Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Purification, chemical modification and immunostimulating activity of polysaccharides from *Tremella aurantialba* fruit bodies*

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Received Dec. 11, 2009; Revision accepted Apr. 1, 2010; Crosschecked May 4, 2010

Abstract: Ultrafiltration and a series of chromatographic steps were used to isolate and purify polysaccharides from *Tremella aurantialba* fruit bodies. Three crude fractions (TAP50w, TAP10–50w, and TAP1–10w), five semi-purified fractions (TAPA–TAPE), and one purified fraction (TAPA1) were obtained. A sulfated derivative of TAPA1 (TAPA1-s) was prepared by chemical modification. The immunostimulating activity of the polysaccharide fractions in vitro was determined using the mouse spleen lymphocyte proliferation assay. Of the three crude fractions tested, cell proliferation rates were increased most by TAP50w. Furthermore, TAPA1-s was markedly more stimulatory than TAPA1, indicating that sulfonation was an effective way to enhance the immunostimulating activity of polysaccharide.

Key words: *Tremella aurantialba*, Purification, Polysaccharide, Sulfation, Immunostimulating activity
doi:10.1631/jzus.B0900402 **Document code:** A **CLC number:** Q93

1 Introduction

Members of the genus *Tremella*, belonging to the order Tremellales and the family Tremellaceae, have attracted growing interest in the biomedical field, mainly due to their reported pharmacological properties, which include immunoenhancing (Ma and Lin, 1992), anti-tumor (Ukai *et al*., 1972), anti-hypoglycemic (Kiho *et al*., 2001; Wang *et al*., 2002) and anti-diabetic activities (Kiho *et al*., 2001; Zhang *et al*., 2004). In China, '*Tremella* polysaccharide' has been used for cancer prevention and for stimulating the immune system (de Baets *et al*., 2002). Compared with other *Tremella* species such as *T. mesenterica*, *T. fuciformis*, and *T. aurantia* (de Baets and Vandamme, 2001), however, relatively little is known about the nature and bioactivity of polysaccharides extracted from fruit bodies of *Tremella aurantialba* Bandoni and Zang, a highly valued edible and medicinal fungus. In order to determine the development potential of this fungus, and since immunomodulation is a basic function associated with many polysaccharides (Su *et al*., 2006), we evaluated the immunomodulatory activity of *T. aurantialba* polysaccharide.

Most traditional methods used to extract and purify fungal polysaccharides are time-consuming and require large volumes of organic solvents. Therefore, in this study we have applied more advanced procedures, including ultrafiltration, anion-exchange chromatography, and gel chromatography, to isolate and purify polysaccharides from *T. aurantialba*. These procedures avoid excessive loss of polysaccharide material and avert structural changes to the samples that occasionally occur when chemical reagents are used.

We have obtained three crude polysaccharide fractions, five partially-purified polysaccharide fractions, and a single purified polysaccharide fraction,

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* Project (No. 2006BAD06B08) supported by the National Key Technology R & D Program of China

TAPA1. Since sulfated polysaccharide is reported to exhibit enhanced and/or more diverse biological properties compared with non-sulfated polysaccharide (Nie *et al*., 2006; Peng *et al*., 2005), a sulfated derivative of TAPA1 (TAPA1-s) was prepared by chemical modification in order to further investigate structure-activity relationships. In vitro immunostimulating activity of each fraction was determined using the mouse spleen lymphocyte proliferation assay.

2 Materials and methods

2.1 Materials

Fruit bodies of *T. aurantialba* were provided by the Kunming Edible Fungi Institute of General National Supply and Marketing Cooperative of the People's Republic of China. DEAE-Sepharose™ Fast Flow and High-Resolution Sephacryl S-500 were purchased from Amersham Pharmacia Company (Sweden). The standard monosaccharides and dextrans were from Sigma-Aldrich Company (USA). Phytohemagglutinin (PHA), penicillin, and streptomycin were from Amersco Company (USA). RPMI-1640 medium and fetal bovine serum (FBS) were from Gibco Company (USA). Alamar Blue™ reagent was from Biosource International Company (USA). All other reagents were of analytical reagent grade and from Chinese sources.

2.2 Isolation and purification of polysaccharides

2.2.1 Extraction of crude polysaccharides

Dried *T. aurantialba* fruit bodies (3 kg) were mechanically chopped into small pieces and immersed in 30 L 95% (v/v) ethanol. After 24 h, the solid residue was collected by filtration and the procedure was repeated twice in order to remove lipid material. The residue was air-dried and then extracted with hot water using a 50-L extractor vessel. Optimum extraction conditions (temperature, solid:water ratio, extraction time, number of extractions) were determined by orthogonal experimentation.

2.2.2 Fractionation of crude polysaccharides by ultrafiltration

Aqueous extracts were combined and fractionated on the basis of molecular weight by ultrafiltration using a Millipore™ ultrafiltration system equipped sequentially with 0.1-m^2 membranes of 500, 100, and 10 kDa molecular weight cut-off (Millipore, USA). The end-point of each ultrafiltration cycle was indicated when the electrical conductance value of the feed solution was almost identical with that of distilled water (39.4 μS/cm). The three crude polysaccharide fractions obtained were designated TAP50w, TAP10–50w, and TAP1–10w, respectively.

2.2.3 Purification of polysaccharides by anion-exchange chromatography

TAP50w was dissolved in distilled water (8 mg/ml), centrifuged (400×*g*, 10 min), and the supernatant was applied to a DEAE-Sepharose™ Fast Flow column (XK 26 mm×100 cm). The column was eluted first with filtered (0.45-μm membrane) distilled water and then with a 0–2.0 mol/L NaCl gradient (2340 ml) at a flow rate of 4 ml/min. Fractions (15 ml) were collected, and polysaccharides were detected using the phenol-sulfuric acid method (Dubois *et al*., 1956). Fractions corresponding to individual peaks were combined and five samples of semi-purified polysaccharides (TAPA–TAPE) were obtained.

2.2.4 Further purification of fraction TAPA by gel chromatography

Fraction TAPA was further purified by gel permeation chromatography using a High-Resolution Sephacryl S-500 column (XK 16 mm×100 cm) attached to an ÄKTA Explorer chromatography system fitted with a refractive index detector (RID-10 A, Shimadzu, Japan). Aliquots (1 ml) were applied to the column, which was eluted with 0.2 mol/L $NaNO_3$ at a flow rate of 0.5 ml/min.

2.3 Determination of purity and molecular weight

Homogeneity and the molecular mass of the purified sample were estimated as described by Du *et al.* (2009).

2.4 Preparation of the sulfated derivative TAPA1-s

TAPA1-s was prepared according to the modified method described previously (Inoue *et al*., 1983; Cui *et al*., 2008). Briefly, TAPA1 (100 mg) was suspended in anhydrous formamide (10 ml) by stirring at room temperature for 15 min followed by addition of 2 ml sulfating reagents (286 μl chlorosulfonic acid

(CSA) and 1.714 ml anhydrous pyridine in a ratio of 1:6 (v/v)). The mixture was maintained at room temperature for 2 h with continuous stirring, and then incubated at 30 °C for 5 h. After rapid cooling to room temperature, the solution was neutralized with 15% (w/v) aqueous NaOH, dialyzed, concentrated, and lyophilized to obtain the sulfated derivative TAPA1-s (Cui *et al*., 2008).

2.5 Determination of the sulfate content in TAPA1-s

The sulfate content of TAPA1-s was estimated according to the $BaCl_2$-gelatin method described previously (Chaidedgumjorn *et al*., 2002). The degree of substitution (DS) was calculated from $162\times C_S/(96–80\times C_S)$, where C_S (%) is the content of SO_4^{2-} (Cui *et al*., 2008).

2.6 Proliferation of mice spleen lymphocytes in vitro

2.6.1 Preparation of mice spleen lymphocytes

C57 BL/6 male mice (aged 8–10 weeks) were killed by cervical dislocation, and the spleens removed and washed three times with phosphate buffered saline, cut into small pieces, and then pressed through a stainless steel mesh (100 mesh) to obtain a single spleen cell suspension. After centrifugation ($400\times g$, 6 min), red cells in the spleen cell suspension were lysed with Tris-HCl-NH_4Cl solution (pH 7.2) (Zhang *et al*., 2002). The cell suspension was further diluted with 5 vols of RPMI-1640 medium and, after mixing and centrifugation, the pelleted cells were resuspended in RPMI-1640 medium to a concentration of 2×10^6 cells/ml.

2.6.2 Spleen cell proliferation assay

Aliquots (180 μl) of the cell suspension and 20 μl of different test agents were added to each well of a 96-well plate. PBS and PHA (6 μg/ml) served as the negative and positive controls, respectively. After incubation at 37 °C in a 5% CO_2 atmosphere for 72 h, 20 μl Alamar blue reagent (Biosource, Nivelles, Belgium) was added to each well and the incubation continued for another 6 h. Absorption values at 570 nm (A_{570}) and 600 nm (A_{600}) were measured using a micro enzyme-linked immunosorbent assay (ELISA) autoreader. The proliferation rate (r) was calculated according to the following formula described previously (Shen *et al*., 2008): r (%)=[117216×A_{570} (sample)−80856×A_{600} (sample)]/[117216×A_{570} (control)−80856×A_{600} (control)]×100%.

2.7 Statistical analysis

All data are presented as mean±standard deviation (SD) of three determinations. Data were analyzed using STST2 statistical software (developed by Nanjing Agricultural University, China). Statistical analyses were performed using the Student's *t*-test.

3 Results and discussion

3.1 Extraction and ultrafiltration of *T. aurantialba* polysaccharides

T. aurantialba fruit bodies were pre-treated with 95% (v/v) ethanol to remove lipophilic substances and low molecular weight materials that might interfere with the polysaccharide extraction process. Orthogonal experiments revealed that the optimum conditions for hot water extraction were as follows: temperature, 100 °C; number of extractions, 3; solid: liquid ratio, 1:10; extraction time, 3 h. Three crude polysaccharide fractions (TAP50w, TAP10–50w, and TAP1–10w) were obtained by subsequent ultrafiltration (Table 1). TAP50w was obtained in highest yield (3.67%), had a high polysaccharide content (62.2%), and was selected for further study.

Table 1 Fractionation of *T. aurantialba* polysaccharides by ultrafiltration

Fraction	Molecular weight range (kDa)	Yield (%)	Polysaccharide content (%)
TAP50w	>500	3.67	62.2
TAP10–50w	100–500	0.03	66.0
TAP1–10w	10–100	0.14	20.4

3.2 Purification of fraction TAP50w

Lyophilized TAP50w fraction yielded five peaks, namely TAPA–TAPE, following chromatography on a DEAE-Sepharose™ Fast Flow anion-exchange column and elution with a NaCl gradient (Fig. 1). Yields of TAPA, TAPB, TAPC, TAPD, and TAPE were 31.6%, 12.1%, 9.8%, 3.3%, and 2.4%, respectively. Further purification of TAPA using Sephacryl S-500 gel chromatography yielded two carbohydrate peaks. The peak eluting first (TAPA1) (Fig. 2) was collected, and appeared as a single symmetrical peak on high performance liquid chromatography (HPLC).

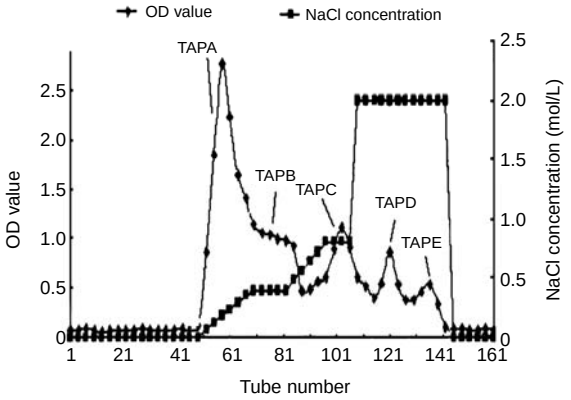

Fig. 1 Elution curve of TAP50w using DEAE-Sepharose™ Fast Flow anion-exchange chromatography

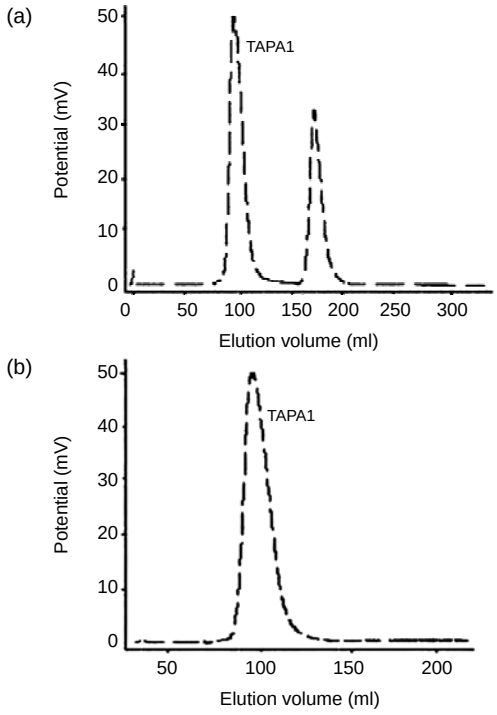

Fig. 2 Elution curves of TAPA using High-Resolution Sephacryl S-500 gel chromatography. (a) The first elution curve; (b) The second elution curve

3.3 Properties of polysaccharide fraction TAPA1

Purified polysaccharide fraction, TAPA1, had a estimated molecular weight of 1.35×10^6 Da by HPLC, and a carbohydrate content of 98.7% by the phenol-sulfuric acid method. Monosaccharide composition analysis, methylation data, and nuclear magnetic resonance (NMR) spectral analysis showed that TAPA1 contained an α-(1→3)-linked mannopyranosyl backbone, partially substituted at position 4 with xylose side chains and at position 2 with side chains composed of either xylose, mannose, and glucuronic acid or of xylose and mannose (Du *et al*., 2009).

3.4 Properties of the sulfated derivative TAPA1-s

The DS of sulfate groups in TAPA1-s, the sulfated derivative of TAPA1, was 0.05 and the sulfate content was 2.88%. Although some polysaccharide degradation usually occurs during the sulfation reaction (Zhang *et al*., 2005; Han *et al*., 2005), the molecular weight of TAPA1-s (1.35×10^6 Da) was almost identical to TAPA1, indicating that no degradation had occurred. This may be due to the use of formamide as the solvent and the moderate temperature (30 °C) used during sulfation (Cui *et al*., 2008). No characteristic absorption bands corresponding to sulfate groups appeared, however, in the infrared (IR) spectrum of TAPA1-s (Zhang *et al*., 2005), and the sulfated position could not be determined by NMR spectrum, possibly due to the low degree of substitution and sulfate content.

3.5 Effect of different *T. aurantialba* polysaccharide fractions on mouse spleen lymphocyte proliferation in vitro

The crude polysaccharide fractions TAP50w, TAP10–50w, and TAP1–10w all significantly stimulated the proliferation of mouse spleen lymphocytes (MSLs) ($P<0.05$) in vitro (Table 2). At 50 μg/ml dose levels, the proliferation rates observed in the presence of the three fractions were (299.96±5.4)%, (278.89±9.4)%, and (250.76±1.7)%, respectively. Cell proliferation rates were increased most by increased concentrations of TAP50w, which exhibited the highest overall effect among the three fractions.

Among the five fractions (TAPA–TAPE) obtained following anion-exchange chromatography of TAP50w, both TAPA and TAPE stimulated the proliferation of MSLs in vitro in a dose-dependent manner (Table 2). At 500 μg/ml concentration, both of these two fractions exhibited a similar potency as 6 μg/ml PHA, which served as the positive control. The effects of fractions TAPC and TAPD on cell proliferation were lower and might reflect differences in structural parameters including monosaccharide composition, and configuration of glycosidic bonds and the glycosidic ring. Cells proliferation rates observed in the presence of 50, 200, and 500 μg/ml

TAPB were (426.90±6.3)%, (460.50±4.2)%, and (423.11±9.0)%, respectively, suggesting that this fraction contained some inhibitory components.

Fraction TAPA1, obtained from TAPA by gel permeation chromatography, was shown to be a homogenous acidic heteropolysaccharide, and to stimulate MSL proliferation more effectively than either of the two fractions (TAP50w and TAPA) from which it was derived (Table 2). At 500 μg/ml, the proliferation rates of TAPA1, TAPA, and TAP50w were (483.15±1.7)%, (454.15±5.4)%, and (474.01±9.0)%, respectively.

Table 2 Effect of different *T. aurantialba* polysaccharide fractions on mouse spleen lymphocyte proliferation in vitro*

Fraction	Proliferation rate (%)		
	50 μg/ml	200 μg/ml	500 μg/ml
TAP50w	299.96±5.4c	453.90±3.8cd	474.01±9.0bc
TAP10–50w	278.89±9.4c	365.28±9.3f	443.83±4.2d
TAP1–10w	250.76±1.7d	329.00±7.9g	324.08±6.3f
TAPA	296.46±2.1c	438.27±9.3de	454.15±5.4d
TAPB	426.90±6.3a	460.50±4.2bc	423.11±9.0e
TAPC	283.04±7.9c	293.44±8.2h	239.61±3.8g
TAPD	213.26±6.1e	248.68±2.5	202.03±6.3h
TAPE	298.46±8.1c	428.27±3.2e	459.97±3.8cd
TAPA1	320.51±9.0b	468.25±1.3b	483.15±1.7b
TAPA1-s	339.67±3.6b	484.10±6.4a	593.98±5.4a

* Positive control: PHA (6 μg/ml), proliferation rate (565.82±15.5)%; negative control: PBS, proliferation rate (100±4.8)%. Values within each column with different letters (a–h) are significantly different at $P<0.05$ using the Student's *t*-test

3.6 Effect of the sulfated derivative, TAPA1-s, on mouse spleen lymphocyte proliferation in vitro

Sulfated modification has been found to be an important method of improving the bioactivity of polysaccharides (Wang *et al*., 2009). Increasing evidence suggests that the bioactivity of sulfated polysaccharides is closely related to structural parameters including molecular weight, DS, position of sulfation, and monosaccharide composition (Duarte *et al*., 2001; Bohn and BeMiller, 1995). Consequently, further studies on the structure-activity relationships are required in the case of sulfated polysaccharides (Wang *et al*., 2009). In this study, we also tested the immunostimulatory activity of the sulfated derivative, TAPA1-s, in vitro using the mouse spleen lymphocyte proliferation assay. TAPA1 was used in control experiments. Recorded cell proliferation rates in the presence of 50, 200, and 500 μg/ml TAPA1-s were (339.67±3.6)%, (484.10±6.4)%, and (593.98±5.4)%, respectively (Table 2). All these values were higher compared with the corresponding values for fraction TAPA1, clearly indicating that sulfation was effective in enhancing immunostimulating activity.

4 Conclusions

Membrane separation technology and a series of chromatographic steps were used to isolate and purify polysaccharides from *T. aurantialba* fruit bodies. Three crude polysaccharide fractions, TAP50w, TAP10–50w, and TAP1–10w, were prepared by ultrafiltration, with the highest yields obtained in the case of TAP50w using a membrane of 500 kDa molecular weight cut-off. Further purification of TAP50w using successive chromatographic steps yielded five semi-purified fractions (TAPA–TAPE) and one purified polysaccharide fraction (TAPA1). A sulfated derivative of TAPA1, TAPA1-s, was prepared by chemical modification in an attempt to improve immune activities. Immunostimulatory activities of all the fractions were estimated in vitro using the mouse spleen lymphocyte proliferation assay, and TAP50w was more stimulatory compared to TAP10–50w and TAP1–10w. TAPA1-s was markedly more stimulatory than TAPA1, indicating that sulfonation was an effective way of enhancing immunostimulating activity. In order to correlate structural features with bioactivity, the structure of TAPA1 has since been investigated in our laboratory using 2D-NMR spectra and methylation analysis, and the results published elsewhere (Du *et al*., 2009). The structural properties of TAPA1-s, especially the position(s) of sulfate substitution and the relationship between the DS and immunostimulating activity, are the subject of continuing research.

5 Acknowledgement

We thank Dr. John BUSWELL, Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, China, for linguistic revision of the manuscript.

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