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Optimization of sulfated modification conditions of tremella polysaccharide and effects of modifiers on cellular infectivity of NDV

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ABSTRACT

Based on our previous research, sulfated modification conditions of Tremella polysaccharide (TPS), the chlorosulfonic acid to pyridine (CSA-Pry) ratio, reaction temperature and time, were optimized by $L_9(3^4)$ orthogonal design taking the yield and degree of sulfation (DS) of modifiers as indexes. Two TPSs, TPS_{tp} and TPS_{70c}, were modified under optimized conditions. The effects of two modifiers, $sTPS_{tp}$ and $sTPS_{70c}$, on cellular infectivity of NDV were determined by MTT method taking the non-modified TPS_{tp}, TPS_{tc} and TPS_{70c} as controls. The results showed that the optimized modification conditions were reaction temperature of 80 °C, CSA-Pry ratio of 1:6 and reaction time of 1.5 h. Five polysaccharides at proper concentrations could significantly inhibit the infectivity of NDV to CEF. The virus inhibitory rates of $sTPS_{tp}$ at 1.563 μg mL^{-1} group were the highest and significantly higher than those of other three non-modified polysaccharide groups in three sample-adding modes. This indicated that sulfated modification could significantly improve the antiviral activity of TPS. $sTPS_{tp}$ possessed the best efficacy and would be as a component of antiviral polysaccharide drug.

1. Introduction

Polysaccharide is a natural macromolecular compound composed of aldoses or ketoses linked by glycosidic bond, and widely exists in plants, microorganisms (bacteria and fungi) and seaweeds. In the past decades, increasing research focused on the biological properties of polysaccharides and their chemical derivatives, especially sulfated derivatives [1]. The chemical modification of polysaccharides, such as sulfation [2], phosphorylation [3], methylation [4] or carboxymethylation method [5], can enhance its antiviral activity and produce new pharmacological agents [6]. Today, sulfated modification is widely used to enhance the biological activities of polysaccharides by reforming its structure, and also enhance its water solubility resulting in convenient application [7]. Besides, much interest has been focused on polysaccharides from fungi. These polysaccharides have important biological activity, such as immunostimulatory and antitumor effects, and have been exploited by food, healthcare and pharmaceutical industry [8].

Tremella (*Tremella fuciformis*) is a popular food and herbal medicine, widely used in Asian countries as a tonic. Tremella polysaccharide (TPS) has received extensive attention. It was reported that TPS exerted an anti-aging effect by increasing the superoxide dismutase (SOD), a key antioxidant enzyme in brain and liver cells [9], and its other pharmacological activities included cytokine-stimulating, anti-tummor, anti-diabetic, anti-inflammatory, vascular-stimulating, cholesterol-lowering, anti-allergic and hepatoprotective effects [10].

In our previous research, the active site of TPS was traced. The crude total TPS (TPS_{tc}) and three crude fractional TPSs, TPS_{60c}, TPS_{70c} and TPS_{80c}, were extracted by water decocting and one-step or stepwise ethanol precipitation method, respectively. Their antiviral and immune-enhancing activities were compared. TPS_{tc} and TPS_{70c} with better efficacy were picked out.

In the present research, sulfated modification conditions of TPS were optimized by orthogonal experiment. TPS_{70c} and purified TPS_t (TPS_{tp}) were modified with the optimized conditions to obtain two sulfated TPSs (sTPSs), $sTPS_{tp}$ and $sTPS_{70c}$, and the effects of the two sTPSs on cellular infectivity of NDV were compared by MTT method taking three non-sulfated TPSs, TPS_{tp}, TPS_{tc} and TPS_{70c} as control. The purpose of this research was to select the optimum modification condition of TPS, probe into the probability of sulfated modification to improve the antiviral activity of TPS, pick out

Abbreviations: TPS, tremella polysaccharide; sTPSs, sulfated tremella polysaccharide; NDV, newcastle disease virus; CEF, chicken embryo fibroblast; DMF, dimethylformamide; MEM, Eagle's minimum essential medium; MM, maintenance medium; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; DS, degree of sulfation; SPF, specified-pathogens free.

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the best sTPS and offer the theoretical evidence for development of antiviral polysaccharide drug.

2. Materials and methods

2.1. Tremella and reagents

Tremella was the product of Fujian Gutian of China, standard no. GB11675-2006.

Eagle's minimum essential medium (MEM) (Gibco) supplemented with penicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 5% fetal bovine serum was used as culturing the cells, for maintenance of medium (MM), the serum concentration was reduced to 2% and used as diluting the polysaccharides and maintaining the cells. Hanks' solution was used to wash the chick embryo tissue shiver and cells. Trypsin (Amresco-0858) was dissolved into 0.25% with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4). 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg mL^{-1} with CMF-PBS (pH 7.4). These reagents were filtered through a 0.22 μm filter. MEM and MM were stored at 4 °C, Trypsin solution, at −20 °C, MTT solution, at 4 °C in dark bottles. Dimethyl sulfoxide (DMSO) was the product of Zhengxing Institute of Chemical Engineering of Suzhou, Chlrosulfonic acid and pyridin, of Sinopharm Chemical Reagent Co., Ltd. Other chemical used in experiment were analytical grade.

2.2. Cells and virus

The chicken embryo fibroblast (CEF) was prepared with 10-day-old specific pathogen free chicken embryo (Nanjing pharmaceutical and apparatus factory of China Animal Husbandry Industry Company). In briefly, after the eggshell was disinfected and opened, the chick embryo was taken out, removed the head, extremities and viscera, washed with Hanks' solution, cut into 1–2 mm^3 pieces, and washed three times with Hanks' solution. The trypsogen solution of 0.25% was added, trypsinized for 30 min at 37 °C and centrifugated. The precipitation was washed three times with Hanks' solution and filtered through a 3-tier gauze. The cells were counted and diluted into 1×10^6 mL^{-1} with 5% MEM and inoculated in 96-well culture plates at 38.5 °C in a humid atmosphere of 5% CO_2 for using.

ND virus (La Sota strain IV, no. 080320) was purchased from Beijing Veterinary Bio-drug Company. $TCID_{50}$ of the virus liquid was 1×10^{-8} by Reed-Mueeh assay. It was diluted into 10^{-6} (100 $TCID_{50}$) with 2% MEM.

2.3. Extraction of TPS

Dried Tremella (1500 g) was added into 35-fold volume 25 °C water and soaked for 30 min, shattered, added into 20-fold volume water of 80 °C to lixiviate for 4 h by sonication (ultrosonic power 400 W, irradiation 40 kHz) and filtered. The filtrate was concentrated into 3000 mL. 1500 mL of drug liquor was used to extract crude total TPS (TPS_{tc}) by one-step precipitation method adding ethanol up to 80% of concentration (v/v), and the precipitation was lyophilized to get TPS_{tc}. Other 1500 mL was used to extract fractional TPS_{70c} by stepwise precipitation [11], firstly adding ethanol up to 60%, removing the precipitation, adding ethanol up to 70% (v/v), and the precipitation was lyophilized to get TPS_{70c}.

2.4. Purification of TPS

TPS_{tc} was dissolved into 10 mg mL^{-1} with distilled water, added into a DEAE-Sepharose Fast-Flow column (1.6 cm × 50 cm), and eluted first with distilled water to obtain the neutral polysaccharide, then gradually with 0–3.0 M NaCl solution to get the acidic polysaccharide. The acidic polysaccharide was added into Sephadex G200 column (1.6 cm × 100 cm), eluted with 0.1 M NaCl (at a flow rate of 0.5 mL min^{-1}) [12]. The eluate was dialyzed in a dialysis sack against tap water for 48 h and against distilled water for 24 h then lyophilized to get purified TPS_t (TPS_{tp}). The polysaccharide contents of TPSs were measured by the phenol–sulfuric acid method [13].

Table 1
Results of $L_9(3^4)$ orthogonal test.

Number	(A) Ratio	(B) Time (h)	(C) Temperature (°C)	Yields (mg)	DS
1	1:2	1.5	50	429.8	0.67
2	1:2	2.5	65	313.1	0.38
3	1:2	3.5	80	512.4	0.67
4	1:4	1.5	65	244.2	0.33
5	1:4	2.5	80	507.0	1.62
6	1:4	3.5	50	260.8	0.30
7	1:6	1.5	80	373.0	1.45
8	1:6	2.5	50	146.8	0.28
9	1:6	3.5	65	255.2	0.99
				Yieldsofproduct	
K_1	418.433	349.000	279.133		
K_2	337.333	322.300	270.833		
K_3	258.333	343.800	464.133		
R	160.100	26.700	193.300		
					DS
k_1	0.573	0.817	0.417		
k_2	0.750	0.760	0.567		
k_3	0.907	0.653	1.247		
R	0.334	0.164	0.830		

2.5. Sulfated modification of TPS

2.5.1. Design of modification condition

Because the effects of the reagent ratio, the reaction time and temperature on sulfated modification were the largest, these three factors were selected. The three levels per factor were used with the ratio of CSA to Pry of 1:2, 1:4 and 1:6, the reaction time of 1.5 h, 2.5 h and 3.5 h, and the reaction temperature of 50 °C, 65 °C and 80 °C, respectively. Nine reacting conditions were designed according to the orthogonal test as L_9 (3^4) (Table 1).

2.5.2. Preparation of sulfating reagent

Chlorsulfonic acid was dropped one by one into pyridine filled in a three-necked flask with agitating and in ice salt water bath. The ratio of CSA to pyridine referred to Table 1. The operations were completed within 40 min and nine sulfating reagents were obtained.

2.5.3. Sulfation reaction

TPS_{tp} (300 mg) was resuspended in N,N-dimethylformamide (DMF) and added into the three-necked flask filled with sulfating reagent, and the mixture was stirred for various durations and temperatures as Table 1. The operations were performed nine times corresponding to nine sulfating reagents. After the reaction, the mixture was cooled to room temperature, pH was adjusted to 7–8 with saturated NaOH solution, and 3-fold volume of dehydrated alcohol was added. The precipitation was re-dissolved with water, dialyzed in dialysis sack against tap water for 48 h and distilled water for 24 h in turn, and dried in vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co., Ltd. Shamen City). Nine kinds of $sTPS_{tp}$ were obtained.

2.5.4. Content determination of sTPSs

The content of carbohydrate was estimated by the phenol–sulfuric acid method taking D-galactose as standard. The degree of sulfation (DS) was established on the basis of the sulfate content determined by barium chloride–gelatin assay [14]. A calibration curve was drawn taking potassium sulfate as