Int. J. Mol. Sci. **2007**, *8*, 670-679

International Journal of
Molecular Sciences
ISSN 1422-0067

www.mdpi.org/ijms/

Full Research Paper

Modification of Low Molecular Weight Polysaccharides from *Tremella Fuciformis* and Their Antioxidant Activity *in Vitro*

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Received: 13 February 2007; in revised form: 31 May 2007 / Accepted: 5 July 2007 / Published: 17 July 2007

Abstract: In this study, sulfated low molecular-weight *Tremella fuciformis* polysaccharides (SLTP) with different sulfate contents were synthesized and their antioxidant activities, including superoxide anion radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical and hydroxyl radical scavenging activities were investigated. The results indicated that, compared to natural *Tremella fuciformis* polysaccharide (TP) and low molecular weight *Tremella fuciformis* polysaccharide (LTP), sulfated LTP (SLTP) exhibited stronger scavenging activity towards superoxide anion, DPPH and hydroxyl radicals. In all the cases the effect was found to be dose dependent. The scavenging activity of SLTP was found to be in parallel with the degree of sulfation of SLTP.

Keywords: *Tremella fuciformis,* polysaccharide, degradation, sulfate, antioxidant.

1. Introduction

Tremella fuciformis is a common nutritional food in China. It is also a traditional Chinese medicine regarded as a tonic for “weakness and aging”. Such dietary and medicinal properties are assumed to be

due solely to the polysaccharides produced by the Tremella species. TP is usually extracted from *Tremella fuciformisis* fruit bodies or fermentation mycelia. Its average molecular weight is in the range of 12~50 kD. TP has a mannose backbone and side chains containing glucosyl, fucosyl xylosyl, and glucuronic acid residues [1]. TP has been found to show several biological activities such as anti-inflammation, anti-cancer, anti-mutation, reducing hypertension, reducing blood sugar and immunity improving properties [2]. The molecular weight of polysaccharides has significant effect on their biological activities. High molecular weight polysaccharides are not conducive to easy crossing of cell-membranes, and exert their biological activities with difficulty. Conceptually, degradation of high molecular weight polysaccharides into low molecular weight ones might improve their biological activities noticeably and therefore, preparation of low molecular weight polysaccharides becomes an interesting method for preparation of new drugs. Chemical modification of polysaccharides provides an opportunity to obtain new pharmacological agents with possible therapeutic uses [3]. It has been reported that sulfate groups of polysaccharides probably contribute to their biological activities [4].

Reactive oxygen species (ROS), capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart disease and many other health problems related to aging [5,6]. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body against free radicals and retard the progress of many chronic diseases [7].

Until now, studies on TP are mainly focused on the extraction and pharmacological activity. However, little information is available concerning the degradation of TP and the modification of LTP. In the present study, sulfation of LTP was done under various conditions to obtain different SLTPs such as SLTPI, SLTPII and SLTPIII and their radical scavenging activity has been compared with the naturally existing TP and LTP and the results obtained are presented.

2. Experimental Section

2.1. Materials

TP (Average molecular weight is 32 kD) was prepared as described by Wu *et al*. [8]. DPPH, nicotinamide adenine dinucleotide–reduced (NADH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), nitro blue tetrazolium (NBT), and Vitamin C (Vc) were purchased from Sigma Chemical Co. All others chemicals and reagents were of analytical grade.

2.2 Preparation of LTP

LTP was obtained by hydrolyzing TP in the following way: the ratio of TP/solvent (w/v) is 1:50, the concentration of HCl is 0.7 M, 80 °C of water bath for 2 h. The average molecular weight of LTP was 1.2 kD. The molecular weight was determined with a Waters gel permeation chromatography (GPC) [9].

2.3. Modification of LTP

SLTP was synthesized by the chlorosulfonic acid-pyridine method [10]. The sulfation agent, pyridine-SO_3 complex, was obtained by dropping chlorosulfonic acid ($HClSO_3$, 2 ml) into pyridine (10 ml) in an ice-water bath. Dry LTP (0.2 g) was added to formamide (FA, 20 ml), and the mixture was

stirred at different temperatures (60, 80, 100 °C) for 30 min to disperse it evenly. Then pyridine-SO_3 complex was added. After 2 h, the mixture was cooled to room temperature, neutralized with 1 M NaOH solution, dialyzed against tap water for 72 h, and precipitated with 95 % ethanol for 24 h. The precipitate was filtered off and washed three times with ethanol, then lyophilized to give SLTP. Three SLTPs with different sulfate content were obtained by changing the reaction temperature with fixed molar ratio of LTP/pyridine-SO_3 complex and reaction time. Sulfate content was determined by using the traditional method of barium chloride-gelatin [11] to evaluate degree of sulphatation (DS).

$$DS = \frac{162 \times (\frac{SO_4^{-2}\%}{98})}{100 - (\frac{96}{98} \times SO_4^{-2}\%)} \quad (1)$$

2.4. Assay for Antioxidant Activity

2.4.1. DPPH Radical Scavenging Activity of SLTP, LTP and TP

The DPPH radical scavenging activity was tested by the method of Li *et al*. [12]. A 0.1 mM solution of DPPH in ethanol was prepared and this solution (1 ml) was added to sample (10-50 μg/ml, 2 ml) in water. After 30 min, absorbance was measured at 517 nm. Decreased absorbance of the reaction mixture indicated higher DPPH radical scavenging activity. The DPPH concentration (C_{DPPH}) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2= 0.9035): Absorbance =-2.9535 × C_{DPPH}.

The scavenging activity of DPPH radical was calculated using the following equation:

$$\text{Scavenging activity}\,(\%) = (1 - \frac{A_{\text{sample at 517 nm}}}{A_{\text{control at 517 nm}}}) \times 100 \quad (2)$$

2.4.2. Hydroxyl Radical Scavenging Activity of SLTP, LTP and TP

The reaction mixture, containing sample (10-50 μg/ml), was incubated with deoxyribose (3.75 mM), EDTA (100 μM), ascorbic acid (100 μM),H_2O_2 (1 mM), and $FeCl_3$ (100 μM) in phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C [13]. The reaction was terminated by adding TBA (1 %, w/v, 1 ml) and TCA (2 %, w/v, 1 ml), then the tube was heated in a boiling water bath for 15 min. After cooled to room temperature, the absorbance of the mixture was measured at 532 nm against a blank. The scavenging activity of hydroxyl radical was calculated using the following equation:

$$\text{Scavenging activity}\,(\%) = (1 - \frac{A_{\text{sample at 532 nm}}}{A_{\text{control at 532 nm}}}) \times 100 \quad (3)$$

2.4.3. Superoxide Anion radical Scavenging Activity of SLTP, LTP and TP

The superoxide radical scavenging activity was assessed by the method of Robak *et al*. [14]. The reaction mixture, containing sample (10-50 μg/ml), Tris–HCl (16 mM, pH 8.0), NADH (338 μM), NBT (72 μM), and PMS (30 μM), was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against a blank. The scavenging activity of superoxide anion radical was calculated using the following equation:

$$\text{Scavenging activity}\,(\%) = (1 - \frac{A_{\text{sample at 560 nm}}}{A_{\text{control at 560 nm}}}) \times 100 \quad (4)$$

2.5. Statistical Analysis

Data were presented as means ± standard deviations (SD) of three determinations and followed by Student's *t* test. Differences were considered to be statistically significant if $P < 0.05$.

3. Results and Discussion

3.1. Modification of LTP

Sulfated LTP were obtained by the chlorosulfonic acid/pyridine method. Three sulfated derivatives named SLTPI, SLTPII and SLTPIII were synthesized at different temperatures. The DS and water solubility are shown in Table 1. With the elevation of the temperature from 60 to 100 °C, the DS of the corresponding derivatives increased from 0.41 to 1.02. This indicated that with the elevation of temperature the DS increased. Water solubility was SLTP > LTP > TP. It showed that the degradation and sulfation improved the water solubility of TP.

The main monosaccharide unit in LTP is mannose. The axial -OH is more likely to be sulfated than equatorial -OH [15]. LTP has few OH groups bound to the C-1 atom. This makes the -OH group on C-2 easy to be sulfated. It has been reported that reactivity of polysaccharide hydroxyl group was C-6 > C-2 > C-4. The low sulfation at C-4 can be attributed to the steric hindrance [16].

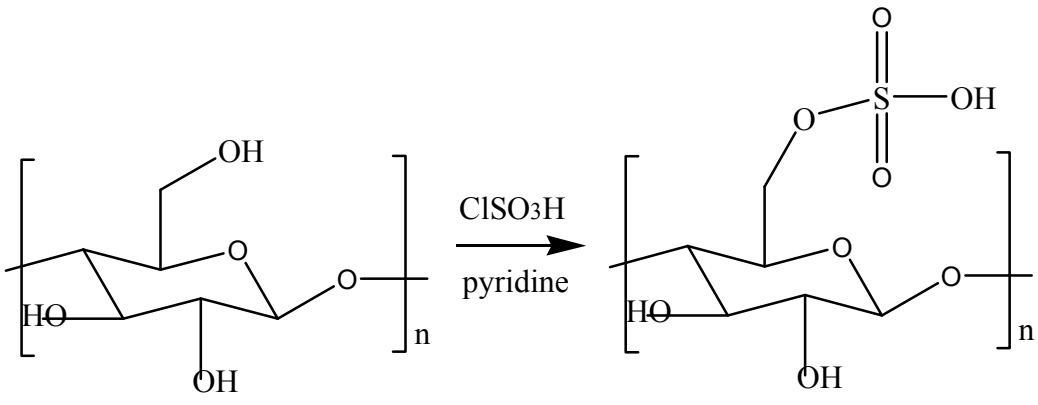

Figure 1. Sulfation of LTP.

Table 1. Sulfation and water solubility of SLTP, LTP and TP.

NO.	Temperature (°C)	Degree of sulphate (DS)	Water solubility (mg/ml)
SLTPI	60	0.41±0.03	23±4.04
SLTPII	80	0.66±0.03	29±2.51
SLTPIII	100	1.02±0.09	41±2.56
LTP			11±2.01
TP			3±0.47

Results are presented as mean ± SD (n = 3).

3.2. Antioxidant activity

3.2.1. DPPH radical scavenging activity of SLTP, LTP and TP

Figure 2 shows the DPPH radical scavenging activity of TP, LTP, SLTP and Vc. The scavenging activity increased with increasing concentration from 10 to 50 μg/ml and sulfate content from 0.41 to 1.12. Among all the samples and Vc, SLTPIII exhibited the strongest scavenging activity against DPPH radical. Moreover, the three SLTPs showed higher scavenging activity than Vc ($P < 0.05$). TP showed the lowest scavenging activity. At the concentration of 10 μg/mL, the scavenging activity was 38.59 % for SLTPIII, while the concentration of SLTPIII was 50 μg/ml, a scavenging activity of 53.8 % was obtained.

Table 2. Absorbance of natural TP, its derivatives and Vitamin C at 517nm.

Concentration	TP	LTP	SLTPI	SLTPII	SLTPIII	Vc	$A_{control}$
10 μg/ml	0.486± 0.012	0.461± 0.017	0.427± 0.006	0.407± 0.013	0.340± 0.008	0.433± 0.008	0.553
20 μg/ml	0.464± 0.015	0.448± 0.019	0.401± 0.007	0.388± 0.008	0.309± 0.006	0.417± 0.007	
50 μg/ml	0.424± 0.008	0.408± 0.011	0.364± 0.012	0.339± 0.014	0.256± 0.009	0.372± 0.012	

Results are presented as mean ± SD (n = 3).

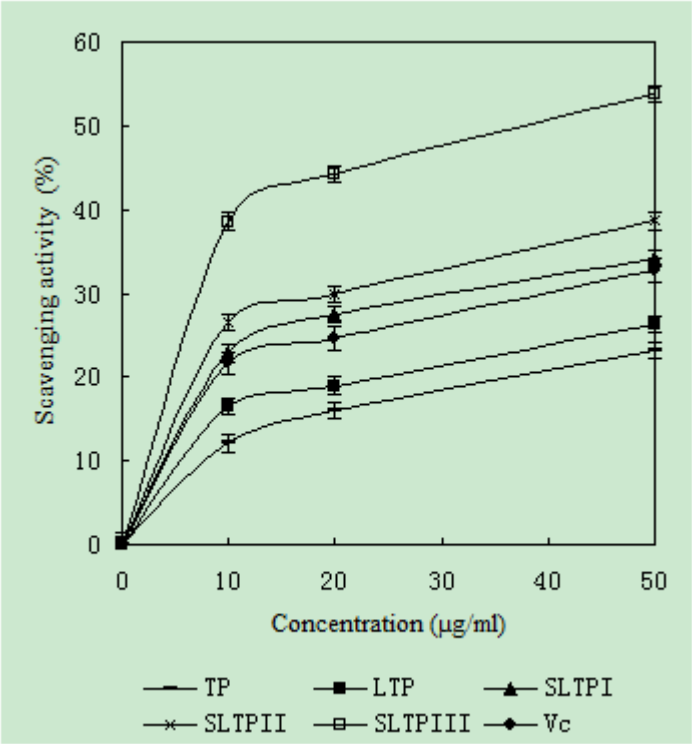

Figure 2. Scavenging activity of natural TP, its derivatives and Vitamin C against DPPH radicals. Values were means ± SD (n = 3).

3.2.2. Hydroxyl radical scavenging activity of SLTP, LTP and TP

Hydroxyl radical and its subsequent radicals are the most harmful ROS and they are responsible for the oxidative injury of biomolecules [17]. The scavenging activity of all samples and Vc are shown in Figure 3. The results suggested that SLTP, possessed more pronounced hydroxyl radical scavenging activity than LTP, TP and Vc ($P < 0.05$), and the scavenging activity increased with increasing the concentration. The scavenging activity of SLTPIII was obviously higher than other polysaccharides, and the highest scavenging activity was 65.9 %. The scavenging activity of SLTPIII at 10 μg/ml was found to be 36.1 %. The same scavenging activity could be obtained for LTP at 50 μg/ml. Vc showed the lowest scavenging activity against hydroxyl radical among all samples tested.

3.2.3. Superoxide anion radical scavenging activity of SLTP, LTP and TP

Among all the reactive oxygen species, superoxide anion radical is generated firstly. Although it is a relatively weak oxidant, it can decompose to form stronger reactive oxidative species, such as single oxygen and hydroxyl radicals [17]. Further, superoxide anion radicals are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals [18]. So, scavenging activity of superoxide anion radical is an important index for antioxidants.

Table 3. Absorbance of natural TP, its derivatives and Vitamin C at 532nm.

Concentration	TP	LTP	SLTPI	SLTPII	SLTPIII	Vc	$A_{control}$
10 μg/ml	0.928±	0.882±	0.842±	0.734±	0.675±	0.958±	1.057
	0.014	0.008	0.012	0.003	0.012	0.010	
20 μg/ml	0.808±	0.778±	0.695±	0.572±	0.503±	0.889±	
	0.006	0.009	0.007	0.009	0.013	0.006	
50 μg/ml	0.736±	0.691±	0.591±	0.487±	0.360±	0.844±	
	0.005	0.003	0.007	0.005	0.006	0.006	

Results are presented as mean ± SD (n = 3).

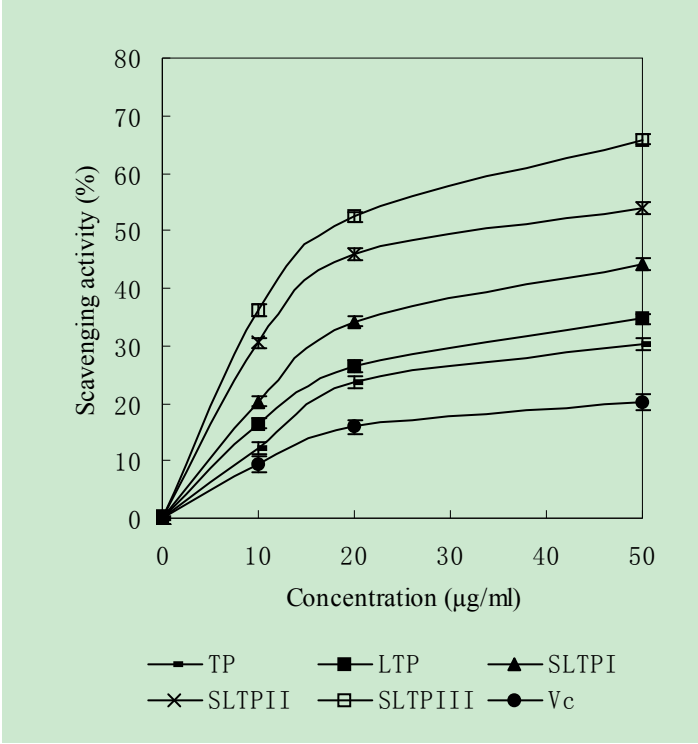

Figure 3. Scavenging activity of natural TP, its derivatives and Vitamin C against hydroxyl radicals. Values were means ± SD (n = 3).

SLTP exhibited a moderate superoxide anion radical scavenging activity than Vc, LTP and TP ($P < 0.05$), and the scavenging activity increased with increasing the concentration. The scavenging activity of SLTPIII at 10 μg/ml was found to be 36.4 %. That of SLTPII at 20 μg/ml was 37.2 %, while a similar value was obtained for LTP at 50 μg/ml. The percentage inhibition of superoxide generation for

SLTPIII at 50 μg/ml was found to be 45 %. The value at this concentration for SLTPII was 40 % (Figure 4).

Table 4. Absorbance of natural TP, its derivatives and Vitamin C at 560nm.

Concentration	TP	LTP	SLTPI	SLTPII	SLTPIII	Vc	$A_{control}$
10 μg/ml	0.588±	0.547±	0.528±	0.503±	0.482±	0.545±	0.758
	0.013	0.009	0.013	0.009	0.010	0.009	
20 μg/ml	0.564±	0.531±	0.495±	0.476±	0.454±	0.510±	
	0.010	0.007	0.008	0.008	0.012	0.007	
50 μg/ml	0.516±	0.491±	0.469±	0.448±	0.411±	0.453±	
	0.010	0.006	0.011	0.004	0.010	0.012	

Results are presented as mean ± SD (n = 3).

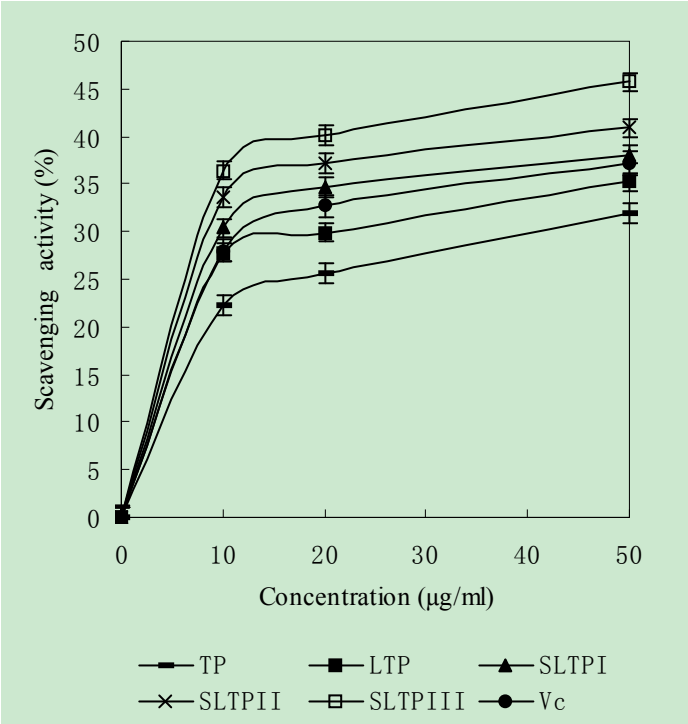

Figure 4. Scavenging activity of natural TP, its derivatives and Vitamin C against superoxide anion radicals. Values were means ± SD (n = 3).

The results of antioxidant assay indicated that SLTP, LTP and TP scavenged free radicals in a concentration-dependent manner. When the concentration was 50 μg/ml, the antioxidant activity became the highest. The antioxidant activity of LTP was higher than those of TP, but lower than those of SLTP. Among three SLTP samples, the highest sulfated content SLTP had the strongest antioxidant

activity. We also studied the antioxidant activity of Vc using the above-mentioned model. SLTP showed stronger scavenging activity against free radical than Vc ($P < 0.05$). It suggested that introduction of sulfonic group into LTP could afford more effective protection against damage. The results in this study suggested that SLTP, possessing pronounced free radical scavenging activity, could be of considerable preventive significance to some life-threatening health problems such as cancer, atherogenesis and Alzheimer's disease, which pathologically initiated by the presence of free radicals leading to the inevitable peroxidation of important biomolecules [17].

One of the mechanisms involving in antioxidant activity is the ability of a molecule to donate hydrogen atom to radical and the propensity of hydrogen donation is the critical factor affecting free radical scavenging activity [19]. The SLTP appeared to function as good hydrogen atom donors and was able to terminate radical chain reactions by converting free radicals to more stale products. Hence, SLTP showed more pronounced antioxidant activity than LTP. Degradation of TP into LTP could also improve the hydrogen atom donation effect. This might be the reason that LTP showed better antioxidant activity than TP.

4. Conclusions

The scavenging activity of superoxide anion radical, DPPH radical and hydroxyl radical were evaluated in this study. The results showed that degradation of TP could improve its water solubility and radical scavenging activity. Sulfation of LTP results in increased antioxidant activity than the natural LTP and TP. The results strongly support the fact that modification of LTP by sulfation will augment the antioxidant activity. Therefore, LTP and SLTP can be taken as potential natural resource of antioxidant, While, the structure-activities of SLTP and the activities *in vivo* need to be further investigated.

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