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Ultrasonic irradiation in the enzymatic extraction of collagen

Defu Li [a,b], Changdao Mu [b], Sumei Cai [a], Wei Lin [a,*]

[a] National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu, 610065 Sichuan, China
[b] Department of Pharmaceutics and Bioengineering, Sichuan University, Chengdu, 610065 Sichuan, China

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ABSTRACT

The application of ultrasonic irradiation (40 KHz, 120 W) in the enzymatic extraction of bovine tendon collagen has been investigated. Our results show that using the ultrasonic irradiation increases the yield of collagen up to ~124% and significantly shortens the extraction time in comparison with the conventional pepsin isolation method. Such improvements are attributed to the enhancement of the enzyme activity and the dissolution of collagen substrate because the ultrasonic irradiation disperses the pepsin aggregates and opens up the collagen fibrils, thus the enzymatic hydrolysis is facilitated. AFM imaging shows the same fibrillar structure of tendon collagens generated from both the methods. The CD and FT-IR measurements reveal that the triple helix structure of collagen remains intact even after the ultrasonic irradiation. This study shows that the mild ultrasound irradiation can effectively improve the efficiency of pepsin extraction of natural collagen without any compromise of the resultant collagen quality.

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1. Introduction

Fibrillar collagen accounts for the majority of the extracellular matrix (ECM) in animals. The molecular packing arrangement of ECM has a significant effect on the maintenance of tissue and cellular shape, strength, and structural integrity [1]. Due to its distinct fibrillar structure, biodegradability, superior biocompatibility and low immunogenicity, collagen is one of the most applicable natural biomaterials for biomedical applications, such as drug delivery systems, wound dressings, and scaffolds [2]. Since the 1980s, the expanding medical application of biomaterials has promoted medical-grade collagen generated from different sources by various methods [3]. Generally, the tissues rich in fibrous collagen such as skin and tendon are used as starting materials to extract collagens by the treatment of neutral salt, acid, alkali, and proteases. Compared with other processing, the method of using pepsin in 0.5 M acetic acid results in higher yields, furthermore, by which the collagen triple helices remain essentially intact though short terminal non-helical regions, namely telopeptides, are selectively cleaved [4,5]. However, these conventional extraction methods are usually time consuming and result in large amounts of collagen tissue residues.

Currently, ultrasonic irradiation is widely used to enhance the mass transference in wet processes that are of importance in mixing, extraction and drying [6–12]. The mechanism for the thermal, mechanical, and chemical effects of ultrasound has been attributed to the rapid formation and collapse of cavitation bubbles [10]. Unfortunately, the cavitational collapse caused by high-intensity ultrasound could create strong shear stresses and high pressures and temperatures in the so-called "hot spots", which may promote proteins/enzymes denaturation [10,13]. It has been documented that trypsin shows decreased activity with increasing ultrasound power from 100 to 500 W at 20 kHz [13]. It is also shown that a significant decrease in the proteolytic activity is resulted from ultrasound treatment at 26.4 kHz and 26 W/cm^2 [14]. On the other hand, some researchers report that the activities of enzymes increase under mild ultrasonic irradiation when the shear force, temperature, pressure, and production of radicals are limited by controlling the power and frequency of ultrasound [7,15,16]. Thus, using a combination of enzyme and ultrasound leads to better treatment of cellulosic textiles [10], faster degradation of phenol in wastewaters [17], and higher COD removal efficiencies of distillery wastewater [18]. However, the knowledge of ultrasound on the activation of enzyme is still very limited.

In this work, a mild ultrasound wave in combination with pepsin has been used to extract type I collagen from bovine tendon. This method is compared with the conventional enzyme method which does not involve ultrasound. The influences of the ultrasonic irradiation on the enzymatic activity and the hydrolysis of bovine

* Corresponding author. Tel.: +86 28 8546 0819; fax: +86 28 8540 5237.
E-mail address: wlin@scu.edu.cn (W. Lin).

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tendon are determined. The extracted collagens are characterized by atomic force microscope (AFM), circular dichroism (CD), and Fourier transform infrared spectroscopy (FT-IR) to verify whether the molecular structures are changed by ultrasonication. Our aim is to investigate the effect of mild ultrasound on biological macromolecules and explore the possibility of ultrasonication as an effective method to improve the pepsin extraction of natural collagen.

2. Experimental

2.1. Isolation of collagen by pepsin treatment

The bovine tendon collagen was extracted using a method similar to the conventional procedure [3]. The fresh adult bovine Achilles tendon was received for the extraction of collagen. After successive rinsing with neutral salt (0.15 M NaCl solution) and acetone to remove non-collagenous proteins and fattiness, the collagen was extracted with pepsin in 0.5 M acetic acid solution by continuous stirring the mixture in the flask at ~20 °C. The increase of collagen concentration in the medium versus time was determined spectrophotometrically. After 2 days of extraction, the residues were filtered off, and the dissolved collagen (Enzyme Soluble Collagen, ESC) was salted out with 2 M sodium chloride twice. Then it was collected by centrifugation under refrigerated condition and further dialyzed in deionized water at ~ 4 °C for 2 days until a constant conductivity was achieved. The collagen was then freeze-dried and kept in refrigerator. The yield of collagen Y_c was calculated by:

$$Y_c = \frac{V_c \times C_c}{W_t} \quad (1)$$

where V_c is the volume of extracted collagen solution in milliliter, C_c is the concentration of the same solution measured by UV–vis in g/mL, and W_t is the lyophilized weight of the bovine tendon in gram. Each set of the test was done in duplicate.

2.2. Isolation of collagen by ultrasound-pepsin treatment

In the ultrasound assisted extraction process, the flask was immersed in a SHP® temperature controlled water bath (Shanghai Hengping, China) with a KQ3200 ultrasonic processor (40 KHz, 120 W, Kunsan, China) at the bottom. The temperature of water bath was kept constant at ~20 °C, and the solution in the flask was also continuously checked by a digital thermometer. In order to avoid forming overheated spot, the ultrasound was operated under a pulsed mode, i.e. 30 min acting time and 30 min resting time during the 2 days of processing period. Other than the additional step of ultrasonic irradiation, the procedure was exactly the same as that in the pepsin treatment method. The extraction efficiency under sonication was also estimated by the determination of collagen concentration in the acetic acid solution at specific time intervals by spectrophotometric analysis. Each set of the test was done in duplicate. The yield of the collagen (Ultrasound-Enzyme Soluble Collagen, U-ESC) was obtained similarly as described above.

2.3. UV–vis measurements

The ultraviolet–visible (UV–vis) spectra of the sample solutions were recorded in the wavelength range of 200–400 nm using a UV–visible Spectrophotometer (UV 2501PC, Shimadzu, Japan). The quantitative measurements were conducted at a specific wavelength of 280 nm. The linear correlation between concentration and absorption value should be fine for collagen solutions at this wavelength.

2.4. Pepsin activity assay

The enzymatic activity of pepsin under the ultrasound was estimated by a conventional spectrophotometric analysis similar to that described by Anson and Mirsky [19], using hemoglobin as substrate. The pepsin in 0.5 M acetic acid solution was irradiated under the same pulsed mode as described in ultrasound-pepsin extraction process. At specific time intervals, 1 mL of pepsin solution was taken from the reactor and added to 10 mL of 1 wt% hemoglobin acidified with 0.5 M acetic acid. After 5 min mixing and digestion, 10 mL of 4 wt% trichloroacetic acid was added to precipitate the undigested hemoglobin. Then the resulting precipitate was filtered off and the filtrate thus contained an amount of digested hemoglobin which was a measure of the amount of effective pepsin. The digested hemoglobin was then dyed with the Brilliant blue G 250 for 10 min and the solution turned blue. The absorbance values of sample solutions against reference solution (0.5 M acetic acid) were measured at 595 nm immediately. The optical intensity was proportional to the activity of pepsin in use. All the operation temperatures were kept at ~20 °C. Each set of the test was done in duplicate.

2.5. Ultrasonication of bovine tendon without pepsin

The influence of the ultrasonic irradiation on bovine tendon was estimated by following the same procedure as that in ultrasound-pepsin treatment but without pepsin. The continuous stirring alone in the dissolution of bovine tendon was also conducted under the similar conditions to compare with the ultrasonication alone.

2.6. AFM measurements

The freshly extracted ESC and U-ESC solutions were used for AFM imaging. After diluting the original solutions 10 times (~0.1 mg/mL) and leaving it to equilibrate at ~4 °C for 12 h, a droplet of collagen solution (~0.5 μL) was deposited onto newly cleaved mica. Then the collagen was spin-coated on the mica at a speed of 1300 rpm for 8 s and then 4000 rpm for 30 s. Topographic images of ESC and U-ESC on mica were captured by a Shimadzu SPM-9600 multimode atomic force microscope in tapping mode with silicon TESP cantilevers.

2.7. CD measurements

The lyophilized ESC and U-ESC samples were freshly dissolved in 0.5 M acetic acid to a concentration of 2.0×10^{-4} g/mL just before use. After the solutions were equilibrated in a refrigerator (*ca.* 4 °C) for 12 h and at room temperature for another 2 h, CD measurements were carried out using a JACSO J-801 spectrometer. The CD spectra from 190 to 240 nm were scanned with a bandwidth of 1 nm under nitrogen atmosphere at 25 °C. The spectrum of 0.5 M acetic acid was used as a blank and subtracted from the average of three spectra to obtain a corrected spectrum for each sample.

2.8. FT-IR measurements

FT-IR spectra of the lyophilized ESC and U-ESC samples were obtained from discs containing ~2.0 mg sample in approximately ~20 mg potassium bromide (KBr). The measurements were carried out on a Perkin–Elmer Spectrum One FT-IR spectrophotometer at the resolution of 4 cm^{-1} in the wave number region 400–4000 cm^{-1}. For contrast, the thermal denatured collagen was prepared by heating pepsin-treated collagen solution to 50 °C for 10 min, and the FT-IR spectra of this lyophilized sample was measured as well.

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