	—	—
4		1.5	1.0	3.5	4.0	25	25	2.0	5.0	1:30	1:30	20	20	—	—
5		—	—	4.0	5.0	30	30	3.0	6.5	1:35	1:35	30	30	—	—
6		—	—	5.0	6.0	35	35	5.0	8.0	—	—	—	—	—	—
7		—	—	—	—	40	40	—	—	—	—	—	—	—	—

Firstly, three different extraction methods including acid method, alkaline method and enzymatic method [12, 16], were compared on their effect of extracting yield of lung collagen. Secondly, being the major factors that influence the collagen yield in enzymatic method, the type and concentration of enzyme and acid solution, the solid/solution ratio, enzymatic pH, temperature, and extraction time were selected (Table 1). The pH was adjusted with either 6 M NaOH or 6 M HCl to obtain the final pH ranging from 2 to 6.

2.5.2. Orthogonal L9 (3^4) Test of the Extracting Technology

On the basis of a single-factor test described above, an orthogonal L9 (3^4) test design with four factors and three levels was used to investigate the optimal enzymatic extraction condition of collagen from *Pelodiscus sinensis*.

As seen from Table 2, twelve extractions were carried out at temperature (A) 32, 35, 38°C, enzyme amount (B) 3, 3.5, 4 $mg \cdot L^{-1}$, the substrate concentration (C) 1:25, 1:30, 1:35, and extraction time 12, 15, 18 h. The collagen yield (%) was the dependent variable. The collagen obtained from the above 12 tests were operated following the method in Section 2.4.

Table 2. *Factors and levels for orthogonal test for collagen extraction*

Variable	Level		
	1	2	3
A, extraction temperature (°C)	32	35	38
B, enzyme dose ($mg \cdot L^{-1}$)	3.0	3.5	4.0
C, ratio of solid to solution (w:v)	1:25	1:30	1:35
D, extraction time (h)	12	15	18

2.6. UV Scanning

The ultraviolet absorption spectra of the collagen samples purified were scanned by protein and nucleic acid analyzer (NanoDrop 2000, Thermo Fisher Scientific, USA) at wavelength interval of 1 nm and wavelength range of 190-400 nm.

2.7. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using the discontinuous Tris-glycine buffer (pH 8.3) system [17] with 5% stacking gel and 10% resolving gel. The electrophoresis was conducted using a vertical cell (MiniPROTEAN 3, Bio-rad, USA) at voltage of 100v for about 100 min (PowerpacBasic, Bio-rad, USA). After electrophoresis, the gel was stained for 30 min with 0.05% (w/v) Coomassie Brilliant Blue R-250 and decolorized for one night. The electrophoresis pattern was analyzed by gel imaging using ultraviolet spectrophotometer (Gel Doc™ XR+, Bio-rad, USA)

2.8. Amino Acid Determination

The collagen samples were hydrolyzed with 6M HCl at 110 °C for 24 h. The amino acid composition was analyzed by an amino acid auto analyzer (L-8900, Hitachi Ltd., Japan). The amino acid content was expressed as the percentage of individual amino acid by the total content. Then the degree of Pro hydroxylation (%) was calculated according to the equation described in Li *et al* [16].

2.9. Statistical Analysis

All statistical analysis was performed by software SPSS 13.0. Differences among means were tested using Duncan's new multiple range test.

3. Results and Discussion

3.1. Nutritional Analysis of the Turtle Tissue

Table 3 shows the nutritional compositions of lung, calipash and muscle of soft-shelled turtle *Pelodiscus sinensis* on the basis of wet weight. The crude protein content of lung was 15.60%, while the content of crude fat was very low. It suggested that the fat may have small influence on collagen extraction. Therefore, the pretreatment of fat was not considered in the test. Moreover, the collagen content of turtle lung was significantly higher than that in muscle ($P<0.05$) (Table 3), and ranked only second to calipash in our preliminary experiment. This is part of the reason that why we choose lung for further collagen extraction optimization.

Table 3. *Table Nutritional composition of soft-shelled turtle Pelodiscus sinensis (on wet basis, means ± SD, n=3)*

Nutrition composition	Lung	Calipash	Muscle
Crude protein (%)	$15.60^{c} \pm 0.30$	$27.29^{a} \pm 0.60$	$18.14^{b} \pm 0.22$
Crude fat (%)	0.45 ± 0.15	0.19 ± 0.08	0.85 ± 0.20
Ash (%)	1.11 ± 0.07	0.94 ± 0.44	1.22 ± 0.28
Moisture (%)	82.65 ± 0.63	71.50 ± 0.38	79.76 ± 0. 82
Collagen ($g \cdot kg^{-1}$)	$326.6^{b} \pm 2.38$	$501.14^{a} \pm 4.48$	$41.54^{c} \pm 1.27$

Values followed by different letters are significantly different at $P < 0.05$.

3.2. Effect of Different Extraction Methods on Collagen Yield

The collagen preparation method varies according to different raw materials. There are about four kinds of extraction methods of aquatic collagen, hot water extraction, acid leaching, alkaline extraction and enzymatic extraction [18]. In the present study, effect of different extraction methods on collagen yield (%) was shown in Figure 1. The results showed that the collagen yield of enzymatic method was significantly higher than that from the other two

methods (P<0.05), and the collagen yield by alkaline method was the lowest.

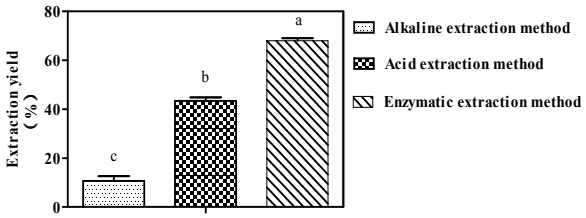

Figure 1. *Effect of different extraction methods on collagen extraction yield. All values are means ± standard deviation of triplicate analysis. Different superscripts indicate significant differences (P<0.05). The same below.*

The collagen extracted by acid method can keep its three spiral structure maximally, which is often used in preparation of the application of biological materials [19], but the product yield is very low [9, 20]. The alkaline method can easily result in the protein denaturation, and it will produce racemic mixture of D- and L-amino acids if excess hydrolysis exists, which may lead to the inhibitation of L- amino acids absorption by D-amino acids, and even bring about carcinogenic, teratogenic and mutagenic effect [21]. Hence, in order to keep the complete structure and safety of collagen, alkaline method was rarely used. By contrast, the enzymatic extraction not only has fast reaction, high extraction efficiency, and also can selectively remove the spiral telopeptide, keep the integrity of the triple helical structure, good physical and biochemical characteristics of collagen [22]. Researchers also reported that one of the methods to increase the yield of collagen extraction is to apply the enzymatic pretreatment of connective tissue by proteolytic enzymes non-specific for collagen such as pepsin [9, 20] or papain [23]. Therefore, in combination with our results, the enzymatic extraction of turtle lung collagen was adopted in further study.

3.3. Optimization of the Enzymatic Extraction Conditions by Single-Factor Test

3.3.1. Effect of Different Acid Types

In the process of enzymatic extraction, we usually conduct the enzymatic method with the aid of acid to improve the extraction efficiency and quality [23]. At present, the enzymes usually used in enzymatic extraction are pepsin, trypsin, papain and ficin, and the types of acid mainly include acetic acid, citric acid, lactic acid and other organic acids [19].

In this study, effects of different acid types (acetic acid, citric acid) on the collagen yield in two enzymatic extractions (pepsin, papain) were investigated and the result was illustrated in Figure 2. The results showed that the effect of combination of pepsin with 1.0 mol·L^{-1} acetic acid on collagen yield was significantly higher than that with 0.5 mol·L^{-1} citric acid (P<0.05). Opposite results were observed for papain. The combination of papain with 0.5 mol·L^{-1} citric acid obtained significantly higher collagen yield than combination of papain with 1.0 mol·L^{-1}acetic acid.

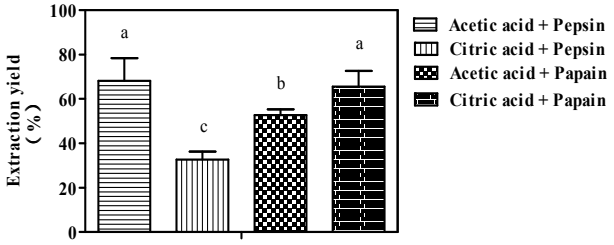

Figure 2. *Effect of different acid solution on the collagen extraction yield*

Therefore, we chose the combination of 1.0 mol·L^{-1} acetic acid with pepsin and 0.5 mol·L^{-1} citric acid with papain as the two collagen enzymatic extraction methods. Li [24] also demonstrated that the extraction effect of acid protease with acetic acid is superior to citric acid, the effect of papain with citric acid is superior to acetic acid. The following single-factor tests were carried out to find the optimum conditions for the two enzymatic extraction methods, respectively, and further to select the best one.

3.3.2. Effect of Acid Concentration

Effects of acetic acid and citric acid concentrations on collagen yield were seen in Figure 3, where other factors were fixed. The results showed that, for pepsin enzymatic extraction, the highest extraction yield of collagen was observed when the concentration of acetic acid was 1.0 mol·L^{-1}, followed by 0.5 mol·L^{-1}. And then there is a drop when the acetic acid concentration increased to 1.5 mol·L^{-1}. As for papain enzymatic extraction, with the citric acid concentration increasing from 0.05 to 1.0 mol·L^{-1}, the yield (%) of collagen continued to increase, reached the peak value at 0.5 mol·L^{-1}. Considering the yield and purity, we selected 1.0 and 0.5 mol·L^{-1} as optimal concentration for pepsin and papain enzymatic extractions, respectively.

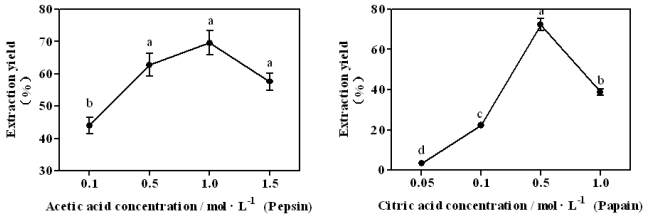

Figure 3. *Effect of acid concentration on the collagen extraction yield*

3.3.3. Effect of Hydrolysis pH

The effect of hydrolysis pH on collagen yield was presented in Figure 4, where other factors were fixed. For both pepsin and papain enzymatic methods, the yield (%) of collagen decreased when the hydrolysis pH increased from 2 to 6. There would be the highest extraction rate when the pH was 3.0 for both pepsin and papain. This result was in agreement with the report of Li *et al* [16] that pepsin soluble collagens (PSC) of Spanish mackerel skin were solubilized to the higher extent in acidic pH range

between 1 and 4, and reached maximums at 3 and 4. Jongjareonrak *et al* [25] also found that PSC from the skin of bigeye snapper exhibited the lowest solubilities at pH 7. The reason might be that collagen undergos denaturation to some extent, leading to impaired solubilities.17 Therefore, we selected pH 3.0 as the appropriate pH value for the two enzymatic methods.

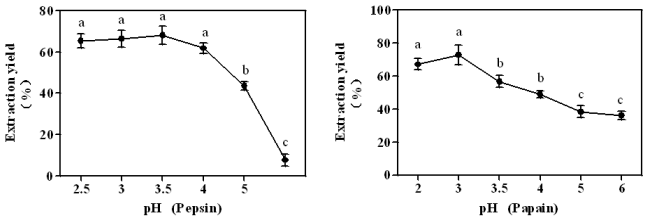

Figure 4. *Effect of hydrolysis pH on the collagen extraction yield*

3.3.4. Effect of Hydrolysis Temperature

Generally, the yield of collagen increases when the extraction temperature increasing [26]. Effect of different hydrolysis temperature on collagen yield was shown in Figure 5, where other factors were fixed. For pepsin enzymatic method, collagen yield increased when the temperature increased from 10 °C to 25 °C, and promptly reached the high value at 30 °C and 40 °C with little drop at 35 °C. For papain enzymatic method, the trend was the same with the exception of change at 30 °C. The results indicated that the dissolution of collagen constantly increase with temperature increasing. According to Li [24], the denaturation temperature of collagen was 40°C− 41°C when pH was neutral, 38°C− 39°C when pH was acid. Therefore, considering the energy efficiency and structural integrity, we adopted 30 °C and 35 °C as extraction temperature for pepsin and papain enzymatic methods, respectively.

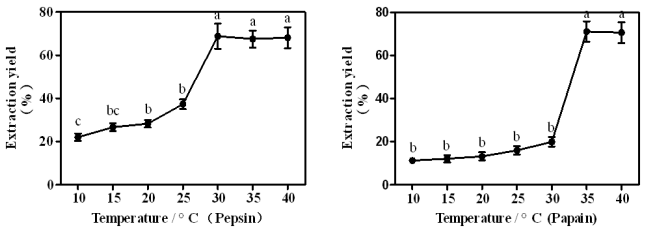

Figure 5. *Effect of enzymatic hydrolysis temperature on the collagen extraction yield*

3.3.5. Effect of Enzyme Addition

Effect of enzyme addition on collagen yield was seen in Figure 6, where other factors were fixed. The yield of collagen increased with increasing enzyme addition and reached the maximum value at 1.5 mg/ml and 3.5 mg/ml enzyme addition of pepsin and papain, respectively. And then both the pepsin and papain extraction yield dropped when enzyme dose was from 1.5 to 5.0 mg/ml, and from 3.5 to 8.0 mg/ml. Taking the extraction cost into consideration, addition of the two types of enzymes should not exceed 1.5 mg/ml and 3.5 mg/ml.

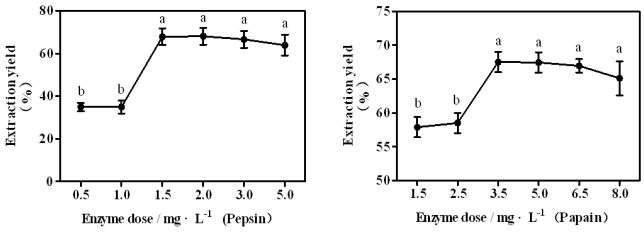

Figure 6. *Effect of enzyme dose on the collagen extraction yield*

3.3.6. Effect of the Ratio of Solid to Solution

Figure 7 shows the effect of the solid/solution ratio on collagen yield, where other factors were fixed. The extraction yield was enhanced with the solid/solution ratio decreasing, and reached to the maximum value at 1:20 and 1:30 for pepsin and papain enzymatic methods, respectively. Afterwards, the yield of collagen dropped a little. The more appropriate ratio of solid to solution would be 1:20 and 1:30, respectively.

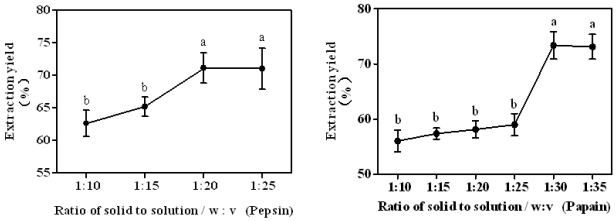

Figure 7. *Effects of ratio of solid to solution on the collagen extraction yield*

3.3.7. Effect of the Hydrolysis Time

Effect of hydrolysis time on collagen yield was seen in Figure 8, where other factors were fixed. The results showed that, when the time extends from 6 to 20 h, the extraction yield increased, and reached the top value at 20 h for pepsin enzymatic method. Similar result was also observed for papain enzymatic method, for which the maximum collagen yield was obtained when time was 15 h. Therefore, hydroylysis time was decided at 20 h and 15 h for pepsin and papain enzymatic methods, respectively.

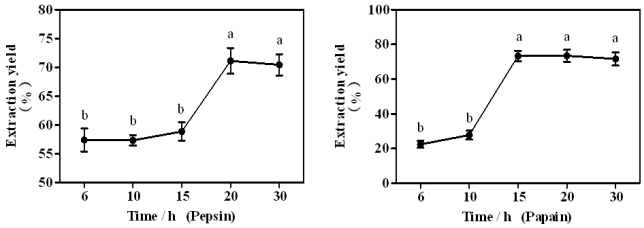

Figure 8. *Effect of hydrolysis time on the collagen extraction yield*

Under the optimized extraction conditions, effects of enzymatic extraction methods using pepsin and papain on collagen yield were compared. Figure 9 shows the effect of enzyme type on extraction of collagen. Clearly, the findings demonstrated that there was no significant difference for the two enzymatic methods ($P>0.05$). However, the papain enzymatic method was more promising method that offers improved efficiency by saving the substrate concentration and reducing hydrolysis time (Figure 7, 8). Mainly studies

have referred to collagen from different aquatic animals extracted by pepsin and acetic acid. Few reports are available for collagen extraction by papain and citric acid. According to previous studies, the papain can help break down part of peptide bonds in collagen molecules and dissolve the collagen [27, 28]. In comparison with pepsin, the use of papain was considered as providing a safe means for treating collagen intended for human use and was the enzyme most commonly used to prepare collagen for biomedical applications, with advantages of reduced immunogenicity, capability of digesting numerous naturally occurring proteins and peptides and lytic effect of elastin, one of the contaminants that is difficult to remove from purified collagen [23].

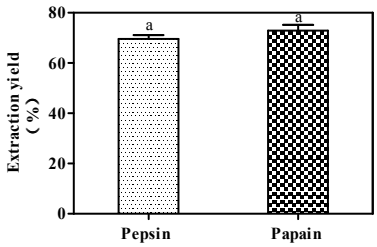

Figure 9. *Effect of enzyme type on the collagen extraction yield*

3.4. Optimization of the Papain Enzymatic Extraction Parameters by Orthogonal Test

Table 4. *Analysis of L_9 $(3)^4$ test results*

Level	Factor				Extraction yield[a] (%)
	A	B	C	D	
1	1	1	1	1	71.88
2	1	2	2	2	75.06
3	1	3	3	3	66.23
4	2	1	2	3	58.9
5	2	2	3	1	70.44
6	2	3	1	2	65.6
7	3	1	3	2	69.18
8	3	2	1	3	50.95
9	3	3	2	1	70.88
k_1 [b]	71.05	66.65	62.81	71.06	
k_2	64.98	65.48	68.28	69.95	
k_3	63.67	67.57	68.62	58.69	
R	7.38	2.09	5.81	12.37	

a Extraction yield (%) = (the amount of extract/ the collagen content in the sample)×100
R refers to the result of extreme analysis

Various parameters affect the optimization of the experimental conditions for the development of papain enzymatic extraction method. The extraction temperature, enzyme dose, ratio of solid to solution, and extraction time are generally considered to be the most important factors that affect the yield (%) of collagen. The investigated levels of each factor were selected depending on the above experimental results of the single-factor and examined using an orthogonal test design L9 (3^4). Independent variables with three variable levels, extraction temperature (32, 35, 38 °C), enzyme dose (3.0, 3.5, 4.0 $mg \cdot L^{-1}$), the ratio of solid to solution (1:25, 1:30, 1:35), and extraction time (12, 15, 18 h) are listed in Table 2. The orthogonal test results and extreme difference analysis are presented in Table 4, with collagen extraction yield (%) as dependent variable.

As seen from the results of Table 4, the maximum yield of collagen (75.06%) was obtained when extraction temperature, enzyme dose, ratio of solid to solution, and extraction time were A1B2C2D2 (32 °C, 3.5 mg/ml, 1:30, 15 h). However, we cannot directly choose the corresponding extraction conditions as the best technology. According to the R values, the influences to the mean extraction yield decrease in the order of D>A>C>B. The yield of collagen was significantly influenced by extraction time and temperature.

In addition, according to the K values, the excellent combination was A1B3C3D1, that's, the temperature 32 °C, enzyme addition 4.0 mg/ml, ratio of solid to solution 1:35, extraction time 12 h. However, this combination did not exist in the nine trials, so additional test was needed to validate the analytical results. With combination of A1B3C3D1, extraction yield of 79.29% was obtained, which was higher than that of A1B2C2D2. Therefore, we finally make the optimum enzymatic extraction technology as follows: extraction temperature, 32 °C, papain enzyme addition, 4.0 mg/ml, and ratio of solid to solution 1:35, and the extraction time, 12 h.

3.5. UV Scanning Spectrum of Turtle Lung Collagen

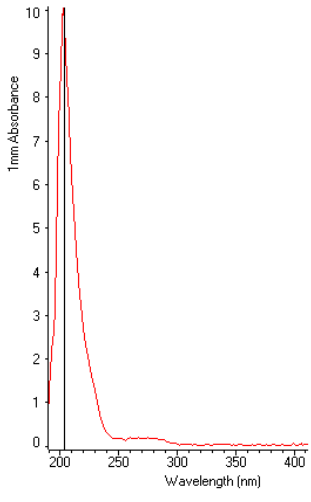

Figure 10. *UV scanning spectra of collagen from turtle lug*

Collagen usually has strong ultraviolet characteristic absorption with absorbance peaks around 220 nm [29]. This property is used to express the integrity of the non-helical telopeptides when collagen is extracted and purified. The spectra were closely related to their higher proteinous

contaminants which were not removed by the extraction procedures [30]. Figure 10 depicts the UV scanning spectrum of lung collagen obtained by the optimized enzymatic extraction method. Samples of lung collagen in our study had the maximum absorption peak at 203 nm by UV scanning, which accords with the characteristics absorption of collagen but was little different from that of turtle calipash which had absorbance peak at 226 nm.14 No absorption band was found around 280 nm, which is from aromatic amino acids, confirming the high purity of the TC-L which has few aromatic amino acids [31].

3.6. Protein Pattern of Turtle Lung Collagen

The electrophoresis patterns of TC-L are shown in Figure 11. There were four bands by dark staining clearly, showing two α chains (likely 2:1 ratio of α1 and α2), β, and γ-components. The molecular weights of α1 and α2 were between 110-130 KDa, and both the molecular weights of β chain and γ chain were about 250 KDa. This observation was similar to the findings reported for deep-sea redfish, yellowfin tuna, etc [32, 33].The patterns were also similar to those of turtle calipash.14 The SDS-PAGE profile proved that the lung collagen from Pelodiscus sinensis were typically structural features of type-I collagen. As observed by previous studies, collagen isolated from the skin of fish species were most likely classified as type I collagen, which consisted of a heterotrimer of two identical α1-chains and one α2-chain with slightly different mobilities [33, 34]. Compared with collagen from soft-shelled turtle calipash (TC-C) that we also self-prepared in our laboratory [14], the band intensity of TC-L was visibly lower. It seemed that the ratio of a1 to a2 chains of collagens from the two different sources was almost the same. Comparing the proportion of high molecular weight (MW) components, particularly γ components between TC-C and TC-L, the former contained the lower band intensity. The result suggested that the intra- and inter-molecular cross-links of collagens were richer in TC-L than in TC-C. We thought the cause might be the difference of extraction enzymes. After digestion by pepsin in turtle calipash treatment, some band γ-components of TC-C might be cleaved into α- or β-components, as evidenced by the increased band intensity of the low MW chains.

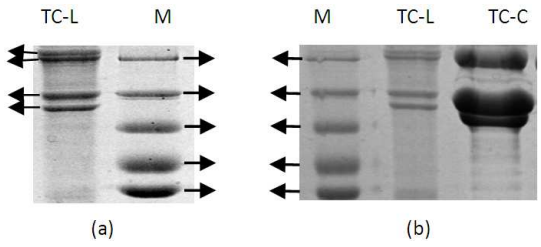

Figure 11. *SDS-PAGE profile of collagen prepared from soft-shelled turtle (a) and its comparison with type I collagen from different animals (b). M: molecular weight marker; TC-C, collagen from soft-shelled turtle calipash (self-prepared); TC-L, collagen from soft-shelled turtle lung (self-prepared).*

3.7. Amino Acid Composition of Turtle Lung Collagen

The amino acid composition directly influenced the collagen's physical-chemical properties, like cross-linking ability and thermal stability [30]. Literatures indicated that the molecular structure of species is different and therefore results in a different structure of collagen. The amino acid analysis of collagen extracted from soft-shelled turtle is illustrated in Table 5. The sample collagen was rich in glutamic acid, glycine, alanine, proline and hydroxyproline, with glycine representing nearly one-fourth of the total amino acid. According to previous studies, glycine was the most abundant amino acid in all collagens [12, 20, 30].

For imino acid content, TC collagen had a 21.8% of proline (Pro) and hydroxyproline (Hyp), representing nearly one-fifth of the total amino acid. Pro and Hyp contents were high at 12.0% and 9.8%, respectively, showing a similar tendency as mammalian collagens and with higher amounts than those of other marine collagen [32, 35, 36]. The hydroxylation degree (44.95%) was close to the collagen from the skin of Amur sturgeon [36], and was comparable to bovine and porcine collagen [16]. Burjanadze [37] previously reported that the content of hydroxyproline varies between species and that the hydroxylation level of proline correlates with the ambient temperature of animals. Difference in heat stability of collagen have been correlated with Pro and Hyp content which are believed to play a substantial role in the stabilization of the triple helix due to the non-covalent bonding with pyrrolidine ring [38]. Therefore, the amounts of Pro + Hyp are important for the structural integrity of collagen. It is speculated that this may be one of the comparative advantages of terrapin animals. However, the thermal stability of turtle lung collagen was not determined and will be evaluated in further study.

Unlike other collagens [39] alanine was found as the third most abundant amino acid in turtle lung collagens. Besides, the sample was low in histidine, tyrosine and methionine, and contained no cysteine and tryptophan. For tyrosine, it was an indicator of telopeptide remained and the value was comparable to other reports in the literatures [30, 40].

Table 5. *Amino acid profile of collagen from soft-shelled turtle lung*

Amino Acids	Residuers/1000 residues	Amino Acids	Residuers/1000 residues
Aspartic acid	55	Leucine	28
Threonine	26	Tyrosine	3
Serine	43	Phenylalanine	21
Glutamic acid	110	Lysine	31
Glycine	233	Histidine	7
Alanine	103	Arginine	81
Cysteine	ND	Proline	120
Valine	20	Hydroxyproline	98
Methionine	7	Tryptophan	ND
Isoleucine	14	Total	1000
Amino Acids	Residuers/1000 residues	Amino Acids	Residuers/1000 residues
Aspartic acid	55	Leucine	28

4. Conclusions

Optimum enzymatic extraction conditions of collagen from the lung of soft-shelled turtle Pelodiscus sinensis were obtained and the chemical properties of the extracted collagen were characterized in the present study. The turtle lung collagen is of high purity, has typical type I collagen characteristics with slightly different two α and β bands, and possessed high content of imino acids. It is suggested that the lung collagen from terrapin animals would be a viable source of mammalian collagen. Further research is presently underway to investigate more physicochemical properties and to prepare for biomaterial applications.

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