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Purification, *in vitro* antioxidant and *in vivo* anti-aging activities of soluble polysaccharides by enzyme-assisted extraction from *Agaricus bisporus*

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ABSTRACT

In present study, the soluble polysaccharides by enzyme-assisted extraction from *Agaricus bisporus* (EnAPS) and its purified fractions of EnAPS-1, -2 and -3 were successfully obtained, and the antioxidant activities and anti-aging effects were investigated. The *in vitro* antioxidant assay demonstrated that EnAPS-2 had superior scavenging activities on DPPH and hydroxyl radicals, chelating activities of Fe^{2+} and reducing power. The *in vivo* animal experiments showed that both EnAPS and its purifies fractions had potential anti-aging effects against the D-galctose-induced aging diseases on liver, kidney, brain and skin, possibly by increasing the antioxidant enzymes, reducing the lipid peroxidation, improving the organ functions and remitting the lipid metabolism. The conclusions demonstrated that the polysaccharides by *A. bisporus* might be suitable for applying functional foods and natural drugs in preventing and delaying the aging and its complications.

1. Introduction

As a well-known complex biological process, the aging is commonly defined as the progressive accumulated changes in an organism diversely and deleteriously, which can increase the risk for disease and death [1]. The free radical theory is recognized as the most plausible and promising mechanism on inducing the aging [2]. With unpaired electrons in the atomic or molecular orbital, the reactive oxygen species (ROS) are generally generated through different pathways due to the exogenous chemical and endogenous metabolic properties in living systems [3]. The ROS-induced oxidative stress, which was induced by disturbing the balance between ROS and antioxidant defenses, can lead to aging and its complications in the cells and tissues [4,5]. Interestingly, cells possess cellular defense systems comprising the endogenous enzymes of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), as well as the non-enzymatic antioxidants of ascorbic acid and melatonin, aiming on maintaining the proper balance between free radicals and antioxidants [6,7]. However, the antioxidant defense system is insufficient for completely preventing oxidative damage caused by excessive free radicals [8]. In this regard, antioxidant plays vital roles in preventing the oxidative stress by safely interacting with free radicals and terminating the chain reactions [9,10]. Compared with the natural antioxidant, the use of synthetic antioxidants is restricted due to their side-effects [11]. On account of this, it seems important significance on seeking natural free radical scavengers for human health protection.

Recently, edible and medicinal mushrooms are rich in dietary fiber, balanced nutrients, and other compounds known to be physiologically beneficial to humans [12]. It has been demonstrated that polysaccharides play important roles as dietary free radical scavengers in the prevention against oxidative damage in living organisms [13]. *Agaricus bisporus*, commonly known as the white button mushroom containing high levels of dietary fibers and polysaccharides, have been widely used as a nutritionally rich foodstuff [14]. Recently, most of the reported polysaccharides isolated

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from water extraction of *A. bisporus* have been proved to have various biological activities including antioxidant [15], hepatoprotective [16] and immunomodulatory [17]. However, no report about the anti-aging effects of soluble *A. bisporus* polysaccharides by enzyme-assisted extraction (EnAPS) has been published up till now.

In this work, the antioxidant activities and anti-aging effects of EnAPS and its purified fractions (EnAPS-1, EnAPS-2 and EnAPS-3) by *A. bisporus* on D-gal-induced aging mice were investigated, aiming to provide references on exploring potential natural antioxidants in functional foods.

2. Materials and methods

2.1. Materials and chemicals

The dried fruiting body of *A. bisporus* SA-01 was obtained from local commercial markets (Taian, China). DEAE-52 cellulose was purchased from Sigma Chemicals Company (St. Louis, USA). The standard monosaccharide samples were provided by Merck Company (Darmstadt, Germany). Assay kits for protein, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and hydroxyproline (HYP) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used in present work were analytical reagent grade and supplied by local chemical suppliers.

2.2. Preparation and purification of EnAPS

The EnAPS by *A. bisporus* was performed according to the method reported previously with slight modifications [18]. The dried *A. bisporus* power and snailase solutions (0.04%) were kept warm in distilled water for 4 h at 40 °C. After centrifugation at 3000 rpm for 15 min, the supernatants were concentrated and precipitated by five volumes ethanol (95%, v/v) overnight (4 °C). The precipitate was collected by centrifugation (3000 rpm, 15 min) and deproteinated by employing the Sevage method. The EnAPS was lyophilized by vacuum freeze drying for further study (Labconco, USA). The percentage of EnAPS yield was calculated according to the following Eq. (1).

$$\text{EnAPS yield}(\%) = W_1/W_0 \times 100 \quad (1)$$

Where W_1 was the polysaccharide weights (g) and W_0 was sample weights (g).

The crude EnAPS (2.0 g) was dissolved in 50 mL distilled water (70 °C), and then the aqueous solutions was applied to a DEAE-52 cellulose column (26 mm × 400 mm), which was equilibrated with Tris-HCl buffer. The column was eluted with 0, 0.1, 0.3, 0.4 and 1.0 mol/L NaCl (2 mL/tube), detected by the phenol-sulfuric acid method [19]. The major fractions were collected, and lyophilized for further analysis.

2.3. Monosaccharide compositions analysis

The monosaccharide compositions of EnAPS and its purified fractions were analyzed by gas chromatography (GC, GC-2010, Shimadzu, Japan) as described previously with slight modifications [24]. The samples (0.1 g) were hydrolyzed with 0.7 mL trifluoroacetic acid (TFA, 2 mol/L) in the boiled water (120 °C) for 4 h. After dissolving completely, 0.3 mL ammonium hydroxide (12 mol/L) and 0.3 mL sodium borohydride (2%, w/v) were added to the above-mentioned reaction solution. The mixture solution (0.4 mL) was acetylated by adding 1-methylimidazole (0.5 mL) and acetic anhydride (4.5 mL). The acetylated monosaccharides (1 μL) were detected on the capillary column (30 mm × 0.25 mm × 0.25 μm), using nitrogen as a carrier gas. The glucose (Glu), fucose (Fuc), arabinose (Ara), xylose (Xyl), ribose (Rib), galactose (Gal), mannose (Man) and rhamnose (Rha) were employed as the standard sugars.

2.4. Antioxidant capacities in vitro

The hydroxyl radical scavenging activity was determined according to the method with slight modifications [20]. One milliliter samples at different concentrations (0.1–1 mg/mL) were mixed with 1 mL ferrous sulfate (9 mmol/L), 1 mL salicylic acid (9 mmol/L) and 1 mL hydrogen peroxide (0.03%, v/v). After the reaction was processed at 37 °C for 60 min, the absorbance was measured at 700 nm. The hydroxyl radical scavenging activity was calculated by using the following Eq. (2).

$$\text{Scavenging ability}(\%) = (1 - A_i/A_o) \times 100 \quad (2)$$

Where A_i was the absorbance of the polysaccharides and A_o was the absorbance of the blank control.

DPPH radical-scavenging activity was measured according to the previously reported method [21]. Two milliliter polysaccharide samples at different concentrations (0.1–1 mg/mL) were mixed with 2 mL DPPH solutions (0.1 mmol/L dissolved in 95% ethanol). The mixture was shaken vigorously and then incubated at room temperature for 30 min in the dark. The absorbance was recorded at 517 nm. The scavenging activity was calculated using the following Eq. (3).

$$\text{Scavenging ability}(\%) = 1 - (A_i - A_j)/A_o \times 100 \quad (3)$$

Where A_i was the absorbance for samples mixed with DPPH, A_j was the absorbance for samples mixed with 2 mL of 95% ethanol, and A_o was the absorbance for 95% ethanol mixed with DPPH solution, respectively.

The reducing power was determined according to the procedure described with some modifications [22]. Each sample (1 mL) was dissolved in 2.5 mL phosphate buffer (0.2 mol/L, pH 6.6) at a series of concentrations (0–1 mg/mL). After adding 2.5 mL potassium ferricyanide (1%, w/v), the mixture was incubated at 50 °C for 20 min. Immediately the reaction was cooled in flowing water, 2.5 mL trichloroacetic acid (10%, w/v) was added. After centrifugation (3000 rpm, 10 min), 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%, w/v). After incubating at room temperature for 10 min, the absorbance was measured at 700 nm.

The Fe^{2+}-chelating rate was determined according to the reported method with slight modification [23]. Two milliliter of polysaccharide samples at a series of concentrations (0–1 mg/mL) were mixed with 0.1 mL of ferrous chloride (2 mmol/L), 0.4 mL of ferrozine solution (5 mmol/L), and 5 mL of distilled water. The reaction was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the mixture was determined at 562 nm. The Fe^{2+}-chelating rate was calculated using the following Eq. (4).

$$Fe^{2+}\text{-chelating rate}(\%) = (A_o - A_j)/A_o \times 100. \quad (4)$$

Where A_o was the absorbance of the polysaccharide samples group, and A_j was the absorbance of the blank control.

2.5. Anti-aging activity in vivo

The male Kunming strain mice (8-week-old, 20 ± 2 g) were used in the present study. All mice were housed under standard environmental conditions (22 ± 0.5 °C, 55 ± 5% humidity and a 12 h light/12 h dark cycle) and maintained with free access to standard laboratory pellet diet and water. The experiments were performed as approved by the Institutional Animal Care and Use Committee