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Rice bran polysaccharides and oligosaccharides modified by *Grifola frondosa* fermentation: Antioxidant activities and effects on the production of NO

Qian Liu [a], Xiujuan Cao [a,b], Xuhui Zhuang [a], Wei Han [a], Weiqun Guo [a], Jian Xiong [b], Xiaolin Zhang [a,*]

[a] Academy of State Administration of Grain, Beijing 100037, PR China
[b] School of Light Industry and Food Science, South China University of Technology, Guangzhou 510640, PR China

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ABSTRACT

Rice bran polysaccharides (RBPSs) are valuable compounds with many biological activities. In this work, a fungus called *Grifola frondosa*, was selected to ferment defatted rice bran water extracts and modify the RBPSs, which were then isolated by ethanol precipitation and deproteinization. GC analysis of fermented products suggested they are composed of glucose, arabinose, galactose, mannose, and xylose at a molar ratio of 9:5:8:2:5, which was 32:4:6:2:5 before fermentation. HPLC analysis revealed that the molecular weight of unfermented RBPS was distributed mainly from 10^3 to 10^4 Da, and it changed to 10^2 to 10^3 Da after fermentation. Antioxidant activities and effects on the production of NO were analyzed and it indicated that the scavenging ratios of hydroxyl and DPPH radicals by the fermented products were significantly enhanced compared to the unfermented ones, and also the products fermented for 9 days exhibited two-way adjusting effects on the production of NO in macrophages.

1. Introduction

Rice is one of the most essential cereals in the world, serving as a staple food in many countries. Rice bran (RB) is the outer layer of milled rice and it is produced as a major co-product in the rice milling industry. RB is a good source of protein, fats, carbohydrates and many other active compounds (e.g. vitamin E and γ-oryzanol) (Iqbal, Bhanger, & Anwar, 2005; Parrado et al., 2006; Pourali, Asghari, & Yoshida, 2010). It has traditionally been primarily utilized in animal feeds, with more and more wide use in human food and nutrition in recent years.

Among these valuable compounds from RB, more attention has been paid to rice bran polysaccharides (RBPSs) due to their various biological benefits including anti-tumor, antioxidant, and anti-inflammatory activities, as well as improvement of diarrhea symptoms and immune function (Choi, Paik, Kwon, & Park, 2014; Schramm, Abadie, Hua, Xu, & Lima, 2007; Zha et al., 2009). As a result, an increasing number of studies have focused on purifying, identifying, and characterizing the activities of RBPSs. Three types of RBPS (PW1, PW2, and PW3) were extracted from RB with hot-water, which exhibited the abilities to scavenge hydroxyl, superoxide, and DPPH radicals (Zha et al., 2009). A heteropolysaccharide, RBPS2a, was obtained from defatted RB and it displayed strong immune-modulating activity relating to host defense response (Wang, Zhang, Zhang, & Chen, 2008). Reports have proven that the molecular mass of polysaccharides, as well as their structure, largely contributes to the biological activities (Ghosh, Chattopadhyay, Karmakar, Mandal, & Ray, 2009; Ray et al., 2013). Therefore, a variety of studies have attempted to modify RBPSs in order to improve their biological activities using physical, chemical, and biological methods. Wang et al. chemically modified RBPSs with sulfur groups and found out that two of the modified RBPSs were in possession of significantly higher anti-tumor activities compared to the unmodified ones (Wang, Li, & Chen, 2009). MGN-3/Biobran is the most widely studied RBPS product and it is obtained by reacting RB hemicellulose with multiple carbohydrate-hydrolyzing enzymes from shiitake mushrooms (Ghoneum, Badr El-Din, Ali, & El-Dein, 2014). MGN-3/Biobran has been shown to induce many powerful biological responses, such as activating dendritic cells, enhancing NK cell activities, modulating cytokines, inducing apoptosis in tumors and augmenting the phagocytic function of macrophages (Ghoneum, El-Din, Fattah, & Tolentino, 2013). While modification could intensely improve the biological activities of RBPSs, the majority of studies on them have been dedicated to extracting, purifying, identifying, and characterizing the activities of unmodified RBPSs. There are relatively few

* Corresponding author at: No. 11 Baiwanzhuang Street, Beijing, 100037, PR China.
E-mail address: zxl@chinagrain.org (X. Zhang).

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studies about the modification of RBPSs, especially those that emphasize on biological modification.

In this present study, the fungus *Grifola frondosa* was used to ferment water extracts from defatted RB, modifying the crude polysaccharides in it. Differences in the content, monosaccharide composition and distribution of molecular weights (as well as antioxidant activities and its effects on the production of NO) between RBPSs and fermented products were investigated in details.

2. Materials and methods

2.1. Materials and reagents

Grifola frondosa was acquired from the College of Biological Science at China Agricultural University. The defatted RB was purchased from Jiamusi (Heilongjiang, China). α-Amylase (50 U/mg), gluco-amylase (70 U/mg) and monosaccharide standards were purchased from Sigma-Aldrich (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Griess reagent, salicylic acid, DPPH, and inositol were also purchased from Sigma-Aldrich. Raw murine macrophages (RAW 264.7) were purchased from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China).

2.2. Preparation of defatted RB water extract

Defatted RB was extracted with water at a ratio of 1:10 (w/v) by microwave treatment (400 W) for 2 min, followed by heating at 100 °C for 20 min. The mixture was cooled to 60 °C and incubated with α-amylase (30 U/mL) and gluco-amylase (200 U/mL) for 4 h to remove the starch. All of the reactions were stopped by heating at 100 °C for 10 min. The extracts were centrifuged at 4000×*g* for 20 min and the sterilized supernatant was used for cultivating *Grifola frondosa*.

2.3. Grifola frondosa fermentation and polysaccharides preparation

Grifola frondosa was cultivated in defatted RB water extract at 28 °C and the samples were collected at several different timepoints (0, 4, 7 and 9 days). The supernatant was collected by filtration and proteins were removed using pepsin. Pepsin solution (20 U/mL) was added to the supernatant at a ratio of 1:100 (v/v) and the mixture was incubated at 37 °C for 1 h. Then the reaction was stopped by heating at 100 °C for 10 min and the supernatant was obtained by centrifugation at 8000×*g* for 20 min. Four volumes of ethanol were added to the supernatant and the mixture was stored at 4 °C for 12 h. The precipitate was collected by centrifugation at 8000×*g* for 20 min and subsequently dissolved in distilled water at a ratio of 1:50 (w/v). The supernatant containing water-soluble crude polysaccharides was freeze-dried for storage.

2.4. Analysis of monosaccharide composition and total carbohydrate content

The polysaccharides or fermented products (10 mg) were dissolved in 2 mL of 1 M sulfuric acid and hydrolyzed at 100 °C for 6 h in a sealed glass tube. The solution was then neutralized by $BaCO_3$, centrifuged to collect the supernatant and then freeze-dried. Polysaccharides hydrolysate and standards (glucose, rhamnose, arabinose, xylose, mannose, and galactose, including inositol as the internal standard) were modified as described by Xu et al. (2012). The derivatives were analyzed by gas chromatography (GC6820, Agilent) equipped with an AT-OV-1701 column (30 m × 0.25 mm × 2 μm) and a flame-ionization detector. The operation was performed using N_2 at a 1.5 mL/min flow-rate, using a program where the temperature was increasing from 180 °C to 240 °C (5 °C/min). The products were identified by the retention times and the percentage of monosaccharide in the sample was calculated based on the peak areas compared to a standard curve. The total carbohydrate levels were measured based on the phenol-sulfuric acid method (Blumenkrantz & Asboe-Hansen, 1973).

2.5. Molecular weight detection

The molecular weights were measured by HPLC using an Agilent PL aquagel-OH column. Each sample was filtered through a 0.22 μm polypropylene filter. The mobile phase containing 0.9% NaCl was degassed in an ultrasonic bath before use. Analysis was carried out using a Wyatt DAWN Heleos-II multi-angle laser light scattering detector, with a column oven temperature of 25 °C and 1 mL/min flow rate.

2.6. Hydroxyl radical scavenging activity

A solution of 0.1 mL polysaccharides or fermented products (0.05–1.0 mg/mL) was mixed with 0.1 mL $FeSO_4$ (6 mM) and 0.1 mL H_2O_2 (2.4 mM). After 10 min of incubation at room temperature, the mixture was incubated with 0.1 mL of 6 mM salicylic acid at 30 °C for 30 min, and hydroxyl radical was detected by monitoring the absorbance at 510 nm. A vitamin C solution (0.05–1.0 mg/mL) lacking polysaccharides served as a positive control. The EC_{50} values were calculated from a linear equation that was determined by plotting the concentration of polysaccharides or fermented products against the percentage of scavenging activity.

2.7. DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity of the extracts was measured as previously described by Butsat and Siriamornpun (2010) with some modifications. DPPH was dissolved in ethanol into a final concentration of 0.04 mg/mL and the 0.1 mL DPPH solution was then mixed with 0.1 mL of polysaccharide or fermented products samples (0.25–2.0 mg/mL). The mixture was incubated at 30 °C for 30 min and then its absorbance was measured at 517 nm. A vitamin C solution (0.25–2.0 mg/mL) lacking polysaccharides served as a positive control. EC_{50} values were calculated from a linear equation that was determined by plotting the concentration of polysaccharides or fermented products against the percentage of scavenging activity.

2.8. Effects on the production of NO in macrophages

RAW 264.7 cells were suspended in a medium containing various concentrations of fermented product fractions (50, 100, 200, 400 μg/mL). The cells were incubated at 37 °C in 5% CO_2 for 24 h, and then 50 μL of the supernatants were transferred and mixed with an equal volume of Griess reagent. After incubating the mixture for 15 min at room temperature, the absorbance was measured at 540 nm using an ELISA reader (Rayto RT-6000, Shenzhen, China). The concentration of nitrite was calculated based on comparison to a $NaNO_2$ standard curve (0–100 μM). Cells incubated in the medium without polysaccharide fractions were used as a negative control group.

2.9. Data analysis

The data were expressed as the mean ± standard deviation. Data in all the bioassays were statistically evaluated by ANOVA and Tukey's test using SPSS (Statistical Package for the Social Sciences).