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Reutilization of *Pelodiscus sinensis* (Chinese Soft-Shelled Turtle) Visceral Waste for cosmeceutical Applications

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Abstract

Pelodiscus sinensis, commonly known as the Chinese soft-shelled turtle, possesses abundant collagen, proteins, and amino acids in its shell and muscle. *Pelodiscus sinensis* has been historically utilized as a valuable traditional Chinese medicine. While the Chinese consume the meat and shell of the turtle, the internal visceral organs are typically discarded. The visceral organs contain valuable components, including 8%-15% collagen and 8% blood. This study focused on extracting collagen from the internal visceral organs, while the remaining turtle parts were condensed into a refined product referred to as "Turtle refined," for cosmeceutical applications. We explored the acid solubilization (ASC) and pepsin solubilization (PSC) techniques to extract collagen. Preliminary findings indicate that the PSC method outperformed the ASC method in terms of collagen extraction. To characterize the collagen derived from the visceral organs and blood, SDS-PAGE, FTIR and DSC analyses were employed. Furthermore, we assessed the antioxidant property of the Turtle refined product, utilizing the DPPH radical scavenging activity test.

Keywords: Collagen; Extraction; *Pelodiscus sinensis*; SDS-PAGE; FTIR; DSC; DPPH assay

1. Introduction

The Chinese soft-shelled turtle (*Pelodiscus sinensis*), also known as water fish or terrapin, is a flattened, oval-shaped creature with a skin-covered shell [1]. It has an inconspicuous pattern and a dark green or yellowish-brown back shell with a thickened connective tissue periphery known as the "skirt."[2]. Throughout history, *Pelodiscus sinensis* has been highly valued and used in traditional Chinese medicine [3].

The Chinese have traditionally consumed both the meat and shell of the turtle, while the internal visceral organs have often been overlooked and discarded. The internal organs of the Chinese soft-shelled turtle contain muscle and fat, and they are connected by a substantial amount of general connective tissue [4]. The extracellular matrix of this connective tissue consists of collagen fibers (white fiber), elastic fibers (yellow fiber), and reticular fibers [5]. As a result, a significant amount of oil and collagen can be extracted. The extract from the turtle visceral organs holds great potential for the development of cosmetic and medical applications.

Collagen is the primary component of the extracellular matrix and a major protein in animal connective tissues [6]. Collagen is widely distributed in the body, contributing to structural support and integrity. Its content in connective tissues ranges from 20% to 30% [7]. Collagen can be classified into various types based on the different combinations of the α chains and their structural characteristics. These types include fibrillary collagen, network-forming collagen, fiber-associated collagens (FACIT), short chain triple helices, beaded fibers, collagen type VII associated with basement membranes, transmembrane domain collagen, and other undefined types. For instance, collagen type I consists of two

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α1 chains and one α2 chain, denoted as [α1(I)]2α2(I). Presently, researchers have identified approximately 27 types of collagen, with type I, II, III, IV, and V being extensively studied, and type I being the most abundant [7]. However, the specific characteristics of certain collagen types are still being investigated and remain unclear.

Collagen has gained significant popularity in the medical and cosmetics industries due to its beneficial effects. [8] It has undergone extensive research and improvement, particularly in the development of composite biomaterials that replicate the surface and physiological structure of biological tissues [9]. These biomaterials possess degradation and repair capabilities and can stimulate cell proliferation by interacting with endothelial cells, making them highly suitable for the creation of biocompatible materials [10]. Notably, type I collagen, widely recognized in the cosmetics industry, can stimulate the synthesis of both type I and type III collagen in the skin, thereby promoting the generation of fibroblasts and providing anti-aging and wrinkle-reducing effects [11]. Consequently, it can be effectively combined with other cosmetic ingredients. Commercial production of collagen primarily has come from the skin and scales of chickens, cows, pigs, and fish. This study sets out to test the feasibility of extracting collagen from turtle internal organs, as a viable alternative source for obtaining collagen.

Experiments conducted by the Chinese National Intellectual Property Administration, the European Patent Office, and Hainan Yang-sheng-tang Pharmaceutical Co., Ltd. have shown that turtle oil offers a range of benefits [12, 13]. These include anti-inflammatory and analgesic effects, [14] promotion of fibroblast proliferation, [9] and significant protection against ultraviolet radiation [9]. As a result, turtle oil can be incorporated into cosmetics as an additive or excipient, providing additional value and desirable properties to cosmetic formulations.

Over the past few years, extensive research has established the effectiveness and diverse applications of collagen in both medicine and cosmetics [9]. Notably, there has been a report on the utilization of Turtle Oil in cosmetics and skincare, which provides support for its potential as an effective additive in cosmetic products [15]. In order to evaluate the practicality of incorporating turtle oil, the main objective of this study was to evaluate methods for extracting collagen from the internal visceral organs of turtles. Additionally, the study aimed to process and condense the remaining parts of the turtle, resulting in a refined product known as "Turtle refined." The potential applications of this refined product in the fields of medicine and functional uses were also investigated.

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2. Material and methods

2.1. Extraction of visceral collagen

The turtle internal organs were collected from a local fish market. The blood from the turtle viscera was washed off using D.D.W (deionized distilled water), and after weighing, 100g portions were packed and frozen in a freezer for later use. According to the experiments conducted by Liu et al. (2015) [16], it was found that collagen extraction efficiency was higher in 0.5M acetic acid compared to other concentrations. Therefore, for this study, 0.5M acetic acid was adopted.

The mixtures of turtle viscera were combined with acetic acid (0.5 M) at a ratio of 1:15 (w/v) and either with pepsin (0.01 mg/ml dry matter) (PSC) or without pepsin (ASC). The mixtures were continuously stirred at 4°C for 48 hours. Afterward, the mixtures underwent filtration through two layers of coarse cotton cloth, and the resulting supernatants were collected through centrifugation. The collagen solution was then mixed with 0.05 M Tris and 2.5 M NaCl, leading to the collection of precipitates by centrifugation at 20,000 g for 60 minutes at 4°C. Next, the precipitate was dissolved in 0.5 M acetic acid and transferred into a dialysis membrane with a molecular weight cut-off of 64 kDa. It was subsequently immersed in 25 volumes of 0.1 M acetic acid for 12 hours. Lastly, the sample was dialyzed in distilled water for 48 hours, and the content percentage was calculated.

2.2. Quantitative Analysis of visceral fat

To quantitatively determine the amount of fat in a sample, a 300 µl sample was taken and combined with 100 µl of ammonia solution, 300 µl of ether, 100 µl of alcohol, and 300 µl of n-hexane. The resulting mixture was thoroughly shaken to ensure homogenous mixing of the components. Subsequently, the supernatant was collected, and an additional 200 µl of alcohol, 300 µl of ether, and 300 µl of n-hexane were added. The mixture was shaken again to achieve

uniformity. Following this, the mixture was subjected to a 75°C water bath for a duration of 1 hour, after which it was transferred to a 90°C oven and left undisturbed for a period of 1 day.

2.3. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was according to the procedure of Laemmli (1970) with slight modification [17]. Samples for were prepared by adding 100 µL of sample buffer (1% dithiothreitol was used instead of β-mercaptoethanol) to 50 µg of protein and heating for 4 min at 100ºC. An aliquot (10 µL) of this mixture was applied to each well in 7% polyacrylamide separating gels. Gels (100 × 750 × 0.75 mm3) and were subjected to electrophoresis at 15 mA using a Mini-Protean II Cell (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, the gels were stained with 0.04% Coomassie Blue in 25% v/v ethanol and 8% v/v acetic acid for 30 min at 60°C. Excess stain was removed with several washes of de-staining solvent (25% v/v ethanol, 8% v/v acetic acid).

2.4. DPPH radical scavenging activity test

The free RSA of the dilute extract was tested using a 1,1-diphenyl-2-picryl hydrazyl (DPPH) technique as described by Shimada et el (1992) with slight modification [18]. Briefly, a 96-well plate was utilized to assess the antioxidant capacity of samples. A mixture of 100µl sodium acetate and 100µl sample dissolved in methanol was prepared and combined with 500 µl of a 0.5 mM DPPH methanol solution. The resulting mixture was then placed in a dark environment for a duration of 30 minutes. As a control, 50 µl of DPPH was replaced with methanol. Subsequently, the absorbance of the samples was measured using an ELISA reader at a wavelength of 517 nm. A lower absorbance value indicates a higher clearance rate and thus stronger antioxidant activity. It is important to note that antioxidants provide hydrogen atoms to the DPPH radical, thereby exhibiting a stronger hydrogen-donating capacity as the clearance rate increases. The following formula was used to compute the percentage of antioxidants or RSA:

% of antioxidant activity= [(Ac−As) ÷Ac] × 100

Sodium acetate, Methyl alcohol, 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) and 2,6-di-tert-butyl-p-cresol (BHT) were purchase from Sigma-Aldrich, Inc. (St. Louis, MO 68178 United States.)

2.5. FTIR Analysis

FTIR spectroscopy was applied to the collagen derived from the visceral organs and blood according to the method described by Chan et al, 1996 with slight modification [19, 20]. Briefly, a film was prepared by pressing 100 mg of KBr using a pellet press, and the background IR spectrum was measured. Subsequently, a mixture of 2 mg of collagen protein and 100 mg of KBr in a 1:50 ratio was pressed into a film using a pellet press. FTIR spectroscopy (Perkin-Elmer FT-IR RX1) was performed by scanning 64 times with a resolution of 4 cm^{-1}, covering the range of 4000 cm^{-1} to 400 cm^{-1}.

2.6. Differential Scanning Calorimeters (DSC)

Differential Scanning Calorimeters (DSC) was used to measure the thermal denaturation temperature of collagen proteins according to the procedure described by Hsieh et al (2017) [21]. The parameters of the setup program were as follows: the initial temperature was set at 20°C, the heating rate was 5°C/min, and the final temperature was set at 200°C.

3. Results and discussion

Currently, industrial production of collagen primarily comes from the skin and scales of chickens [22], cows [23], pigs [24], and fish [25]. Acid, alkali, and enzyme methods are commonly used for collagen extraction [26]. Acid and alkali methods are simple and cost-effective but may lead to structural damage to the collagen molecules [27]. Recently, the highest extraction rate has been achieved using pepsin/0.5M acetic acid solution, which has become the most commonly used method for extracting collagen from animal tissues [28].

Enzymes used in the extraction process primarily function by breaking down collagen and cleaving its cross-linking bonds. They can be classified into plant-based and animal-based enzymes [29]. Papain: 25°C, pH 6.2[30], bromelain: 25°C, pH 4.6[31], and pepsin: 37°C, pH 2.0[32] are the most widely used enzymes due to their easy accessibility and low cost. Our study has successfully demonstrated the feasibility of extracting collagen from turtle internal organs, highlighting its potential as a viable alternative source for obtaining collagen.

3.1. Extraction of protein and turtle oil of from turtle internal organs

The mixtures of turtle viscera were combined with acetic acid (0.5 M) at a ratio of 1:15 (w/v) and either with pepsin (0.01 mg/ml dry matter) (PSC) or without pepsin (ASC). The resulting protein and turtle oil content of the extracted visceral collagen from turtle internal organs were quantified, and the yield percentages were calculated using the formulas:

Protein Yield % = (Final Product / Total Organ Weight) * 100

Turtle Oil Yield % = (Final Product / Total Organ Weight) * 100

The protein yield ranged from 10.22% to 11.65%, while the turtle oil yield ranged from 5.72% to 6.90% (Table 1).

Table 1 The protein and turtle oil content of the extracted visceral organ

Total Weight (g)	Turtle Oil (g)	Turtle Oil (%)	Collagen (g)	Collagen (%)
130	8.96	6.9%	15.15	11.65%
300	17.17	5.72%	30.68	10.22%

The data presented in Table 1 shows notable differences between the results obtained from a total weight of 300g and 130g. The lower yield obtained from the higher quantity suggests that a longer extraction time may be necessary for achieving optimal results. It is worth considering the potential impact of seasonal variations as well. Turtles tend to have increased food consumption during the summer and autumn seasons, whereas in winter, their activity levels decrease as they enter a dormant state when water temperatures drop below 20°C. These seasonal factors could contribute to the observed variations in the extraction process.

Fat exhibits poor solubility in water but high solubility in non-polar compounds like benzene or ether. To quantitatively determine the amount of turtle oil, a non-polar solvent was used to extract the sample, followed by evaporation of the organic solvent. The remaining residue obtained after evaporation represents the fat content. However, it is important to note that the organic solvent not only extracts fat but also other non-polar components present in the sample, including free fatty acids, organic acids, pigments, alkaloids, cholesterol, and fat-soluble vitamins. Consequently, the turtle oil measured using this method is commonly referred to as crude turtle oil.

3.2. SDS-PAGE of Chinese soft-shell turtle proteins

The protein extracted from turtle internal organs was analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure1). By comparing with standard collagen protein, it can be seen in Figure1 that bands corresponding to molecular weights of 138 kDa (αl) and 115 kDa (αll) are present. Type I collagen includes α-chains, β-chains, and a fraction that is covalently cross-linked. The intensity of α1 band is higher than α2 band. The triple helix structure of type I collagen is composed of two A1 chains and one A2 chain. These results are consistent with the presence of type I collagen obtained from the viscera.

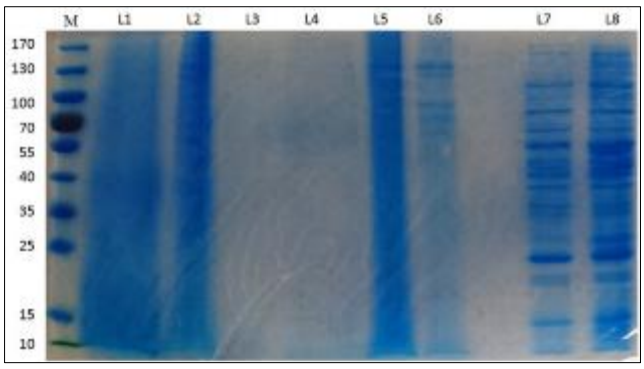

Figure 1 SDS-PAGE of Chinese soft-shell turtle proteins

Molecular weights of subunits of acid soluble collagen (ASC) were estimated using molecular weight standards from Thermo-Fisher pre-stained protein ladder. Protein bands corresponding to molecular weights of 138 kDa (αI) and 115 kDa (αII) are present. M: Marker, L1: ASC supernatant, L2: ASC precipitate, L3: PSC mixture, L4: PSC supernatant, L5: PSC precipitate, L6: Lyophilized PSC, L7, L8: Lysed E. coli.

3.3. Fourier Transform Infrared (FTIR) analysis of soft-shell turtle collagens

To identify and characterize the functional groups and chemical bonds present in soft-shell turtle collagens, the soft soft-shell turtle ASC (acid-solubilized collagen) and PSC (pepsin-solubilized collagen) were analyzed by FTIR (Figure 2). In Figure 2, it can be seen that the vibration frequencies of ASC and PSC for turtle are similar to the peaks of amide I, II, III, A, and B, which are characteristic of collagen proteins.

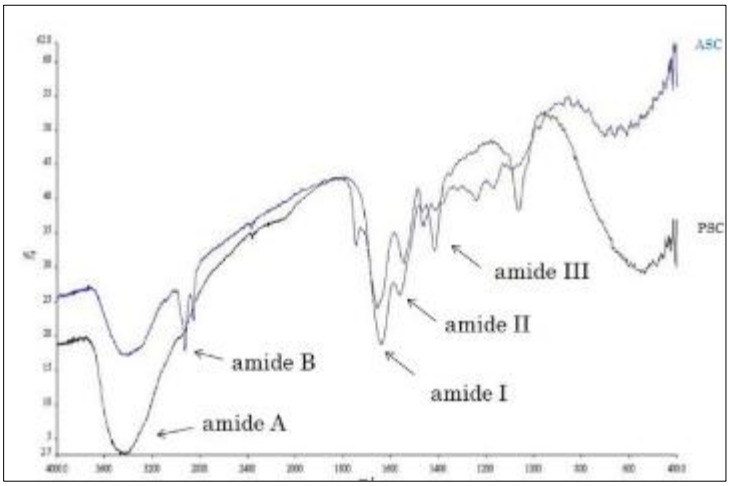

Figure 2 FTIR spectra of soft-shell turtle collagens

FTIR spectroscopy was performed by scanning 64 times with a resolution of 4 cm-1, covering the range of 4000 cm^{-1} to 400 cm^{-1}(Perkin-Elmer FT-IR RX1). The FTIR spectra of ASC and PSC for soft-shell turtle exhibit similar vibration frequencies to the characteristic peaks of amide I, II, III, A, and B, which are typical of collagen proteins

3.4. Differential Scanning Calorimetry analysis of the visceral collagens.

To understand the triple helical structure of the extracted collagen proteins, DSC was used to measure the temperature at which the structure of collagen protein is disrupted. When proteins are heated to a certain temperature, their structure is disrupted due to the heat. The helical chains of collagen unfold and transition from an extended fibrous state to an irregularly coiled state, known as collagen denaturation. From Figure3, it can be seen that collagen protein exhibits an endothermic peak during the heating process. The first endothermic peak occurs at a temperature of approximately 55°C, which represents the thermal denaturation and subsequent heat degradation of collagen protein.

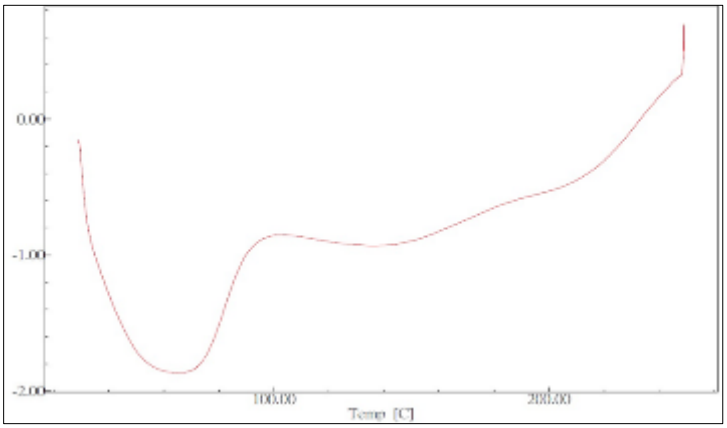

Figure 3 Differential Scanning Calorimetry of the Collagen

The extracted collagen protein was found to have a Td value of 55°C, indicating the temperature at which thermal denaturation occurs for the collagen protein. The curve represents the temperature on the horizontal axis and the rate of heat absorption and release by the sample on the vertical axis. The parameters of the setup program were as follows: the initial temperature was set at 20°C, the heating rate was 5°C/min, and the final temperature was set at 200°C.

3.5. DPPH radical scavenging activity of Turtle refined

To evaluate the antioxidant capacity of Turtle refined, we conducted a DPPH radical scavenging activity test. The test was conducted at various concentrations. Figure 4 illustrates the results, indicating that Turtle refined possesses antioxidant ability. At a concentration of 25% (0.1g/ml), the removal rate of DPPH radicals was approximately 8%. Increasing the concentration to 50% (0.2g/ml) resulted in a removal rate of around 32%. At a concentration of 75% (0.3g/ml), the removal rate increased to approximately 66%. Finally, at 100% concentration (0.4g/ml), the removal rate reached 86%.

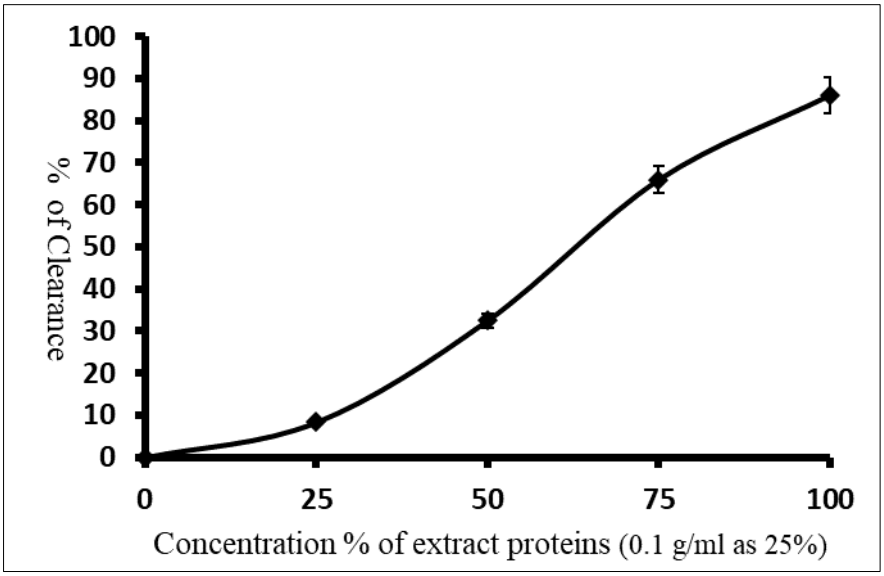

Figure 4 DPPH radical scavenging activity of Turtle refined

The free RSA of the dilute extract was tested using DPPH technique as described by Shimada et el (1992) [18]. A 96-well plate was utilized to assess the antioxidant capacity of samples. The absorbance of the samples was measured using an ELISA reader at a wavelength of 517 nm. The following formula was used to compute the percentage of antioxidants or RSA:

% of antioxidant activity= [(Ac−As) ÷Ac] × 100

4. Conclusion

To validate the protein structure of purified collagen obtained from acid extraction, the triple helical structure was further confirmed using FTIR as a supplementary analysis. The FTIR spectrum of the purified soft-shell turtle collagen extracted with acid and pepsin/0.5M acetic acid exhibit similar vibration frequencies to the characteristic peaks of amide I, II, III, A, and B, which are typical of collagen proteins (Fig 2).

The absorption characteristics of the amide A band are typically observed in the N-H region, with stretching vibrations occurring at 3400-3440 cm^{-1}. The amide B bond is associated with the asymmetric stretching of CH2 and can be identified at 2925 cm^{-1}. The amide I band is characterized by peaks in the range of 1600-1700 cm^{-1}, representing the stretching vibrations of the main chain peptide bonds (C=O) in the collagen backbone. The amide II band typically represents the bending vibrations of N-H bonds and is commonly observed in the range of 1500-1600 cm^{-1}. The amide III band (1220-1320 cm^{-1}) is associated with the deformation of N-H bonds and the stretching and bending vibrations of C-N bonds [34].

By comparing these spectra with the FTIR spectra of bovine skin AEC and PEC (Masilamani et al. 2016) [23], chicken skin AEC, PEC, and SSC (Zhou et al. 2016) [22], and fish scale PEC (Chen et al. 2016) [33], it can be concluded that collagen proteins extracted using ASC and PSC methods exhibit a triple helix structure and share similar properties to terrestrial animals.

Differential Scanning Calorimetry (DSC) is an analytical technique used to study the thermal properties and behavior of materials. DSC measures the heat flow associated with temperature changes in a sample, providing information about its phase transitions, thermal stability, melting and crystallization behavior, and other thermal events. By comparing the heat flow of a sample to a reference material, DSC can reveal changes in heat capacity, enthalpy, and other thermodynamic parameters, allowing for the characterization and analysis of ASC and PSC extracted from soft-shell turtle. The helical chains of collagen unfold and transition from an extended fibrous state to an irregularly coiled state, which is known as collagen denaturation. From Figure 3, it can be seen that collagen protein exhibits an endothermic peak during the heating process. The first endothermic peak occurs at a temperature of approximately 55°C, which represents the thermal denaturation and subsequent heat degradation of collagen protein. This confirms that the triple helical structure of the extracted collagen protein undergoes thermal denaturation during the heating process.

In closing, we have successfully extracted collagen from turtle internal organs, and the FTIR and DSC analyses confirmed the bona fide collagen signature structures of the extracted protein, thus demonstrating its potential as a viable alternative source for obtaining collagen. We also showed the antioxidant capacity of Turtle refined by DPPH radical scavenging activity test. Together, the turtle visceral organs had great potential for the development of cosmetic and medical applications.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to disclosed.

References

[1] Sánchez-Villagra MR, Müller H, Sheil CA, Scheyer TM, Nagashima H, Kuratani S. Skeletal development in the Chinese soft-shelled turtle Pelodiscus sinensis (Testudines: Trionychidae). Journal of Morphology. 2009;270(11):1381-99.

[2] Zou Y, Xu P, Li P, Cai P, Zhang M, Sun Z, et al. Effect of ultrasound pre-treatment on the characterization and properties of collagen extracted from soft-shelled turtle (Pelodiscus sinensis). LWT-Food Science and Technology. 2017;82:72-81.

[3] Chen T-H, Chang H-C, Lue K-Y. Unregulated trade in turtle shells for Chinese traditional medicine in East and Southeast Asia: the case of Taiwan. Chelonian Conservation and Biology. 2009;8(1):11-8.

[4] Li C, Song W, Wu J, Lu M, Zhao Q, Fang C, et al. Thermal stable characteristics of acid-and pepsin-soluble collagens from the carapace tissue of Chinese soft-shelled turtle (Pelodiscus sinensis). Tissue and Cell. 2020;67:101424.

[5] Chen B-j, Niu C-j, Yuan L. Ascorbic acid regulation in stress responses during acute cold exposure and following recovery in juvenile Chinese soft-shelled turtle (Pelodiscus sinensis). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2015;184:20-6.

[6] Sun B. The mechanics of fibrillar collagen extracellular matrix. Cell Reports Physical Science. 2021;2(8).

[7] Gelse K, Pöschl E, Aigner T. Collagens—structure, function, and biosynthesis. Advanced drug delivery reviews. 2003;55(12):1531-46.

[8] Sionkowska A, Skrzyński S, Śmiechowski K, Kołodziejczak A. The review of versatile application of collagen. Polymers for Advanced Technologies. 2017;28(1):4-9.

[9] Kim SK. Marine cosmeceuticals. Journal of cosmetic dermatology. 2014;13(1):56-67.

[10] Gould LJ. Topical collagen-based biomaterials for chronic wounds: rationale and clinical application. Advances in wound care. 2016;5(1):19-31.

[11] Graf J. Anti-aging skin care ingredient technologies. Cosmetic dermatology: Springer; 2005. p. 17-28.

[12] Li P, Li D, Hong Y, Chen M, Zhang X, Hu L, et al. Combining DNA mini-barcoding and species-specific primers PCR technology for identification of heosemys grandis. Frontiers in Ecology and Evolution. 2022;10:822871.

[13] Berdick M. The role of fats and oils in cosmetics. Journal of the American Oil Chemists' Society. 1972;49(7):406-8.

[14] Alioglu B. In Vitro Effects of Emollient Substances on Release of Ketorolac Tromethamine. 2008.

[15] Kim S-K, Wijesakara I. Cosmeceuticals from marine resources. Marine Cosmeceuticals: Trends and Prospects; Kim, S-K, Ed. 2011:1-9.

[16] Liu D, Wei G, Li T, Hu J, Lu N, Regenstein JM, et al. Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (Ctenopharyngodon idella) skin. Food Chem. 2015;172:836-43.

[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature. 1970;227(5259):680-5.

[18] Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. Journal of agricultural and food chemistry. 1992;40(6):945-8.

[19] Chan H-K, Ongpipattanakul B, Au-Yeung J. Aggregation of rhDNase occurred during the compression of KBr pellets used for FTIR spectroscopy. Pharmaceutical research. 1996;13:238-42.

[20] Lin H-H, Liu H-H. FTIR analysis of biodegradation of polystyrene by intestinal bacteria isolated from Zophobas morio and Tenebrio molitor. Proceedings of Engineering and Technology Innovation. 2021;17:50-7.

[21] Hsieh W-C, Hsu C-C, Shiu L-Y, Zeng Y-J. Biocompatible testing and physical properties of curdlan-grafted poly (vinyl alcohol) scaffold for bone tissue engineering. Carbohydrate polymers. 2017;157:1341-8.

[22] Zhou C, Li Y, Yu X, Yang H, Ma H, Yagoub AEA, et al. Extraction and characterization of chicken feet soluble collagen. LWT - Food Science and Technology. 2016;74:145-53.

[23] Masilamani D, Madhan B, Shanmugam G, Palanivel S, Narayan B. Extraction of collagen from raw trimming wastes of tannery: a waste to wealth approach. Journal of Cleaner Production. 2016;113:338-44.

[24] Morimura S, Nagata H, Uemura Y, Fahmi A, Shigematsu T, Kida K. Development of an effective process for utilization of collagen from livestock and fish waste. Process Biochemistry. 2002;37(12):1403-12.

[25] O'Sullivan A, Shaw N, Murphy S, Van de Vis J, van Pelt-Heerschap H, Kerry J. Extraction of collagen from fish skins and its use in the manufacture of biopolymer films. Journal of aquatic food product technology. 2006;15(3):21-32.

[26] Matinong AME, Chisti Y, Pickering KL, Haverkamp RG. Collagen extraction from animal skin. Biology. 2022;11(6):905.

[27] Feng X, Liu T, Ma L, Dai H, Fu Y, Yu Y, et al. A green extraction method for gelatin and its molecular mechanism. Food Hydrocolloids. 2022;124:107344.

[28] Lin Y-K, Lin T-Y, Su H-P. Extraction and characterisation of telopeptide-poor collagen from porcine lung. Food Chemistry. 2011;124(4):1583-8.

[29] Samatra MY, Noor NQIM, Razali UHM, Bakar J, Shaarani SM. Bovidae-based gelatin: Extractions method, physicochemical and functional properties, applications, and future trends. Comprehensive Reviews in Food Science and Food Safety. 2022;21(4):3153-76.

[30] KILINÇ A, ÖNAL S, TELEFONCU A. Stabilization of papain by modification with chitosan. Turkish Journal of Chemistry. 2002;26(3):311-6.

[31] Campos DA, Valetti NW, Oliveira A, Pastrana-Castro LM, Teixeira JA, Pintado MM, et al. Platform design for extraction and isolation of Bromelain: Complex formation and precipitation with carrageenan. Process Biochemistry. 2017;54:156-61.

[32] Ahmad M, Benjakul S. Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (Aluterus monocerous). Food Chemistry. 2010;120(3):817-24.

[33] Chen S, Chen H, Xie Q, Hong B, Chen J, Hua F, et al. Rapid isolation of high purity pepsin-soluble type I collagen from scales of red drum fish (Sciaenops ocellatus). Food Hydrocolloids. 2016;52:468-77.

[34] https://www.sigmaaldrich.com/TW/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table