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Effect of ultrasound pre-treatment on the characterization and properties of collagen extracted from soft-shelled turtle (*Pelodiscus sinensis*)

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ABSTRACT

The aim of the present study was to evaluate the effect of ultrasound pre-treatment on the characterization and *in-vitro* digestion properties of collagen from turtle calipash and the antioxidant activity of its hydrolysates. The results showed that ultrasound significantly increased the collagen yield compared with that from conventional extraction (ASC, $P < 0.05$). Containing α1 and α2 chains, acid-soluble collagen (ASC) and the collagen with ultrasound pre-treatment (UASC) were characterized as type I collagen in SDS-PAGE patterns and UASC also maintained their structural integrity in the circular dichroism spectra. At the same pH, UASC had smaller particle size and larger absolute value of net charge than ASC. Moreover, ultrasound could induce apparent physical changes as looser, more porous and homogenous in microstructures imagines. Influenced by various pH, UASC showed higher solubility than ASC. Additionally, UASC was more prone to enzymolysis *in vitro* digestion and their hydrolysates showed significant increases antioxidant properties over ASC ($P < 0.05$), indicating that UASC would be a potential substrate to generate bioactive peptides. These results suggested that UASC would be a potential new material for biomedical applications and functional supplement in food industries.

1. Introduction

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total protein in vertebrates, which is generally found to support and protect the body and organs (Birk & Bruckner, 2005). It has been widely used in food, medicine, cosmetics, and cell cultures and the consumption has increased with development of new industrial applications (Rasika, Ranadheera, & Vidanarachchi, 2013; Woo, Yu, Cho, Lee, & Kim, 2008), which was used as dermal filler, as hemostat, for drug delivery, skin substitutes, expandable intra-arterial stents and cell attachment substrate (Senaratne, Park, & Kim, 2006; Veeruraj, Arumugam, Ajithkumar, & Balasubramanian, 2015). In addition to that, collagen has been used for producing edible casings for meat processing industries (Simões et al., 2014; Veeruraj, Arumugam, & Balasubramanian, 2013).

In recent years, many papers focused on the practical utilization of various kinds of aquatic species and their by-products such as skins, bones and scales, mostly, to produce collagen. These are the rich in collagen materials from aquatic species. The main differences of aquatic species collagen were mainly from different collagen sources (freshwater species, deep-sea species and so on) and processing conditions (acid-aided process, pepsin-aided process and so on) (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). The soft-shelled turtle (*Pelodiscus sinensis*), is a commercially important and delicious aquatic species in Asian countries including Taiwan, China, Japan and Korea etc., due to its high nutritional (high protein and low fat) and medicinal values (such as

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decreasing blood pressure, antioxidation, anticancer), or sold as pet. Additionally, the global aquaculture production of soft-shelled turtle was up to 355,000 tons in 2014 (Zhang, Xu, He, Zheng, & Shao, 2017). Turtle shell surrounding connective tissue is often referred to as the calipash, which has high collagen content and is the most nourishing and medicinal part. And now, aquatic collagen has been investigated broadly in many countries, for example, America, Germany, Malaysia, et al. However, there has been little report on the collagen from turtle calipash.

The collagens are mostly extracted by solubilisation using acetic acid (Elango et al., 2016). Some researchers have previously reported the extraction and characterization of collagen from turtle calipash (Yang, Li, Song, Wang, & Qian, 2016) and a novel collagen gene fragment of 756 bp was cloned from a soft shelled turtle (Xu et al., 2016), whereas there is no information regarding the effect of ultrasound-assisted processing on characteristics and application of calipash from turtle. Ultrasound is generally considered to be safe, inexpensive, reproducible and reliable, and environmentally friendly in our previous study (Zou, Ding et al., 2016). These give the use of ultrasound a major advantage over other techniques and ultrasound has been proven to be a very effective tool for improving the operating efficiency (Goula, 2013; Sharmila et al., 2016). Moreover, the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave could partly alter the physicochemical and functional properties by our previous study (Zou et al., 2017). To our knowledge, there are very limited data available on the effects of ultrasound-assisted processing on collagen isolation especially for countercurrent and pulsed ultrasound.

Therefore, the aim of this study is to develop an effective process for countercurrent and pulsed ultrasound-assisted processing of collagen from soft-shelled turtle calipash, and also to compare their yield of collagen and the physicochemical properties between the collagen by ultrasound pre-treatment (UASC) and from the conventional extraction (ASC), and further to investigate the simulated *in vitro* digestive properties of ASC and UASC and the antioxidant activity of its hydrolysates.

2. Materials and methods

2.1. Materials and chemical reagents

Fresh soft-shelled turtles were provided by Banquet King aquatic product Foods Ltd. in Suqian, Jiangsu, China (body weight of 500 ± 50 g). They were put in the fresh box with ice bag when transported to our laboratory by air express. The calipash tissues were dissected by using clean scalpel on crushed ice, cut into small pieces (1 × 0.5 cm), smashed in a blender until smooth and stored at −20 °C for further use. All experimental procedures were approved by the Animal Ethics Committee of Jiangsu Academy of Agricultural Sciences. All reagents used in this study were analytical grade (Nanjing chemical reagent co., LTD, Nanjing, China).

2.2. Proximate composition of calipash

The crude protein (988.05), crude fat (960.39a), moisture (950.46B) and ash (920.153) content of turtle calipash were estimated by the AOAC (2003) 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 at 105 °C for 4–5 h. The ash content was determined by using muffle furnace at 550–600 °C. The analyses were replicated three times.

2.3. Sample collection and calipash preparation

The frozen calipash was first thawed at 4 °C and cut into small pieces, soaked in a solution of NaCl (200 g/L) in Tris-HCl (0.05 mol/L, pH 7.5) at a ratio of 1:20 (g/mL) and stirred continuously for 12 h in magnetic stirring apparatus at 20 °C (78-1; Changzhou Guohua Electric Appliance Co., Ltd., Jiangsu, China). The mixture was centrifuged at 13,400 *g* for 20 min by a centrifuge (Vnicen MR, Herolab, Ludwig-Wagner, Germany) and the precipitate was stirred continuously for 24 h with a solution of Na_2CO_3 (0.5 mol/L) at a ratio of 1:20 (g/mL). The Na_2CO_3 solution was renewed every 8 h. Calipash was de-mineralized using EDTA-2Na (0.3 mol/L, pH 7.4) at a ratio of 1:20 (g/mL) with stirring for 24 h using a magnetic stirrer. At last, the pretreated calipash sample was kept in a −20 °C until use.

2.4. Extraction and purification of collagen

2.4.1. Conventional extraction and purification of acid-soluble collagen (ASC)

The acid collagen was extracted from soft-shelled turtle calipash according to the method described previously (Liu, Zhou, Li, & Regenstein, 2014) with minor modification. The pretreated calipash particles were suspended in acetic acid (0.5 mol/L) at liquid-to-solid ratios of 20:1 (mL/g) for 24 h. Subsequently, samples were centrifuged at 13,400 *g* for 15 min and the supernatant was collected. The residue from the above centrifugation was extracted again as the same process. The supernatants of calipash ACS were collected together and added with NaCl to salting-out for 12 h in a final concentration of 2.5 mol/L and 1.0 mol/L, respectively. The precipitate was collected after centrifugation at 13,400 *g* for 15 min. The precipitate from NaCl solution was dissolved in 0.5 mol/L acetic acid (1:10, g/mL) and then dialyzed in 0.1 mol/L acetic acid (1:25, g/mL), followed by distilled water for 48 h with changes in distilled water every 12 h. All processes were carried out at 4 °C. ASC was lyophilized and then stored at −20 °C until further use.

2.4.2. Extraction and purification of acid-soluble collagen with ultrasound pre-treatment (UASC)

The solution samples were treated by an ultrasonic reactor (SCIENTZ-IID, Ningbo Xinzhi ultrasonic technology co., LTD, Zhejiang, China) with a 1.5 cm flat tip probe operating in a pulsed on-time 2 s and off-time 3 s. The pretreated sample solution passed through the probe by countercurrent method which two peristaltic pumps used to keep the material solutions in a counter-current flow state. The reaction worked with ultrasonic power of 200 W having a single frequency of 24 kHz in the ultrasound generator for 5 min. A temperature controlled steel jacket passed through cold water was used in the ultrasonic processer. The cooling water temperature was set at 20 °C to avoid heating effects. The next step was indicated as section 2.4.1. The acid-soluble collagen by ultrasound-assisted processing (UASC) was lyophilized and then stored at −20 °C until further use.

2.5. Ultraviolet (UV) absorption spectrum

The ultraviolet absorption spectra of the ASC and UASC collagen samples were scanned by UV spectrophotometer (UV-6100, Meipuda instrument Co., Ltd., Shanghai, China) between 190 and 400 nm with an interval of 1 nm. The sample was dissolved in 0.5 mol/L acetic acid and then the viscous solution was centrifuged at 13,400 *g* for 20 min to remove the undissolved collagen fibre. The supernatants were used in this analysis.