Carbohydrate Polymers 82 (2010) 1278–1283

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

Jun Liu[a,b], Jianguang Luo[a], Hong Ye[a], Yi Sun[a], Zhaoxin Lu[a], Xiaoxiong Zeng[a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, Weigang 1, Nanjing 210095, Jiangsu, PR China
[b] College of Food Science and Engineering, Yangzhou University, Yangzhou 225127, Jiangsu, PR China

ARTICLE INFO

Article history:
Received 24 December 2009
Received in revised form 1 May 2010
Accepted 2 July 2010
Available online 13 July 2010

Keywords:
Antioxidant activity
Endophytic bacterium
Exopolysacchrides
In vitro
In vivo
Paenibacillus polymyxa

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)

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.07.008

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:

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

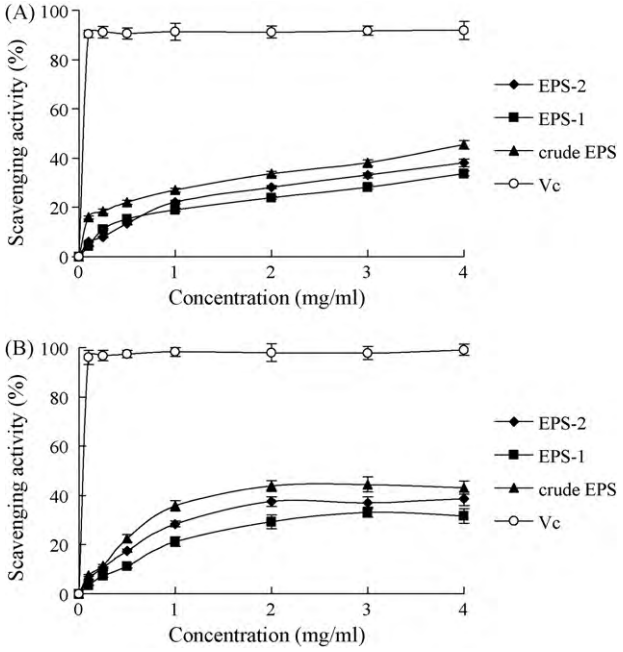

Fig. 1. The DPPH radical (A) and H_2O_2 (B) scavenging activities of crude EPS, EPS-1 and EPS-2 from *P. polymyxa* EJS-3 with V_C as the positive control. Data were presented as means ± SD of triplicates.

The biochemical assays were carried out according to the instructions of kits purchased from Nanjing Jiancheng Bioengineering Institute. Briefly, SOD activity was measured through the inhibition of hydroxylamine oxidation by the superoxide radicals generated in the xanthine–xanthine oxidase system. GSH-Px activity was measured on the basis of the reaction of GSH and 5,5′-dithiobis-(2-nitrobenzoic acid). CAT activity was determined by measuring the absorbance of the yellow H_2O_2–ammonium molybdate complex at 405 nm. TAOC was measured by FRAP assay. All above activities were expressed as unit per milliliter (U/ml) in serum or unit per milligram of protein (U/mg protein) in liver. In addition, MDA level was measured by the TBARS method and expressed as nmol per milliliter (nmol/ml) in serum or nmol per milligram of protein (nmol/mg protein) in liver. The protein content in liver supernatant was determined by the Bradford method using bovine serum albumin as the standard.

2.4. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The Duncan test and one-way analysis of variance (ANOVA) were used for multiple comparisons by the SPSS 13.0 software package. Difference was considered to be statistically significant if $P < 0.05$.

3. Results and discussions

3.1. Antioxidant activities in vitro of EPS

3.1.1. DPPH scavenging activity of EPS

The model of scavenging DPPH radical is a widely used method to evaluate the free radical scavenging activities of antioxidants (Yuan et al., 2005a). In the DPPH assay, the antioxidants are able to reduce the stable DPPH radical (purple) to the non-radical form DPPH-H (yellow). The DPPH scavenging activities of antioxidants are attributed to their hydrogen donating abilities.

As shown in Fig. 1(A), the scavenging activities on DPPH radicals of crude EPS, EPS-1, and EPS-2 increased with the increase of concentrations for all the samples. At a concentration of 4.0 mg/ml, the DPPH scavenging activities for crude EPS, EPS-1, and EPS-2 were 45.40%, 33.74%, and 38.04%, respectively. It should be noted that the scavenging activity of crude EPS was higher than its purified fractions of EPS-1 and EPS-2 ($P < 0.05$). This was probably due to the presence of other antioxidant components in the crude EPS, such as proteins, amino acids, peptides, organic acids and microelements. In addition, the scavenging activity of EPS-2 was higher than that of EPS-1 ($P < 0.05$), which was probably due to the differences in the monosaccharide composition and molecular weight between them. In our previous report, EPS-1 was found to be composed of mannose, fructose and glucose in a molar ratio of 2.59:29.83:1 with molecular weight of 1.22×10^6 Da, whereas EPS-2 was composed of mannose, fructose and glucose in a molar ratio of 4.23:36.59:1 with molecular weight of 8.69×10^5 Da (Liu et al., 2009). It should be noted that the scavenging activities of all the polysaccharide samples tested were much lower than that of V_C ($P < 0.05$). These results indicated that EPS had moderate DPPH scavenging activities. And the scavenging activities of EPS on DPPH radicals were probably due to their hydrogen donating abilities.

3.1.2. H_2O_2 scavenging activity of EPS

It has been reported that H_2O_2 plays an important role as the radical-forming intermediate in the production of ROS molecules (Barbouti, Doulias, Nousis, Tenopoulou, & Galaris, 2002). Although H_2O_2 is not very reactive, its high penetrability in cellular membrane leads to hydroxyl radical formation when it reacts with Fe^{2+} or superoxide anion radical in the cell (Liu et al., 2007). Thus, H_2O_2 is considered as one of main inducers of cellular aging and could attack many cellular energy-producing systems.

As depicted in Fig. 1(B), crude EPS, EPS-1 and EPS-2 all exerted concentration-dependent H_2O_2 scavenging activities. At a concentration of 4.0 mg/ml, the H_2O_2 scavenging activities for crude EPS, EPS-1 and EPS-2 were 43.17%, 31.67% and 38.61%, respectively. However, V_C exhibited a much higher H_2O_2 scavenging activity of 96.1% at 0.1 mg/ml ($P < 0.05$). The results suggested that EPS had moderate H_2O_2 scavenging activities.

3.1.3. Fe^{2+} chelating activity of EPS

The Fe^{2+} chelating activity is also considered as an important antioxidant property. The transition metal of Fe^{2+} can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction and accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, thereby driving the chain reaction of lipid peroxidation (Benedet & Shibamoto, 2008). It has been recognized that chelating agents may inhibit lipid oxidation by stabilizing transition metals. In the Fe^{2+} chelating assay, the chelating activities of the antioxidants are assayed by inhibiting the formation of red-colored ferrozine–Fe^{2+} complex.

As shown in Fig. 2(A), the Fe^{2+} chelating activities of crude EPS, EPS-1 and EPS-2 were all concentration related. At a concentration of 1.0 mg/ml, chelating activities for crude EPS, EPS-1 and EPS-2 were 92.4%, 81.1% and 86.5%, respectively. Notably, EDTA-2Na was an excellent chelating agent for Fe^{2+} and its chelating activity was 98.4% at 0.1 mg/ml. The results showed that EPS exhibited strong Fe^{2+} chelating activities at high concentrations. It has been reported that compounds with metal chelating activities usually contain two or more of the following functional groups: –OH, –SH, –COOH, $-PO_3H_2$, C=O, $-NR_2$, –S– and –O– (Yuan, Bone, & Carrington, 2005). EPS-1 and EPS-2 were both levan type polysaccharides, which were mainly composed of β-(2→6)-linked β-D-fructofuranosyl residues backbone with (2→1)-linked branches (Liu et al., 2010). Accordingly, the Fe^{2+} chelating activities of EPS were partially due to the presences of –OH and –O– groups in their structures. And the exact mechanism should be further investigated.

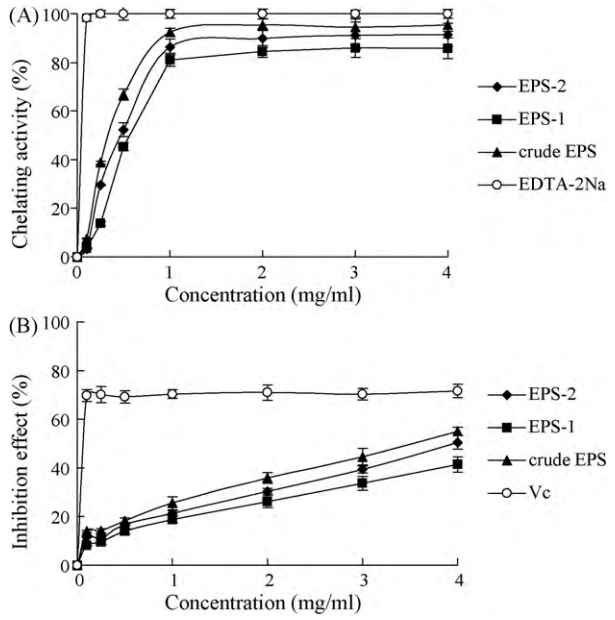

Fig. 2. The Fe^{2+} chelating activities (A) and lipid peroxidation inhibition effects (B) of crude EPS, EPS-1 and EPS-2 from *P. polymyxa* EJS-3 with EDTA-2Na and V_C as the positive control. Data were presented as means ± SD of triplicates.

3.1.4. Lipid peroxidation inhibition effect of EPS

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products (Abuja & Albertini, 2001). MDA is one of the products of lipid peroxidation, which can react with TBA, yielding a pinkish red chromogen with the maximum absorbance at 532 nm. In the present study, $FeCl_2$–H_2O_2 system was used to induce lipid peroxidation in mouse liver homogenate.

The lipid peroxidation inhibition effects of crude EPS, EPS-1 and EPS-2 increased with the increase of sample concentrations as shown in Fig. 2(B). At a concentration of 4.0 mg/ml, the inhibition effects of crude EPS, EPS-1 and EPS-2 were 55.07%, 41.45% and 50.43%, respectively. As the positive control, V_C showed a plateau of inhibition effect ranging from 69.28% to 71.59% at 0.1–4.0 mg/ml. These results indicated that EPS had moderate inhibition effects on lipid peroxidation compared with V_C. It has been reported that the inhibition effects on lipid peroxidation of antioxidants might be attributed to their scavenging abilities on hydroxyl radical and H_2O_2 produced by $FeCl_2$–H_2O_2 in the reaction system (Wang, Gao, Zhou, Cai, & Yao, 2008). On the other hand, Liu et al. (2007) suggested that the inhibition effects on lipid peroxidation of polysaccharides were due to their metal ion chelating activities, which could interfere with the free radical reaction chains. In the present study, EPS were found to have scavenging abilities on hydroxyl radical and H_2O_2 as well as metal ion chelating activities. In our opinion, therefore, the lipid peroxidation inhibition effects of EPS were attributed to their Fe^{2+} chelating abilities as well as hydroxyl radical and H_2O_2 scavenging abilities.

3.1.5. FRAP of EPS

FRAP assay is a simple and direct way to measure antioxidant capacity. It is based on the ability of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm (Benzie & Strain, 1996). The reducing potentials of antioxidants are associated with their electron donating abilities to break the free radical chain reactions.

In the present study, FRAP value was applied to evaluate the antioxidant power of EPS. Based on the calibration curve of $FeSO_4$ solution, the FRAP values for crude EPS, EPS-1 and EPS-2 were calculated to be 10.79, 3.37, and 4.38 mM $FeSO_4$/g, respectively. However, V_C showed a much higher FRAP value of 11,919 mM $FeSO_4$/g, suggesting that the antioxidant power of V_C was much stronger than that of EPS. The results indicated that EPS might act as electron donors and react with free radicals to convert them into more stable products to terminate the free radical chain reactions.

Table 1
Effects of EPS-1 on spleen and thymus indices in Gal induced aging mice.[a].

Group	Dose (mg/kg)	Thymus index (mg/g)	Spleen index (mg/g)
NCG		3.57 ± 0.24	3.44 ± 0.22
ACG		3.18 ± 0.29[b]	3.03 ± 0.27[b]
PCG	100	3.51 ± 0.58	3.71 ± 0.30[d]
EPS-1 100	100	3.43 ± 0.26	3.49 ± 0.39[c]
EPS-1 200	200	3.58 ± 0.15[c]	4.56 ± 0.41[d]
EPS-1 400	400	3.72 ± 0.26[d]	4.50 ± 0.57[d]

[a] Data were presented as means ± SD ($n = 6$) and evaluated by one-way ANOVA. Differences were considered to be statistically significant if $P < 0.05$.
[b] $P < 0.05$, compared with NCG.
[c] $P < 0.05$, compared with ACG.
[d] $P < 0.01$, compared with ACG.

3.2. Antioxidant activities in vivo of EPS-1

3.2.1. Establishment of D-Gal induced aging mice model

In the present study, the D-Gal induced aging mice model was used to evaluate the antioxidant activity *in vivo* of EPS-1. D-Gal is a reducing sugar, which can be metabolized by D-galactokinase and galactose-1-phosphate uridyltransferase in animals. However, overdose of D-Gal beyond the capacities of those two enzymes will allow aldose reductase to catalyze the accumulated D-Gal into galactitol, which cannot be metabolized and will accumulate in the cell, leading to osmotic stress and generation of ROS (Hsieh, Wu, & Hu, 2009). In addition, D-Gal can react with the free amines of amino acids in proteins and peptides to form advanced glycation endproducts (AGE). The accumulated AGE *in vivo* can also accelerate the aging process (Song, Bao, Li, & Li, 1999). In order to establish the D-Gal induced aging mice model, mice were subcutaneously injected with D-Gal (100 mg/kg body weight per day) for 6 weeks and simultaneously administered with physiological saline via gavage in this study. As shown in Table 1, the spleen and thymus indices of mice in ACG decreased remarkably compared to NCG, indicating that the immune function was diminished when the mice were treated with D-Gal. Furthermore, D-Gal injected mice in ACG showed a marked increase in the MDA level and significant decreases in the activities of SOD, CAT, GSH-Px and TAOC in both serums and livers as compared to NCG (Tables 2 and 3). It has been reported that the aging mice model, induced by high D-Gal diets or D-Gal injection, shows neurological impairment, increased production of free radicals, decreased activities of antioxidant enzymes and poor immune responses in mice (Song et al., 1999; Zhong, Ge, Qu, Li, & Ma, 2009). Thus, the results suggested that the aging mice model in the present study was established successfully.

3.2.2. Effect of EPS-1 on thymus and spleen indices in D-Gal induced aging mice

It is well known that thymus and spleen are important immune organs, and the thymus and spleen indices could partially reflect the immune function of the organism. As shown in Table 1, mice treated with EPS-1 and V_C at the dose of 100 mg/kg showed a significant increase in spleen index compared to ACG. In addition, mice treated with EPS-1 at the dose of 200 and 400 mg/kg showed a marked increase in thymus and spleen indices compared to ACG. However, the treatment of EPS-1 and V_C at the dose of 100 mg/kg had no significant effect on thymus index compared to ACG. These results

Table 2
Effects of EPS-1 on the activities of SOD (U/ml), CAT (U/ml), GSH-Px (U/ml), TAOC (U/ml) and levels of MDA (nmol/ml) in serums of Gal induced aging mice.[a].

Group	SOD	CAT	GSH-Px	TAOC	MDA
NCG	263.47 ± 27.72	49.46 ± 6.39	5096.25 ± 548.33	27.85 ± 2.66	12.33 ± 1.24
ACG	191.09 ± 29.75[b]	33.57 ± 3.09[b]	4059.38 ± 214.36[b]	21.99 ± 2.09[b]	15.99 ± 1.88[b]
PCG	237.99 ± 20.29[d]	54.28 ± 6.82[d]	4767.81 ± 468.95[d]	26.39 ± 0.91[d]	11.54 ± 1.10[d]
EPS-1 100	209.52 ± 21.37	40.05 ± 4.49[c]	4577.34 ± 179.37[d]	24.36 ± 2.64	10.17 ± 1.66[d]
EPS-1 200	265.62 ± 24.30[d]	60.67 ± 5.59[d]	5835.94 ± 189.55[d]	28.47 ± 1.63[d]	9.84 ± 0.86[d]
EPS-1 400	303.47 ± 35.55[d]	65.79 ± 6.13[d]	5790.94 ± 103.15[d]	29.50 ± 2.57[d]	8.52 ± 0.59[d]

[a] Data were expressed as means ± SD ($n = 6$) and evaluated by one-way ANOVA. Differences were considered to be statistically significant if $P < 0.05$.
[b] $P < 0.01$, compared with NCG.
[c] $P < 0.05$, compared with ACG.
[d] $P < 0.01$, compared with ACG.

Table 3
Effects of EPS-1 on the activities of SOD (U/mg protein), CAT (U/mg protein), GSH-Px (U/mg protein), TAOC (U/mg protein) and levels of MDA (nmol/mg protein) in livers of Gal induced aging mice.[a].

Group	SOD	CAT	GSH-Px	TAOC	MDA
NCG	489.84 ± 27.59	47.12 ± 3.30	708.57 ± 22.71	1.18 ± 0.07	2.20 ± 0.15
ACG	375.00 ± 46.00[b]	35.12 ± 4.95[b]	585.24 ± 43.87[b]	0.87 ± 0.07[b]	2.77 ± 0.39[b]
PCG	432.58 ± 41.45[c]	48.23 ± 4.11[d]	690.04 ± 56.45[d]	1.27 ± 0.10[d]	1.91 ± 0.28[d]
EPS-1 100	466.80 ± 31.41[d]	46.41 ± 3.64[d]	657.52 ± 75.54	1.29 ± 0.14[d]	1.76 ± 0.28[d]
EPS-1 200	525.19 ± 56.60[d]	51.75 ± 5.82[d]	740.98 ± 47.01[d]	1.76 ± 0.12[d]	1.37 ± 0.11[d]
EPS-1 400	532.93 ± 32.52[d]	55.86 ± 6.49[d]	785.34 ± 60.84[d]	1.97 ± 0.06[d]	1.34 ± 0.22[d]

[a] Data were expressed as means ± SD ($n = 6$) and evaluated by one-way ANOVA. Differences were considered to be statistically significant if $P < 0.05$.
[b] $P < 0.01$, compared with NCG.
[c] $P < 0.05$, compared with ACG.
[d] $P < 0.01$, compared with ACG.

suggested that middle and high doses of EPS-1 might stimulate the immune mechanism of aging mice.

3.2.3. *Effect of EPS-1 on the activities of antioxidant enzymes in serums and livers of* D-*Gal induced aging mice*

One of the aging theories is the free radical damage hypothesis which states that the generation of ROS or free radicals can lead to cell and tissue damage paralleled by alterations in the function of genetic apparatus, resulting in aging and untimely cell death (Hsieh et al., 2009; Zhang et al., 2003). SOD, GSH-Px and CAT are the most important antioxidant enzymes to inhibit free radical formation and usually used as biomarkers to indicate ROS production. Effects of EPS-1 and V_C on activities of SOD, CAT and GSH-Px in serums and livers of aging mice were shown in Tables 2 and 3. In both serums and livers, EPS-1 at the dose of 200 or 400 mg/kg and V_C treatment groups significantly increased the activities of antioxidant enzymes (SOD, CAT and GSH-Px) as compared to ACG. The enhanced activities of antioxidant enzymes may provide an effective defense from the damaging effects of free radicals. As reported, the enhanced activities of antioxidant enzymes were partially due to the increased mRNA expression of these enzymes (Zhong, Yan, Lim, & Oberley, 1999). Furthermore, the improved activities of the antioxidant enzymes may be closely related to the immune activity of polysaccharide (Yuan et al., 2009). Certainly, the exact mechanism of EPS-1 on the activities of antioxidant enzymes needs to be further investigated.

3.2.4. *Effect of EPS-1 on the levels of MDA in serums and livers of* D-*Gal induced aging mice*

Lipid peroxidation is the process that involves the chain reactions of free radicals with polyunsaturated fatty acid. These reactions lead to hydroperoxide generation and lipid breakdown into lower molecular weight fragments such as ketones, alcohol, hydrocarbon, acids and epoxides. Therefore, inhibition of lipid peroxidation is of great importance in disease processes involving free radicals (Abuja & Albertini, 2001). As MDA is a naturally occurring product of lipid peroxidation, the level of MDA was measured in the present study. As shown in Tables 2 and 3, the levels of MDA in EPS-1 and V_C treatment groups all decreased significantly in both serums and livers. The results indicated that the administration of EPS-1 and V_C resulted in effective inhibition effects on lipid peroxidation in aging mice. Notably, although EPS-1 showed much lower lipid peroxidation inhibition effect than V_C *in vitro*, EPS-1 exhibited similar or stronger inhibition effects than V_C *in vivo* at the same concentration. This result was consistent with that of many other investigators (Anderson & Phillips, 1999; Fardet, Rock, & Rémésy, 2008). It was probably due to the differences in the affecting factors of antioxidant activities between *in vitro* and *in vivo* assays. In antioxidant assay *in vitro*, the chemical composition of culture media and the dose of the compound are major factors affecting the antioxidant activities (Anderson & Phillips, 1999). However, in antioxidant assay *in vivo*, numerous factors including digestibility, bioavailability and metabolism of the compound may influence the antioxidant activities. In addition, antioxidant assay *in vitro* usually ignores the biological actions *in vivo*, such as the activities of antioxidant enzymes, the oxidative-related metabolic pathways as well as the activation or repression of gene expression of antioxidant compounds and enzymes (Fardet et al., 2008). Therefore, antioxidant assays *in vivo* are more essential to evaluate the real antioxidant potential of a compound.

3.2.5. *Effect of EPS-1 on TAOC in serums and livers of* D-*Gal induced aging mice*

TAOC reflects the capacity of the non-enzymatic antioxidant defense system. In the present study, FRAP assay was used to evaluate the TAOC of antioxidants *in vivo*. As shown in Tables 2 and 3, EPS-1 and V_C increased effectively the TAOC levels in both serums and livers, indicating that the non-enzymatic antioxidant defense system of aging mice had been enhanced. Furthermore, EPS-1 showed similar or higher TAOC level than V_C in antioxidant *in vivo* assay at the same concentration. This result was not consistent with that of antioxidant *in vitro* assay, and the reasons should be the same as mentioned above (Section 3.2.4). In addition, the improved TAOC by EPS-1 might also be due to the increase of non-enzymatic antioxidants *in vivo*, such as glutathione, thiols, some vitamins and metals (Seifried et al., 2007).

4. Conclusion

In the present study, both *in vitro* and *in vivo* antioxidant activities of EPS from *P. polymyxa* EJS-3 have been investigated. EPS showed moderate DPPH radical scavenging activity, H_2O_2 scavenging activity, lipid peroxidation inhibition effect, and strong Fe^{2+} chelating activity. Furthermore, antioxidant assays *in vivo* demonstrated that EPS-1 administration significantly increased the activities of antioxidant enzymes, decreased MDA levels and improved TAOC in serums and livers of D-Gal induced aging mice. Notably, EPS-1 exhibited similar or strong lipid peroxidation inhibition effects and similar or higher TAOC level than V_C *in vivo*, probably due to the differences in the affecting factors of antioxidant activities between *in vitro* and *in vivo* assays. These results suggested that EPS from *P. polymyxa* EJS-3 had potent antioxidant activities and could be utilized as new natural antioxidant in food and therapeutics.

Acknowledgement

This work was partly supported by Grants-in-Aid for scientific research from the National Natural Science Foundation of China (Nos. 30570415 and 30870547).

References

Abuja, P. M., & Albertini, R. (2001). Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. *Clinica Chimica Acta*, *306*, 1–17.

Anderson, D., & Phillips, B. J. (1999). Comparative in vitro and in vivo effects of antioxidants. *Food and Chemical Toxicology*, *37*, 1015–1025.

Barbouti, A., Doulias, P.-T., Nousis, L., Tenopoulou, M., & Galaris, D. (2002). DNA damage and apoptosis in hydrogen peroxide-exposed Jurkat cells: Bolus addition versus continuous generation of H_2O_2. *Free Radical Biology & Medicine*, *33*, 691–702.

Benedet, J. A., & Shibamoto, T. (2008). Role of transition metals, Fe(II), Cr(II), Pb(II), and Cd(II) in lipid peroxidation. *Food Chemistry*, *107*, 165–168.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Fan, Y., He, X., Zhou, S., Luo, A., He, T., & Chun, Z. (2009). Composition analysis and antioxidant activity of polysaccharide from *Dendrobium denneanum*. *International Journal of Biological Macromolecules*, *45*, 169–173.

Fardet, A., Rock, E., & Rémésy, C. (2008). Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *Journal of Cereal Science*, *48*, 258–276.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of aging. *Nature*, *408*, 239–247.

Hsieh, H.-M., Wu, W.-M., & Hu, M.-L. (2009). Soy isoflavones attenuate oxidative stress and improve parameters related to aging and Alzheimer's disease in C57BL/6J mice treated with D-galactose. *Food and Chemical Toxicology*, *47*, 625–632.

Lin, C. L., Wang, C. C., Chang, S. C., Inbaraj, B. S., & Chen, B. H. (2009). Antioxidative activity of polysaccharide fractions isolated from *Lycium barbarum* Linnaeus. *International Journal of Biological Macromolecules*, *45*, 146–151.

Liu, C., Wang, C., Xu, Z., & Wang, Y. (2007). Isolation, chemical characterization and antioxidant activities of two polysaccharides from the gel and the skin of *Aloe barbadensis* Miller irrigated with sea water. *Process Biochemistry*, *42*, 961–970.

Liu, J., Luo, J., Ye, H., Sun, Y., Lu, Z., & Zeng, X. (2009). Production, characterization and antioxidant activities in vitro of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydrate Polymers*, *78*, 275–281.

Liu, J., Luo, J., Ye, H., Sun, Y., Lu, Z., & Zeng, X. (2010). Medium optimization and structural characterization of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydrate Polymers*, *79*, 206–213.

Lu, F., Sun, L., Lu, Z., Bie, X., Fang, Y., & Liu, S. (2007). Isolation and identification of an endophytic strain EJS-3 producing novel fibrinolytic enzymes. *Current Microbiology*, *54*, 435–439.

Luo, D., & Fang, B. (2008). Structural identification of ginseng polysaccharides and testing of their antioxidant activities. *Carbohydrate Polymers*, *72*, 376–381.

Qiao, D., Ke, C., Hu, B., Luo, J., Ye, H., Sun, Y., et al. (2009). Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. *Carbohydrate Polymers*, *78*, 199–204.

Ruch, R. J., Cheng, S. J., & Klauning, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, *10*, 1003–1008.

Seifried, H. E., Anderson, D. E., Fisher, E. I., & Milner, J. A. (2007). A review of the interaction among dietary antioxidants and reactive oxygen species. *Journal of Nutritional Biochemistry*, *18*, 567–579.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, *40*, 945–948.

Song, X., Bao, M., Li, D., & Li, Y. M. (1999). Advanced glycation in D-galactose induced mouse aging model. *Mechanisms of Ageing and Development*, *108*, 239–251.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*, 44–84.

Wang, H., Gao, X. D., Zhou, G. C., Cai, L., & Yao, W. B. (2008). In vitro and in vivo antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. *Food Chemistry*, *106*, 888–895.

Yen, G. C., & Hsieh, C. L. (1998). Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) towards various lipid peroxidation models in vitro. *Journal of Agricultural and Food Chemistry*, *46*, 3952–3957.

Yuan, C., Huang, X., Cheng, L., Bu, Y., Liu, G., Yi, F., et al. (2009). Evaluation of antioxidant and immune activity of *Phellinus ribis* glucan in mice. *Food Chemistry*, *115*, 581–584.

Yuan, H., Zhang, W., Li, X., Lü, X., Li, N., Gao, X., et al. (2005). Preparation and in vitro antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohydrate Research*, *340*, 685–692.

Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated in vitro. *Food Chemistry*, *91*, 485–494.

Zhang, Q., Li, N., Zhou, G., Lu, X., Xu, Z., & Li, Z. (2003). In vivo antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodephyta) in aging mice. *Pharmacological Research*, *48*, 151–155.

Zhong, S.-Z., Ge, Q.-H., Qu, R., Li, Q., & Ma, S.-P. (2009). Paeonol attenuates neurotoxicity and ameliorates cognitive impairment induced by D-galactose in ICR mice. *Journal of the Neurological Sciences*, *277*, 58–64.

Zhong, W., Yan, T., Lim, R., & Oberley, L. W. (1999). Expression of superoxide dismutases, catalase, and glutathione peroxidase in glioma cells. *Free Radical Biology & Medicine*, *27*, 1334–1345.