Carbohydrate Polymers 97 (2013) 849–854

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Antioxidant activity of phosphorylated exopolysaccharide produced by *Lactococcus lactis* subsp. *lactis*

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ARTICLE INFO

Article history:
Received 10 April 2013
Received in revised form 23 May 2013
Accepted 19 June 2013
Available online 26 June 2013

Keywords:
Lactococcus lactis subsp. *lactis*
Exopolysaccharide
Phosphorylate
Antioxidation activity

ABSTRACT

Exopolysaccharide (EPS) of *Lactococcus lactis* subsp. *lactis* was isolated and purified from MRS culture broth. Phosphorylated exopolysaccharide (P-EPS) was synthesized by using the purified EPS and sodium hexametaphosphate (SHMP). The *in vitro* and *in vivo* antioxidant activity of EPS and P-EPS was analyzed. Both EPS and P-EPS displayed superoxide anion ($O^{2-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and DPPH scavenging activity. Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity increased in serum and the livers of mice treated with EPS and P-EPS, while malondialdehyde (MDA) levels decreased. P-EPS was shown to prevent the progression of D-galactose-induced oxidative stress in hepatocytes *in vivo*. P-EPS showed stronger *in vitro* and *in vivo* antioxidant activity than EPS.

1. Introduction

Microbial exopolysaccharides (EPS) are glycopolymers present on the surface of bacteria, which can either be covalently linked to the bacterial surface forming a capsule or can be secreted into the environment in the form of slime (López et al., 2012). EPS produced by food-grade lactic acid bacteria (LAB) has been widely studied (Ai et al., 2008). EPS produced by several LAB has been found to have potential health benefits, such as antioxidant activity, antihypertensive activity, immunomodulatory effects and antimicrobial properties (Ai et al., 2008; Kanmani et al., 2011).

Research has demonstrated that molecular modification can increase the biological activities of polysaccharides (Chen et al., 2011a). For example, Yuan et al. found the substituted groups such as sulfate, acetyl and phosphate can enhance the antioxidant activity of polysaccharide *in vitro* (Yuan et al., 2005). However, studies on oligosaccharide modification have been focused on algal and plant polysaccharides (Chen et al., 2011a; Liu, Wan, Shi, & Lu, 2011; Suarez, Kralovec, & Grindley, 2010; Yuan et al., 2005). Studies focused on modified EPS of LAB are comparatively rare. Our purpose was to develop a nontoxic and highly active antioxidant compound for use in the food and pharmaceutical industries. In our previous research, we reported that EPS produced by *Lactococcus lactis* subsp. *lactis* showed antioxidant activity (Pan & Mei, 2010). In this study, EPS of *Lactococcus lactis* subsp. *lactis* was purified and phosphorylated. The *in vitro* and *in vivo* antioxidant activities of EPS and P-EPS were evaluated. The aim of this study was to evaluate the antioxidant activity of P-EPS, and to characterize the effect of phosphorylation on antioxidant ability.

2. Materials and methods

2.1. EPS production and purification

2.1.1. Isolation of EPS of *Lactococcus lactis* subsp. *lactis*

Lactococcus lactis subsp. *lactis* (conserved at Nanjing Normal University) was grown in BLX medium (glucose 10.0 g, fructose 10.0 g, peptone 12.5 g, tryptone 12.5 g, distilled water 1000 mL, pH 6.0). The fermentation liquid was centrifuged (6000 rpm, 15 min, 15 °C) after cultivation for 24 h at 37 °C. The supernatant was collected. The mixture contained 95% (v/v) cold ethanol. The collected supernatant (3:1) was kept at −4 °C overnight. After centrifugation (6000 rpm, 15 min, 4 °C), the precipitates were collected and dissolved in distilled water. Ten percent TCA was added to the dissolved precipitates to deproteinize the mixture. The supernatant was collected by centrifugation (6000 rpm, 30 min, 4 °C), mixed with 4 volumes of 95% (v/v) cold ethanol and kept at −4 °C overnight. The mixture was centrifuged (6000 rpm, 15 min, 4 °C) to produce precipitate which was washed with anhydrous ethanol, acetone and ether. The crude EPS were obtained after vacuum freeze drying.

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http://dx.doi.org/10.1016/j.carbpol.2013.06.024

2.1.2. Purification of EPS

Lyophilized crude EPS (100 mg in 1 mL distilled water) was subjected to a DEAE-Cellulose-52 (2.6 cm × 20 cm; Amersham Biosciences, Uppsala, Sweden). DEAE-Cellulose-52 was eluted with PBS buffer (pH 6.8) and 0.1–1.0 mol/L NaCl at a flow rate of 3.5 mL/min. The total carbohydrate content of the collected fractions was measured according to the phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The fraction that had the highest carbohydrate content was subjected to a Sepharose 4B gel filtration column (1.6 cm × 50 cm; Amersham Biosciences, Uppsala, Sweden). The column was eluted with distilled water at a flow rate of 2 mL/min. The fractions were pooled and lyophilized for total carbohydrate content testing.

2.2. Preparation of phosphorylated-exopolysaccharides (P-EPS)

EPS was phosphorylated by sodium hexametaphosphate (SHMP). One gram EPS and 6 g SHEP (pH 6.0) was mixed for 4 h at 90 °C. The reaction mixture was cooled to room temperature and then washed with ethanol. Vacuum evaporation was used to remove the ethanol. Small molecular substances were removed using dialysis against distilled water. The solution was lyophilized and solid P-EPS was obtained. The phosphorus content was determined by the method outlined by Chen, Toribara, and Warner (1956).

2.3. UV–vis absorption spectra and infrared (IR) spectra of EPS and P-EPS

UV–vis absorption spectroscopy was used to measure the purity of EPS. Infrared (IR) spectra were used to analyze the structure of the EPS and P-EPS. UV–vis absorption spectra and IR spectra were done according to the method described by Pan and Mei (2010).

2.4. EPS and P-EPS in vitro antioxidant activity

2.4.1. EPS and P-EPS superoxide anion and hydroxyl radical scavenging activity

The nitrotetrazolium blue chloride (NBT) method was used to measure superoxide anion scavenging activity. The hydroxyl radical (•OH) scavenging activity produced by Fenton's reaction was measured to analyze hydroxyl radical scavenging activity. These methods were carried out as described by Pan and Mei (2010).

2.4.2. EPS and P-EPS DPPH radical scavenging activity assay

Two milliliters of sample with different concentrations were mixed with 2.0 mL of 0.1 mmol/L DPPH (dissolved in 95% ethanol). The mixture was kept in the dark for 30 min. The absorbance of the mixture was measured at 517 nm (You, Zhao, Regenstein, & Ren, 2011).

$$\text{Scavenging activity}(\%) = \frac{A_0 - A_X}{A_0} \times 100\%$$

where A_X is the value of the sample solution mixed with the DPPH solution; and A_0 is the value of 2 mL of 95% ethanol mixed with the DPPH solution.

2.5. EPS and P-EPS in vivo antioxidant activity

2.5.1. Animal preparation and experimental design

Male ICR mice (18 22 g, 8 weeks old, SCXK(Su)2008-0004) were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China). Mice were kept in cages under controlled conditions [SYXK(Su)2008-0007] of 22 ± 1 °C and 55 ± 5% relative humidity with a 12 h light/dark cycle. During the experiments, mice ate a standard diet and water. After acclimation for 7 days, the mice were randomly assigned to seven groups. Mice received water by intragastric administration (normal group, group 1); mice treated with D-galactose (800 mg/kg/day body weight) by abdominal injection (model group, group 2); mice treated with D-galactose (800 mg/kg/day body weight) by abdominal injection who received a pill containing six ingredients with rehmannia (0.68 g/kg/day body weight) by oral administration (positive control group, group 3); mice treated with D-galactose who received 20 or 100 mg/kg body weight EPS by intragastric administration (groups 4 and 5); mice treated with D-galactose who received 20 or 100 mg/kg body weight P-EPS by intragastric administration (groups 6, 7). All groups were administered their respective doses intragastrically once per day at approximately the same time interval of 24 h for 48 consecutive days.

2.5.2. Organ indices

The organ indices were calculated according to the following formula: organ indices (mg/g) = organ weight (mg)/body weight (g).

2.5.3. Measurement of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, and the malondialdehyde (MDA) level in serum and the livers of mice

After the last intragastric administration, mice in all groups were sacrificed. Serum was obtained after the blood of the mice was centrifuged (3000 rpm, 10 min, 4 °C). The antioxidant enzyme activities (CAT, SOD, GSH-Px) and MDA levels in the serums were analyzed.

The liver of each mouse was removed and washed with ice-cold physiological saline. Ten percent (w/v) liver, which was suspended in ice-cold physiological saline, was homogenized. Homogenate was centrifuged (3000 rpm, 4 °C, 10 min) to obtain the supernatant. The antioxidant enzyme activity (CAT, SOD and GSH-Px) and the MDA levels were measured in the supernatant of the liver homogenate.

The antioxidant enzyme activity (CAT, SOD and GSH-Px) and the MDA levels were analyzed by using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the instructions of manufacturer.

2.5.4. Ultramicrostructure of hepatic tissue

Livers of mice were treated according to the previous method described by Sun, Pan, Guo, and Li (2012). A JEOL JEM-1010 electron microscope (JEOL, Tokyo, Japan) was used to capture the micrographs at an acceleration voltage of 80 kV.

2.6. Statistical analysis

The data was expressed as mean ± standard deviation (SD). One-way ANOVA, Duncan's multiple range test and Student's *t*-test were used to analyze the data using SPSS 12.0 software.

3. Results and discussion

3.1. UV and IR analysis of EPS and P-EPS

Purified EPS and P-EPS had no proteins or nucleic acids because there was no absorption at 280 or 260 nm in the UV spectrum (Fig. 1).

Fig. 1 shows IR analysis of purified EPS and P-EPS. The bands at 3400 cm^{-1} suggested hydroxyl stretching vibrations. The IR absorption bands in the 2927 cm^{-1} region and at 1650 cm^{-1} corresponded to, respectively, C—H stretching vibrations and —OH bending vibrations. The absorptions at 1078 cm^{-1} suggested a pyranose form of glucosyl residue. For P-EPS, the absorptions at 1257 cm^{-1} indicated P=O stretching vibrations. The absorptions at 680,511 cm^{-1} suggested P—O—C stretching vibrations. The above

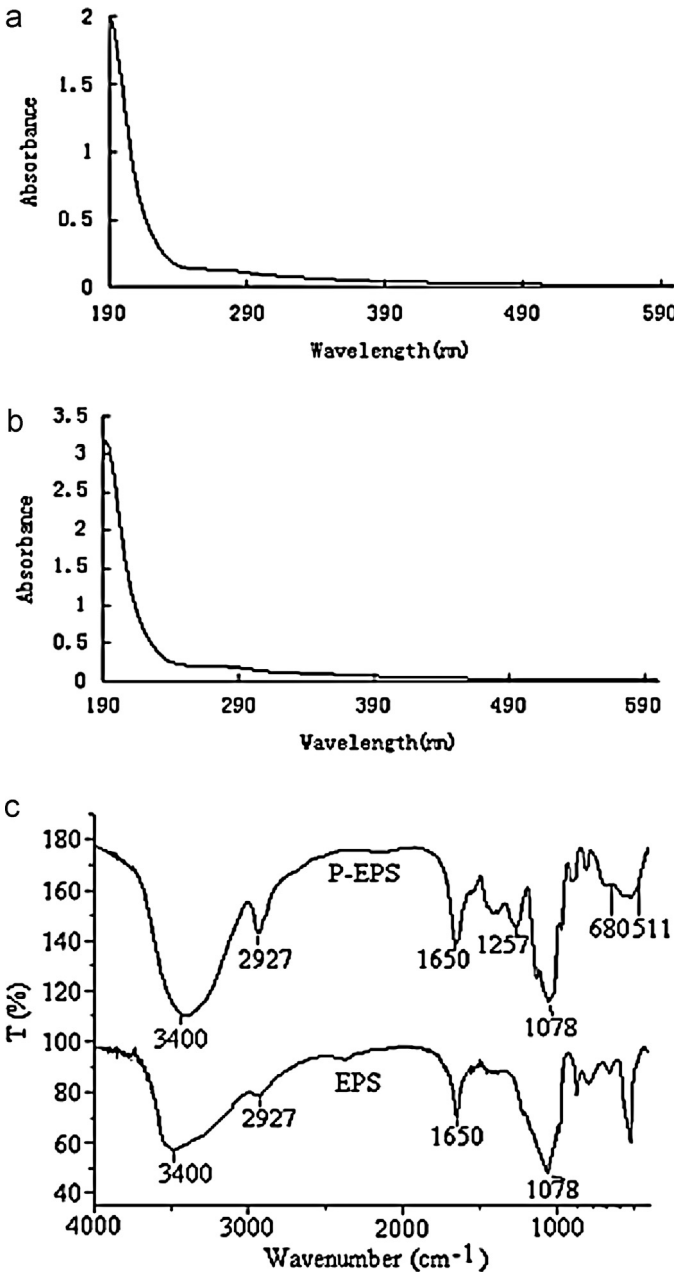

Fig. 1. UV–vis absorption spectra of EPS (a) and P-EPS (b); infrared (IR) analysis of EPS and P-EPS (c).

results showed that P-EPS has the same general structure as EPS. The addition of SHMP did not change the general structure of EPS, and the synthesis of P-EPS was successful. The phosphorus content of EPS was 1.639 mg/g.

Table 2
O^{2-}• scavenging ability of EPS and P-EPS.

Scavenging ability (U/L)	Concentration (mg/mL)					
	0.25	1.0	2.0	4.0	8.0	12.0
EPS	32.81	36.07	42.83	50.78	96.09	116.40
P-EPS	17.97	20.42	24.51	32.03	117.97	199.20

3.2. EPS and P-EPS in vitro antioxidant activity

3.2.1. EPS and P-EPS hydroxyl radical (OH•) scavenging activity

Hydroxyl radicals (OH•) are potent oxidants that can react with biological molecules and cause serious damage to living cells (Pan & Mei, 2010). Table 1 illustrates that EPS and P-EPS exhibited hydroxyl radical scavenging activity. The hydroxyl radical scavenging activity of P-EPS was greater than that of EPS at the same concentration. The hydroxyl radical scavenging activity of P-EPS was concentration dependent.

3.2.2. EPS and P-EPS superoxide anion (O^{2-}•) scavenging activity

Superoxide anions (O^{2-}•) are precursors of active free radicals, which can induce tissue damage by reacting with biological macromolecules (Halliwell & Gutteridge, 1984). The superoxide anion scavenging activity of EPS and P-EPS increased in a concentration-dependent manner (Table 2). P-EPS exhibited greater superoxide anion scavenging activity (at 8.0 and 12.0 mg/mL) than EPS at the same concentration.

3.2.3. EPS and P-EPS DPPH scavenging activity

EPS and P-EPS DPPH scavenging activity at different concentrations is shown in Table 3. EPS and P-EPS DPPH scavenging activity were concentration dependent. P-EPS exhibited greater DPPH scavenging activity than EPS.

3.3. Antioxidant activities of EPS and P-EPS in vivo

3.3.1. Effects of EPS and P-EPS on organ indices

The D-galactose treated group (2) had the lowest organ index, which demonstrated that the model was successfully built (Table 4). The EPS and P-EPS treatment groups (4, 5, 6, and 7) showed a significant increase ($P < 0.05$) in the liver, spleen and thymus indices compared with the model control group (2). The high-dose EPS treatment group (group 6) showed a significant increase ($P < 0.05$) in the liver and spleen indices compared with the positive control group (3). The high-dose P-EPS treatment group (7) showed a significant increase ($P < 0.05$) in the liver, kidney and spleen indices compared with the positive control group (3). These results indicated that EPS and P-EPS can repair the organ damage caused by D-galactose.

3.3.2. Effects of EPS and P-EPS on SOD, CAT and GSH-Px activity, and MDA content in serum of mice

As shown in Table 5, the EPS and P-EPS groups (4, 5, 6 and 7) showed a significant increase ($P < 0.05$) in enzymatic activity (SOD,

Table 1
Hydroxyl radical scavenging ability of EPS and P-EPS.

Scavenging ability (%)	Concentration (mg/mL)				
	1.0	3.0	7.0	10.0	20.0
EPS	23.73 ± 0.37*	24.17 ± 0.43*	26.80 ± 0.15*	29.12 ± 0.31*	33.97 ± 0.11*
P-EPS	29.01 ± 0.55*	38.56 ± 0.61*	74.09 ± 0.32*	87.61 ± 0.51*	98.52 ± 0.39
Vc	92.17 ± 0.17	97.57 ± 0.62	99.91 ± 0.35	100.07 ± 0.13	100.14 ± 0.22

Tests were carried out in triplicate for all experiments. All data was presented as means ± standard deviations (SD). Significant differences between treatments were tested by analysis of variance (ANOVA) followed by a comparison between treatments using Fisher's least significant difference (LSD) method, with levels of significance of $P < 0.05$ and *$P < 0.01$ compared with the Vc group.

Table 3
DPPH radical scavenging ability of EPS and P-EPS.

Scavenging ability (%)	Concentration (mg/mL)					EC_{50}
	0.1	0.2	0.4	1.0	4.0	
EPS	10.80 ± 0.86*	11.07 ± 0.94*	11.19 ± 1.37*	17.43 ± 0.69*	49.42 ± 0.98*	4.09
P-EPS	13.34 ± 1.03*	15.16 ± 0.49*	18.81 ± 0.61*	24.88 ± 1.72*	56.27 ± 1.05*	4.72
Vc	94.11 ± 0.13	94.12 ± 0.54	94.44 ± 1.15	94.65 ± 0.93	94.46 ± 0.87	0.05

Tests were carried out in triplicate for all experiments. All data was presented as means ± standard deviations (SD). Significant differences between treatments were tested by ANOVA followed by a comparison between treatments performed using Fisher's LSD method, with levels of significance of $P < 0.05$ and $^{*}P < 0.01$ compared with the Vc group.

Table 4
Effects of EPS-2 and P-EPS on organ indices of mice (mg/g).

Group	Heart	Liver	Kidney	Spleen	Thymus
1	5.01 ± 0.68	46.17 ± 1.18**	14.89 ± 0.89	4.14 ± 0.56	1.65 ± 0.48**
2	4.63 ± 0.58	41.54 ± 1.14##	14.39 ± 1.57	3.72 ± 0.34	1.16 ± 0.08
3	4.87 ± 0.60	46.21 ± 1.52**	14.60 ± 1.41	3.97 ± 0.66	1.52 ± 0.37
4	4.82 ± 0.39	45.03 ± 0.94**	14.54 ± 0.81	3.91 ± 0.25*	1.39 ± 0.39
5	4.89 ± 0.42	51.68 ± 1.65**,##	14.83 ± 1.30	4.21 ± 0.60**,#	1.55 ± 0.21*
6	4.88 ± 0.64	45.24 ± 0.63**	14.74 ± 0.59	4.39 ± 1.36**,#	1.53 ± 0.31*
7	5.08 ± 0.35	53.29 ± 0.98**,##	15.70 ± 1.00##	4.71 ± 0.78**,#	1.64 ± 0.49**

Tests were carried out in triplicate for all experiments. All data was presented as means ± standard deviations (SD). Significant differences between treatments were tested by ANOVA followed by a comparison between treatments performed using Fisher's LSD method. Differences were considered to be statistically significant if $P < 0.05$.
* $P < 0.05$, compared with the model group (group 2).
** $P < 0.01$, compared with the model group (2).
# $P < 0.05$ compared with the positive control group (3).
$P < 0.01$ compared with the positive control group (3).

Table 5
Effect of EPS-2 and P-EPS on SOD, CAT and GSH-Px activity and MDA content in mice serum.

Group	SOD (U/mL)	GSH-Px (U/mL)	CAT (U/mg Hb)	MDA (nmol/mL)
1	356.26 ± 16.28**	303.57 ± 19.41**,##	58.49 ± 3.62**	3.91 ± 0.64
2	282.09 ± 6.29##	277.14 ± 15.14##	49.77 ± 3.71##	4.20 ± 0.39
3	340.92 ± 6.82**	334.69 ± 13.50**	58.06 ± 6.61**	3.96 ± 0.34
4	311.39 ± 7.58**,##	301.84 ± 11.45**,##	55.56 ± 7.51*	3.97 ± 0.30
5	327.69 ± 16.01**	318.16 ± 15.11**,#	58.47 ± 5.28**	3.83 ± 0.28*
6	327.08 ± 18.88**	334.08 ± 10.38**	57.78 ± 3.96**	3.89 ± 0.27
7	341.73 ± 9.49**	360.00 ± 13.68**,#	62.89 ± 6.01**	3.77 ± 0.40*

Tests were carried out in triplicate for all experiments. All data was presented as means ± standard deviations (SD). Significant differences between treatments were tested by ANOVA followed by a comparison between treatments performed using Fisher's LSD method. Differences were considered to be statistically significant if $P < 0.05$.
* $P < 0.05$, compared with the model group (2).
** $P < 0.01$, compared with the model group (2).
# $P < 0.05$ compared with the positive control group (3).
$P < 0.01$ compared with the positive control group (3).

Table 6
Effects of EPS and P-EPS on SOD, CAT and GSH-Px activity, and MDA levels in mice livers.

Group	SOD (U/mL)	GSH-Px (U/mL)	CAT (U/mg Hb)	MDA (nmol/mL)
1	19.05 ± 0.79**	96.72 ± 9.67	22.61 ± 1.93**	1.00 ± 0.18**
2	17.20 ± 0.38##	91.55 ± 3.50	20.33 ± 1.10#	1.20 ± 0.10##
3	18.88 ± 0.26**	96.30 ± 7.81	21.94 ± 1.23*	0.93 ± 0.25**
4	19.19 ± 0.47**	98.94 ± 7.12*	22.18 ± 1.51**	1.01 ± 0.15*
5	19.43 ± 0.54**	104.10 ± 6.72**,#	22.94 ± 0.97**	0.86 ± 0.10**
6	19.54 ± 0.76**	100.24 ± 3.66**	22.76 ± 0.55**	0.91 ± 0.15**
7	20.42 ± 1.52**,##	105.55 ± 3.16**,##	23.26 ± 1.36**,#	0.83 ± 0.06**

Tests were carried out in triplicate for all experiments. All data was presented as means ± standard deviations (SD). Significant differences between treatments were tested by ANOVA followed by a comparison between treatments performed using Fisher's LSD method. Differences were considered to be statistically significant if $P < 0.05$.
* $P < 0.05$, compared with the model group (2).
** $P < 0.01$, compared with the model group (2).
# $P < 0.05$ compared with the positive control group (3).
$P < 0.01$ compared with the positive control group (3).

CAT and GSH-Px) in serum compared with the model mice (group 2). The MDA levels in the serum of the EPS and P-EPS groups (4, 5, 6 and 7) significantly decreased ($P < 0.05$) compared with the model group (2).

As shown in Table 6, the EPS and P-EPS groups (4, 5, 6 and 7) showed a significant ($P < 0.05$) increase in enzymatic activity (SOD, CAT and GSH-Px) and a decrease in the MDA levels in their livers compared to the model mice (group 2). The high-dose P-EPS group

showed a significant increase in SOD, CAT and GSH-Px activity compared to the positive control group (3), which indicated that P-EPS is a good antioxidant.

Superoxide radicals are decomposed into hydrogen peroxide (H_2O_2) by SOD. Hydrogen peroxide is decomposed into water and oxygen by CAT and GSH-Px, which can prevent the formation of hydroxyl radicals (Pan & Mei, 2010).

MDA is a hallmark of lipid peroxidation and displays cytotoxic activity. The amount of MDA reflects the formation of lipid peroxides, which can damage cell membranes and hepatic tissue (Chen et al., 2011b).

In our study, we found that EPS and P-EPS displayed antioxidant activities *in vivo*, which showed that they play an important role in protecting biological systems.

3.4. Protective effect of EPS and P-EPS against oxidative stress during D-galactose-induced aging in mice

The cell stayed in good condition in the normal control group (1). The nuclear membrane boundaries and nuclear pores were clearly observed. A significant amount of rough endoplasmic reticulum was seen in good order. The rough endoplasmic reticulum was studded with ribosomes on the cytosolic face. The architecture of the mitochondria was intact. The mitochondrial bilayers and cristae can be clearly seen in Fig. 2.

In the model group (2), the hepatocyte was damaged by D-galactose. The nuclear membrane was vague and had irregular sunken sections in certain locations. The rough endoplasmic reticulum was fractured and had even disappeared in places. The mitochondria swelled and large vacuoles were observed.

In the positive control group (3), the nuclear membrane boundaries and nuclear pores were clearly observed. The rough endoplasmic reticulum was neatly arranged and it was studded with ribosomes. The ultrastructure of the cell was similar to that seen in the normal group (1). However, some vacuoles were observed in group 3.

In the low-dose and high-dose EPS groups (4 and 5), the nuclear membrane boundaries and nuclear pores were observed more clearly than in group 3, although there were some vacuoles seen in the low-dose EPS group (4). The cells in the high-dose EPS group (5) were in good condition. Little vacuoles were observed in group 5. These findings indicated that a high dose of EPS more effectively protected cells than a low dose of EPS.

The cells in the low- and high-dose P-EPS groups (6 and 7) showed good properties, although the cells in the high-dose group were in the best condition. Rough endoplasmic reticula were clearly seen in groups 6 and 7. Compared with the normal control group, little vacuoles were seen in groups 6 and 7. The mitochondria had an intact architecture and the mitochondrial bilayers and cristae were clearly observed. The microscope images suggested that high doses of EPS and P-EPS played a role in preventing the progression of D-galactose-induced oxidative stress of hepatocytes *in vivo*. P-EPS appeared to protect against oxidative damage caused by D-galactose.

In this study, we found that compared to EPS, P-EPS exhibited more effective *in vitro* and *in vivo* antioxidant activity. The phosphorylated polysaccharides enhanced the biological function of the polysaccharides. Chen et al. reported that phosphorylated polysaccharide from *Portulaca oleracea* L. showed greater antioxidant activity (Chen et al., 2011b). In our previous research, we reported that selenium exopolysaccharide produced by *Lactococcus lactis* subsp. *lactis* showed higher antioxidant and immunomodulatory activity than EPS (Guo et al., 2013). With further development, P-EPS could be used as a new antioxidant in the food and pharmaceutical industries.

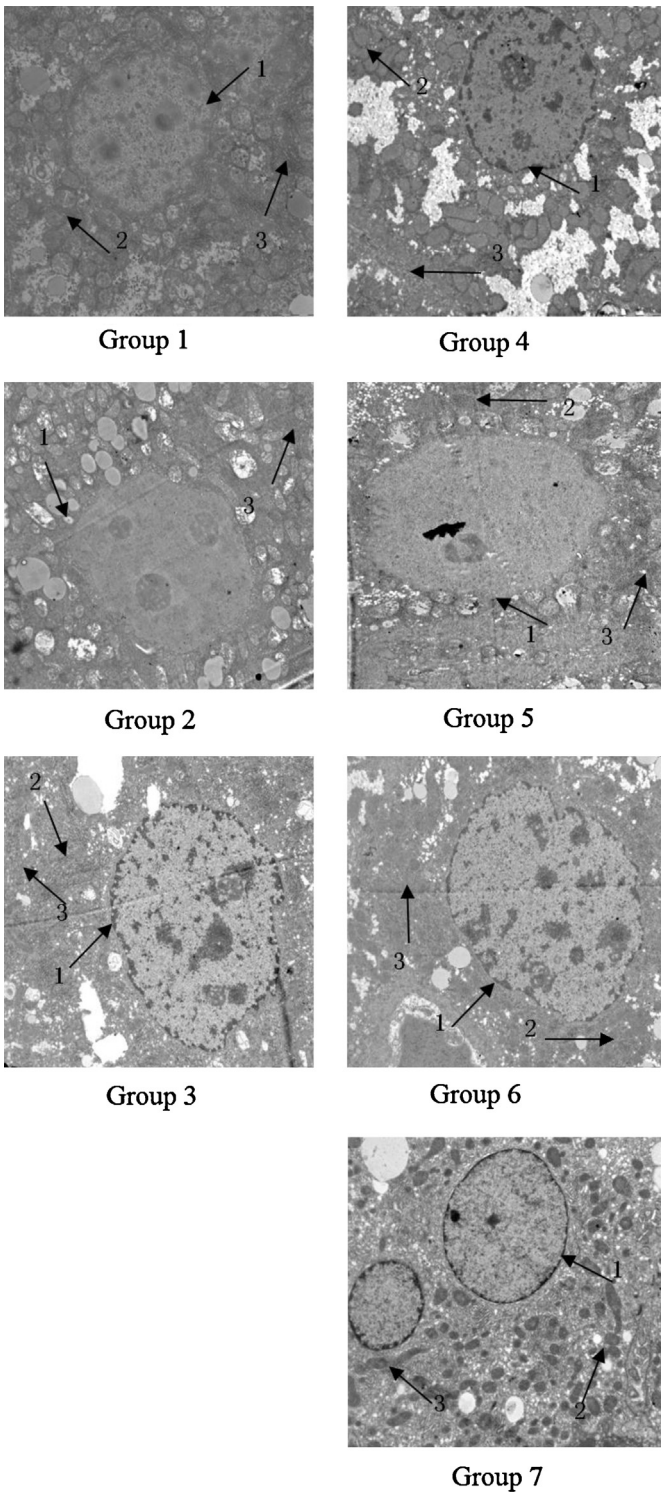

Fig. 2. Antioxidant activity of EPS and P-EPS *in vivo*. (1) Cell nuclear membrane; (2) mitochondria; (3) rough endoplasmic reticulum. Magnification: 10,000×.

4. Conclusions

P-EPS was synthesized by the EPS of *Lactococcus lactis* subsp. *lactis* and sodium hexametaphosphate (SHMP). *In vitro* antioxidant testing showed that EPS and P-EPS displayed significant superoxide anion ($O^{2-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and DPPH scavenging activity. EPS and P-EPS also increased CAT, SOD and GSH-Px activity in the

serum and livers of mice, as well as decreased MDA levels in serum and mice livers in an *in vivo* experiment. High doses of EPS and P-EPS can prevent the progression of D-galactose-induced oxidative stress of hepatocytes *in vivo*. P-EPS appeared to protect against oxidative damage caused by D-galactose.

Acknowledgements

This work was supported by the Natural Science Foundation of Zhejiang Province (Z3110211 and LQ12C20003) and of China (41276121 and 31101314), the State Science and Technology Ministry of the People's Republic of China (2012BAK08B01), the Key Agriculture Project of Major Science and Technology Projects of Zhejiang Province (2012C12016-1), the Project of Ningbo Science and Technology Department (2011C11017) and the K.C. Wong Magna Fund in Ningbo University. This work was also supported by the Natural Science Foundation of Jiangsu Province (Grant BK2011787).

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