Ultrasonics Sonochemistry 29 (2016) 495–501

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Effect of ultrasonic pretreatment on kinetics of gelatin hydrolysis by collagenase and its mechanism

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ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form 3 November 2015
Accepted 3 November 2015
Available online 4 November 2015

Keywords:
Gelatin
Hydrolysis
Ultrasonic pretreatment
Kinetics
Mechanism

ABSTRACT

Gelatin is a mixture of soluble proteins prepared by partial hydrolysis of native collagen. Gelatin can be enzymatically hydrolyzed to produce bioactive hydrolysates. However, the preparation of gelatin peptide with expected activity is usually a time-consuming process. The production efficiency of gelatin hydrolysates needs to be improved. In present work, effect of ultrasonic pretreatment on kinetic parameters of gelatin hydrolysis by collagenase was investigated based on an established kinetic model. With ultrasonic pretreatment, reaction rate constant and enzyme inactivation constant were increased by 27.5% and 27.8%, respectively. Meanwhile, hydrolysis activation energy and enzyme inactivation energy were reduced by 36.3% and 43.0%, respectively. In order to explore its possible mechanism, influence of sonication on structural properties of gelatin was determined using atomic force microscopy, particle size analyzer, fluorescence spectroscopy, protein solubility test and Fourier transform infrared spectroscopy. Moreover, hydrogen peroxide was used as a positive control for potential sonochemical effect. It was found that reduction of gelatin particle size was mainly caused by physical effect of ultrasound. Increased solubility and variation in β-sheet and random coil elements of gelatin were due to sonochemical effect. Both physical and chemical effects of sonication contributed to the change in α-helix and β-turn structures. The current results suggest that ultrasound can be potentially applied to stimulate the production efficiency of gelatin peptides, mainly due to its effects on modification of protein structures.

1. Introduction

Gelatin is a mixture of soluble proteins produced by partial hydrolysis of native collagen [1]. In recent years, due to its distinctive functional properties, gelatin has gained an increasing popularity in many fields. In pharmaceutical industry, it has been widely used for wound care, tablet coating and production of soft and hard capsules [2]. In food industry, gelatin can be applied in confectionery, jelly deserts, baked goods and meat products by providing them with chewiness, texture, water-binding capacity, emulsification and foam stabilization [3]. Moreover, it can also be enzymatically hydrolyzed to prepare bioactive peptides, which are perceived as an important source of food additive [4].

Over the last few decades, biological activities of gelatin hydrolysates have been studied extensively. Some typical activities of peptides sourced from gelatin were reported. Gómez-Guillén found that hydrolysates from fish skin gelatin had antimicrobial property against 18 strains of Gram-positive and Gram-negative bacteria [4]. Kim et al. demonstrated that antioxidant gelatin peptide was effective to scavenge free radicals and inhibit lipid peroxidation [5]. Moreover, antihypertensive gelatin peptides were capable of inhibiting angiotensin converting enzyme (ACE) [6]. However, the preparation of gelatin hydrolysate with expected bioactivity is usually a time-consuming process. Some ACE inhibitory peptides were produced by one-step hydrolysis in 6 h [5,7]. Active hydrolysates from duck skin gelatin were prepared by two-step hydrolysis in 16 h [8]. So it is essential to find an effective way to improve the production efficiency of gelatin peptides.

Power ultrasound is an emerging and promising technology that has been applied in a variety of fields [9]. Power ultrasound is known to accelerate some chemical reactions and industrial processes due to its effects of shearing, mass transfer and generation of reactive radicals [10]. Recently, enhancement of peptide production by ultrasound has become a focus of research in food industry. With ultrasonic pretreatment of substrates, the

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http://dx.doi.org/10.1016/j.ultsonch.2015.11.004
1350-4177/

enzymatic hydrolysis of wheat germ protein can be significantly stimulated [11]. In addition, hydrolysis of a food protein can also be enhanced by using sonicated enzymes [12]. The ultrasonic treatment seems to be a useful tool to accelerate the release of peptides. Nevertheless, to the best of our knowledge, effect of ultrasonic pretreatment on gelatin hydrolysis has barely been reported.

In this work, the ultrasound-induced variation in kinetic parameters of gelatin hydrolysis was investigated using an established model. To explore action mechanism of this phenomenon, impact of sonication on structural properties of gelatin was measured by atomic force microscopy (AFM), particle size analyzer, fluorescence spectroscopy, protein solubility assay and Fourier transform infrared (FTIR) spectroscopy.

2. Materials and methods

2.1. Materials

Hydrogen peroxide and bovine serum albumin (BSA) were purchased from Aladdin Inc. (Shanghai, China). Collagenase was obtained from Sigma–Aldrich (St. Louis, USA). Pigskin gelatin was provided by Kelong Corporation (Chengdu, China). All other chemicals were sourced commercially and used without further purification. Deionized water used in the experiments was purified by a Milli-Q Element system (Jin Yi, Shanghai, China).

2.2. Enzymatic hydrolysis of gelatin

The enzymatic hydrolysis of gelatin by collagenase was conducted in a 100 mL reactor with magnetic stirring and controlled pH value and temperature (30, 37 and 45 °C). Protein solutions (17.39, 26.08, 34.78, 43.47 and 52.16 g/L) were prepared by deionized water and adjusted to pH 7.0 with 0.2 M of sodium hydroxide (NaOH). Collagenase (1.02, 2.05, 3.07, 4.10 and 5.12 × 10^3 U/L) was added to start the hydrolysis under stirring (300 rpm). Degree of hydrolysis (DH) was determined using the pH-stat method [13]. The pH value of reaction system was kept by titrating 0.6 M of NaOH. The extent of hydrolytic degradation was calculated using the equation: DH (%) = $[(B \times N_b)/(M_P \times \alpha \times h_{total})] \times 100\%$, where B is the volume of NaOH used for titration (mL), N_b is the normality of NaOH, M_P is the mass of protein (g), h_{total} determined by amino acid composition analysis is 8.4 meqv/g and α is the average degree of dissociation of α-amino groups.

2.3. Ultrasonic pretreatment

In all the experiments, sonicated gelatin solution was treated by a KQ-300VDB ultrasonic equipment (Kunshan Instruments, China). The ultrasonic bath was filled with deionized water, fixed with the transducer at the bottom (40 kHz), and capable of outputting acoustic power in the range of 0–300 W. Sample solution in an air-tight glass container was placed in the middle of the bath and treated with following conditions: power, 300 W; frequency, 40 kHz; time, 15 min; temperature, 35 °C. The ultrasonic condition was optimized according to a series of one-factor and orthogonal experiments, and the actual power was determined to be 294.0 ± 16.3 W by a calorimetric method [14]. The temperature of ultrasonic bath (35 ± 1 °C) was controlled by discharging the overheated water and refilling with the cool water. All the controls were incubated in a water bath (35 °C) without ultrasonic treatment for 15 min.

2.4. Modeling of gelatin enzymatic hydrolysis

According to a previous report [15], the kinetic model of gelatin hydrolysis by collagenase was established and the DH is displayed as following: DH (%) = $1/b[\ln(1 + abt)]$, where a and b are constants and t is the hydrolysis time. In the model, the parameter a is expressed as ke_0/s_0, where k is the reaction rate constant, e_0 is the initial enzyme concentration and s_0 is the initial substrate concentration. The parameter b is expressed as k_d/k, where k_d is the enzyme inactivation constant.

2.5. Determination of sonochemical effect

The sonochemical effect of ultrasound was evaluated by measurement of the hydrogen peroxide (H_2O_2) generation [16]. In brief, 2.5 mL of solution A (10^{-3} M ammonium molybdate, 0.05 M sodium hydroxide and 0.4 M potassium iodide), 2.5 mL of solution B (0.1 M potassium biphthalate), and 5 mL of deionised water were mixed and sonicated. Then, absorbance of the mixture was measured at 350 nm. The mixture without sonication was used as the blank. The yield of H_2O_2 concentration was calculated from a standard curve.

According to the results, 25 μg/L of H_2O_2 solution (approximately twice the determined value) was used as the positive control to investigate the chemical effect of ultrasound on gelatin.

2.6. AFM observation

A SPM-9600 scanning probe microscope (Shimadzu, Japan) was used to observe the microstructure of gelatin. The sample solution (500 μg/mL) was dropped on the sample carrier, naturally dried for 24 h and then analyzed. The tapping-mode was operated with the imaging force of 3–4 nN at a scan rate of 1 Hz.

2.7. Measurement of particle size distribution

Particle size analysis was performed using a Nano-ZS ZEN3600 instrument (Malvern Instruments, UK) with dynamic light scattering detection. Gelatin solution (0.1 mg/mL) was passed through a membrane filter (pore size, 0.45 μm), placed in a DTS1060C sample cell (volume, 1 mL) and then analyzed at 37 °C.

2.8. Determination of gelatin solubility

Gelatin solubility was determined by a method reported by Zhao, Xiong, & McNear [17]. The sample solution (10 mg/mL) was prepared by dissolving gelatin in a series of citrate (25 mM; pH, 3–5) and phosphate (25 mM; pH, 6–8) buffers and then centrifuged at 5000 rpm for 10 min. The solubility of gelatin was obtained with following equation: Solubility (%) = $(C_{supernatant}/C_{sample}) \times 100\%$, where C_{sample} is the protein concentration in non-centrifuged sample and $C_{supernatant}$ is the protein concentration in the supernatant calculated by using BSA as the standard.

2.9. Spectra analysis of gelatin

Fluorescence spectrum was recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA) at a rate of 300 nm/min. Sample solution (2 mg/mL) was measured at excitation wavelength of 280 nm (slit, 5 nm) and emission wavelength of 300–500 nm (slit, 5 nm). The deionised water was used as the blank.

As for infrared spectrum determination, gelatin solution (1 mg/mL) was dropped on a sampler, dried to form a protein membrane and analyzed by a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, USA). Each spectrum was obtained in