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Purification, optimization and physicochemical properties of collagen from soft-shelled turtle calipash

Ya'nan Yang, Caiyan Li*, Wei Song, Wei Wang, Guoying Qian**

College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo 315100, China

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ABSTRACT

The present work was to optimize the purification conditions for soft-shelled turtle (*Pelodiscus sinensis*) calipash collagen (STCC) isolated by pepsin and to explore collagen physicochemical properties for potential biomaterial applications. Single-factor test and orthogonal method $L_9(3^4)$ were employed with the STCC recovery yield as indicator. The optimum purification conditions were obtained when NaCl concentration, collagen concentration and purification time were 2 M, 8 g/L, and 24 h, respectively. Purified STCC were characterized by SDS-PAGE, UV scanning, FTIR, solubility, thermal behavior and amino acid analysis. The results showed that STCC contained high hydroxyproline content than that of other fishery skins, belonging to typical type I collagen in form of $[\alpha_1(I)]_2\alpha_2(I)$. FTIR spectra of STCC were quite similar to other aquatic animals' collagens. It has the lowest solubility at pH 6, and when NaCl concentration decreased from 2% to 6% (w/v), solubility dropped. The denaturation temperature (Td) and melting temperature (Tm) were 35.1 °C and 105.14 °C, respectively. Morphology of STCC depicted as regular and porous network structure by SEM. In general, the results suggested that turtle calipash can be exploited as alternatives to mammalian collagen and could also be used for biomedical applications as a potential new material.

1. Introduction

Collagen is a very important major structural protein supporting connective tissues, which has a unique triple-helical structure formed by three polypeptide chains [1]. Collagen possesses many superior features, such as high tensile strength, good biocompatibility, low antigenicity, low irritation and low cytotoxicity [2], and can be used in burns, trauma, corneal disease, orthopedic, hard tissue repair [3], soft tissue repair [4], wound healing [5] and other extensive medical and health purposes, attributing to its fibril forming ability which is useful in biomaterial applications. The excellent biological properties have demonstrated its potential as an ideal biomaterial. However, the improvement of biological properties of collagen molecules is of great importance in the research and development of novel collagen-based biomaterial. Despite the advantage, the application of collagen as a promising biomaterial also has some drawbacks. For instance, the preparation and purification procedures of collagen are always complicated.

The traditional collagen in biomaterial applications was mainly extracted from pig, cattle and other terrestrial animals [6]. However, the collagen product from mammalian animals was restricted due to mad cow disease, foot-and -mouth disease and other problems [7]. In recent years, aquatic collagen as an alternative has received increased attention because of more biological safety [8]. Collagens were extracted from the skin, muscle or bones of various kinds of fishes [9–12]. The soft-shelled turtles, *Pelodiscus sinensis*, which is a commercially important and delicious aquatic species in Asian countries, especially in China, Japan and southern Taiwan area, have been used for collagen extraction [13–16]. Compared to other coldwater fishes, the soft-shelled turtle is an aquatic animal living in relatively higher ambient environment at around 30 °C [13]. The turtle collagens have higher denaturation temperature (Td) and may have the advantage of higher thermal stability in biomedical applications. Additionally, the global aquaculture production of *P. sinensis* was up to 347,587 tons in 2013 and approximately more than 140,000 t were produced from 2005 in China alone [17,18]. Our colleagues and other researchers have previously reported the extraction and characterization of collagen from turtle skin [13], lung [14] and calipash [15,16]. And optimum extraction conditions were also obtained. The present study was further

* Corresponding author at: College of Biological and Environmental Sciences, Zhejiang Wanli University, South Qianhu Road 8, Ningbo 315100, China.
** Corresponding author.
E-mail addresses: licy82@163.com, licy@zwu.edu.cn (C. Li), qiangy@zwu.edu.cn (G. Qian).

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attempted to determine the purification conditions for extracted soft-shelled turtle calipash collagen (STCC) and to characterize the collagen by several physicochemical methods in order to discuss the potential of STCC in biomaterial application area.

2. Materials and methods

2.1. Materials and chemical reagents

The soft-shelled turtles were provided by the turtle farm in Ningbo, China (body weight of 500 ± 50 g). They were put in the nylon mesh bag under normal temperature when transported to our laboratory. The calipash tissues were dissected under iced conditions, cut into small pieces and stored at −20 °C for further use. All experimental procedures were approved by the Animal Ethics Committee of Zhejiang Wanli University. The chemical reagents including pepsin (3000 U/g) and L-hydroxyproline standard were purchased from Sangon Biotech (Shanghai) Co., Ltd. All reagents used in this study were analytical grade.

2.2. Extraction and purification of STCC

The collagen was extracted by pepsin from soft-shelled turtle calipash. The tissues were pretreated in 0.1 M NaOH for 24 h at 4 °C to remove non-collagenous proteins and then with 10% isopropyl alcohol to remove fat. Collagen was crudely extracted from calipash in 25 vol (v/w) of 0.5 M acetic acid containing 2 mg/mL pepsin for 24 h with continuous stirring. Supernatant was collected by centrifugation and then the crude collagen solution was salted-out by the addition of NaCl.

The salted out STCC precipitate was further obtained by centrifugation at 10,000*g* for 30 min. As for the calculation of recovery yield of STCC in salting out technology optimization, the precipitate could be used directly for collagen content determination without the remove of salt. But for the STCC characterization, the pellets were dissolved in 0.5 M acetic acid and the resulting solution was dialyzed against distilled water for 24 h under 4 °C to remove salt. The resulting dialysates was lyophilized for further use [19].

2.3. Optimization of salting out technology

2.3.1. Single-factor test

Effects of three factors including salting out time, STCC concentration and NaCl concentration were investigated on STCC recovery yield, respectively. The design of single factor experiment was shown in Table 1. In order to avoid the denaturation of collagen solution and ensure the purity and yield of collagen, according to the previous references [20] and our preliminary experiment, the fixed levels of salting out time, NaCl concentration and STCC concentration were selected at 24 h, 3 M, 10 g/L respectively. One factor was changed from the lowest level to the highest, while the other two factors were fixed at one level.

The recovery yield of STCC was calculated and expressed as collagen content in salted out solution/crude solution of STCC. The collagen content was determined by measuring the content of hydroxyproline, which is the characteristic amino acid in collagen, by using spectrophotometric analyzer (NanoDrop2000, Thermo Fisher Scientific, USA). The procedures were described in our previous study [14].

2.3.2. Orthogonal L9 (3^4) test

On the basis of the single-factor test described in Section 2.3.1, an orthogonal L_9 (3^4) test design with four factors and three levels was used to investigate the optimal salting out condition of STCC. As seen from Table 2, nine groups of salting out experiment were carried out at salting out time (A) 12, 24, 36 h, NaCl concentration (B) 2–4 M, STCC concentration (C) 6, 8, 10 g/L. The STCC recovery yield (%) was the dependent variable, and the data of the orthogonal experiment was analyzed by SPSS 19.0 software.

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

The electrophoresis was performed by using the discontinuous Tris-HCl/glycine buffer system with 8% separation gel and 5% stacking gel using a vertical cell (MiniPROTEAN3, Bio-rad, USA) at voltage of 100 v for about 100 min (PowerpacBasic, Bio-rad, USA). After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 10% (v/v) acetic acid for 30 min and destained with 30% (v/v) ethanol and 10% (v/v) acetic acid. High molecular weight markers were used to estimate the molecular weight of proteins. The electrophoresis pattern was imaged by ultraviolet spectrophotometer (Gel Doc^{TM} XR+, Bio-rad, USA). The crude and purified collagens were both used for SDS-PAGE to compare the purification effects and the composition.

2.5. UV scanning

The ultraviolet absorption spectra of the purified collagen samples were scanned by UV spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA) between 190 and 400 nm with an interval of 1 nm.

2.6. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of purified STCC were obtained by Fourier Transform Infrared Spectrometer (VERTEX 70, Bruker, Germany). One-two mg of collagen samples were tableted with KBr and then scanned in a range of 400–4000 cm^{-1} with 32 scans per sample at a resolution of 4 cm^{-1} [11].

2.7. Amino-acid composition

STCC samples and calipash of *P. Sinensis* were both hydrolyzed under standard conditions. 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.

2.8. Collagen solubility

Effect of pH and NaCl on collagen solubility was measured according to the method described in Ahmad and Benjakul [21] with slight modification. Briefly, STCC was dissolved in 0.5 M acetic acid to obtain a final of 3 mg/mL or 6 mg/mL and the mixture was stirred at 4 °C until STCC was completely solubilised.

STCC solution (8 mL, 3 mg/mL) was transferred to a series of centrifuge tubes to obtain pH values ranging from 1.0 to 10.0 by addition of 6 M NaOH or 6 M HCl. The final volume was adjusted to 10 mL with distilled water. The mixture was stirred and centrifuged at 10,000*g* for 30 min. Protein concentration of the supernatant was measured by the Lowry et al. method [22] using bovine serum albumin as a standard. Relative solubility was calculated by dividing the highest solubility value by the solubility of the test sample.

In the determination of STCC solubility at different NaCl concentration, 5 mL of 6 mg/mL STCC solution was mixed with six groups of 5 mL NaCl solution (0%, 2%, 4%, 6%, 8%, 10% and 12% (w/v)), separately, to obtain a series of STCC concentrations (0%, 1%, 2%, 3%, 4%, 5% and 6% (w/v)). The protein content and collagen solubility was measured as described above.