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Supercritical CO_2 extraction and pre-column derivatization of polysaccharides from *Artemisia sphaerocephala* Krasch. seeds via gas chromatography

Jian Chen[a], Juan Li[a], Ai-dong Sun[a], Bo-lin Zhang[a], Shu-gao Qin[b], Yu-qing Zhang[b,*]

[a] *Department of Food Science and Engineering, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China*
[b] *Yanchi Research Station, College of Soil and Water Conservation, Beijing Forestry University, Beijing 100083, China*

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ABSTRACT

Optimal process conditions for supercritical CO_2 extraction of polysaccharides from *Artemisia sphaerocephala* Krasch. seeds and their monosaccharides were analyzed to provide a theoretical basis for identification and development. Single-factor experiments and orthogonal designs were performed to determine the effects of extraction conditions on polysaccharide yields. The polysaccharides were fractionated through ion-exchange chromatography, and were shown to be homogeneous via high-performance size-exclusion chromatography (HPSEC). The resultant polysaccharide samples were hydrolyzed with trifluoroacetic acid, and then acetylated and analyzed for monosaccharide composition by gas chromatography. The optimum extraction parameters were established with extraction pressure of 45 MPa, extraction temperature of 45 °C, flow rate of CO_2 at 20 L/h, extraction time of 2 h, separation pressure of 10 MPa, and separation temperature of 56 °C. The corresponding *A. sphaerocephala* Krasch. seeds polysaccharides (ASKR) yield was 18.59% (w/w). The polysaccharide contained mannose, rhamnose, galactose, glucose, arabinose, xylose, and fucose. The predominant monosaccharides in ASKR were glucose (38.48 mg/g) and xylose (16.2 mg/g). The average molecular weight of ASKR was 551.3 ± 11.2 kDa, and its radius of gyration was 22.6 ± 0.5 nm based on HPSEC–MALLS–RI system.

1. Introduction

Artemisia sphaerocephala Krasch. (Compositae) is a perennial psammophyte subshrub species grown in desert regions in China (Guo et al., 2011, 2013). The plant is widely distributed throughout the region, and is rich in resources. Polysaccharides comprise a class of complex and large molecular structures of polymer carbohydrates that are compounded by monosaccharide polymerization. The molecular weight of polysaccharides ranges from 10 kDa to 1000 kDa (Guo et al., 2012). *A. sphaerocephala* Krasch. seed polysaccharide (ASKR) is a reducing polysaccharide extracted from its seed oil residues. ASKR possesses a medium molecular weight (Hao et al., 2010; Wang et al., 2010). Studies have confirmed that ASKR exhibits medicinal effects, including anticancer properties, antioxidant properties (Xing et al., 2009), and immunity enhancement (Wang et al., 2009; Zhang et al., 2006).

Water extraction, microwave, and ultrasonic methods have been used to extract ASKR (Xing et al., 2009; Zhang et al., 2012). However, the traditional methods are time consuming, cause undesirable decomposition of ASKR contents, and possess low extraction efficiency. Supercritical extraction determines the special properties of a substance near the critical point (Chen et al., 2010; Jin and Tong, 2014). Appropriate entrainers or modifying agents (e.g., methanol and ethanol) are used in supercritical CO_2 systems to improve the extraction efficiency of strong-polarity and large-molecule materials, as well as to increase the probability of polysaccharide extraction (del Valle et al., 2008; Diaz-Reinoso et al., 2006). Previous studies have used supercritical CO_2 extraction for specific plant polysaccharides (Diaz-Reinoso et al., 2006; Pradhan et al., 2010; Raeissi et al., 2008). However, data on ASKR extraction remain limited (Guo et al., 2013; Wang et al., 2010).

In the current research, we used supercritical CO_2 to extract ASKR through initial hydrolysis with trifluoroacetic acid. The substance was derivatized with acetic anhydride, gas chromatograph with capillary column, and external standard method to detect the physical properties of hydrolyzed ASKR. We also provided details for efficient ASKR extraction.

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[*] Corresponding author. Tel.: +86 010 62338221.
E-mail address: zhangyqbjfu@gmail.com (Y.-q. Zhang).

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2. Materials and methods

2.1. Materials and reagents

A. sphaerocephala Krasch. seeds were collected in Ningxia, China. The seeds were air dried, sealed in plastic bags, and stored in the refrigerator at 4 °C. CO_2 (food grade, purity >99%), methanol, ethanol, methylene chloride, dimethyl sulfoxide, sulfuric acid, glucose, 1-methyl imidazole, anthrone, acetic anhydride, diethylaminoethanol (DEAE)–Sepharose fast flow ion exchange resin, and trifluoroacetic acid were purchased from Beijing Chemical Reagent Co. (Beijing, China). Standard monosaccharides (L-rhamnose, D-galactose, D-arabinose, D-mannose, D-fucose, xylose, and glucose) were purchased from Sigma–Aldrich Co. (USA). All other reagents were of analytical grade. Deionized water was used throughout the experiment.

2.2. Extraction and purification of ASKR

Two hundred grams of ASKR was placed into an extraction kettle. The process parameters of supercritical CO_2 extraction were set; cooling and kettle-heating devices were opened. The main pressure pump was opened after the temperature reached the desired value. CO_2 flow and extraction time were 20 L/h and 2 h, respectively. The pressure and temperature of kettle II were 4 MPa and 36 °C, respectively. Influential factors of ASKR yield included extraction pressure, extraction temperature, as well as pressure and temperature of kettle I. The extracted ASKR was collected from a recycled bottle. Chloroform and n-butanol (ratio, 4:1) were added to the polysaccharide. The ASKR-containing bottle was fully shaken for 30 min and centrifuged at 5000 rpm for 20 min. The protein was replaced 10 times from the upper water phase. The dialysis supernatant was placed into a fluid bag; dialysis was performed using tap and distilled water for 48 and 24 h, respectively. The solution was concentrated and freeze-dried under vacuum to obtain crude ASKR (Guthalugu et al., 2006; Nascimento da Fonseca Contado et al., 2010).

Crude ASKR was dissolved and added to the DEAE–Sepharose fast flow ion exchange resin. The polysaccharide solution was eluted with distilled water and different concentrations of NaCl solution at a flow rate of 1.5 mL/min. The eluent was collected (30 min/pipe), and detection was performed using sulfuric acid phenol. Approximately 0.2 mL of the sample solution was drawn and distilled up to 2.0 mL. Then, 1.0 mL of 6% phenol, as well as 1.0 and 5.0 mL of concentrated sulfuric acid, was added. The resulting mixture was shaken and cooled for 20 min and then left to stand at room temperature. The absorbance was then measured at 490 nm, and the polysaccharide content was calculated based on the glucose standard curve. Similar flow points were merged, and the polysaccharide solution was concentrated. Dialysis was then performed on the solution. Crude ASKR solution was freeze-dried under vacuum to yield various polysaccharide fractions (Wang et al., 2010).

2.3. Determination of ASKR content

2.3.1. Glucose standard curve scheme

Seven large tubes were respectively added with 0, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8 mL glucose standard solutions (100 μg/mL) and filled with 1 mL distilled water.

Four milliliters of anthrone reagent (0.2 g anthrone dissolved in 100 mL sulfuric acid) was immediately placed into each tube. The glass tubes were then soaked in ice water for rapid cooling. The tubes containing anthrone solution were immersed in boiling water for 10 min; nozzle and reflux funnel were used to prevent evaporation. The solution was cooled with flowing water and placed at room temperature for 10 min. The absorbance at 620 nm was determined, and the standard curve was obtained (Chen et al., 2012).

2.3.2. Determination of soluble polysaccharide content

Fifty milliliters of ASKR was dissolved in 5 mL of 30% ethanol solution; the polysaccharide solution was diluted 100 times. One milliliter of the diluent was taken from the polysaccharide solution, and 4.0 mL anthrone reagent was immediately added into each tube. The tubes were soaked in ice water for rapid cooling. The tubes containing anthrone solution were immersed in boiling water for 10 min; evaporation was inhibited by using a nozzle and reflux funnel. The solution was cooled with flowing water and placed at room temperature for 10 min. Subsequently, the absorbance at 620 nm was determined. The standard curve was used to calculate polysaccharide concentration (C). The polysaccharide content in ASKR extract (Liu et al., 2012; Xin et al., 2012) was calculated as follows:

$$\text{polysaccharide content} = \frac{C(\mu\text{g/mL}) \times 5\,\text{mL} \times 5 \times 100 \times 10^{-6}\,\text{g}/\mu\text{g}}{0.05\,\text{g}} \times 100$$

2.4. Homogeneity of ASKR and determination of average molecular weight

Polysaccharide fractions were filtered with soluble cellulose acetate membrane (3.0 μm). High-performance size-exclusion chromatography–multi-angle laser light scattering–differential detector coupled system (HPSEC–MALLS–RI) was used, and comprised three chromatographic column series: TSK G5000 PW (7.5 mm × 600 mm), TSK G3000 PWxl (7.8 mm × 300 mm), and TSK G2500PWxl (7.8 mm × 300 mm). Mobile phases included 0.15 mol/L $NaNO_3$ and 0.02% NaN_3; the velocity was 0.4 mL/min. The average molecular weight of the polysaccharide was based on data from MALLS and RI detectors; the molecular weight was directly calculated by ASTRA 5.3 software (Gengec and Kobya, 2013; Maina et al., 2014).

2.5. Monosaccharide characteristics in ASKR prior to column derivatization via gas chromatography (GC)

2.5.1. Preparation of ASKR hydrolysis solution

Fifty milliliters of ASKR was added in the test tube containing 2 mL of 4 mol/L trifluoroacetic acid. The solution was hydrolyzed at 120 °C for 2 h, and the test tube was cooled to room temperature. Anhydrous methanol was added for distillation. Trifluoroacetic acid was removed and yielded the ASKR hydrolysis solution. Each sample underwent the process three times.

2.5.2. Preparation of standard monosaccharide mixture

Ten milliliters of monosaccharide standards (L-rhamnose, D-galactose, D-arabinose, D-mannose, D-fucose, xylose, and glucose) were used. The solution was mixed and dissolved in 10 mL volumetric flask; the mixture was diluted with water to 5.00, 3.00, 2.00, 1.50, 1.00, 0.50, and 0.25 mg/mL (Xin et al., 2012).

2.5.3. Column derivatization

Ten microliters of monosaccharide standard mixture and polysaccharide hydrolysis solution were taken; two milliliters of sodium borohydride solution was added in the mixture and oscillated at 42 °C for 90 min. The solution was cooled to room temperature. About 0.5 mL of acetic acid, 50 μL of 1-methyl imidazole, and 1 mL of acetic anhydride were added and allowed to react in the solution at room temperature for 10 min. Eight milliliters of water was added to the system and cooled to room temperature. Four milliliters of methylene chloride methane was then added to the system and oscillated. The upper water phase was discarded, and