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Phosphorylated modification and *in vitro* antioxidant activity of *Radix Hedysari* polysaccharide

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Abstract Phosphorylated modification of a polysaccharide obtained from *Radix Hedysari* (RHP) was studied. Three phosphorylated polysaccharides (RHPP) with variable degrees of substitution (DS_p) were obtained with 4-dimethylaminopyridine (DMAP) and *N*, *N*′ Dicyclocarbodiimide (DCC) as catalyst. The structures of RHPP were characterized by FT-IR spectra and ^{13}C NMR spectra. Depending on different reaction time, RHPP showed different DS_p ranging from 0.30 to 0.66, and different Mw ranging from 86.6 to 89.7 KDa. Compared with RHP, RHPP exhibited superior antioxidant activities *in vitro*, which indicated that phosphorylated modification could enhance antioxidant activities of RHP. Furthermore, it was obvious that the DS_p had a significant effect on the antioxidant activity.

Keywords *Radix Hedysari* · Polysaccharide · Phosphorylated modification · Antioxidant activity

Introduction

Reactive oxygen species (ROS), including superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2), are generated by normal metabolic processes or exogenous factors in biological systems [1]. However, a vast amount of evidence indicate that their uncontrolled generation can cause damage to a wide range of essential biomolecules, such as DNA, and they have been associated with atherosclerosis, carcinogenesis, coronary heart disease and many other diseases related to advancing age [2, 3]. Antioxidants are able to prevent the radical chain reactions of oxidation by interrupting the free-radical chain of oxidation and donating hydrogen, so forming stable free radicals will not initiate or propagate further oxidation of lipids [4]. Therefore, it is essential to utilize and develop effective antioxidants so that they can scavenge free radicals in the human body. However, many of them are suspected to have some toxic effects or carcinogens, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). In recent years, it has become an important branch to explore potent natural compounds with low cytotoxicity from plants as antioxidants.

Plant polysaccharides have exhibited strong antioxidant properties and can be explored as novel potential antioxidants. Antioxidant activities of polysaccharide depend on sugar unit, glycosidic bonds in the main chain, the types and polymerization degree of the branch, flexibility and configuration of the chains [5], which can be modified by chemical derivatization in such a polymer [6, 7]. Therefore, molecular modification and structure improvement of polysaccharide are considered as a way to enhance the antioxidant activities of polysaccharides. Most studies have demonstrated that antioxidant activities of polysaccharides were greatly increased by molecular modification [8, 9]. For example, the phosphorylated modification of polysaccharides could not only enhance the water solubility but also change the chain conformation, resulting in alteration of their antioxidant activities [8]. The phosphate groups on the polysaccharides played an important part in enhancing their antioxidant

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activities. The phosphorylated modification of polysaccharide was an esterification process essentially, which was very difficult to accomplish and obtained lower the degree of substitution (DS) due to the complicated structures of polysaccharides [10]. To solve this problem, catalyst was added into the reaction system for increasing the reaction rate and DS of phosphorylated polysaccharide.

Radix Hedysari (RH), known as "Hongqi" (HQ) in China, is the dried root of *Hedysarum polybotrys* Hand.-Mazz., which belong to the Fabaceae family. RH is one of the most popular herbal medicines known worldwide and has a long history in the treatment of many diseases in China. Recently, polysaccharide obtained from RH (RHP) had attracted great attention owing to its antioxidant properties, immunomodulatory effect, antitumor activities and antiviral activities [11, 12]. Hui reported that RHP was composed of rhamnose, xylose, arabinose, glucose and galactose, and their molar ratio was 0.3:0.2:2.7:16.1:2.0. The glucosidic bonds of RHP had mainly β-configuration [13].

In the study, we extracted RHP and synthesized its phosphorylated derivatives (RHPP) with *N*, *N*′ Dicyclocarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as catalyst. RHPP was studied including DS_p, weight-average molecular mass (Mw), chain conformation and antioxidant activities *in vitro*. The effects of chain conformation and phosphate groups on the antioxidant activities of RHPP were discussed.

Materials and methods

Materials and reagents

The root of RH was collected from the mountain area in Wudu City, Gansu Province, China. The crude RHP was obtained from the laboratory of School of Basic Medical Science at Lanzhou University.

Sephadex G-25 was from Pharmacia Co. (Sweden). Papain was from Sigma-Aldrich Co. (Switzerland). *N*,*N*-dimethylformamide (DMF) were analytical grade and were obtained from Jiangsu Haimen Chemical Industry Co., Ltd. (China). 3-Phosphonopropionic acid was purchased from Alfa Aesar (Shanghai, China). 4-Dimethylaminopyridine (DMAP) and *N*, *N*′ Dicyclocarbodiimide (DCC) was purchased from Shanghai Meryer Chemical Reagent Co. (China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid (V_C), BHA, ethylene diamine tetra-acetic acid (EDTA), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and deoxyribose were purchased from Sigma Chemical Co. (USA). Nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS) and nitroblue tetrazolium (NBT) were purchased from E. Merck (Darmstadt, Germany). H_2O_2 and potassium ferricyanide was purchased from Tianjin Guangfu Chemical Research Co. Ltd (Tianjin, China).

Purification of RHP

The crude RHP was purified as follows: according to the reference [14], the protein was removed by the Sevage method, combined with papain. After centrifugation, the supernatant was dialyzed against deionized water using a dialysis tubing (molecular weight cut-off of 8.0 kDa) for 36 h. Through Sephadex G-25 column, the purified polysaccharide (RHP) was obtained after lyophilizing, and then kept in dryness box. Mw of RHP was 84.7 kDa. The content of RHP was measured by Vitriol–Phenol taking anhydrous glucose as standard control.

Phosphorylated modification of RHP

Phosphorylated modification of RHP was carried out by reference with some modifications [15]. RHP (500 mg) was suspended in anhydrous DMF (20 mL) at room temperature with stirring for 30 min. Then 3-phosphonopropionic acid (2.0 g), DCC (2.7 g) and DMAP (3.1 g) were added. To obtain RHPP with variable DS_p, the reaction was accomplished during different periods of time (6 h, 12 h and 24 h), respectively. The mixture was dialyzed against deionized water using a dialysis tubing (molecular weight cutoff 8.0 kDa) for 36 h to remove DMF, DMAP and potential degradation products. Three kinds of RHPP (RAPP-1 to RAPP-3) with different DS_p were obtained after lyophilizing, and then kept in dryness box. RHPP-4 was obtained without catalyst and used as negative control.

Characterization of RHPP

Mw of RHPP was determined by high-performance gel-filtration chromatography (HPGFC) on a Waters 2695 instrument. 0.5 mg/mL sample solution was injected in HPGFC system. The mobile phase was 0.05 mol/L Na_2SO_4 aqueous solution at a flow rate of 0.6 mL/min. The HPGFC system was calibrated with T-series Dextran standards.

The P content of the phosphorylated polysaccharide was determined on an inductively coupled plasma atomic emission spectrophotometer (ICP-AES) IRIS ER/S (TJA Company, USA), and DS_p which represented the average number of phosphate groups on each monosaccharide residue, was calculated using the following formula:

$$DS_p = (162 \times P\%)/(3100 - 84 \times P\%)$$

FT-IR spectra of RHP and RHPP were recorded by a Nicolet 20 NEXUS 670 FT-IR spectrophotometer (Ramsey,

MA, USA) using KBr pellets. ^{13}C NMR spectra of the samples were recorded on a Bruker AVANCE 600 MHz spectrometer (Rheinstetten, Germany) using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard and D_2O as the solvents at 25°C.

Assay for antioxidant activities

Superoxide anion scavenging activity

The superoxide radical scavenging ability of RHP and RHPP was assessed by the method of Qi *et al.* [16]. Superoxide radicals were generated in 5.0 mL of Tris–HCl buffer solution (16 mM, pH 8.0) containing 0.5 mL of NBT (72 μM) solution, 0.5 mL of NADH (388 μM) solution, 0.5 mL of PMS (42 μM) solution and varying concentrations of samples (0.1–6.0 mg/mL). The reaction mixture was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against a blank. V_C was used as positive control. The scavenging capability of RHP and RHPP to superoxide radical was calculated using the formula:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

Where A_0 was the absorbance of mixture solution without sample; A was the absorbance of the test sample mixed with reaction solution.

Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging ability of RHP and RHPP were measured by the method of Ghiselli with a minor modification [17]. Samples were dissolved in distilled water at the varying concentrations (0.1–6.0 mg/mL). The sample solution (0.1 mL) was mixed with 0.6 mL of reaction reagent, including 50 mM phosphate buffer (pH 7.4), 2.7 mM deoxyribose and 0.15 mM EDTA. Then 0.2 mL of 0.2 mM ferrous sulfate, 0.05 mL of 1.5 mM Vc and 0.05 mL of 20 mM H_2O_2 were added. The reaction solution was incubated for 30 min at 37°C, and then 1.0 mL of 1 % TBA and 1.0 mL of 1.5 % TCA were added into the reaction. The mixture was heated in boiling water for 15 min and cooled to room temperature. The absorbance of the mixture was measured at 532 nm against blank. V_C was used as positive control. Scavenging activity of hydroxyl radical was calculated by using the formula:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

Where A_0 was the absorbance of mixture solution without sample; A was the absorbance of the test sample mixed with reaction solution.

DPPH radical scavenging activity

The DPPH radical scavenging effect of RHP and RHPP was measured according to the method of Shimada with some modifications [18]. Briefly, a 0.2 mM solution of DPPH in 50 % methanol was incubated with different concentrations of the samples (0.1–6.0 mg/mL). The reaction mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance was then recorded at 517 nm against a blank. BHA was used as positive control. The DPPH radical scavenging effect was calculated as the formula:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

Where A_0 was the absorbance of DPPH solution without sample; A was the absorbance of the sample mixed with DPPH solution.

Reducing power

The reducing power of RHP and RHPP was determined by Li *et al.* [19]. 2.0 mL of different concentrations of samples (0.1–6.0 mg/mL) in phosphate buffer (200 mM, pH 6.6) were mixed with 2.0 mL of potassium ferricyanide (1 %, w/v), and incubated at 50°C for 20 min. Then TCA (10 %, w/v) was added to the mixture to terminate the reaction. Finally, the solution was mixed with ferric chloride (0.1 %, w/v). The absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. BHA was used as positive control.

Results and discussion

Characterization of RHPP

Mw and DS_p of RHPP were shown in Table 1. Mw of RHPP increased from 86.6 to 89.7 kDa, and DS_p increased from 0.30 to 0.66, which showed that hydroxyl groups were substituted successfully by phosphate groups on the polysaccharide. The molecular mass and DS_p of polysaccharide after phosphorylated modification increased obviously. Furthermore, the reaction could achieve dynamic equilibrium

Table 1 Mw and DS_p of RHPP

RHPP	Time (h)	Mw (kDa)	P%	DS_p
RHPP-1	6	86.6	4.98	0.30
RHPP-2	12	87.2	6.71	0.43
RHPP-3	24	89.7	9.44	0.66
RHPP-4	24	84.2	0.72	0.04

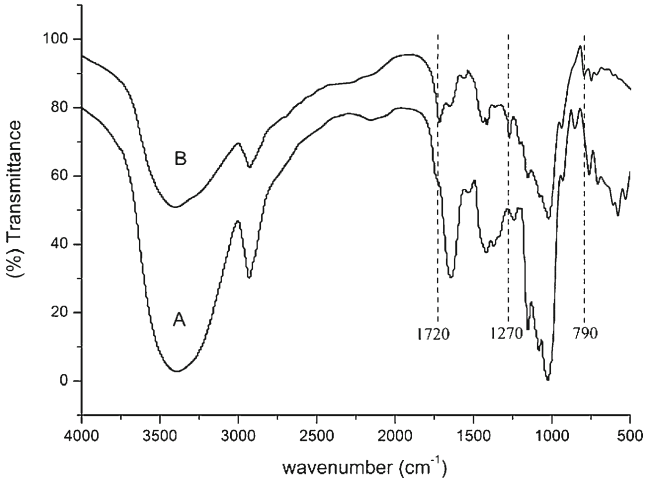

Fig. 1 FT-IR spectra of RHP and its phosphorylated derivatives. (**a**) RHP; (**b**) RHPP-3

quickly after the addition of DCC and DMAP. However, RHPP-4 had lower DS_p without catalyst.

The FT-IR spectra of RHP and RHPP were shown in Fig. 1. Compared with RHP, three new strong absorption peaks appeared at 1720 cm^{-1}, 1270 cm^{-1} and 790 cm^{-1} for RHPP, assigned to the C = O stretching vibration, the P = O asymmetric stretching and C–O–P symmetric vibrations, respectively. These absorptions indicated that the phosphorylated modification in RHPP had actually occurred.

The phosphorylated position of polysaccharide was usually determined by ^{13}C NMR spectra. The ^{13}C NMR spectra of RHP and RHPP are shown in Fig. 2. Compared with the signals of RHP, new signals appeared due to the existence of phosphate groups in RHPP-3. If the carbon is attached to an electron-withdrawing phosphate group, it would shift to a lower field position. In contrast, the carbon indirectly attached to phosphate group would shift to higher field position [20]. The new peak of RHPP-3 at 58.5 ppm represented the signal of C-6p; the peak at 72.5 ppm represented the signal of C-2p. The peak was weakened at 60.9 ppm, which showed that C-6 had been substituted by the phosphate group, but C-2 had been substituted partially. We believed that the C-6 position was more reactive than the C-2 position due to the steric hindrance.

Antioxidant activity analysis

Scavenging activity of superoxide radical

The scavenging ability of superoxide radicals is extremely important to antioxidant work. Superoxide radicals were determined by the PMS–NADH superoxide generating system and the results are shown in Fig. 3a. All of RHPP exhibited stronger superoxide radicals scavenging ability than that of RHP except RHPP-4. Furthermore, the scavenging effect of RHP and RHPP was increased with the concentration increasing. At the concentration of 6 mg/mL, the scavenging capacity of RHPP-3 was 74.4 %, which was comparable to V_c (85.1 %). These results indicated that the difference in the scavenging ability may be attributed to their different DS_p. It was found that polysaccharide with higher DS_p exhibited stronger scavenging activity of superoxide radical. We believed that presence of phosphate groups could change the three-dimensional structure of polysaccharide, and a lot of hydroxyl groups would emerge, which would affect the antioxidant ability.

Scavenging activity of hydroxyl radical

Hydroxyl radical, which is well known as the most reactive free radical, could be formed from superoxide anion and H_2O_2 in the presence of copper or iron, and be also thought to initiate cell damage *in vivo* [21]. Hydroxyl radical could be generated in several ways, but the most important mechanism was the Fenton reaction [22]. Figure 3b depicts the hydroxyl radical-scavenging ability of all samples. All samples showed a scavenging ability on superxoide radicals in a dose dependent manner (0.1–6.0 mg/mL). Compared with the RHP, all of RHPP exhibited strong scavenging ability except RHPP-4. Furthermore, RHPP-3 showed the best

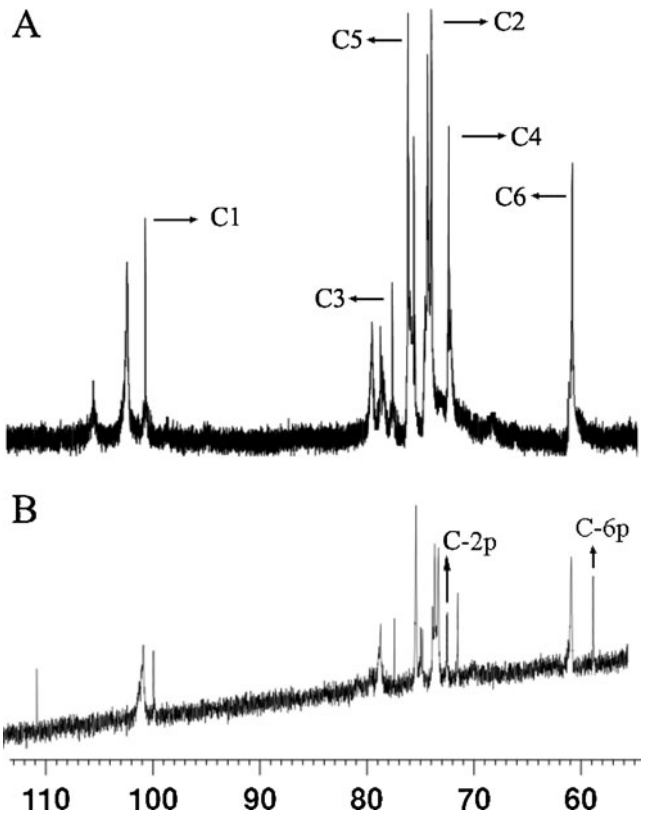

Fig. 2 ^{13}C NMR spectra of RHP and its phosphorylated derivatives. (**a**) RHP; (**b**) RHPP-3

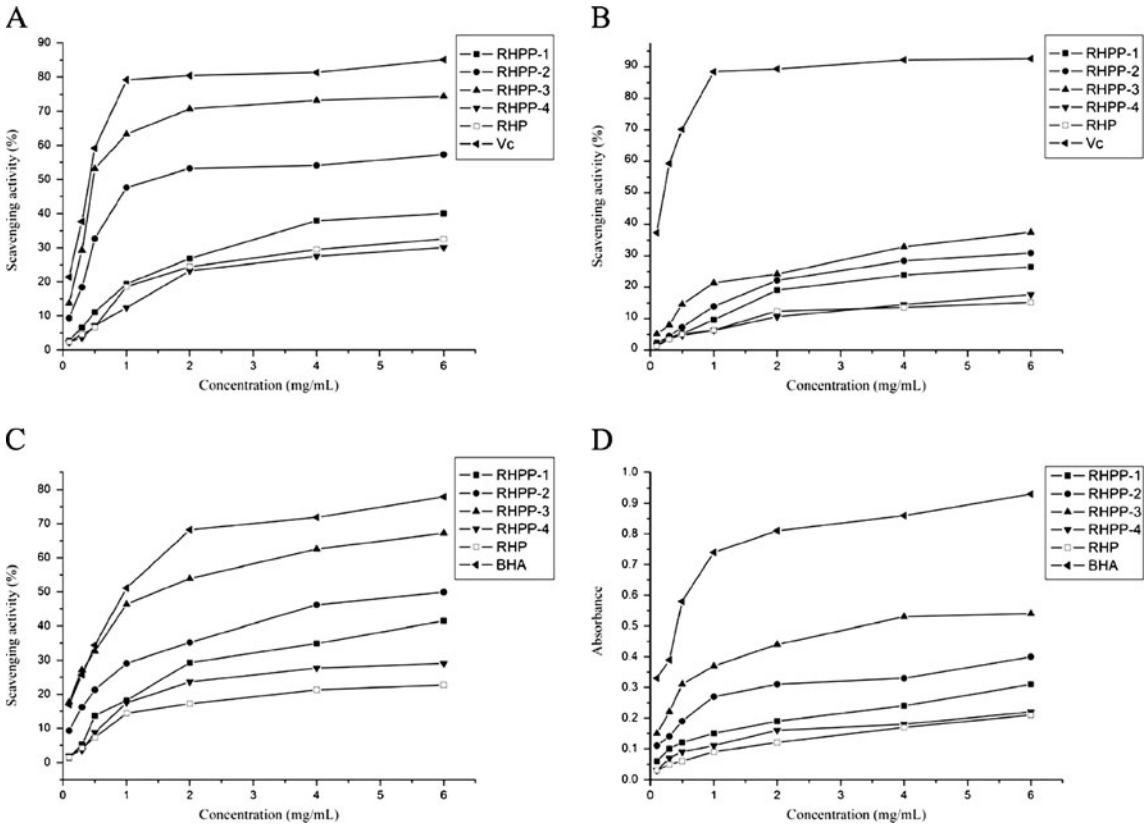

Fig. 3 Antioxidant effect of RHP and RHPP: (**a**) scavenging activity of superoxide radicals; (**b**) scavenging activity of hydroxyl radicals; (**c**) scavenging activity of DPPH radicals; (**d**) reducing power

scavenging ability, and the scavenging ability was 37.5 % at the concentration of 6.0 mg/mL. These results indicated that phosphate groups in the molecule may play an important role in the strong scavenging ability of polysaccharide.

It was reported that the scavenging activity of hydroxyl radical was due to the inhibition of hydroxyl radical generation by chelating ions, such as Fe^{2+} [23]. Hydroxyl radicals could be generated by the reaction of Fe^{2+} and H_2O_2 ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$), and since the phosphate groups of RHPP have a high nucleophilic characteristic and chelating ability with metal ions, the hydroxyl radical scavenging activities of phosphorylated derivatives (RHPP) were much stronger than of RHP.

Scavenging activity of DPPH radicals

DPPH was a stable radical, the model of scavenging DPPH radical was a widely method to evaluate antioxidant activities. As shown in Fig. 3c, the scavenging ability of all samples was concentration related. RHPP-3 had strong antioxidant activity, the scavenging effects were 67.2 % at a dose of 6 mg/mL, higher than RHP but lower than BHA. However, the scavenging effects of RHPP-4 were 29.1 % at the concentration of 6 mg/mL, which was weaker than that of RHPP-1, RHPP-2 and RHPP-3.

DPPH radical was scavenged by antioxidant through donation of hydrogen to form a stable DPPH-H molecule when accepted an electron or hydrogen radical. The effect of antioxidant was due to their hydrogen-donating ability. Compared with RHP, RHPP showed excellent scavenging activity on DPPH radical, which might be attributable to its strong hydrogen-donating ability. In our opinion, the presence of phosphate groups in RHPP could activate the hydrogen atom of the anomeric carbon. Thus, RHPP at different DS_p could lead to different scavenging ability.

Reducing power

The reducing capacity of a compound might serve as a significant indicator of its potential antioxidant activity. Figure 3d depicted the reducing power of all samples. All of RHPP exhibited stronger reducing power than that of RHP except RHPP-4. Furthermore, RHPP-3 had the strongest reducing power, which was 0.54 at the concentration of 6 mg/mL, but lower than that of BHA. On the contrary, the reducing power of RHP and RHPP-4 was only 0.21 and 0.22 at the concentration of 6 mg/mL, respectively.

The reducing property was generally associated with reductones, a strong antioxidant, which could break free-radical chain by donating a hydrogen atom. Reductone was

also reported to react with certain precursors of peroxide, thus preventing peroxide formation. RHPP with high donating-hydrogen ability showed excellent reducing power, the same to the ability of scavenging DPPH radical.

Conclusions

Three phosphorylated derivatives of RHP from RH with different DS_p were synthesized using DMAP and DCC as catalyst. Depending on different reaction time, RHPP showed different DS_p ranging from 0.30 to 0.66, and different Mw ranging from 86.6 to 89.7 KDa. The antioxidant activities of RHPP were evaluated *in vitro*. Compared with RHP, RHPP showed greater antioxidant activities, which were affected by different DS_p. In conclusion, phosphorylated derivatives of RHP would have an assistant role as an antioxidant in the further.

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