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The optimization of sulfation modification conditions for ophiopogonpolysaccharide based on antiviral activity

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ABSTRACT

Ophiopogonpolysaccharide (OPS) was extracted by water decoction and ethanol precipitation, purified through eliminating protein by trichloroacetic acid method and column chromatography of DEAE-Cellulose-52, then sulfatedly modified by chlorosulfonic acid–pyridine method according to three-factors, ratio of chlorosulfonic acid to pyridine, reaction temperature and reaction time, and three level $L_9(3^4)$ orthogonal designed to obtain nine sulfated OPSs, $sOPS_1$–$sOPS_9$. Their effects on NDV to infect chick embryo fibroblast were compared by MTT assay taking the non-modified OPS as control. The results showed that sulfation modification could significantly enhance the antiviral activity of OPS, $sOPS_3$ presented best effect and the optimal modification conditions were the ratio of chlorosulfonic acid to pyridine of 1:4, the reaction temperature of 60 °C and the reaction time of 2 h.

1. Introduction

Polysaccharide is a class of crucial biomacromolecule besides protein and nucleic acid in organism, it is also a class of high molecular compound conglomerated by monosaccharide and exists in animal, plants and microorganisms. It has many biological activities, such as anti-viral, anti-tumor, anti-oxidation, immunologic enhancement and so on [1–6]. The biological activity of polysaccharide mainly depends on its molecular structure, especially when some chemical group are inserted, the flexibility and space structures of polysaccharide chains are changed, which will make polysaccharide activity change or generate new activity. Therefore, the chemical modification becomes a hot spot in polysaccharides research field [7–10].

The chemical modification method of polysaccharide mainly includes sulfation-, phosphorylation- and selenylation-method, in which sulfation modification is commonly used, because it can significantly improve the antiviral activities of polysaccharides. Sulfation modification is to change the hydroxyl of polysaccharide residue into sulfate radical. There are concentrated sulfuric acid method, chlorosulfonic acid–pyridine (CSA–Pry) method, sulfur trioxide–pyridine and so on, in which CSA–Pry method is commonly used since the reagent preparation is easy, reaction condition is simple and production recovery is convenient. The main affection factors of CSA–Pry method include CSA–Pry ratio, reaction temperature and reaction time [11,12].

Ophiopogonis japonicus (*Radix Ophiopogonis Japonici*) is a traditional Chinese medicine with the function of nourishing *yin* and moisturizing lung. It is applied in treatment of stagnation of vital energy in the chest and abdomen, cough, tussiculation, thirsty, constipation and insomnia due to lung dryness, asthenia consumption and bodyfluid loss. Ophiopogonis japonicus contains a lot of active ingredients including steroid saponin, ophiopogonpolysaccharide (OPS), homoisoflavone amino acid and so on. The most active ingredient is OPS and it possesses the functions of enhancing immunity, anti-myocardial ischemia, lowering blood glucose, anti-hypoxia and ananaphylaxis [13–16].

In this study OPS was extracted by water decoction and ethanol precipitation, purified through eliminating protein by trichloroacetic acid method and column chromatography of DEAE-Cellulose-52, then modified by CSA–Pry method according to three-factors, CSA–Pry ratio, reaction temperature and reaction time, and three-level $L_9(3^4)$ orthogonal designed to obtain nine sulfated OPSs (sOPSs), $sOPS_1$–$sOPS_9$. Their effects on NDV to infect chick embryo fibroblast (CEF) were compared by MTT assay taking the non-modified OPS as control. The aim of this study is to explore

Abbreviations: OPS, ophiopogonpolysaccharide; sOPS, sulfated ophiopogonpolysaccharide; NDV, newcastle disease virus; CEF, chicken embryo fibroblast; CSA–Pry, chlorosulfonic acid–pyridine; 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; DMF, dimethylformamide; DS, degree of sulfation; SPF, specified-pathogens free.

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Table 1
The modification condition, yield, DS and carbohydrate content of sOPSs.

sOPSs	A CSA:Pyr	B Temperature (°C)	C Time (h)	Yield (mg)	DS (%)	Carbohydrate content (%)
$sOPS_1$	1:4	95	1	831	1.52	49.6
$sOPS_2$	1:4	80	3	874	1.62	45.0
$sOPS_3$	1:4	60	2	831	1.49	48.1
$sOPS_4$	1:6	95	2	581	0.83	50.3
$sOPS_5$	1:6	80	1	872	1.57	38.4
$sOPS_6$	1:6	60	3	792	0.97	46.2
$sOPS_7$	1:8	95	3	760	1.36	46.7
$sOPS_8$	1:8	80	2	762	1.49	39.7
$sOPS_9$	1:8	60	1	542	1.36	26.0

the probability of sulfation modification to improve the antiviral activity of OPS, choice out the best sOPS and optimal modification condition, and offer theoretical evidence for the development of antiviral polysaccharide drug.

2. Materials and methods

2.1. Ophiopogon and reagents

Ophiopogon japonicus was bought from Dahua Pharmacy of Chinese Medicine in Nanjing, Jiangsu province., Lot no. 090115.

Eagle's minimum essential medium (MEM) (Gibco) supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 5% fetal bovine serum was used as nutritive medium. For maintenance medium (MM), the serum concentration was reduced to 2%. For Hanks' solution, pH was adjusted to 7.4 using 5.6% $NaHCO_3$, supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1}. Trypsin was dissolved into 0.25% with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco) was dissolved with CMF-PBS into 5 mg mL^{-1}. These three reagents were filtered through a 0.22 μm filter. Trypsin solution was stored at −20 °C, the others were at 4 °C and MTT solution was in dark bottle.

Dimethyl sulfoxide (DMSO, Lot no. 060902) and chlorsulfonic acid (CSA, Lot no. 080421) were the product of Shanghai Ling Feng Chemical Company. Pyridine (Pyr, Lot no. T20091105) and N,N-dimethylformamide (DMF, Lot no. T20090105) were bought from Sinopharm Group Chemical Company.

ND virus (La Sota strain IV, no. 081011) was purchased from Beijing Veterinary Bio-drug Company. TC ID_{50} of the virus liquid was 1×10^{-7} by Reed–Mueeh assay. It was diluted into 10^{-5} (100 TC ID_{50}) with 2% MEM.

2.2. Extraction and purification of OPS

Dried ophiopogon japonicus (1000 g) was crushed into 0.3–1 cm^3 small block by muller, soaked 12 h with 2000 mL 95% ethanol, reflowed for 1.5 h twice in water bath of 80 °C. After aired for 12 h, the drug was decocted with 9-fold volume water 3 times for 90, 60 and 60 min in turn. The physic liquor was filtrated through two-layers gauze, concentrated into 1000 mL, centrifugated for 20 min at 2500 rpm and added with 95% ethanol up to 80% of concentration (v/v), after standing 24 h, the precipitation was lyophilized to get crude OPS.

Protein was eliminated from the crude OPS by trichloroacetic acid method, then the OPS was dissolved into 50 mg mL^{-1} with distilled water, added into a chromatographic column of DEAE-Cellulose-52 (2.5 cm × 30 cm) and eluted with distilled water [17]. The flow rate was maintained at 1 mL min^{-1}, the eluent was collected by automatic fraction collector, 5 mL per tube, and measured for polysaccharide by the phenol–sulfuric acid method. Finally the liquor was freeze-dried. The carbohydrate concentration (%) of OPS was 97.59 comparing with D-glucose.

2.3. Sulfation modification of OPS

2.3.1. Design of modification condition

Since ratio of CSA to Pry (A), reaction temperature (B), and reaction time (C) have an important effect on sulfation modification [18–20], so these three factors were selected. The three levels per factor were used with A of 1:4, 1:6 and 1:8, B of 95 °C, 80 °C and 60 °C, C of 1 h, 2 h and 3 h, respectively. Nine reacting conditions were designed according to the orthogonal test as $L_9(3^4)$ (Table 1).

2.3.2. Preparation of esterification reagent

The pyridine of 25 mL was added into a three-necked flask with agitating device on ice salt water bath [21], then according to the ratio of CSA to pyridine in Table 1, the CSA was added in droplet completing within 40 min. The continuous agitation reaction was performed. When a great quantity of nankeen solid appeared the reaction was stopped. Nine sulfating reagents were obtained.

2.3.3. Sulfation reaction

OPS of 3.6 g was divided equally into 9 portion, respectively resuspended in N,N-dimethylformamide (DMF) and added into the three-necked flask filled with sulfating reagent. The mixture was stirred for various durations and temperatures as Table 1. After the reaction, the mixture was added with 100 mL ice water to terminate reaction, cooled to room temperature and the pH was adjusted to 7–8 with saturated NaOH solution, then 3-fold volume of dehydrated alcohol was added. After standing for 24 h, 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 by vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co., Ltd. Shamen City). Nine sulfated OPSs (sOPSs) named $sOPS_1$–$sOPS_9$ were obtained.

2.3.4. Content determination of sOPSs

The carbohydrate contents of sOPSs were estimated by the phenol–sulfuric acid method taking D-galactose as standard [22,23]. The sulfur contents of sOPSs were determined by barium chloride–gelatin method [24] and the degree of sulfation (DS) was calculated according to the equation:

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%}$$

2.4. Antiviral assays

2.4.1. Preparation of CEF

The chicken embryo fibroblast (CEF) was prepared with 10-day-old specified-pathogens free (SPF) chicken embryo (Nanjing pharmaceutical and apparatus factory of China Animal Husbandry Industry Company). In briefly, after the eggshell was disinfected