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Effect of phthaloylation on radical-scavenging and moisture-preserving activities of polysaccharide from *Enteromorpha linza*

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ABSTRACT

In this study, phthaloyl polysaccharide from *Enteromorpha linza* (PHEP) has been prepared in a homogeneous process by using 1,2-benzenedicarboxylic anhydride and 4-Dimethylaminopyridine as phthaloyl agent and catalyst, respectively. And then the radical-scavenging and moisture-preserving activities of the samples were investigated. The results of chemical composition and FT-IR analysis showed the phthaloyl modifications of polysaccharide were successful. And moreover, the derivative PHEP showed excellent radical-scavenging and moisture-preserving activities, so this derivative needs to be attention and studied in further.

1. Introduction

The activity of polysaccharide was usually affected by molecular weight and functional groups such as carboxyl, hydroxyl, amino, acetyl, sulfate groups (Ramesh & Tharanathan, 2003). Although natural polysaccharide shows a large number of bioactivities, the presence of various functional groups provides a broad space to chemical structural modification. And in addition, the modification is also important for the research of the structure-activity relationship of polysaccharide. Therefore, deep research into the structural modification method and mechanism could promote the application of polysaccharide. As a matter of fact, there were many reports about the study of sulfated, acetylated, phosphated, carboxymethylated derivatives of polysaccharide (Lee, Koizumi, Hayashi, & Hayashi, 2010; Liu et al., 2012; Silva et al., 2004; Suflet, Chitanu, & Desbrières, 2010). Besides the above methods, the phthaloylation was also previously studied although the reports were much for small molecular compounds rather than polysaccharide (Aziz, Majid, & Arof, 2012; Casimir, Guichard, & Briand, 2002; Saxena, Saxena, & Rai, 1992). However, the phthaloylation has been used as an important method for the modification of chitosan, one of the naturally abundant polysaccharides (Deemak, Wanichwecharungruang, Nonthabenjawan, & Jornjangjun, 2011; Feng & Dong, 2007; Kurita, Tomita, Tada, Nishimura, & Ishii, 1993; Torii, Ikeda, Shimojoh, & Kurita, 2009; Yalinca, Yilmaz, Taneri, & Bullici, 2013). And moreover, the phthaloylation of chitosan or other polysaccharides was reported to significantly improve the bioactivities of the derivatives (Badawy & Rabea, 2011; Qi et al., 2006; Wang, Jia, Song, Xie, & Jiang, 2013; Yi, Yang, Ying, Chen, & Wang, 2006).

In previous study, polysaccharide from *Enteromorpha linza*, a kind of wild green algae on the coast of intertidal zone in China, 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* (EP), several methods was used to obtain different derivatives. The low molecular weight polysaccharide (LEP) and its sulfated derivative (SEP) and phosphorylated derivative (PEP) were prepared in previous reports (Wang, Zhang, Yao, Zhao, & Qi, 2013a, 2013b; Zhang, Wang, Mo, & Qi, 2013). The results indicated that these derivatives possess higher bioactivities than raw material LEP.

In this study, the phthaloyl derivative of LEP was prepared and antioxidant activity and moisture-preserving of the derivative was analyzed. The relationship between the nature of functionalized groups and the chemically modified derivatives and their activity were also discussed.

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2. Materials and methods

2.1. Materials

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. The polysaccharide from *E. linza* (EP) and its low molecular weight polysaccharide (LEP) were prepared with the methods in previous reports (Zhang et al., 2013).

2.2. Analytical methods

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. Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard (Bitter & Muir, 1962).

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.

2.3. Phthaloylation of polysaccharide

A mixture of LEP (2.0 g) and formamide (80 mL) was heated at 60 °C for 30 min. Then, 4-Dimethylaminopyridine (DMAP) (0.1 g) and p-toluenesulfonyl chloride (p-TsCl) (7.0 g) was added and followed by dropwise addition of 1,2-benzenedicarboxylic anhydride (18 g). After reaction for 4 h at 60 °C, the mixture was terminated by pouring 50 mL of distilled water, cooled to room temperature, and precipitated with 85% ethanol. The precipitate was filtered off and washed three times with ethanol, and then dissolved in 100 mL distilled water. The solution was dialyzed against tap water for 48 h and distilled water for 24 h using 3600 Da Mw cutoff dialysis membranes. The product was then concentrated and lyophilized to give the product phthaloyl polysaccharide (PHEP).

2.4. Determination of phthaloyl group content

The phthaloyl group content was determined with the methods of ultraviolet spectrophotometer. 50 mg of phthalate was added into saturated NaOH to 10 mL standard solution. 3, 2, 1.6, 1.2, 0.8 and 0.4 mL standard solution was diluted with saturated NaOH to 5 mL and then the absorbance of these solutions was measured at 289 nm. The results were used to make the standard curve. 0.1 g of PHEP was dissolved in 10 mL water, and dropped into 90 mL saturated NaOH. The mixture reacts at 70–80 °C for 4 h. Then the absorbance was measured at 289 nm. The phthaloyl group content was determined according to the standard curve (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982).

2.5. Radical-scavenging 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:

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

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.

2.6. Moisture-preserving activities

2.6.1. Moisture absorption activity

All the samples were ground into fine powder and oven-dried at 100 °C for 4 h. Then they were placed in a sealed humidity chamber maintained by saturated K_2CO_3 (43% relative humidity; RH) and saturated $(NH_4)_2SO_4$ (81% RH) at 25 °C for indicated times. The weights of all the samples were obtained after 0 h, 4 h, 24 h, 48 h, 72 h and 96 h. The moisture absorption rate (Ra) of a sample was evaluated by the gain of weight:

$$\text{Ra}\ (\%) = \frac{W_t - W_0}{W_0} \times 100,$$

where W_0 was the weight of an oven-dried sample and W_t was the weight of the sample after moisture absorption for a specific time in the humidity chamber.

2.6.2. Moisture retention activity

The oven-dried samples (100 mg each) were added in distilled water and then placed in a humidification chamber dried silica gel containing saturated K_2CO_3 (43% RH) to dehydrate at 25 °C for the indicated times. The moisture retention rate (Rr) was evaluated by weight loss of the water after 0 h, 4 h, 24 h, 48 h, 72 h and 96 h.

$$\text{Rr}\ (\%) = \frac{W_t}{W_0} \times 100,$$

where W_0 was the weight of added distilled water, W_t was the weight of the water after a specific time.

3. Results and discussion

3.1. Phthaloylation of LEP

Phthaloylation is the introduction of the aryl radical $[C_6H_5(COO)_2^-]$ into a molecule. Dichlorophthalic anhydride or 1,2-benzenedicarboxylic anhydride in DMF solution is most common used for phthaloylation (Deemak et al., 2011; Yalinca et al., 2013). Yi et al. (2006) reported N-phthaloyl-chitosan was synthesized rapidly in acetic acid solution with pyridine as catalyst. Both two methods were not suitable for LEP because it were insoluble in DMF and acetic acid solution. In our previous paper, the synthesis of sulfated LEP in a homogeneous process by using sulfur trioxide as sulfating agent was reported. It was found that the functionalization reaction can be well controlled

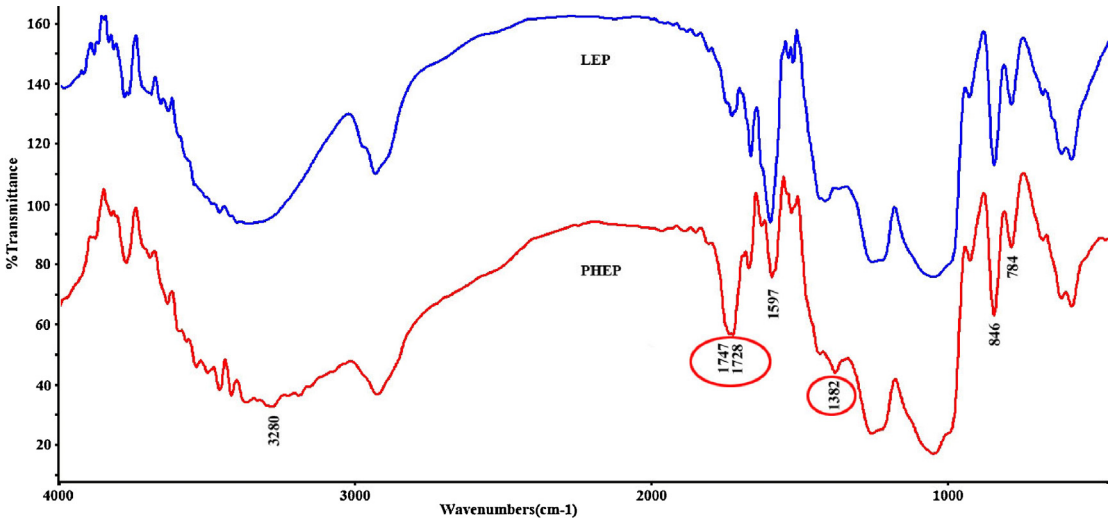

Fig. 1. FT-IR spectra of samples LEP and PHEP.

and carried out to a high degree of sulfation (Wang et al., 2013). In present study, this procedure was followed to prepare phthaloyl LEP in its well-solution agent formamide. In the phthaloylation experiments, p-TsCl was used to activate the hydroxyl groups and make it into an easy leaving group, which help in replacing —OH of the LEP with phthaloyl groups (Chen, Zhu, Cheng, Lu, & Chen, 2004; Tosh, Saikia, & Dass, 2000).

3.2. Chemical analysis

The chemical compositions of LEP and PHEP were determined and compared. From the results, the total sugar (51.03%), sulfate content (10.94%) and uronic acid (9.26%) of the derivative PHEP were lower than LEP (57.61%, 12.18%, 16.32%) because of the addition of phthaloyl group.

The phthaloyl group content was determined according to ultraviolet absorption of benzene ring. Benzene is a closed conjugate system, but when the benzene ring was linked with a substituent, electronic distribution will be changed. The most ultraviolet absorption wave number would be larger. In this study, phthaloyl group showed the strong absorption peak at 289 nm because of π–π conjugate between C=O with benzene ring. In present study, phthaloyl group content in PHEP was 13.27%.

Fig. 1 showed the FT-IR spectrums of the products. Typical signals of polysaccharide at 3464, 2934, 1667, 1418, 1257 and 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 or esters, 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. For PHEP, a doublet was observed at 1728 and 1747 cm^{-1}, which, as in the case of other cyclic dicarbonyl compounds, was the result of the interaction of vibrations of two carbonyl groups with a resonance splitting (Tosh et al., 2000). Further evidence of phthaloylation was provided by observing the intensity of the band at about 1382 cm^{-1}, due to the vibrations of —COO— for carbonyl (Bellamy, 1958).

3.3. Radical-scavenging activities

Fig. 2 depicted the inhibitory effects on the superoxide radical of two samples. Significant scavenging (53–86%) of superoxide radicals was evident at all the tested concentrations of LEP and PHEP (4.1–77.5 μg/mL). The effect on oxidative damage, induced

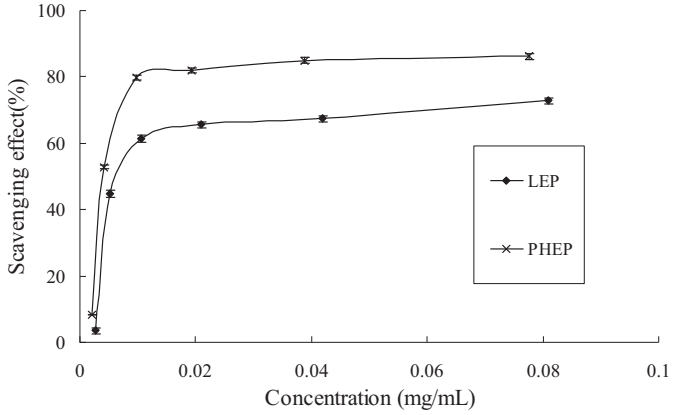

Fig. 2. Scavenging effects of LEP and PHEP on superoxide radical. Values are means ± S.D. (n = 3).

by Fe^{3+}/H_2O_2 on deoxyribose, as measured by the thiobarbituric acid method, was plotted in Fig. 3. While a marginal inhibition was evident at the lowest concentration, nearly 77.5% inhibition was observed at the highest concentration. As for PHEP, the IC_{50} values of two radical-scavenging effects were 3.71 μg/mL and 0.98 mg/mL, respectively, much lower than the data (7.02 μg/mL and 1.64 mg/mL) observed with raw material LEP.

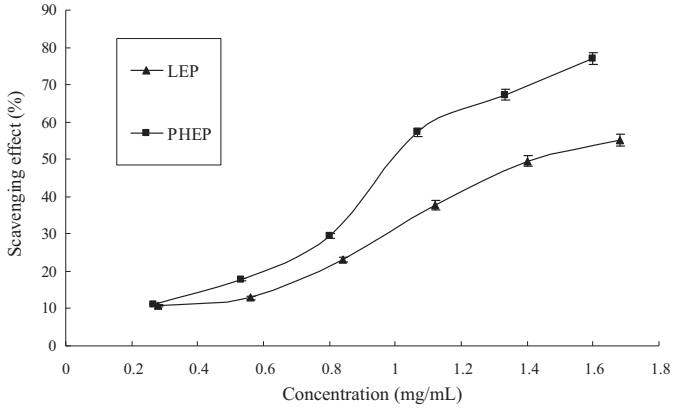

Fig. 3. Scavenging effects of LEP and PHEP on hydroxyl radical. Values are means ± S.D. (n = 3).

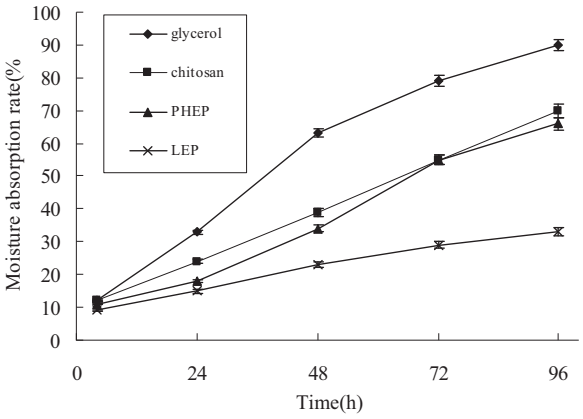

Fig. 4. Moisture absorption by samples at RH = 81% ($n = 3$).

It was reported that addition of electron-withdrawing groups to the pyrrole enhanced antioxidant activity (Yanagimoto, Lee, Ochi, & Shibamoto, 2002). In this study, carbonyl was electron-withdrawing group and the benzene ring stronger this electron-withdrawing effect, which could significantly increase the activity of scavenging radicals (Chataigner, Panel, Gérard, & Piettre, 2007).

For hydroxyl radical, one of antioxidation mechanism is that the antioxidant 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). In the present study, substituent carbonyl was strong electron-withdrawing group, which make benzene to have high nucleophilic characteristic and easily chelate with metal ion (Bunnett & Evitt, 1948), so the hydroxyl radical scavenging activities of PHEP was much stronger than LEP.

3.4. Moisture absorption and retention properties

Figs. 4 and 5 showed the moisture absorption and retention properties of glycerol, chitosan, LEP and PHEP. From the figures, the moisture absorption rate (Ra) of all the samples at 81% RH and 43% RH increased significantly in the first 24 h but slowly after that. However, the Ra of LEP and PHEP increased much fast. After exposed to 81% RH for 96 h, the Ra of PHEP (66.4%) was much higher than that of LEP (33.7%), which was similar with that at 43% RH. After 96 h, the Ra of glycerol, chitosan, PHEP and LEP at 81% RH was 90.3%, 70.5%, 66.8 and 33.7%, and at 43% RH that was 29.1%, 22.6%, 20.2 and 11.5%, respectively.

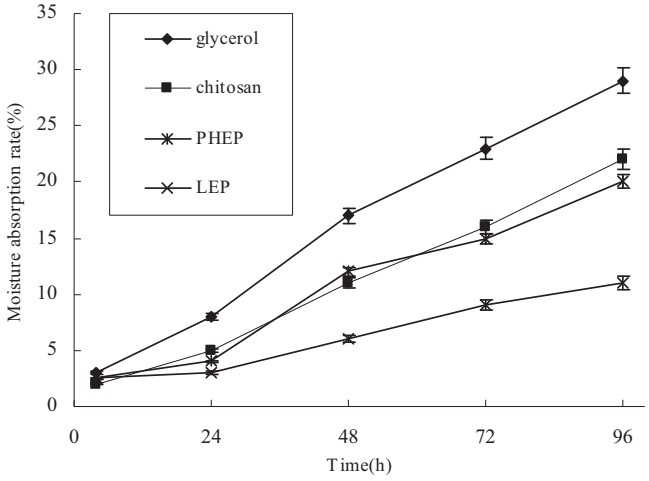

Fig. 5. Moisture absorption by samples at RH = 43% ($n = 3$).

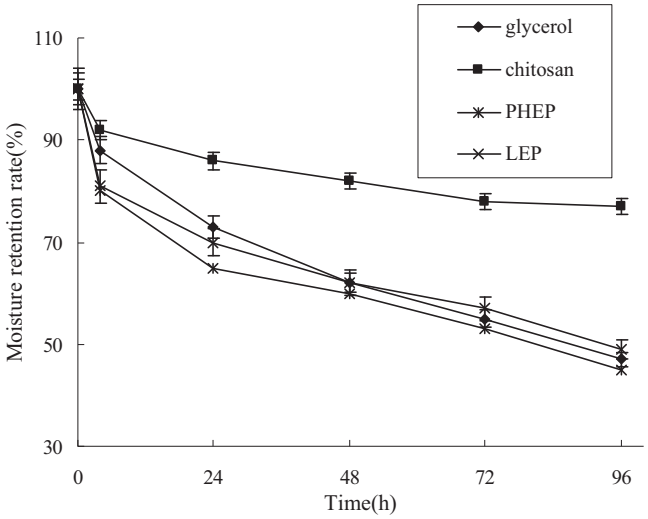

Fig. 6. Moisture retention by samples at RH = 43% ($n = 3$).

The moisture-retention properties of four samples were placed and evaluated in a water-humidified chamber to absorb moisture for 96 h at 43% RH. As shown in Fig. 6, water was lost gradually in all the samples but much slower in glycerol than in other samples. After test for 96 h at 43% RH, the moisture retention rate (Rr) of PHEP was 49.3%, which were slightly higher than chitosan (47.0%). These results indicated the significant moisture-retention capacity of PHEP as compared to chitosan and glycerol.

As the bioactive macromolecules, polysaccharides are really important component of the cortex in human skin. Many polysaccharides play a great role in metabolism of the skin. At present, polysaccharide has been one important aspect of the biological beauty due to its moisture absorption, anti-aging and antioxidant activities (Wang, Li, Guo, & Wei, 2004). The moisture content is firstly important to the skin health, and so the guarantees of the water moisturizer have been one of skin care cosmetics most main research subjects. The hydroxyl, the carboxyl group and other polar group in the polysaccharide may form the hydrogen bond with the H_2O to unify the massive moisture contents, and simultaneously, the polysaccharide chain can also mutually interweaves to the lattice, which plays very strongly a role in guarantying the moisture content (Sebti & Coma, 2002). In this study, PHEP showed the significant moisture absorption and retention properties because of the carboxyl group in phthaloyl and uronic acid. The moisture-retention competence of PHEP may stem from its chemical structure and three-dimensional network: the presence of special peripheral groups such as uronic acid may help to modulate its rheological properties (Helm et al., 2000), and the stereoscopic network may be beneficial to its moisture absorption and retention (Shaw et al., 2003).

Secondly, radical-scavenging was also the effective way of anti-aging. The free radical theory suggested peroxidation could fundamentally cause skin aging, while the peroxidation is mainly caused by oxygen free radicals (Herrling, Jung, & Fuchs, 2008). In present study, PHEP showed higher radical-scavenging effects than LEP, which indicated it was an ideal antioxidant for skin. And what is more, polysaccharide could whitening skin due to its UV absorption effect. PHEP can absorb the strong ultraviolet rays at about 210, 230, 290 nm because of π–π conjugate between C=O with benzene ring. Based on this, PHEP can be used in sunscreen cosmetics.

4. Conclusion

The results of the present work indicated that phthaloyl derivative of low-molecular-weight polysaccharide from *E. linza*

possessed antioxidant activities and moisture absorption and retention capacities. Therefore, PHEP is an ideal moisture-retention additive in biomaterials including living cells and tissues. It may have a use as a possible supplement in the food, pharmaceutical and cosmetic industries. However, factors effecting and attributing to radical scavenging effect and moisture absorption and retention capacities need to be further studied.

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