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Research on acetylation and antioxidant activity of *Russula alutacea* Fr. water-soluble polysaccharides

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There is a large amount of alkali-soluble polysaccharides in *Russula alutacea* Fr., but insolubility in water limits its development. Chemical modification can improve the solubility of natural polysaccharides limiting their activities, and enlarge their application range. Water-soluble polysaccharide was extracted by water and precipitated with alcohol in this study. It was further modified by acetylation, thus improving its water solubility. The scavenging activity of water-soluble polysaccharide, acetylated polysaccharide and vitamin C on the hydroxyl radical (OH•), 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion(O_2^-•) was analysed and compared. The results showed that water-soluble polysaccharide, acetylated modified polysaccharide and vitamin C have a strong scavenging effect on O_2^-• which increases with the increase with mass concentration in the range of 0.34 ~ 1.7 mg/mL of mass concentration. The yield of water-soluble polysaccharide was 1.75%. The mass concentration of acetic acid was 0.63 mg/ml, the degree of substitution (DS) was 0.44%. According to its EC50 value, it can be concluded that the order of OH• scavenging ability was: VC>acetylated polysaccharide>water-soluble polysaccharide; the order of DPPH scavenging ability was: acetylated polysaccharide>VC>water-soluble polysaccharide; the order of O_2^-• scavenging ability was: VC>acetylated polysaccharide>water-soluble polysaccharide.

Key words: *Russula alutacea* Fr., Acetylation, Hydroxyl radical (OH•), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Superoxide anion (**O_2^-•**).

INTRODUCTION

Russula alutacea Fr. is a kind of ectomycorrhizal fungi with forest tree symbiosis [1,2]. It has an important economic value. It can be used not only for food but also for medicine purposes. When eated, it is rich in nutrition and delicious; it has blood enriching, nourishing Yin, cooling, detoxificating, cholesterol lowering, liver protecting, anticancer effects [3]. The polysaccharide structure modification is an important branch of polysaccharides pharmacology. To improve the biological activity of polysaccharides, molecular modification and structural modification of polysaccharides are of great significance, and acetylation of polysaccharides is one of the most important methods for their chemical modification. Acetylation can change the stretch of sugar chain, make hydroxyl (OH•) exposed, increase solubility in water.

It regulates the body's immune function mainly through the reticuloendothelial system (RES), macrophages (MO), lymphocytes and so on, and improves the immune capacity of the body. More and more polysaccharide preparations have been widely used in clinical practice, and have achieved encouraging results in autoimmune diseases, immune dysfunction, cancer treatment, etc. Among them, the polysaccharide extracted from traditional Chinese edible and medicinal fungi is the most important one [4]. The anti-tumor effect of fungal polysaccharides is the most important biological activity, and also the most active part of the study. Pharmacological and clinical trials have found that 178 kinds of extract from the 50 genus of higher fungi have biological effects of inhibiting the cell growth of mouse sarcoma and Ehrlich Ascites tumor [5]. Polysaccharide Krestin (PSK) and *Grifola Frondosa* polysaccharide have been used as clinical antitumor drugs, have showed a broad spectrum of anti-tumor effects, and are quite remarkable anticancer drugs in recent years [6]. Modern research shows that *Russula alutacea* Fr. contains 5 kinds of polysaccharides. The polysaccharide content is about 2.47%, among them, monosaccharides and oligosaccharides account for 33.9% of total sugars; it contains anti-cancer polysaccharide substances, is conducive to blood circulation, reduces the cholesterol in the blood, suppresses metastasis of cancer cells, improves immunity, fights off viruses, also has certain curative effect in the treatment of acute spinal optic neuropathy. At present, the research is mainly focused on *Russulavinosa* in *Russula*, but studies on *Russula alutacea* Fr. of Yunnan Province are rarely reported [7]. However, a lot of polysaccharides extracted with alkali solution from

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the fruiting body and mycelium are of interest because of their water solubility. Therefore, its water solubility should be increased by chemical modification, which will significantly improve the biological activity of the polysaccharides and extend its application range. At present, the extracting methods of polysaccharides are mainly using water extraction and ethanol precipitation, alkali extraction, and enzymatic hydrolysis [8,9]. The reaction of alkali extraction is intense, the extraction fluid needs to be neutralized, which could destroy the structure of the polysaccharide, decrease the molecular weight of *Russula alutacea* Fr. *Velutipes* polysaccharide, lead to a decline in its medicinal value [10]. Although the reaction time is short, the enzymatic hydrolysis method can greatly increase the extraction rate of polysaccharide, but the price of enzyme is higher. Although the extraction time of traditional water extraction is long, the extraction effect is better, and easily available materials are used. In this paper, the extraction of *Russula alutacea* Fr. polysaccharide, the antioxidant activity of the polysaccharide, the preparation of water-soluble polysaccharide by acetylation, its structural characterization, activity, etc. were studied. The scavenging ability of water-soluble polysaccharide, acetyl polysaccharide and vitamin C towards OH•, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion (O_2^-•) was compared and valuable information on the modification/structure activity relationship of *Russula alutacea* Fr. polysaccharide was accumulated.

EXPERIMENTAL

Materials

Analytical methods

Fresh *Russula alutacea* Fr. fruiting bodies were purchased from a local market in Jinggu County, Yunnan. The fresh bodies were air-dried and shattered with mini plant sample grinder (DFT-100, Beijing KeSi Jia Technology Co., Ltd.), and then the powder was sieved with a 100-mesh screen.

Preparation of of Russula alutacea Fr. water-soluble polysaccharide

Russula alutacea Fr. samples (40 g) were weighed and extracted three times with water at a solid-to-liquid ratio of 1:20 at 80℃ for 2 h. The combined extracts were centrifuged and the residue was discarded. 1% (w/v) activated carbon was added to the water-soluble crude polysaccharide in the last step, the mixture was shaken at 75 ℃ for 2.5 h, centrifuged and the precipitate was discarded. Add 5 times sevage solution (n-butyl alcohol to chloroform, 1:5) into the decolorized supernatant, shake for 20~30 min, centrifuge the obtained supernatant then add 4 times absolute alcohol into it, place in the refrigerator (4℃) for 4 h. After centrifugation and drying of the precipitate pure *Russula alutacea* Fr. water-soluble polysaccharide was obtained.

The content of crude polysaccharide was determined by the phenol-sulfuric acid method [11]. The polysaccharide content in the extract was determined by the absorption values at OD490 which was the maximum absorption wavelength of the polysaccharide.

Acetylation modification of Russula alutacea Fr. polysaccharide

Acetylation modification was used [12]. The acetylation substitution degree was determined [13]. The content of total carbohydrates was determined using a phenol-sulfuric acid colorimetric method with glucose as a standard [14].

FT-IR analysis

For IR spectroscopy, polysaccharide powders were mixed with KBr, grinded, and pressed into 1 mm pellets. Then scanning measurement was performed in the range from 4000 to 400 cm^{-1}.

In vitro analysis of the antioxidant activity

Reducing power was evaluated with a slight modification of the published method [15]. The scavenging effect of superoxide anion (O_2^-•) was determined by the pyrogallol autooxidation method [16]. The scavenging rate of DPPH was determined according to [17]. The scavenging ability of hydroxyl free radical (OH•) was determined according to [18].

RESULTS AND DISCUSSION

The standard curve of *Russula alutacea* Fr. polysaccharide content was obtained by the phenol-sulfuric acid method in the range of 40.00-200.00 μg/ml, the regression equation is $y=7.8436x+0.0138$ ($R^2=0.9993$) and has good linear relationship. The polysaccharide rate was 1.75%. The regression equation of the standard curve of acetyl using a standard sample of acetylcholine chloride was $y=0.4575x-0.0007$ ($R^2=0.9997$), and has good linear relationship within the determination range. According to the regression equation the calculated acetyl concentration of the acetylated polysaccharide was 0.63 mg/ml, and the substitution degree was 0.44%. 1 mg of dry sample

and 150 mg of KBr were pressed onto a pellet, then scanning measurement was performed in the range of 4000-400 cm^{-1}.The modified polysaccharides exhibited a strong stretching vibration characteristic absorption peak of O-H and a bending vibration absorption peak of C-O at 3400 cm^{-1} and 1105 cm^{-1}, respectively, with typical absorption characteristics of polysaccharides. Acetylation products, in addition to retaining the characteristic absorption of the original polysaccharide, also remarkably enhanced the C=O stretching vibration characteristic absorption peak of the ester group at 1731 to 1743 cm^{-1} and the C-O stretching vibration absorption characteristic peak of the ester group at about 1246 cm^{-1}. The above results showed that the polysaccharide was successfully modified by acetylation (Figs. 1 and 2).

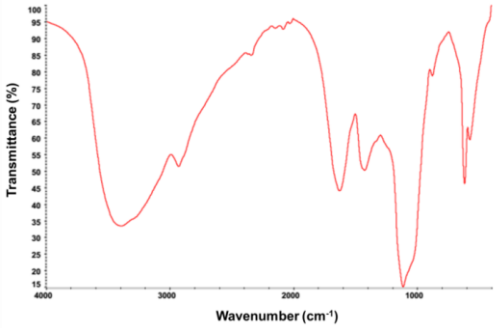

Fig.1. Infrared spectrum of unmodified polysaccharides

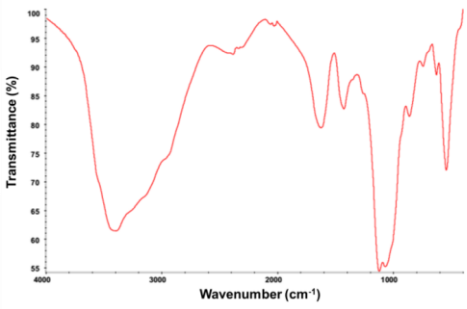

Fig.2. Infrared spectrum of modified polysaccharides

Reducing force is one of the indices to evaluate the antioxidant activity of the material. The antioxidant mechanism is: material with reducing force can react with free radicals to stabilize free radicals, for interrupting the lipid peroxidation of the chain reaction.

From table 1 it is seen that before and after acetylation modification, the *Russula alutacea* Fr. polysaccharide concentration and ascorbic acid were positively correlated with reducing ability.

Table 2 shows that water-soluble polysaccharide, acetylation-modified polysaccharide and vitamin C have strong scavenging effect on $O_2^{-}\bullet$, which increases with the increase in mass concentration in the range of 0.34 ~ 1.7 mg/mL.

Table 1. Effect of acetylation cation on reducing power

Sample	Concentration (mg/mL)	Absorbance
Polysaccharides	0.34	0.151
	0.68	0.403
	1.02	0.55
	1.36	0.733
	1.70	0.87
Acetylated polysaccharides	0.34	0.196
	0.68	0.681
	1.02	0.857
	1.36	0.979
	1.70	1.218
Ascorbic acid (vitamin C)	0.34	0.295
	0.68	0.631
	1.02	1.02
	1.36	1.454
	1.70	1.839

Table 2. Scavenging effect on superoxide anions

Sample	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
Polysaccharides	0.34	2.2	1.4725
	0.68	19.9	
	1.02	27.9	
	1.36	48.2	
	1.70	58.3	
Acetylated polysaccharides	0.34	29.1	0.6621
	0.68	58.9	
	1.02	68.8	
	1.36	76.3	
	1.70	86.8	
Ascorbic acid (vitamin C)	0.34	7.3	1.3701
	0.68	24.8	
	1.02	35.6	
	1.36	51.6	
	1.70	61.3	

Table 3. Scavenging effect on DPPH free radicals

Sample	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
Polysaccharides	0.34	19.6	1.2543
	0.68	24.6	
	1.02	30.5	
	1.36	58.4	
	1.70	70.3	
Acetylated polysaccharides	0.34	30.7	0.8498
	0.68	42.8	
	1.02	61.9	
	1.36	66.5	
	1.70	77.4	
Ascorbic acid (vitamin C)	0.34	37.8	0.5473
	0.68	54.6	
	1.02	76.9	
	1.36	92.9	
	1.70	97.7	

In the range of 0.34~1.7 mg/mL of mass concentration, the scavenging ability of acetylation-modified polysaccharide on $O_2^{-}\bullet$ is greater compared with water-soluble polysaccharide and

vitamin C, and the scavenging ability of vitamin C O_2^{-}• is greater than that of water-soluble polysaccharide.

As can be seen from table 2, the highest scavenging rates of water-soluble polysaccharide, acetylation-modified polysaccharide and vitamin C are: 86.8%, 58.3%, 61.3%, respectively. According to the value of EC50 the scavenging ability on O_2^{-}• is: acetylation-modified polysaccharide>vitamin C>water-soluble polysaccharide. After acetylation modification, the activity of water-soluble polysaccharide is enhanced.

From table 3 it can be seen that water-soluble polysaccharide, acetylation- modified polysaccharide and vitamin C have a strong scavenging effect on DPPH which increases with the increase in mass concentration in the range of 0.34 ~ 0.7 mg/mL. In this range, the scavenging ability of vitamin C on DPPH is greater compared to water-soluble polysaccharide and acetylation-modified polysaccharide, and the scavenging ability of acetylation-modified polysaccharide on DPPH is greater than that of water-soluble polysaccharide. As can be seen from table 3, the highest scavenging rates of water-soluble polysaccharide, acetylation-modified polysaccharide, vitamin C are 70.3%, 77.4%, 97.7%, respectively. According to the value of the scavenging ability on DPPH is: vitamin C > acetylation- modified polysaccharide>water-soluble polysaccharide. After acetylation modification, the activity of the water-soluble polysaccharide is enhanced.

Table 4. Scavenging effect on hydroxyl free radicals

Sample	Concentration (mg/mL)	Scavenging activity (%)	EC_{50} (mg/ml)
Polysaccharides	0.34	5.6	1.3072
	0.68	24.9	
	1.02	36.2	
	1.36	56.2	
	1.70	64.3	
Acetylated polysaccharides	0.34	14.9	1.1152
	0.68	31.1	
	1.02	48.7	
	1.36	61.4	
	1.70	73.3	
Ascorbic acid (vitamin C)	0.34	25.4	0.8948
	0.68	44.2	
	1.02	56.2	
	1.36	64.5	
	1.70	85.6	

From table 4 it can be seen that water-soluble polysaccharide, acetylated polysaccharide and vitamin C have strong scavenging effect on OH• which increases with the increase in mass concentration in the range of 0.34 ~ 1.7 mg/mL. In this range, the scavenging ability of vitamin C on OH• is greater in comparison with water-soluble polysaccharide and acetylated polysaccharide, and the scavenging ability of acetylated polysaccharide on OH • is greater than that of water-soluble polysaccharide.

As can be seen from table 4, the highest scavenging rates of water-soluble polysaccharide, acetylation modified polysaccharide, vitamin C are 64.3%, 73.3%, 85.6%, respectively. According to the value of EC50 the scavenging ability on OH• is: vitamin C> acetylated polysaccharide>water-soluble polysaccharide. After acetylation modification, the activity of water-soluble polysaccharide is enhanced.

CONCLUSION

In this paper, *Russula alutacea* Fr. water-soluble polysaccharides were extracted. Acetylation technology was applied to the study of *Russula alutacea* Fr. polysaccharide. Acetic anhydride was used as an acylation reagent to perform the acetylation modification of *Russula alutacea* Fr. polysaccharide, Then the antioxidant activity of *Russula alutacea* Fr. polysaccharide and its acetylated products was studied by *in vitro* test method to investigate the effect of acetylation on the biological activity of *Russula alutacea* Fr. polysaccharide. *Russula alutacea* Fr. processed by water extraction and ethanol precipitation yielded water-soluble polysaccharide and acetylation-modified polysaccharide. They have a good scavenging effect on OH•, O_2^{-}• and DPPH. According to the EC50 value, the scavenging ability on superoxide anion was: acetylation-modified polysaccharide > vitamin C> water-soluble polysaccharide; the scavenging ability on DPPH was: vitamin C > acetylation-modified polysaccharide > water-soluble polysaccharide; the scavenging ability on OH• was: vitamin C> acetylation-modified polysaccharide>water-soluble polysaccharide. After acetylation modification, the scavenging ability of water-soluble polysaccharide on OH•, O_2^{-}• and DPPH were enhanced.

Acetylation is one of the most important methods in the study of the antioxidant activity of polysaccharides. Jiao *et al.* [19] studied the molecular modification and biological activity of jujube polysaccharide. On the basis of extraction and purification of jujube polysaccharide, in this paper, molecular modification techniques were applied to the study of jujube polysaccharide. By ultrasonic treatment, hydrochloric acid degradation and sulfatation, carboxyl methylation and

acetylation modification, its molecule was further modified, and then the antioxidant activities, hypoglycemic activity and inhibitory effect on hyaluronic acid enzyme of jujube polysaccharide and its molecular modification products were studied by *in vitro* test method. It confirmed that jujube polysaccharides have inhibitory effect on α-amylase, α-glucosidase, hyaluronidase and nonenzymatic glucosylation reaction.

Molecular modification can significantly change the biological activity of jujube polysaccharide, and this change is more related to the change of the advanced structure of jujube polysaccharide caused by molecular modification. Jujube polysaccharide conformational changes caused by molecular modification are more important for its biological activity effect than physical and chemical changes. To maintain a certain length and space conformation is the key to keep its activity. Xie [20] studied the molecular modification and biological activity of *Cyclocarya paliurus* (Batal) Iljinskaja polysaccharide. The study of molecular modification, including sulfatization, hydroxyl methylation, phosphorylation, and so on, has gradually become one of the hot topics in the study of polysaccharides. Among them, because of its good antioxidant and antiviral activity, sulfated polysaccharides have been widely concerned by researchers. Wang *et al.* [21] studied the preparation and antioxidant activity of acetylated fucoidan from *Laminaria japonica* and determined the scavenging ability on OH• and O_2^-•, DPPH and reduction ability of acetylation derivatives under different conditions. The results showed that the acylating agent dosage and reaction temperature have significant effects on fucoidan acetylation ($P<0.05$). The scavenging activities on OH• and O_2^-•, DPPH and reduction ability of acetyl derivatives were different. It was concluded that NBS as a catalyst for acetylation of Fucoidan from *Laminaria japonica* is feasible, could replace the traditional strong toxicity of pyridine fucoidan from *Laminaria japonica* by acetylation of hydroxyl. The antioxidant activity of the polysaccharide was obviously enhanced after acetylation. It is of great significance to carry out further research on it.

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