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Free-radical degradation by Fe^{2+}/Vc/H_2O_2 and antioxidant activity of polysaccharide from *Tremella fuciformis*

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

[a] Department of Pharmacology, Huzhou Teachers College, Huzhou 313000, China
[b] School of Pharmaceutical and Biological Sciences, Weifang Medical University, Weifang 261053, China

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ABSTRACT

The free-radical degradation and antioxidant activity of polysaccharide from *Tremella fuciformis* was investigated. In present study, the combination of Fe^{2+}, ascorbic acid and H_2O_2 was used as degradation regents in order to obtain the lower molecular weight product. The result ascertained oxidative-reduce degradation did not change the main structure of polysaccharides in the test conditions. Five degraded polysaccharides were selected to evaluate their antioxidant activities *in vitro*. It was found that the degraded sample with lower molecular weight possessed the higher antioxidant activities. It was possible that free-radical degradation is an effective way for enhancing antioxidant activity to decrease molecular weight of polysaccharides.

1. Introduction

In recent years, polysaccharides have been widely used in food and cosmetics industry. And more and more researchers show that polysaccharides possess good biological activity, such as anti-tumor, immunity, hypoglycemic, anti-aging and so on, which indicate they have a very wide application prospect in the field of medicine (Jhamandas, Wie, Harris, MacTavish, & Kar, 2005; Kim & Joo, 2008; Martinichen-Herrero, Carbonero, Gorin, & Iacomini, 2005; Mazumder et al., 2002; Wang, Zhang, Li, Hou, & Zeng, 2004). However, the high molecular weight of many polysaccharides limited their properties which refer to both physical characteristics, such as solution viscosity, and more complex properties, such as biological activity (Zhou, Yu, Zhang, He, & Ma, 2012). The larger molecular weight resulted in the polysaccharides being great difficult in spanning membranes to exert their biological effects. Therefore, the clinical application of natural polysaccharides has been restricted due to their disadvantages of having large molecular weights and low solubility. It plays an important role in studying on the degradation of polysaccharides.The white jelly mushroom *Tremella fuciformis*, also called 'silver ear' or 'snow fungus', is found mainly in subtropical regions but it has also been reported from tropical and temperate regions and even frigid zones. It grows on the decayed trunk of hardwood trees such as oak and willow (Chang, 1998). In recent years, study on the structure an pharmacological activities of polysaccharide from *T. fuciformis* has received extensive attention (Cheung, 1996; Cho, Oh, Chang, & Yun, 2006; Du, Zhang, Yang, Tang, Jia, & Pan, 2010; Reshetnikov, Wasser, Duckman, & Tsukor, 2000). From the reports already described of these yeast polysaccharides, it can certainly be concluded that they deserve detailed further study. However, the lager molecular weight of polysaccharide presents the researchers with a dilemma. At present, this yield still is quite low and practically no work has been done on the degradation of polysaccharides from *T. fuciformis*. Just as the mention above, once this is solved, the specific and unique properties of these polymers may lead to a wide application range and an identifiable market share.

Among the methods of polysaccharides degradation, oxidation method was used more and more recently, such as H_2O_2 method, $NaBO_3$ method, ClO_2 method and Cl_2 method (Du, Lai, & Yan, 2008; Hjerde, Stokke, Smidsrød, & Christensen, 1998; Kubota, Tatsumoto, Sano, & Toya, 2000). Especially in Japan, there were a number of studies reported in the literature every year, in which H_2O_2 method was mostly used. Based on this, several methods were developed, such as H_2O_2–Vc, H_2O_2–UV, H_2O_2–$NaClO_2$ and H_2O_2–HAc method (Huang, Wang, Wang, & Huang, 2004; Qin, Du, Liu, & Xiao, 2001; Zhang, Wang, Mo, & Qi, 2013). In this study, the iron–H_2O_2–Vc method was described for the iron-oxidative degradation of natural polysaccharide from *T. fuciformis*. These degraded polysaccharides were selected to evaluate their antioxidant activities *in vitro* and characterized the relationship between antioxidant activity and chemical characteristics. It was expected that this investigation

* Corresponding author. Tel.: +86 572 2321166; fax: +86 572 2321166.
E-mail addresses: zhzs@hutc.zj.cn, shan.911@163.com (Z. Zhang).

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Table 1
Effect of the concentrations of Fe^{2+}, ascorbic acid and H_2O_2 on polysaccharide degradation.

Samples	Fe^{2+} (mM)	c (H_2O_2) (mM)	c (Ascorbic acid) (mM)	Intrinsic viscosity (mL/g)	Decrease in viscosity (%)
TP	–	–	–	285.24	–
LTP 1	0	5	5	251.20	11.93
LTP 2	0	10	10	182.42	36.05
LTP 3	0	20	20	177.69	37.70
LTP 4	2	2	20	112.75	60.47
LTP 5	2	5	10	157.36	44.83
LTP 6	2	5	20	98.22	65.56
LTP 7	5	5	10	132.10	53.68
LTP 8	5	5	20	115.21	59.61
LTP 9	5	5	50	65.96	76.87
LTP 10	5	10	10	195.99	31.28
LTP 11	5	10	20	132.68	53.48
LTP 12	10	5	5	239.18	16.14
LTP 13	10	10	10	153.65	46.13
LTP 14	10	20	100	65.39	77.07

would provide encouragement for further exploration into degradation of natural polysaccharide.

2. Materials and methods

2.1. Materials

T. fuciformis was collected in Sichuan, China. Ethylene diamine tetraacetic acid (EDTA), ascorbic acid, H_2O_2, sodium citrate and other reagents were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3500 Da.

2.2. Analytical methods

Total sugar content was determined by phenol–sulfuric acid method using rhamnose as standard (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956). Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard (Bitter and Muir, 1962).

Infrared spectrums were measured by a Nicolet Magna-Avatar 360 with KBr disks.

The intrinsic viscosity (η_r) of reaction mixtures was determined in an Ubbelohde viscosimeter at 25 °C. The result was measured as the following equation [$\eta_r = (\ln t/t_0)/c$], where t is the solution flow time (s), t_0 the solvent flow time (s) and c is the concentration of solution in distilled water (g/mL).

Molecular weight of all samples was determined by HP-GPC on a Waters 515 GPC system at 35 °C, where 0.7% Na_2SO_4 solution was used as mobile phase with a flow rate of 0.5 mL/min. TSK G3000 column (300 mm × 7.8 mm) and 2140 refractive index detector was used. A series of different molecular weight dextrans purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China) were used as standard.

2.3. Extraction of polysaccharides

Dried *T. fuciformis* was soaked into water for 0.5 h and then shattered. The thick liquid was added into distilled water and triturated for 4 h at 100 °C. The solution was dialyzed against distilled water for 48 h, and then concentrated, precipitated by the addition of ethanol to a final concentration of 75% (v/v). The precipitate was air-dried to give *T. fuciformis* polysaccharide (named after TP) as a white powder.

2.4. Degradation of TP

A solution of raw polysaccharide TP (1.0% m/v) was added in ascorbic acid, H_2O_2 and $FeSO_4$ solution according to groups arrangement in Table 1. After stirred for 2 h at 25 °C, the solution was dialyzed and then precipitated by 75% alcohol and centrifuged to obtain the degraded product.

2.5. Antioxidant activities

2.5.1. Hydroxyl radical assay

The reaction mixture, containing all different samples (0.6–7.0 mg/mL), was incubated with EDTA-Fe^{2+} (2 mM), saffron (360 μg/mL), and H_2O_2 (3%) in potassium phosphate buffer (150 mM, pH 7.4), 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 sample.

2.5.2. 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 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:

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

2.5.3. Reducing power assay

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, 1.0 mL 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 potassium ferricyanide (1%, w/v), and was incubated at 50 °C for 20 min. Afterwards, 2.0 mL 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. Degradation of TP

In present study, iron, H_2O_2 and Vc were used to be degradation regent, which was based on the mechanism of action of ascorbic acid and H_2O_2 appears to be via $^{\bullet}OH$ since they fulfilled the requirements for a Fenton reaction in plants (Fry, 1998). Vc(AH_2) reduces oxygen in vivo to hydrogen and simultaneously reduce metal ions to ferrous. And then hydrogen peroxide and ferrous conduct Fenton reaction to generate hydroxyl radical generation. These hydroxyl radicals are very reactive and will react with the hydrogen atoms of polysaccharides on plant cell (hydrogen abstraction reaction), which causes the glycoside bond cleavage. This process could be shown with the following equations:

$$AH_2 + O_2 \rightarrow A + H_2O_2 \quad (1)$$

$$AH_2 + 2Fe^{3+} \rightarrow A + 2H^+ + 2Fe^{2+} \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (3)$$

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \quad (4)$$

3.2. Influence of concentration of the degradation regent

An aqueous solution of TP (1%) was degraded within 2 h completely. Table 1 showed the change of viscosity of polysaccharide solution under the effect of different degradation regent. From the table, the ferrous, H_2O_2 and Vc concentration have a great impact on the degradation of polysaccharides. From LTP1 to LTP3, when there was no case of ferrous, the higher the absolute concentrations of H_2O_2 and Vc were, the lower the viscosity of solution was. However, the intrinsic viscosity of solution unobvious reduced when the concentration increased from 10 to 20 mM. From LTP4 to LTP14, the intrinsic viscosity of solution significantly reduced with the concentration of ferrous. Similarly, from LTP7-LTP9, the result was similar with the concentration of H_2O_2 in case of fixed concentration of ferrous and Vc. It suggested that ferrous and H_2O_2 were important factor for the degradation of TP. From LTP4 and LTP6, LTP7 and LTP9, Vc slightly affects the viscosity of solution. And moreover, from LTP12, degradation system weakened when the concentration of ferrous was higher than H_2O_2 and Vc. To be sure, when the concentration of H_2O_2 was higher than or equal to 20 mM, the greater the degradation effect, the higher the absolute concentrations of ferrous and Vc were. The intrinsic viscosity of solution reduced 77.1% for the sample LTP14, which suggested that the degradation was successful.

According to Fenton reaction, there were three reagents, oxidant, reducer and metal ion. However, there was several nature metal ion (such as Fe, Zn, Cu) in polysaccharide. Therefore, in present study, degradation of TP was still in progress without Fe. But just as LTP3 in Table 1, the viscosity of solution was still relatively large even if the concentration of H_2O_2 and Vc was 20 mM. From the results, the lower viscosity arises in the case of Fe. In present study, a few of Fe^{2+} would limit the reaction rate (Eq. (3)). And so, the addition of Fe^{2+} could speed up the degradation of TP. Moreover, if the absolute concentrations of ferrous higher than H_2O_2 and Vc, Eq. (3) occurred quickly (Suh, Zhu, & Frei, 2003). As a result, a part of Vc was used to reduce Fe^{3+} (Eq. (2)), which lead to the inhibition of H_2O_2 (Eq. (1)). Finally, the Fenton reaction was inhibited and then $^{\bullet}OH$ was not generated. That was why LTP12 showed the higher viscosity.

3.3. Characteristics of samples

Raw material TP and Five degraded samples, LTP1, LTP5, LTP6, LTP9 and LTP10, were selectively prepared and their chemical analysis results were shown in Table 2. From the table, five degraded products contained similar total sugars and uronic acid content to that of natural polysaccharide. It indicated that the degradation could not result in destruction of main chain.

Table 2
The chemical characteristics of the samples.

Samples	Total sugar (%)	Uronic acid (%)	M_w (Da)	M_n (Da)	M_w/M_n
TP	47.63	2.37	599,580	231,721	2.59
LTP 1	43.12	2.58	289,749	181,011	1.60
LTP 5	49.31	3.98	155,863	62,147	2.51
LTP 6	40.49	3.20	85,223	38,218	2.23
LTP 9	49.32	3.77	26,895	12,571	2.14
LTP 10	46.04	2.88	141,992	102,530	1.38

M_w, weight average molecular weight; M_n, number average molecular weight.

For the FT-IR spectrums in Fig. 1, typical signals of polysaccharide from *Tremella fuciformis* at 3425, 2925, 1633, 1419, 1251 and 1071 cm^{-1} were clear for all the samples. 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, C—H variable angle vibration and C—O—C stretching vibrations in ring. These results suggested that no major functional group transformations happened during the degradation.

3.4. Molecular weight distribution of samples

Table 2 shows the mean molecular weight distribution of degraded polysaccharides determined by HPGPC. The molecular weights were obtained from their retention time with the calibration curves of dextran. From the table, molecular weight (M_W) and molecular number (M_n) of each polysaccharide was in concordant to its viscosity. Each sample showed a relatively large symmetrical peak although some tailing was observed. The dispersity index (M_w/M_n) was used to show the range of molecular weight distribution and sharp degree of chromatographic peak after the polysaccharide degradation. For the single dispersion, M_w/M_n was 1. But when the molecular weight distribution becomes wider, M_w/M_n becomes larger. From Table 2, the dispersity indexes of the samples LTP1 and LTP10 were lower than 2, which showed their molecular weight distributions were in a narrow range. However, that of the other samples were higher than 2, which indicated they were in a broad range.

And in addition, M_w of these samples decreased with different concentration of degraded agents. A lower molecular weight of 26,895 Da was obtained, which suggested the degradation method was sound.

3.5. Antioxidant activities

Fig. 2 shows the concentration-dependent hydroxyl radical scavenging activity of TP and its degraded samples. All the samples exhibited varying degrees of antioxidant activity. Especially, LTP9 possessed significant scavenging effects, which was 64.2% at a concentration of 1.52 mg/mL. At the concentration over 0.21 mg/mL, the scavenging activities of all the degraded samples were stronger than that of raw polysaccharide TP. The results indicated that lower molecular weight polysaccharides did show stronger scavenging activity on superoxide radicals.

The scavenging abilities on superoxide radicals of all samples were shown significant in a concentration-dependent fashion in Fig. 3. At the concentration below 0.05 mg/mL, the scavenging effect significantly increased with increasing concentration. At a higher concentration 0.42 mg/mL, the inhibitory effects of LTP1, LTP5, LTP6, LTP9 and LTP10 were 44.5%, 56.7%, 67.8%, 66.1%, and 45.3% respectively. When the concentration was higher than 0.42 mg/mL, the scavenging effect increased slowly.

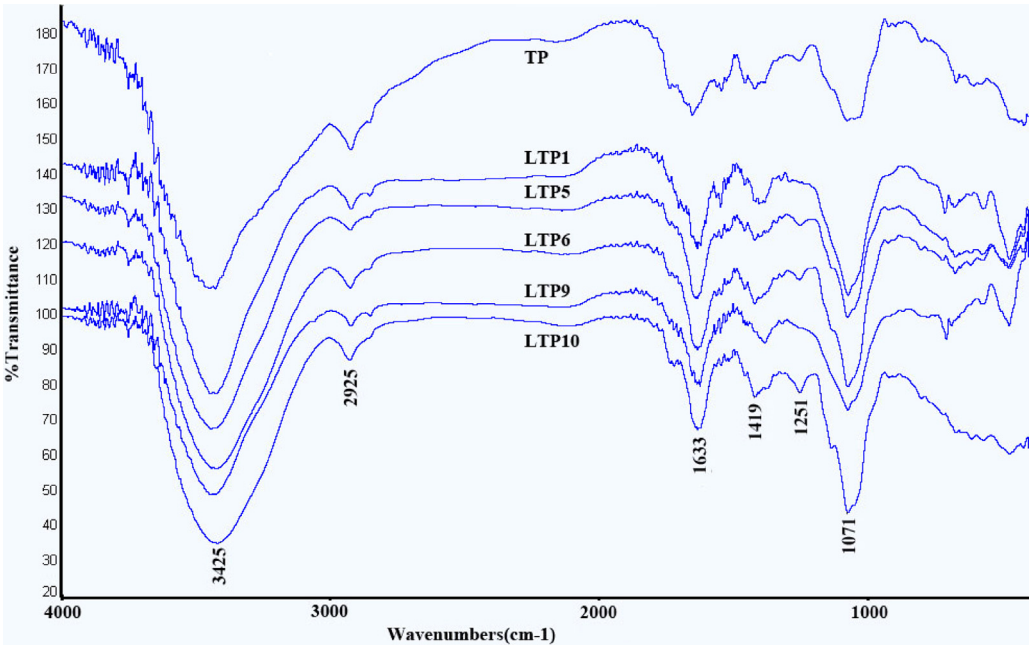

Fig. 1. FT-IR spectrums of the samples TP and its degraded samples.

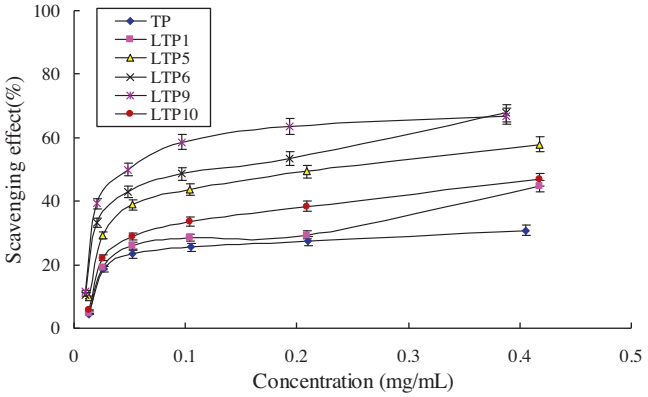

Fig. 2. Scavenging effects of the samples on hydroxyl radical. Values are means ± S.D. ($n = 3$).

The reducing power of all samples was shown in Fig. 4. From the figure, the reducing power of the degraded samples correlated well with increasing concentration, which showed stronger reducing powers. However, the absorbance of TP was only 0.112 at 0.24 mg/mL because of the slow rate of increasing power with increasing concentration.

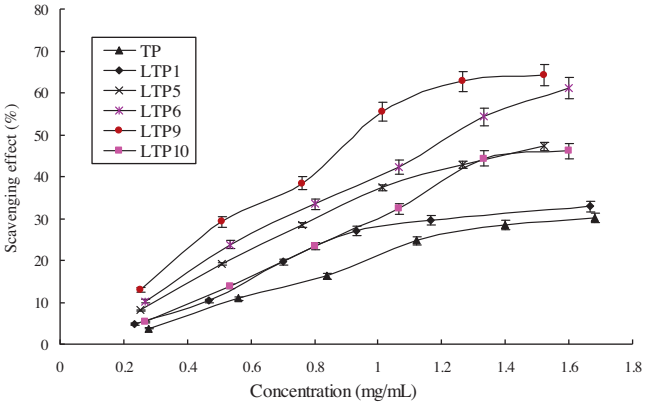

Fig. 3. Scavenging effects of the samples on superoxide radical. Values are means ± S.D. ($n = 3$).

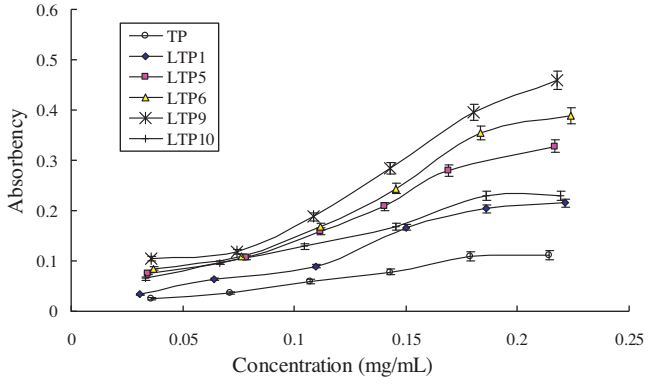

Fig. 4. Reducing power assay of the samples. Values are means ± S.D. ($n = 3$).

In the antioxidant activity test, the molecular weight significantly affected the antioxidant activity. The lower molecular weight sample contained more free hydroxyl groups, which may have a very important effect on the antioxidant activities. It was supposed that the chemical groups in degraded sample have more chance to contact with radical because of the better water-soluble and more surface area. The further studies are needed to improve our understanding of antioxidant activities mechanism.

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