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Physicochemical characterization and *in vitro* hypoglycemic activities of polysaccharides from *Sargassum pallidum* by microwave-assisted aqueous two-phase extraction

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ABSTRACT

Microwave-assisted aqueous two-phase extraction (MAATPE) was applied for simultaneous extraction and separation of polysaccharides from *Sargassum pallidum* (SPPs). The optimal extraction parameters, physicochemical properties, and hypoglycemic activities *in vitro* of SPPs were investigated. The results revealed that the optimal extraction conditions were as follows: 21.0% ethanol (w/w) and 22.0% ammonium sulfate (w/w) for ATPS, ratio of material to liquid 1:60 (g/mL), extraction time 15 min, microwave power 830 W, and extraction temperature 95 °C. Under the optimal these conditions, the maximum yields of SPPs were 0.75 ± 0.04% of the top phase (SPP-1) and 6.81 ± 0.33% of the bottom phase (SPP-2). SPP-1 and SPP-2 were homogeneous with molecular weights of 1518.6 and 50.6 kDa, respectively. SPP-1 mainly consisted of fucose, galactose, mannose, and glucuronic acid with a molar ratio of 4.97:9.75:6.44:6.07, whereas SPP-2 was mainly composed of fucose, galactose, glucose, and mannose with a molar ratio of 4.20:2.88:18.05:7.83. SPP-1 and SPP-2 exhibited favorable α-amylase and α-glucosidase inhibitory activities, and could remarkably improve glucose consumption in insulin resistance (IR) model cells. Notably, SPP-1 exhibited stronger α-glucosidase inhibitory activity than SPP-2, and even was comparable with acarbose.

1. Introduction

Sargassum pallidum (Turn.) C. Agardh, a species in the family of *Sargassum*, has been commonly known as an edible and medicinal resource in China, Japan, and other Asian countries. It is officially used as "*Hai Zao*" in Chinese Pharmacopeia and claimed to have functions of treating goiter, scrofula and edema [1]. *S. pallidum* have well been demonstrated to possess various phytochemicals such as polysaccharides, triacylglycerols, plastoquinones, chromenes, sterols, dihomogammalinolenic acid, vitamins, and *etc* [2,3]. Polysaccharides, as one of the most major active compounds in *S. pallidum*, are found to exert antioxidant, antiproliferative, hypoglycemic and adjuvant activities [4–6]. In recent years, more and more attention has been paid to *S. pallidum* polysaccharides because of its multiple pharmacological activities [6].

Extraction is an essential step for studying natural resources. For obtaining high extraction yield and efficiency, some novel techniques have been developed including ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), infrared-assisted extraction (IAE) and others [7–9]. These methods can achieve a higher extraction yield in a shorter extraction time. Comparatively, MAE has been demonstrated to an efficient and green method that takes the advantages of high extraction efficiency, low solvent consumption, and decreased energy consumption [7]. However, this method only yields a narrow window of polysaccharides in a single solvent [10]. In recent decades, aqueous two-phase extraction (ATPE), a liquid–liquid extraction fractionation method, has been extensively applied to the simultaneous separation, concentration and purification of biomolecules due to its high yield, their process integration capability, easy scale up parameters, and environment-friendly features [11–13]. ATPE systems were generally constructed by two structurally different polymers (such as a polymer and a salt, an ionic liquid and a salt, or a low molecular weight alcohol and a salt) mixed above the limit concentration according to the phase diagram to form two immiscible phases [14]. On account of biphasic extraction capacity, ATPE is popularly adapted to separate and purify lots of compounds in a single-step process [15]. Combining of MAE and ATPE has been becoming a novel and promising method for the extraction of polysaccharides

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natural resources due to its high yield and multiphase separation [16,17].

Therefore, the objective of this study was to apply microwave-assisted aqueous two-phase extraction (MAATPE) for simultaneous extraction and separation of polysaccharides from *S. pallidum*. The extraction conditions including the composition of ethanol and ammonia sulfate, extraction time, temperature, and microwave power were optimized by response surface methodology (RSM) and Box-Behnken design (BBD). The physicochemical characteristics and hypoglycemic activities *in vitro* of two polysaccharides from top phase (SPP-1) and bottom phase (SPP-2) were comparatively investigated.

2. Materials and methods

2.1. Materials and chemical reagents

S. pallidum was collected from Qingdao City (Shandong, China) on May 5, 2017. The sample was cleaned, dried and pulverized into powder using a cutting mill (FW135, Taisite, Tianjin, China) and then passed through an 80-mesh sieve to obtain fine powder (5.0% moisture content). Laminarin, bovine serum albumin (BSA), trifluoroacetic acid (TFA), acarbose, dextran standards, porcine pancreatic α-amylase and α-glucosidase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HepG2 cell line (ATCC® HB-8065) was purchased from American Type Culture Collection (Manassas, VA, USA). Glucose test kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). All chemicals and solvents were analytical grade or better.

2.2. Optimization of MAATPE of polysaccharides from S. pallidum

2.2.1. Preparation of ATPS

The ATPS of ethanol/ammonium sulfate was prepared according to the report of Chen et al. with some modifications [10]. Anhydrous ethanol, ammonium sulfate and deionized water were employed to form ATPS in this work. The preparation process of ATPS can be summarized as follows: a certain amount of ammonium sulfate was dissolved in a given mass of deionized water, added a predetermined volume of anhydrous ethanol, then mixed using a vortex mixer at 20 °C and stabilized until two phases formed. The ATPS of ethanol/ammonium sulfate were prepared according to the phase diagram. In brief, 6.60 g of ammonium sulfate was dissolved in 12.94 mL of deionized water. The salt solution was mixed with 8.02 mL of ethanol by a vortex stirrer. ATPS containing 22.0% ammonium sulfate (w/w) and 21.0% ethanol was formed when the mixture showed two phase separation above the phase diagram.

2.2.2. Phase diagram

According to the turbidimetric titration method reported in the previous reports, the phase diagram was prepared at 20 ± 0.1 °C with slight modifications [18]. In brief, absolute ethanol of known mass was added into a 50 mL conical flask, and then an ammonium sulfate solution of known mass fraction was added drop-wise and mixed thoroughly. It can be observed that the solution was clear at first, with the adding of a certain amount of ammonium sulfate solution, one further drop made the solution turbid and then separated into two phases. The mass fraction of ethanol and ammonium sulfate was noted precisely. Finally, a few drops of deionized water was drop-wise added which made the mixture clear again. The above procedures were repeated many times to obtain sufficient data to construct the phase diagrams.

2.2.3. Extraction of polysaccharides with MAATPE

The MAATPE was performed on a microwave extraction device (MARS6, CEM Company, USA). Extraction of polysaccharides from *S. pallidum* with ATPS was done on a microwave extraction chamber (MARS6, CEM Company, USA) equipped with a digital timer, temperature and power controller. The ammonium sulfate mass of ATPS was tested according to the phase diagram. Briefly, a given amount of ammonium sulfate was dissolved in 3.0 mL of deionized water, then added 3.0 mL of absolute ethanol and 1.0 mL of polysaccharides water solution with a predetermined concentration. The whole system was stirred fully on a heating magnetic Stirrer (JK-MSH-Pro-500A, Shanghai, China) at 20 ± 0.1 °C, making ammonium sulfate dissolve completely and system separate subsequently. The dosages of ethanol and ammonium sulfate were also investigated in the system: the concentrations of ethanol were set as 19, 21, 23, 25, and 27% (w/w), and ammonium sulfate of 20, 21, 22, 23, and 24% (w/w).

The two phases solution were concentrated and dialyzed using dialysis tubes (molecular weight cutoff = 3000 Da, Mym Bio-logical Technology Company Limited, USA) in distilled water for 48 h to remove salt and small molecule impurities for 24 h, followed by filtration and vacuum concentration. Then concentrated supernatant was adjusted to a concentration of 80% (v/v) ethanol and kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 10 min and freeze-dried. The products were named as SPP-1 for the top phase and SPP-2 for the bottom phase. Both of the two phases were collected and the total sugar content was determined by the phenol–sulphuric method in triplicate. The yield of total polysaccharides was determined as following equation:

$$\text{Yield}(\%) = (C_t V_t + C_b V_b)/M_s \times 100 \quad (1)$$

where C_t and V_t were the measured concentration (mg/L) and volume (L) of the top phase respectively; C_b and V_b were the measured concentration (mg/L) and volume (L) of the bottom phase respectively; and M_S was the mass of sample powders (mg).

2.2.4. Single-factor experiment

The single-factor design was used to determine the preliminary range of the extraction factors including A (extraction time: 5, 10, 15, 20, 25 min), B (extraction temperature: 50, 60, 70, 80, 90, 100 °C), and C (microwave power: 200, 400, 600, 800, 1000 W). The ratio of sample solid to the ATPS was set as 1:60 according to the reported literature [8].

2.2.5. Experimental design

On the basis of single-factor experiments, in which the preliminary range of three variables including extraction time, temperature and microwave power are determined, RSM was employed to further optimize the conditions of MAATPE [17,19]. The BBD with three independent variables at three levels were conducted shown in Table 1. The detailed range and levels of the independent variables were depicted in Table 1. In view of the evaluation of pure error sum of squares, three replicates at the center of the complete experimental design (a total of 17 experimental points) were conducted. Furthermore, all the experiments were deliberately conducted in a random order to minimize the effects resulted from some unexpected variables [20]. Data from the BBD were fitted to a quadratic polynomial model, the following quadratic Eq. (2) can be right for explaining the model *via* the Design-Expert software of 7.1.3.

$$Y = \beta k0 + \sum_{i=1}^{3} \beta kiXi + \sum_{i=1}^{3} \beta kiiX_i^2 + \sum_{i<j=2}^{3} \beta kiiXiXj \quad (2)$$

where Y was the yield of polysaccharides, β_{k0} was the constant coefficient, β_i, β_{ii}, and β_{ij} were the coefficients which represented the linear, quadratic, and cross-product effects of the factors, respectively.