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Preparation and Antioxidant Activity of Phosphorylated Polysaccharides from *Russula Alutacea* Fr.

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Abstract

As a large amount of alkali-soluble polysaccharides is present in *Russula alutacea* Fr., their insolubility limits their application. Chemical modification can improve the solubility of natural polysaccharides to enhance their activities, and enlarge their application range. In this study, water-soluble polysaccharides of *Russula alutacea* Fr. were modified by phosphoric-acid esterification reaction. The antioxidant activity and structural properties of modified and unmodified polysaccharides were measured. The scavenging ability of hydroxyl radical, superoxide anion and DPPH radical scavenging were analyzed and the properties of the modified and unmodified polysaccharides comprehensively compared. The results showed that antioxidant activity of the phosphorylated polysaccharides was higher than that of unmodified polysaccharides from *Russula alutacea* Fr.

Keywords: *Russula alutacea* Fr., polysaccharides, antioxidation, reducibility

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INTRODUCTION

Russula alutacea Fr. is a fungus of the family *basidiomycetes, agaricales, russulaceae* (Zhao et al. 2012) and has important economic value. It can be used not only as food but also as a medicine. When eaten, it is rich in nutrition and delicious. It has effects of enriching blood, nourishing Yin, cooling, detoxicating, cholesterol lowering, liver protecting, anticancer (Guo et al. 2011). *Russula alutacea* Fr. is generally large, shows deep red amaranth, fresh purple or dark purple color, smooth edge or not obvious stripe cap of diameter 6 cm ~ 16 cm, white flesh, soft taste, gill pale reddish brown white, often with red front. White nearly cylindrical stipe length of 3.5 cm ~ 13 cm, thickness of 1.5 cm ~ 3.5 cm. Summer and autumn, when rainfall is more intense and the relative humidity is higher, are rich periods of *Russula alutacea* Fr. (Gan et al. 2005). *Russula alutacea* Fr. is tepid, not only delicious but also has a very high nutritional value. At present, the research of *Russula* is mainly about *Russula vinosa* produced by Fujian and others (Li et al. 1999, Yu et al. 2006), but the research on the polysaccharides of *Russula alutacea* Fr. in Yunnan is rarely reported. The United States, Japan, North Korea, Russia are the major foreign distributors, while Fujian, Liaoning, Jiangsu, Yunnan, Anhui, Hebei, Guangxi and other provinces are the country's main distribution areas (Wang and Shi 2004). *Russula alutacea* Fr. is a famous edible and medicinal fungus, Ectomycorrhizal fungus (ECM fungus).

Polysaccharides, also known as proteoglycans, are high-molecular compounds composed of monosaccharides. In the plant, polysaccharide components include starch, cellulose, fructan, hemicelluloses, gum, mucus and baregin, etc. In addition to their action as plant storage nutrients and skeleton components, in some plants polysaccharide compounds have a unique physiological antitumor, anti-vascular disease, anti-aging activity. In the past, in studies of the active components of plants, polysaccharides were often regarded as ineffective ingredients and abandoned. In 1969, Japanese scholars using hot-water extract of mushrooms were the first to confirm the anti-tumor activity. Qian *et al.* further researched and confirmed that the active ingredient is lentinus edodes polysaccharide. Since then, extraction of antitumor active components from fungi set off a

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craze all over the world. Especially in recent years, along with the rapid development of biology, chemistry and other related subjects, glycol conjugates and polysaccharide compounds have been deeply studied.

The structure of monosaccharides and their complexes was derived by a chemical method, making their active or toxic functional groups to reduce toxicity after optimization, improve the biological activity and bioavailability; or by modifying to change their physical and chemical properties, broaden their application field, and improve their application effect—this is the chemical modification of the carbohydrate. At present, it is an important means for the study of structure-activity relationship of polysaccharides to molecular modification of polysaccharides, also an important way to discover and develop polysaccharide drugs (Wu and Dai 2010). Therefore, the chemical modification of carbohydrates and their complexes is not only of interest for synthetic chemists, but also for scientists in the fields of pharmacochemistry, biology, medicine, etc. This is the main method of molecular modification of the polysaccharides, such as sulfation, phosphoric esterification, acetylation, carboxymethylation, graft copolymerization, etc.

The properties of the main chain and advanced structures of branched polysaccharide molecules are the structural factor affecting the biological activity of polysaccharides. It directly determines the activity of polysaccharide in carbohydrate compositions of the polysaccharide chain, category of glycosidic bond, type of polysaccharide branched chain, degree of polymerization, distribution and substitution degree of branched chain in polysaccharide chains determine the activity of polysaccharide. The advanced structure of the polysaccharide molecules, the flexibility of the chain and the space conformation are closely related to the activity of the polysaccharide (Yang et al. 2011). Completed studies confirmed that molecular structure directly or indirectly affects the activity of polysaccharides. Changing the activity of polysaccharides can modify the molecular structure of polysaccharides appropriately (Zhang et al. 2007). At present, there are few reports about phosphorylation modification of *Russula alutacea* Fr. polysaccharides. In this paper, a mixed phosphate reagent was used to modify *Russula alutacea* Fr. polysaccharides by phosphorylation. The preparation, antioxidant properties and structure characterization of *Russula alutacea* Fr were studied. Water-soluble polysaccharide and its esterified-derivatives, accumulate valuable information for the study of modification and structure activity relationship of *Russula alutacea* Fr. polysaccharides, thus, provide a theoretical basis for the exploitation and development of new functional foods or drugs from *Russula alutacea* Fr. polysaccharides.

METHODOLOGY

Russula alutacea Fr. was purchased from Jinggu County, Yunnan Province. After drying, it was crushed by a small plant sample mill, sifted through 80 mesh, and stored in a sealed dryer.

After the pretreatment of *Russula alutacea* Fr. samples at a solid-to-liquid ratio of 1:20, 80℃ for 2.5 h and extracted three times with water, the supernatant obtained was centrifuged, and the residue was kept. Absolute alcohol was added 3 times to supernatant, placed in a refrigerator (4℃) for 12 h, centrifuged, the supernatant was discarded; the water-soluble crude polysaccharide was dried out. The dry crude polysaccharide was dissolved in 100 ml of distilled water, 1% (w/v) activated carbon was added, oscillated for 2h at a temperature of 75℃, centrifuged and discarded. Sewage solution was added 5 times to the decolorized supernatant, the supernatant was centrifuged, then absolute alcohol was added oscillated for 20~30 min, placed in a refrigerator (4℃) for 4 h. After centrifugation, the dried out precipitate obtained was *Russula alutacea* Fr. water-soluble refined polysaccharide. The content of crude polysaccharide was determined by the phenol-sulfuric acid method (Wang et al. 2010).

The polysaccharide content in the *R. alutacea* extract was determined by the absorption values of OD490 at the maximum absorption wavelength of polysaccharide. Then, the polysaccharide extraction rate was calculated by Eq. (1):

$$Polysaccharide\ extraction\ rate\ (\%) = (C \times V \times M\_1)/1000/m \times 100 \quad (1)$$

where, C is the mass concentration of *Russula alutacea* Fr. polysaccharides in the extract solution (mg/ml); V is constant volume (ml); M1 is the weight of the extracted polysaccharides (g); m is the weight of sample (g).

An exactly weighed amount of *Russula alutacea* Fr. polysaccharides was dissolved in distilled water at a ratio of 1:10. Add a certain amount of phosphoric acid reagent to the reaction sample solution ($Na_5P_3O_{10}$:$NaPO_3)_3$= 6:2 and 5% Na_2SO_4) to reduce the foaming phenomenon in reaction, and carry out the reaction at a certain temperature after adjusting pH. After reaction, add 3 times of 95% ethanol to the sample solution to precipitate for 24 h. Alcohol-precipitated

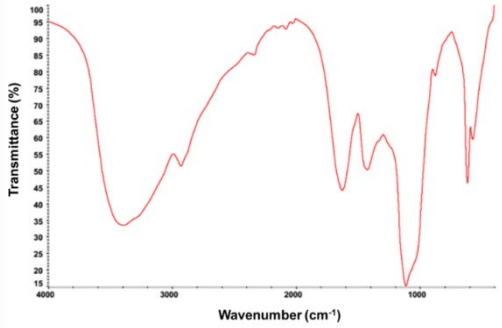

Fig. 1. Infrared spectrum of unmodified polysaccharides

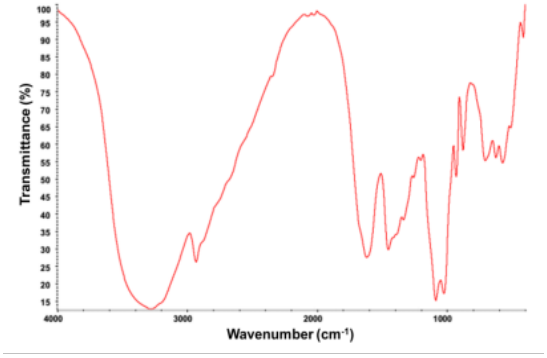

Fig. 2. Infrared spectrum of modified polysaccharides

polysaccharide was vacuum dried at 30 ℃. Remove The residual ethanol was removed and the precipitate was rehydrated at 50℃ in a water bath. Then the solution was dialyzed in a dialysis bag for 24 h. Finally, the reaction solution was dried, and phosphorylated polysaccharide derivatives were obtained. Reaction temperature: 90℃, pH: 9, reaction time: 5 h and amount of phosphoric acid esterification reagent was 20%. At the above conditions, phosphoric acid esterification modification reaction was carried out.

0.1 g of *Russula alutacea* Fr. water-soluble polysaccharide phosphate was dissolved in water to 100 mL, the absorbance value was measured by the standard curve method, and the content of phosphoric acid was calculated according to a standard curve.

Substituting degree formula: DS=(BX162/30.974)/[100-(3.8734XD+3.2922XB)] (B is combined phosphorus (%); D is free phosphorus (%)) (Li et al. 2007).

For IR spectroscopy, polysaccharide powders were mixed with KBr, ground, and pressed into 1- mm pellets. Then scanning measurement was performed in the range from 4000 to 400^{-1} (Sun et al. 2013).

Reducing power was evaluated by the published method (Li 2006) with a slight modification. The scavenging effect of superoxide anion (O_2^{-}•) was determined by pyrogallol autoxidation method (Li et al. 2007). The scavenging rate of DPPH was used (Sun et al. 2013). The scavenging ability of hydroxyl free radical (OH•) was used (Jiang et al. 1999).

RESULTS AND DISCUSSION

Russula alutacea Fr. water-soluble polysaccharide phosphate was preparated by phosphorylation. The unmodified phosphorus content in *Russula alutacea* Fr. polysaccharide was measured, it was 6.863 mg/g. The modified phosphorus content in Russula alutacea Fr. polysaccharide was 7.101 mg/g. Degree of substitution (DS) was 1.258%.

Figs. 1 and **2** show the infrared spectra of phosphorylated *Russula alutacea* Fr. polysaccharide and *Russula alutacea* Fr. polysaccharide. From these figures it can be concluded that apart from phosphorylated *Russula alutacea* Fr. polysaccharide keeping the characteristic absorption of *Russula alutacea* Fr. polysaccharide, it also displays stretching vibrations of phosphate at 1050-950 cm^{-1} and 1350-1160 cm^{-1}. The above results show that the polysaccharide has formed a phosphate.

It can be seen from the figures that water-soluble polysaccharide, *Russula alutacea* Fr. water-soluble polysaccharide phosphate and vitamin C(VC) have a strong scavenging effect on OH, which increases with the increase in mass concentration in the range of 0.1 ~ 0.50 mg/ml, and it exhibits a dose-dependent effect. For water-soluble polysaccharide, *Russula alutacea* Fr., water-soluble polysaccharide phosphate and VC, free radical scavenging ratios are as high as 74.84%, 81.70%, 73.04%, respectively. The scavenging rate of phosphate polysaccharide is higher than that of water-soluble polysaccharide in the range of 0.1～0.50 mg/ml of mass concentration. When the concentration is higher than 0.5 mg/ml, the scavenging rates of phosphate polysaccharide and water-soluble polysaccharide are higher than that of VC. According to its EC50 value, the order of the OH· scavenging ability is: phosphate polysaccharide >VC> water-soluble polysaccharide (**Table 1**).

Table 1. Effect of polysaccharides on hydroxyl free radicals

No.	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
	0.1	4.08	
	0.2	14.71	
Polysaccharides	0.3	23.53	0.3893
	0.4	52.78	
	0.5	74.84	
	0.1	5.88	
Phosphorylated	0.2	17.32	
polysaccharides	0.3	44.44	0.3381
	0.4	66.83	
	0.5	81.7	
	0.1	8.33	
Ascorbic acid	0.2	16.99	
(Vc)	0.3	41.5	0.3574
	0.4	62.91	
	0.5	73.04	

Table 2. Scavenging effect on DPPH free radicals

No.	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
	0.1	7.14	
	0.2	14.27	
Polysaccharides	0.3	42.86	0.3827
	0.4	57.14	
	0.5	64.29	
	0.1	10.71	
Phosphorylated	0.2	15	
polysaccharides	0.3	46.43	0.3643
	0.4	59.29	
	0.5	69.29	
	0.1	14.29	
Ascorbic acid	0.2	21.43	
(Vc)	0.3	64.29	0.3001
	0.4	71.43	
	0.5	78.57	

Table 3. Scavenging effect on superoxide anion

No.	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
	0.1	4.49	
	0.2	10.11	
Polysaccharides	0.3	17.98	0.4404
	0.4	39.33	
	0.5	65.17	
	0.1	13.48	
Phosphorylated	0.2	22.47	
polysaccharides	0.3	28.09	0.3792
	0.4	52.81	
	0.5	75.28	
	0.1	15.73	
Ascorbic acid	0.2	20.22	
(Vc)	0.3	30.34	0.3788
	0.4	47.19	
	0.5	79.78	

It can be found out from the figures, that the scavenging rate of phosphate polysaccharide on DPPH is higher than that of water-soluble polysaccharide; both are lower than that of VC. According to its EC50 value, the order of the DPPH-scavenging ability is VC > phosphate polysaccharide > water-soluble polysaccharide (**Table 2**).

It can be seen from the figures that the scavenging rate of phosphate polysaccharides on superoxide anion is higher than that of water-soluble polysaccharide.

When the concentration is higher than 0.5 mg/ml, the scavenging rate of VC is higher than that of phosphate polysaccharides and water-soluble

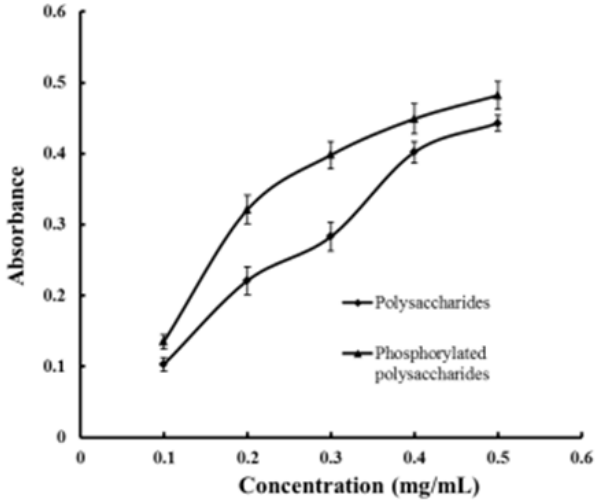

Fig. 3. Effect of enzymatic modification on reduction power

polysaccharide. According to its EC50 value, the order of the $O_2^{-\cdot}$ scavenging activity is VC > phosphate polysaccharide > water-soluble polysaccharide (**Table 3**).

As seen from the **Fig. 3**, the reduction ability of phosphate polysaccharide and water-soluble polysaccharide increases with the increasing mass concentration of polysaccharide. The reduction ability of phosphate polysaccharide is higher than that of water-soluble polysaccharide.

CONCLUSION

Phosphoric esterification of *Russula alutacea* Fr. polysaccharides was carried out through phosphoric acid esterification modification of *Russula alutacea* Fr. water-soluble polysaccharides (Liu et al. 2011). The *in vitro* antioxidant experiment showed that the scavenging effect of DPPH, $OH^{\cdot}$, $O_2^{-\cdot}$ and the reduction ability for phosphoric esterification of *Russula alutacea* Fr. polysaccharides are better than that before modification. This study provides a theoretical basis for expanding the application range of *Russula alutacea* Fr. water-soluble polysaccharides. Polysaccharides were subjected to phosphoric esterification by sodium tripolyphosphate ($Na_5P_3O_{10}$), orthophosphate, phosphorus oxychloride. The ability of acetylation of $POCl_3$ is strong; a product with high degree of substitution can be obtained. But the uniformity of the product is usually not good. When sodium tripolyphosphate and orthophosphate are used in the phosphoric esterification reaction, the reaction temperature is relatively high, but a monoester can be synthesized. In this experiment, using sodium tripolyphosphate and trimetaphosphate grade III,

trisodium water-soluble *Russula alutacea* Fr. phosphate polysaccharide was obtained. With the continuous development of new technologies, the research of polysaccharides and their derivatives becomes more thorough, and biological activity, chemical structure and structure activity relationships of polysaccharides are further understood, which makes the polysaccharides more widely used in the fields of health care, fermentation industry and video industry.

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