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Phosphorylation of low-molecular-weight polysaccharide from *Enteromorpha linza* with antioxidant activity

Xiaomei Wang[a,b,*], Zhongshan Zhang[a,b], Qin Yao[a], Mingxing Zhao[a], Huimin Qi[c]

[a] Department of Medicine, Huzhou Teachers College, Huzhou 313000, China
[b] The Key Laboratory for Chemical Biology of Fujian Province, Xiamen 361000, China
[c] School of Pharmaceutical and Biological Sciences, Weifang Medical University, Weifang 261053, China

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ABSTRACT

In this study, the phosphorylated derivative of low-molecular-weight polysaccharide from *Enteromorpha linza* was prepared. And then the antioxidant activities of all the samples were investigated including scavenging effects of superoxide and hydroxyl radicals and reducing power. The results of chemical composition, FT-IR spectrum and DSC analysis showed the modifications of polysaccharide were successful. And moreover, the phosphorylated derivative showed excellent antioxidant activity in these three assays, so this derivative needs to be attention in further.

1. Introduction

Polysaccharide phosphate ester was an important derivative of polysaccharide. It has been widely reported existing in plant, animal and microorganism (Casteren et al., 1999; Parolis, Parolis, Kenne, Meldal, & Bock, 1998; Senchenkova et al., 2004; Severn, Furneaux, Falshaw, & Atkinson, 1998; Wang et al., 1999). However, there was few amount and kind of oligosaccharide or polysaccharide phosphate ester naturally extracted from organisms, which affected the study about the activity and function of sugar phosphate ester. Therefore, the researchers artificially synthesize a wide variety of sugar phosphate ester and their analogs to systematically study their structure–activity. As a matter of fact, during the past thirty years it has been reported monosaccharide, oligosaccharide and polysaccharide phosphate ester derivative exhibited good antitumor (Chen, Xu, Zhang, & Zeng, 2009; Huang & Zhang, 2011), antibacterial (Ruttens & Kováč, 2006), antiviral (Liu, Wan, Shi, & Lu, 2011) and antioxidant activity (Wu & Dai, 2010). It was also reported that phosphated crosslinked guar was prepared for colon-specific drug delivery aimed at localizing drugs in the distal portions of the small bowel (Gliko-Kabir, Yagen, Penhasi, & Rubinstein, 2000). And highly phosphorylated cellulose gels were synthesized and demonstrated their biocompatibility and ability to promote the formation of calcium phosphates (Granja et al., 2000). Thus it can be seen the study about sugar phosphate ester is essential to find the regularity of phosphorylation and design the phosphate ester derivative with better bioactivity.

Enteromorpha linza is a kind of wild green algae on the coast of intertidal zone with its rich resource and nutrient, food and medicinal value (Jamieson & Reid, 1972). It is reported that it could tolerate a wide range of salinities and has often been used as a heavy metal monitor (Say, Burrows, & Whitton, 1986). However, to our knowledge, little information is available on the polysaccharides from this alga. In our previous study, polysaccharide from *E. linza* was isolated, purified and evaluated as a novel antioxidant and humectants (Shi et al., 2009). In order to deeply study the structure–activity of polysaccharide from *E. linza*, several methods was used to obtain different derivatives. In the present study, a phosphorylated method was developed to improve water solubility of polysaccharide from *E. linza*. Antioxidant activity of the resulted phosphorylated derivative was analyzed to evaluate its water solubility. The relationship between the nature of functionalized groups and the chemically modified derivatives and their antioxidant activity were also discussed.

2. Materials and methods

2.1. Chemicals

E. linza was collected on the coast of Ningbo in October 2011. The fresh alga was soon washed; sun dried and kept in plastic bags at room temperature for use.

* Corresponding author at: Department of Medicine, Huzhou Teachers College, Huzhou 313000, China. Tel.: +86 572 2321166; fax: +86 572 2321166.
E-mail address: wangxiaomei@hutc.zj.cn (X. Wang).

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Ferrozine, nicotinamide adenine dinucleotidereduced (NADH), hydrogen peroxide (H_2O_2), ethylene diamine tetra-acetic acid (EDTA), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ferric chloride and potassium ferricyanide were purchased from Sigma Chemicals Co. All other chemicals and reagents, unless otherwise specified, were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3600 Da.

Sulfate content was determined by barium chloride method (Kawai, Seno, & Anno, 1966). Total sugar content was determined by phenol-sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956) using rhamnose as standard. The total phosphate was determined by the wet ashing method (Blennow, Nielsen, Baunsgaard, Mikkelsena, & Engelsen, 2002).

The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} region.

2.2. Polysaccharide and degradation

The nature polysaccharide was extracted from *E. linza* in hot water with the method of previous study. A solution of nature polysaccharide (10 mg/mL) was added in 10 mM ascorbic acid and 10 mM H_2O_2, stirred for 2 h, and then precipitated to give the degraded product named after LEP (Zhang, Wang, Mo, & Qi, 2013).

2.3. Phosphorylation of polysaccharide

The phosphorylation of polysaccharide was prepared by the modified method of Inoue, Kawamoto, Nakajima, Kohno, and Kadoya (1983). 2.0 g of LEP dissolved in 100 mL of FA was added into tributylamine (15 mL) and polyphosphoric acid (6.0 g). The mixture was stirred for 20 h at room temperature, and then poured into ethanol (600 mL). The resulting precipitate was collected by centrifugation, and then dissolved in water, dialyzed against distilled water for 24 h. The resultant was concentrated and lyophilized to give phosphorylated polysaccharide (PEP).

2.4. DSC thermogram

A Perkin-Elmer Diamond differential scanning calorimeter was used to obtain the DSC thermogram of the samples using a mass of approx. 1.00 g and a scan rate of 10 °C/min in the temperature range from 25 to 600 °C.

2.5. Antioxidant activity

2.5.1. Superoxide radical assay

The superoxide radical scavenging abilities of all samples were assessed by the modified method of Nishimiki, Rao, and Yagi (1972). In this experiment, the sample (0.5–50.0 μg/mL) in 4.5 mL of Tris–HCl buffer (16 mM, pH 8.0) was added into 0.5 mL of NBT (300 μM) solution, 0.5 mL of NADH (468 μM) and 0.5 mL of PMS (60 μM). The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left(\frac{1 - A_{\text{sample}\,560}}{A_{\text{control}\,560}}\right) \times 100$$

2.5.2. Hydroxyl radical assay

The reaction mixture, containing all different derivatives (0.6–7.0 mg/mL), was incubated with EDTA-Fe^{2+} (2.0 mM), saffron (360 μg/mL), and H_2O_2 (3%) in potassium phosphate buffer (150 mM, pH 7.4), and was incubated for 30 min at 37 °C (Wang et al., 1994). The absorbance was read at 520 nm against a blank. Hydroxyl radical bleached the saffron, so decreased absorbance of the reaction mixture indicated a decrease in hydroxyl radical scavenging ability. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

where A_0 is the absorbance of the control (without samples) and A_1 is the absorbance of the mixture containing samples.

2.5.3. Reducing power assay

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, 1.0 mL of different concentration of samples (0.47–6.0 mg/mL) in phosphate buffer (0.2 M, pH 6.6) was mixed with 1.0 mL of potassium ferricyanide (1%, w/v), and was incubated at 50 °C for 20 min. Afterwards, 2.0 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. Then the solution was mixed with 1.2 mL ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated increased reducing power.

3. Results and discussion

3.1. Phosphorylation of LEP

A neutral polysaccharide (EP) extracted from green algae *E. linza* was degraded with previous method. The degraded product LEP was readily phosphorylated with polyphosphoric acid to obtain derivative PEP. Until now, several methods regarding the synthesis of phosphorylated derivatives were reported such as polyphosphoric acid/tributylamine/dimethyl formamide method (Heinze, Liebert, Heublein, & Hornig, 2006), H_3PO_4/faty acids esters method (Suzuki, Suzuki, & Matsumoto, 1975), H_3PO_4/palmitic acid/tertiary amines method (Heinze et al., 2006), H_3PO_4/urea method (Makarova, Aptova, Litvak, & Raldugina, 1978) and H_3PO_4/formamide method (Sato et al., 2004). Among these methods, H_3PO_4 was commonly used as phosphorylated regent on higher temperature, which usually lead to the degraded of polysaccharide (Williams et al., 1991). In addition, the polysaccharide from *E. linza* was complicated and only soluble in FA among the organic solvents. Therefore, polyphosphoric acid/tributylamine/formamide method was used in this study. The results suggested that when FA acted as the solvent in the reaction system, degree of phosphorylation in product increased obviously.

3.2. Chemical analysis

The chemical compositions of LEP and PEP were given in Table 1. Relatively to LEP, a slightly decrease of the molecular weight for PEP was observed. As shown in table, the total sugar and sulfate contents of the derivative PEP were lower than LEP because of the addition of phosphate groups.

Fig. 1 showed the FT-IR spectrums of the products. Typical signals of polysaccharide at 3464, 2934, 1667, 1418, 1257 and

Table 1
Yield and chemical composition of the samples (% w/w of dry weight).

Samples	Total sugar (%)	Sulfate (%)	Phosphate (%)	Uronic acids (%)	Molecular weight (Da)
LEP	62.7	21.8	–	12.1	11,618
PEP	56.5	17.3	4.05	10.6	10,230

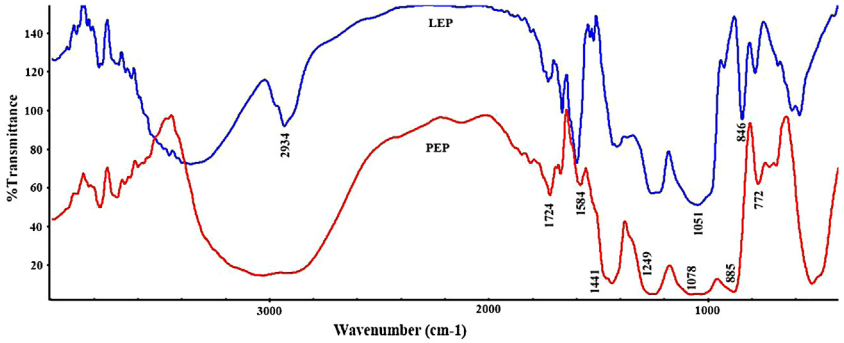

Fig. 1. FT-IR spectra of samples LEP and PEP.

1051 cm^{-1} were clear for LEP. They correspond to the O—H stretching vibrations, the C—H stretching vibrations, the carbonyl C=O vibrations in uronic acid, the carbonyl C—O stretching vibrations, the S=O asymmetric stretching vibration of sulfate group, and the C—O—H in glucosidal bond or C—O—C stretching vibrations in ring. The weak signal at 920 cm^{-1} stand for β-pyranose ring vibration. The strong signal at 845 cm^{-1} may be C—H deformation angle vibration in α-marginal group, or C—O—S in sulfate group. For the PEP, the signal at 1222 cm^{-1} indicated the P=O stretching vibration was not evident because they overlapped with the signals of LEP. In the spectrum of PEP several new bands appeared, a band at a shoulder at 1024 cm^{-1} attributable to the P—OH bond, and a band at 885 cm^{-1} corresponding to the P—O—C bonds (Hesse, Meier, & Zeeh, 1984).

3.3. DSC thermogram

DSC was usually used to investigate the possibility of interaction between both components and measure the extent of disruption of the hydrogen bonds as well as quantify the heat energy (Bilideris, Maurice, & Vose, 1980; Fringant, Desbrieres, & Rinaudo, 1996; Zhang et al., 2012). The effect of phosphorylation on the thermal properties of LEP was examined by DSC in the temperature range from 20 to 600 °C. The results obtained from the DSC curves of LEP and phosphorylated sample PEP was shown in Fig. 2. As observed from figure, an endothermic depression can be observed at about 34 °C, where the elimination of water has taken place, and they started to decompose at 221 and 236 °C. The exothermic peak, which represents heat released from the sample, was observed at a maximum temperature of 271 and 248 °C for LEP and PEP. This indicated that the PEP was a new substance instead of raw material, and the nature sample was more stable than the modified derivative. This lower thermal stability of the PEP was undoubtedly due to the disintegration of intramolecular interactions such as hydrogen bonds between polymer molecules. And in addition, the two exothermic peaks were asymmetric due to overlapping endothermic response from both unreacted sample and the reaction product (Zhuang & Steiner, 1993).

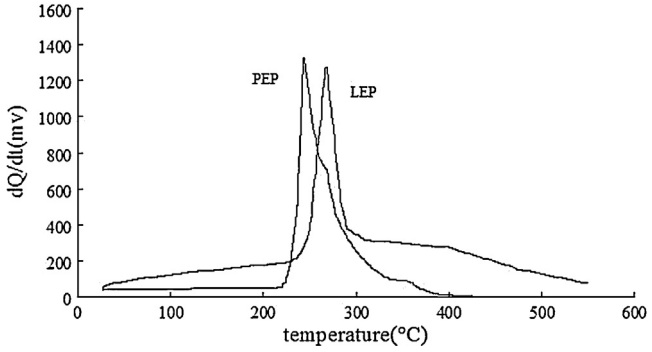

Fig. 2. DSC thermograms of samples LEP and PEP.

3.4. Superoxide radical assay

The superoxide radical ($^{\bullet}O_2^-$) was a highly toxic species that was generated in a PMS/NADH system for being assayed in the reduction of NBT (Banerjee, Dasgupta, & De, 2005). Fig. 3 depicted the inhibitory effects on the superoxide radical of two samples. For two samples, at the concentration below 42 μg/mL, the scavenging effects significantly increased with increasing concentration, and at the concentration over 5 μg/mL, the scavenging effects increased slowly. The IC_{50} values of LEP and PEP were 12.6 and 18.7 μg/mL, respectively.

It was reported that addition of electron-withdrawing groups to the pyrrole enhanced antioxidant activity (Yanagimoto, Lee, Ochi, & Shibamoto, 2002). In this study both sulfate and phosphate group were strong electron-withdrawing group, which could significantly increase the activity of scavenging radicals. Although superoxide was a relatively weak oxidant, it decomposed to form stronger, reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids. Furthermore, superoxides were also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Dahl & Richardson, 1978). The results above indicated that the antioxidant activities of two samples were related to their abilities to scavenge superoxide.

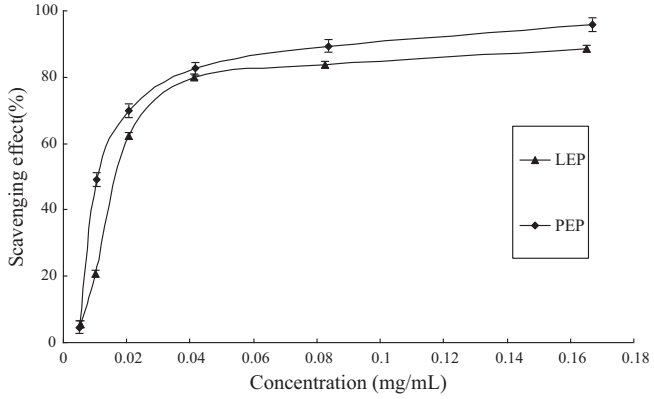

Fig. 3. Scavenging effects of LEP and PEP on superoxide radical. Values are means ± S.D. ($n = 3$).

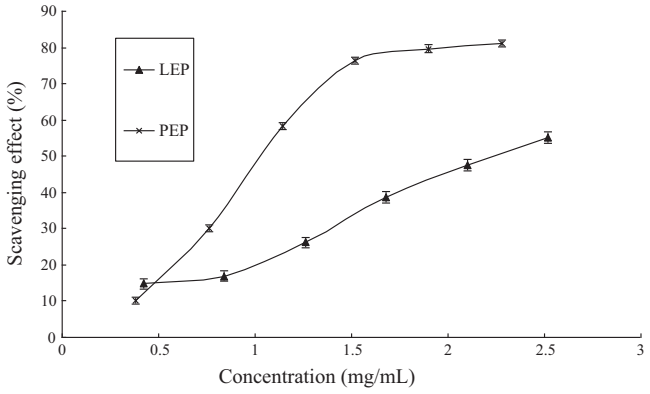

Fig. 4. Scavenging effects of LEP and PEP on hydroxyl radical. Values are means ± S.D. ($n = 3$).

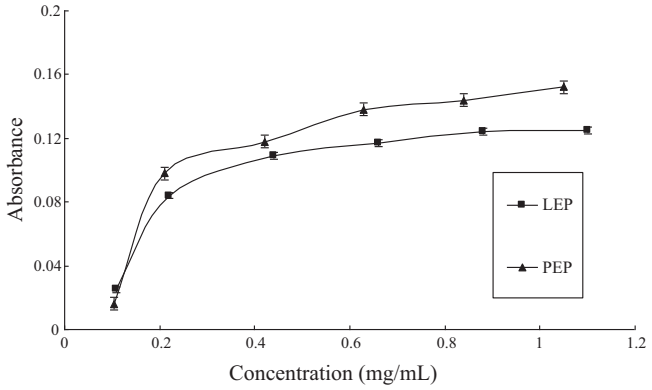

Fig. 5. Reducing power of LEP and PEP. Values are means ± S.D. ($n = 3$).

3.5. Hydroxyl radical assay

The scavenging effect of all samples was shown in Fig. 4. The hydroxyl radical, known to be generated through the Fenton reaction in this system, was scavenged by polysaccharide samples. For all the samples, the effects of scavenging hydroxyl radicals were in a concentration-dependent manner. The IC_{50} values of LEP and PEP were 0.82 and 2.33 mg/mL, respectively. PEP showed excellent scavenging effects and at the concentration of 2.28 mg/mL, the scavenging effects were 81.2%, which suggested the modification of the phosphate group take strong effect on scavenging action on the hydroxyl radicals.

For hydroxyl radical, there were two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may ligate to the metal ions which react with H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give hydroxyl radicals (Ueda, Saito, Shimazu, & Ozawa, 1996). Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases. Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage (Singh & Rajini, 2004). In the present study, phosphate groups had high nucleophilic characteristic and could chelate with metal ion, so the hydroxyl radical scavenging activities of phosphorylated derivatives were much stronger than LEP.

3.6. Reducing power assay

The reducing power of two samples was shown in Fig. 5. As shown in the figure, the reducing power of the samples correlated well with increasing concentrations. The reducing powers of LEP and PEP were 0.125 and 0.153 at the concentration of 1.15 and 1.08 mg/mL, respectively.

It has been previously reported that there was a direct correlation between antioxidant activities and reducing power of certain plant extracts (Duh, Du, & Yen, 1999). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, PEP showed excellent reducing power probably because of its high donating-hydrogen ability. It was considered that H on the phosphate group was easily contributed to reduce the Fe^{3+}/ferricyanide complex and then close the reaction.

4. Conclusion

The results of the present work indicated that phosphorylated derivative of low-molecular-weight polysaccharide from *E. linza* possessed antioxidant activities in certain assays. PEP may have a use as a possible supplement in the food and pharmaceutical industries. However, factors effecting and attributing to radical scavenging effect need to be further studied.

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