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Ultrasonic-assisted enzymatic extraction of polysaccharide from *Corbicula fluminea*: Characterization and antioxidant activity☆

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ABSTRACT

Ultrasonic-assisted enzymatic extraction (UAEE) technology was used to prepare *Corbicula fluminea* polysaccharide. Response surface methodology (RSM) and Doehlert matrix (DM) design were used to determine the optimum extraction conditions. The optimum extraction conditions consisted of the extraction time 32 min, ultrasound power 300 W, extraction temperature 62 °C and ratio of water to raw material 35 mL/g. The UAEE and enzyme-assisted extraction (EAE) methods were compared. The yield of UAEE for 32 min was higher than that of EAE for 4 h, which suggested that UAEE method has higher extraction efficiencies. The structures of crude polysaccharides obtained from UAEE and EAE were analyzed with FT-IR and HPLC methods. Their antioxidant activities were also evaluated on the basis of free radical scavenging. Compared with the polysaccharide (EP) extracted by EAE, the polysaccharide (EP-us) extracted by UAEE had lower molecular weight, higher sulfate content, and higher superoxide radical scavenging activity.

1. Introduction

Polysaccharides have been extensively studied as additives in food and pharmaceutical applications due to their unique bioactive properties and chemical structures (Bendjeddou, Lalaoui, & Satta, 2003; Zhou, Song, Feng, & Tan, 2011). The polysaccharides present in mollusks have several bioactive properties including anti-tumor, antioxidant, anti-diabetic, and immunomodulatory activities (Jiang, Wang, Liu, Gan, & Zeng, 2011; Miller, Dodd, Ormrod, & Geddes, 1993; Xu et al., 2008).

Asian clam (*Corbicula fluminea*), a freshwater bivalve mollusk native to Asia, is widely used in East Asian countries as a tonic and health food. Besides the aforementioned bioactive properties, this mollusk also shows hepato-protective, anti-hypertensive, and hypocholesterolemic activities (Chen & Lin, 2003; Chijimatsu et al., 2011; Han, Qiu, & Li, 2009; Lin et al., 2012; Qiu, Dai, & Li, 2009; Tsai, Lin, Chen, & Pan, 2006). Recent studies have reported that a bioactive glycoprotein extracted from fresh *C. fluminea* has anti-tumor activities by inducing apoptosis in human hepatoma BEL7404 cells (Zhu, Lin, Wu, & Lin, 2004). Polysaccharides are the main bioactive components of this shellfish. However, to the best of the authors' knowledge, there are very few studies on extraction methods of *C. fluminea* polysaccharides. Thus an efficient method for the extraction of *C. fluminea* polysaccharides is required.

Several conventional extraction methods (e.g., enzyme-assisted extraction, maceration, mechanical rabbling, and heat reflux) have been used for the extraction of target compounds from raw materials. Enzyme-assisted extraction (EAE) is the main and most conventional extraction method for polysaccharides (Puri, Sharma, & Barrow, 2012; Sowbhagya & Chitra, 2010). However, EAE, which requires long extraction time and high temperatures, has low extraction efficiencies (Le & Le, 2012). Ultrasonic-assisted extraction has been widely employed in the extraction of target compounds from different materials. Through super agitation at low frequencies, this technique allows effective mass transfer between immiscible phases (Hromádková & Ebringerová, 2003). Additionally, Ultrasonic-assisted extraction is highly reproducible and requires short extraction time and low energy inputs. Furthermore, Ultrasonic-

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assisted extraction is easy to use and requires low solvent volumes (Hromádková, Ebringerová, & Valachovic, 2002). Thus the conventional extraction method (EAA) coupled with ultrasound irradiation may be an effective method for polysaccharide extraction.

Recently, Doehlert matrix (DM) design can be easily applied to optimize processing parameters, which requires fewer experiments (Doehlert, 1970). Response surface methodology (RSM) allows an easier and more efficient interpretation of the experiments (Ferreira, dos Santos, Quintella, Neto, & Bosque-Sendra, 2004). In this study, crude polysaccharide was extracted from freshwater clam (*C. fluminea*) using ultrasonic-assisted enzymatic extraction (UAEE) method. RSM was used with a DM design to optimize the extraction parameters (e.g., water to raw material ratio, ultrasound power, extraction time, and extraction temperature). Two methods (UAEE and EAE) were compared on the yields of polysaccharides from the *C. fluminea* soft body. In addition, the chemical composition and antioxidant activity of the extracted polysaccharides by UAEE and EAE were evaluated.

2. Materials and methods

2.1. Materials and reagents

C. fluminea was supplied by Fenren Foodstuff Co., Ltd. (Huzhou, China). Cysteine (Cys) was purchased from Fluka (Seelze, Germany). The derivatization reagent, 1-phenyl-3-methyl-5-pyrazolone (PMP), was from Sinopharm Chemical Reagents (Shanghai, China). Standard monosaccharide, lactose, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), ferrozine, nitroblue tetrazolium (NBT) and papain (1800 Unit per milligram, PU mg^{-1}) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Extraction methods

2.2.1. EAE of C. fluminea polysaccharides

The extraction method has been previously described in a study on sea cucumber sulfated fucans (Chen et al., 2011). The *C. fluminea* shell was removed, and the whole soft body was defatted using three 24 h extractions with acetone. The fat-free dried tissue was cut into a fine powder using a pulverizer (FW-100, Tianfeng, Shanghai, China). Approximately 5 g of dried, defatted, pulverized powder of whole *C. fluminea* soft body was suspended in sodium carbonate buffer (50 mL, 0.1 mol/L, and pH 6.0) and digested with different amounts of papain. The enzyme concentrations were 0, 1.7, 3.3, 5.0, 6.7, 8.3, 10.0 and 11.7 mg/mL. These values were equivalent to 0, 30, 60, 90, 120, 150, 180, and 210 papain unit per milligram (PU mg^{-1}) of diluted *C. fluminea* tissue homogenate, respectively.

Each digestion mixture was then incubated for 4 h at 60 °C. Trichloroacetic acid was added to the mixture to remove protein. The digestion mixture was centrifuged at 8000 × *g* for 20 min. The polysaccharide solution was concentrated in a rotary evaporator, precipitated with four volumes of 95% ethanol at 4 °C for 48 h, and centrifuged at 8000 × *g* for 30 min. The precipitate was washed in acetone, dissolved in water, and dialyzed against 100 volumes of water. The dialyzate was freeze-dried to obtain crude polysaccharide named EP. Carbohydrates were detected using the phenol/sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Polysaccharide yield (%) was then calculated using the following equation:

$$\text{Polysaccharide yield}(\%) = \frac{C \times N \times V}{W \times 1000} \times 100\% \quad (1)$$

where C is the polysaccharide concentration obtained from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of the extraction solution (mL); and W is the weight of the raw material (g).

2.2.2. UAEE of C. fluminea polysaccharides

The UAEE method used was that described by Chen, Li, Liu, Yang, and Li (2012) with some modifications. Dried, defatted, pulverized powder of whole *C. fluminea* soft body was extracted with sodium carbonate buffer (0.1 mol/L, pH 6.0) and sonicated in the ultrasonic generator (JY99-IIDN, Scientz, Shanghai, China). The steps that followed were similar to the steps in EAE. The crude polysaccharide obtained by UAEE was named EP-us.

2.2.3. Optimization of UAEE conditions

Based on the preliminary experimental results, an RSM was used to optimize the UAEE conditions. Table S1 shows the extraction parameters and DM design (Ferreira et al., 2004). The fitted response surface plots and contour plots for the model were generated using the MATLAB software (Version 7.11.0.584, The MathWorks, Inc., USA). The water to raw material ratio (X_1, mL/g), extraction temperature (X_2, °C), ultrasound power (X_3, W), and extraction time (X_4, min) were the independent (input) variables; crude polysaccharide yield (Y, %) was the dependent (output) variable. The complete quadratic equation used was.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the dependent variable (EP-us yield); β_0, β_i, β_{ii}, and β_{ij} represent the regression coefficients; and X_i and X_j are the independent variables ($i \neq j$). The variables were coded according to the equation

$$x_i = \left(\frac{X_i - X_{oi}}{\Delta X_i}\right)\alpha_i \quad (3)$$

For statistical calculations the variables X_i were coded as x_i, which represents the coded value of the ith factor, X_i is the natural value, X_{oi} is the middle point value, ΔX_i the change in the natural value, and α_i is the maximum value of the coded factor (i.e., 1.0, 0.866, 0.817, and 0.791 for five, seven, seven, and five levels, respectively).

2.3. Analytical methods

2.3.1. Chemical analysis

Total carbohydrate was determined by the phenol–sulfuric acid colorimetric method using D-glucose as the standard (Dubois et al., 1956). Protein was determined by the Lowry method (Lowry, Roseerbrough, Farr, & Randall, 1951). Sulfate content was determined using the $BaCl_2$/gelatin method and ion-exchange chromatography (Ohira & Toda, 2006). Uronic acid content was determined by the *m*-hydroxydiphenyl method with galacturonic acid as the standard (Kimberley & Jock, 1992).

2.3.2. Scanning electron microscope (SEM)

SEM images of *C. fluminea* soft body dried powder were obtained for untreated samples (control) and for samples subjected to EAE or UAEE. The powder was fixed to the specimen mount with aluminum tape and sputtered with gold using an ion sputter coater (Hitachi, Japan). All specimens were examined with a Philips Model-S 3400 SEM (Philips, Netherlands) under high vacuum conditions, an accelerating voltage of 15.0 kV, and a working distance of 10–11 mm.

2.3.3. Molecular weight determination

The homogeneity and molecular weight (M_W) of EP and EP-us were determined using Agilent 1100 liquid chromatography (Palo