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In vitro and *in vivo* antioxidant activity of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3

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ABSTRACT

The antioxidant activities of exopolysaccharides (EPS) from endophytic bacterium *Paenibacillus polymyxa* EJS-3 were evaluated by various methods *in vitro* and *in vivo*. In antioxidant assays *in vitro*, both crude EPS and its purified fractions (EPS-1 and EPS-2) were found to have moderate 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, hydrogen peroxide scavenging activity, lipid peroxidation inhibition effect, and strong ferrous ion chelating activity. And the antioxidant activities *in vitro* of EPS decreased in the order of crude EPS > EPS-2 > EPS-1. In antioxidant assays *in vivo*, mice were subcutaneously injected with D-galactose (D-Gal) for 6 weeks and administered EPS-1 via gavage simultaneously. As a result, administration of EPS-1 significantly increased the thymus and spleen indices of D-Gal induced aging mice. Moreover, EPS-1 administration significantly enhanced the activities of antioxidant enzymes and total antioxidant capacity and decreased the levels of malondialdehyde in both serums and livers of aging mice. These results suggested that EPS had potent antioxidant activities and could be explored as novel natural antioxidant.

1. Introduction

Increasing evidence highlights that reactive oxygen species (ROS) and oxygen-derived free radicals may contribute to a variety of pathological effects (e.g. DNA damages, carcinogenesis and cellular degeneration) and induce many diseases including aging, cancer, atherosclerosis, diabetes and rheumatoid arthritis (Finkel & Holbrook, 2000; Seifried, Anderson, Fisher, & Milner, 2007; Valko et al., 2007). Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases. In the search of newer and more effective natural antioxidants, a number of polysaccharides obtained from plants, animals and microorganisms have been demonstrated to possess potent antioxidant activities and to be explored as novel potential antioxidants (Fan et al., 2009; Lin, Wang, Chang, Inbaraj, & Chen, 2009; Luo & Fang, 2008; Qiao et al., 2009).

Recently, we have reported the culture conditions, structural characterization and antioxidant activities *in vitro* of exopolysaccharides (EPS) from endophytic bacterium *Paenibacillus polymyxa* EJS-3 isolated from the root tissue of *Stemona japonica* (Blume) Miquel, a traditional Chinese medicine (Liu et al., 2009, 2010; Lu et al., 2007). We found that *P. polymyxa* EJS-3 could produce a high level of EPS (35.26 g/L) with strong scavenging activity on superoxide and hydroxyl radicals *in vitro* (Liu et al., 2009). In addition, the purified EPS fractions (EPS-1 and EPS-2) have been demonstrated to be levan type polysaccharides (Liu et al., 2010). In order to evaluate the antioxidant activity of EPS from *P. polymyxa* EJS-3 systematically, the other antioxidant activities of EPS *in vitro* were further investigated by measuring 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and hydrogen peroxide (H_2O_2) scavenging activity, ferrous ion (Fe^{2+}) chelating activity, lipid peroxidation inhibition effect, and ferric reducing antioxidant power (FRAP). Moreover, the potential antioxidant activity of EPS-1 *in vivo* was investigated by using the D-galactose (D-Gal) induced aging mice model since EPS-1 was the main fraction (53.6%) of crude EPS from *P. polymyxa* EJS-3.

2. Materials and methods

2.1. Materials and reagents

Crude EPS and its purified fractions of EPS-1 and EPS-2 were prepared as described in our previous report (Liu et al., 2009). DPPH, ferrozine, D-Gal, and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits used for determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), protein, malondialdehyde (MDA), and total antioxidant capacity (TAOC)

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were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade.

2.2. Assay of antioxidant activities in vitro of EPS

2.2.1. Assay of DPPH radical scavenging activity

The DPPH radical scavenging activity was assayed according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modifications. Briefly, 0.2 ml of DPPH solution (0.4 mM DPPH in methanol) was mixed with 1.0 ml of sample (0.1–4.0 mg/ml) and 1.8 ml of water. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance of the mixture was measured at 517 nm. Vitamin C (V_C) was used as the positive control. The DPPH radical scavenging activity was calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = \frac{1-(A_1-A_2)}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (water instead of DPPH).

2.2.2. Assay of H_2O_2 scavenging activity

The H_2O_2 scavenging activity was determined according to the method of Ruch, Cheng, and Klauning (1989) with some modifications. The mixture containing 1.0 ml of sample (0.1–4.0 mg/ml), 2.4 ml of phosphate buffer (0.1 M, pH 7.4) and 0.6 ml of H_2O_2 solution (40 mM) was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. V_C was used as the positive control. The H_2O_2 scavenging activity was calculated as follows:

$$\text{Scavenging activity}\,(\%) = \frac{1-(A_1-A_2)}{A_0} \times 100 \quad (2)$$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (water instead of H_2O_2 solution).

2.2.3. Assay of Fe^{2+} chelating activity

The Fe^{2+} chelating activity was determined according to the method of Liu, Wang, Xu, and Wang (2007). The reaction mixture, containing 1.0 ml of sample (0.1–4.0 mg/ml), 0.05 ml of ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 ml of ferrozine solution (5 mM) and 2.75 ml of water, was shaken vigorously and incubated at room temperature for 10 min. The absorbance of the mixture was then measured at 562 nm. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as the positive control. The Fe^{2+} chelating activity was calculated by the following formula:

$$\text{Chelating activity}\ (\%) = \frac{1-A_1-A_2}{A_0} \times 100 \quad (3)$$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (water instead of $FeCl_2$ solution).

2.2.4. Assay of lipid peroxidation inhibition effect

The lipid peroxidation inhibition effect was determined by thiobarbituric acid-reactive-substances (TBARS) assay using mouse liver homogenate as the lipid rich media with some modification (Yen & Hsieh, 1998). Briefly, 1.0 ml of sample (0.1–4.0 mg/ml) was mixed with 1.0 ml of 1% liver homogenate (each 100 ml homogenate solution contains 1.0 g mouse liver), then 0.05 ml of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) were added to initiate lipid peroxidation. After incubation at 37 °C for 60 min, 1.5 ml of trichloroacetic acid (20%, w/v) and 1.5 ml of thiobarbituric acid (TBA) solution (0.8%, w/v) were added to quench the reaction. The resulting mixture was heated at 100 °C for 15 min, and then centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm.V_C was used as the positive control. The inhibition effect on lipid peroxidation was calculated as follows:

$$\text{Inhibition effect}\,(\%) = \frac{1-A_1-A_2}{A_0} \times 100 \quad (4)$$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (water instead of liver homogenate).

2.2.5. Assay of FRAP

The FRAP assay was determined according to the method of Benzie and Strain (1996) with some modifications. Briefly, the FRAP reagent was freshly prepared by mixing 100 ml of acetate buffer (0.3 M, pH 3.6), 10 ml of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 10 ml of $FeCl_3$ (20 mM). Then, 1.0 ml of sample (0.1–4.0 mg/ml) was added to 5.0 ml of FRAP reagent, and the reaction mixture was incubated at 37 °C for 10 min. The absorbance of the mixture was measured at 593 nm. $FeSO_4$ solutions ranging from 0.05 mM to 0.3 mM were used to perform the calibration curve. V_C was used as the positive control. The antioxidant power of sample was calculated from the calibration curve of $FeSO_4$ solution and expressed as mmol $FeSO_4$ equivalent per gram of sample on dry weight.

2.3. Assay of antioxidant activities in vivo of EPS-1

2.3.1. Animals and experimental design

Male Kunming mice (weighing 20 ± 2 g, 8 weeks old) were purchased from Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). Mice were housed in cages at an ambient temperature of 21 ± 1 °C with 50–60% relative humidity in a 12 h light/dark cycle. They had free access to the standard pellet diet and drinking water during the experiments. After adaptation for one week, the mice were randomly divided into six groups (six mice per group): normal control group (NCG), D-Gal aging control group (ACG), V_C positive control group (PCG) and EPS-1 treatment groups. Mice in NCG were fed with physiological saline (10 ml/kg body weight per day) by gavage and subcutaneous injection. Mice in ACG were fed with physiological saline (10 ml/kg body weight per day) by gavage and D-Gal (100 mg/kg body weight per day) by subcutaneous injection. Mice in PCG were fed with V_C (100 mg/kg body weight per day) by gavage and D-Gal (100 mg/kg body weight per day) by subcutaneous injection. Mice in EPS treatment groups were respectively fed with EPS-1 in three different doses (100, 200 and 400 mg/kg body weight per day) by gavage and D-Gal (100 mg/kg body weight per day) by subcutaneous injection. All groups were performed once daily for 42 consecutive days.

2.3.2. Biochemical assay

Twenty-four hours after the last drug administration, mice were weighed and sacrificed. Blood samples were collected and centrifuged at 4000 × g at 4 °C for 10 min to afford the serums. The liver was removed rapidly, washed and homogenized in ice-cold physiological saline to prepare 10% (w/v) homogenate. Then, the homogenate was centrifuged at 4000 × g at 4 °C for 10 min to remove cellular debris, and the supernatant was collected for analysis. The thymus and spleen were also excised from mice and weighed immediately to afford the index of spleen and thymus. The thymus and spleen indices were calculated by the following formula:

$$\text{Thymus or spleen index}\ (\text{mg/g}) = \frac{\text{Weight of thymus or spleen}}{\text{Body weight}} \quad (5)$$