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Antivirus and immune enhancement activities of sulfated polysaccharide from *Angelica* sinensis

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ABSTRACT

This study is to synthesize sulfated Angelica polysaccharides (APSs) and investigate the activity of one of the sulfated derivatives APS-1 on murine leukemia virus *in vivo*. Six sulfated derivatives with degree of sulfation ranging from 0.68 to 1.91 were obtained. And the virus replication was inhibited by APS-1 at the dose of 10 and 30 mg/kg (26% and 30% inhibition respectively). Furthermore, both the percentage of $CD4^+$ cells and $CD4^+/CD8^+$ ratio in peripheral blood cells were significantly enhanced by APS-1 at 3–30 mg/kg. In addition, the reduced thymus/body weight index by murine leukemia virus infection was increased by ASP-1 in a dose dependent manner. These results suggest that APS-1 could not only inhibit virus replication, but also improve the immune function. APS-1 may be a potential new and better antiviral drug.

1. Introduction

Many viruses display affinity for heparin sulfate proteoglycans on the cell surface with biological relevance in virus entry. This raises the possibility of a new antiviral therapy by applying sulfated polysaccharide to interfere with the virus entry. Actually, the antiviral effects of sulfated polysaccharide have been known for almost 60 years [1]. Since then, heparin and several other sulfated carbohydrate polymers, such as dextran, fucoidan, galactan and xylomannan have been reported to inhibit HSV and other enveloped viruses [2–5], which make them the new potential pharmaceutical candidates in antiviral chemotherapy. However, these macromolecules did not create the ideal results in efficacy trials so far [6], although some reports with new concepts or approaches in clinical trials offered improved prospects for efficacy [7]. Since sulfated polysaccharides are safe and have no obvious side effects [8], it has the great potential to become a new antivirus drug candidate.

Sulfated polysaccharide, a kind of polysaccharide with sulfated group in its hydroxyls, includes natural sulfated polysaccharides and synthesized sulfated derivative of polysaccharide. Many studies have confirmed that the sulfated polysaccharide exerted more potent biological properties than its non-sulfated polysaccharide, suggesting that sulfation of polysaccharide could promote its antiviral and other biological functions. In our previous studies, we have analyzed the structural characteristics, immune enhancement and antitumor activity of the polysaccharide isolated from *Angelica* sinensis (AP), which is a traditional Chinese medicine having been used in the treatment of various diseases for thousands of years. Accordingly, it is interesting to further know if the sulfation of AP can improve its antiviral activities. In the present study, the effects of one of sulfated derivatives of AP on murine leukemia virus, a kind of retrovirus, were evaluated *in vivo*.

2. Materials and methods

2.1. Isolation and size exclusion chromatography of polysaccharide

The fresh roots of *Angelica* sinensis Diels were collected in autumn from Minxian County, Gansu Province, China. After isolation by boiling water extraction followed by ethanol precipitation, total polysaccharides obtained were chromatographed on a Saphacry S400 HR column (3.5 cm × 100 cm) eluted with 0.1 mol/L sodium chloride. After being dialyzed and lyophilized, the major fraction was used for this experiment (AP). The yield was 1.1% relative to the fresh material. It had a molecular weight of approximately 50,000, which was evaluated by HPLC, and contained protein (3.0%) and carbohydrate (97.0%). In carbohydrate, 8.6% was uronic acid [9] and it consisted of glucose and arabinose with a molar ratio of 13.8:1 [10].

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2.2. Sulfation

AP was sulfated using the chlorosulfonic acid-pyridine method as described [11]. Briefly, chlorosulfonic acid was slowly dropped into ice-cold pyridine with stirring to prepare chlorosulfonic acid-pyridine complexes. 4 g of AP, suspended in DMF, was added quickly into the complex, and the mixture was heated at 100 °C for 1 h with stirring. Then the mixture was cooled to room temperature, poured into iced-water, neutralized with saturated NaOH solution, and precipitated with ethanol. After the precipitation was re-resolved with water, dialyzed against distilled water, lyophilized, the light-yellow powder of *Angilica* polysaccharide sulfates (APSs) were obtained.

2.3. Sulfate estimation

Sulfate groups were confirmed by IR-spectrometric and sulfate content was determined by modified turbidometric barium chloride methods as described [12].

2.4. Cells and viruses

L6565 leukemia cells containing murine leukemia virus (MuLV) were grown as suspension in RPMI-1640 medium supplemented with 10% inactivated calf serum. The cells were collected when cell concentration reached 3×10^6/ml, and freeze-thawed for 3 times, centrifugated at 2000 r/min for 10 min. Supernatant containing L6565 MuLV was obtained [13].

2.5. In vivo antiviral assay

Neonatal Balb/c mice were infected with MuLV by subcutaneously injection on the back with 0.1 ml/mouse of MuLV supernatant, totally twice with 1 day interval. 4 weeks after infection, mice were treated with APS-1 intraperitoneally for 28 days and its antiviral effects were compared to the classic antivirus drug, Combivir (zidovudine/lamivudine, GlaxoSmithKline Australia Pty Ltd), which was administered intragastrically for the same periods (28 days).

2.6. RT-PCR for plasma viral load

TRIzol reagent was used to extract viral RNA from mouse plasma. Reverse transcription (RT) of RNA to cDNA was synthesized by using TaKaRa mRNA selective PCR Kit. The mixture was incubated at 42 °C for 45 min, followed by incubation at 95 °C for 5 min. Subsequently, in the same tube, PCR was carried out with 5 units of Taq DNA polymerase and 5 pmols of primers as follows: sense: 5′-GAGACTGTTGGACCAGGGAA-3′, anti-sense: 5′-TTGTCCTGAGATTCCCAT-3′. All reagents were added according to the protocol of the kit. PCR was performed in a GeneAmp PCR system (Perkin Elmer, USA) starting with a 90 s incubation step at 95 °C, followed by a three-step temperature cycle (45 s at 92 °C, 45 s at 57 °C, and 1 min at 72 °C). This cycle was repeated 28 times and concluded with 5 min incubation step at 72 °C to complete polymerization. PCR products were electrophoresed on a 2% agarose gel and photographed after staining with ethidium bromide.

2.7. Flow cytometric analysis for $CD4^+$ and $CD8^+$ cells

Mouse blood samples were collected and anticoagulated with heparin. Then it was treated with mouse CD4-FITC or CD8-FITC monoclonal antibody followed by RBC lysing solution. The cells obtained by centrifugation were washed with phosphate buffer saline to remove unbounded antibody. The percentage of $CD4^+$ or $CD8^+$ cell in total cells was measured by flow cytometric method using Coulter ELTTE ESP (BECKMAN, USA).

Table 1
Effects of chlorosulfonic acid concentration on sulfation of *Angelica* polysaccharide.

	Sulfating complex (volume ratio[a])	Sulfur content (%)	Yield (%)	DS[b]
APS-1	0.33:1	17.14	76.5	1.91
APS-2	0.25:1	16.32	82.1	1.72
APS-3	0.20:1	15.61	81.6	1.57
APS-4	0.12:1	12.95	84.9	1.12
APS-5	0.10:1	12.52	87.2	1.05
APS-6	0.07:1	9.37	88.4	0.68

[a] Volume of chlorosulfonic acid/volume of pyridine.
[b] Degree of sulfation, represents the average number of sulfonyl groups on each monosaccharide residue; DS = [1.62 × S(%)]/[32–1.02 × S(%)].

2.8. Statistics

Data were expressed as mean ± SD and statistical analysis was performed by the one-way ANOVA method.

3. Results

3.1. Sulfation of AP

To test the influence of amount of chlorosulfonic acid on degree of sulfation (DS, represents the average number of sulfonyl groups on each monosaccharide residue), the major fractions of Angelica polysaccharide (AP) were sulfated under various conditions to yield several sulfated derivatives, APS-1, APS-2, APS-3, APS-4, APS-5 and APS-6, as white, fluffy, water-soluble compounds. Their reaction conditions and DS are given in Table 1.

The degree of sulfation of these 6 derivatives is ranging from 0.68 to 1.91. By increasing the ratio of chlorosulfonic acid in sulfating complex, the DS can be enhanced accordingly. Characteristic absorptions derived from sulfo groups in the IR spectrum at 820 and 1240 cm^{-1} were assigned to C—O—S and S=O respectively (Fig. 1).

In our previous study, NMR spectrum confirmed that AP had a backbone composed of 1,4-α-D-glucopyranosyl residues, with branches attached to O-6 of some residues. The branches were composed of 1,6-α-D-Glcp residues and terminated with β-L-arabinofuranose residues. ^{1}H NMR (500 MHz, D_2O) δ: 3.35, 3.44, 3.48, 3.55, 3.57, 3.60, 3.62, 3.67, 3.70, 3.76, 3.78, 3.83, 3.87, 3.89, 3.94, 4.01, 4.11, 4.90, 5.33. ^{13}C NMR (125 MHz, D_2O) δ: 59.9, 60.4, 63.4, 65.5, 65.9, 69.5, 70.2, 71.2, 71.5, 71.8, 73.1, 73.4, 75.2, 76.2, 76.7, 80.3, 97.7, 98.0, 98.5, 99.6, 100.0 [14]. Since hydroxyl at C-6 was first sulfated, then C-2 and C-4 hydroxyl [15], the sulfate position in APS was mainly at C-6 hydroxyl of the main chain and then C-2, C-4 hydroxyl of the branches depending on its DS.

3.2. Bioactivity of sulfated Agelica polysaccharide

3.2.1. Antivirus activity in vivo

DS affects the antiviral activity of polysaccharides. In general, for a particular class of polysaccharide, the more the charge density, the higher is its antiviral activity [16]. In this study, we chosed APS-1, which has the highest DS of the six derivatives, to test the antiviral activities *in vivo*.

The antiviral results of APS-1 on L6565 MuLV were summarized in Fig. 2. APS-1 exhibited a potent inhibitory effect against L6565 MuLV in the range of 10 (26% inhibition) and 30 mg/kg (30% inhibition) with the plasma viral loads, which was significantly lower when compared with model. The inhibitory effects of APS-1 showed a dose dependent manner in the range of 3–30 mg/kg of