Isolation, Characterization, and Antitumor Activity of a Novel Heteroglycan from Cultured Mycelia of Cordyceps sinensis

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Abstract

A novel heteroglycan, Cordyceps sinensis polysaccharide 1 (molecular weight 17 × 10^5 Da), was isolated and purified from mycelia of the fungus C. sinensis obtained by solid-state culture. Structural characterization by chemical analysis, GC-MS, FTIR, and NMR spectroscopy showed that C. sinensis polysaccharide 1 was mainly composed of (1→6)-linked α-D-Glc and α-D-Gal, with minor β-(1→4)-D-Xyl and β-(1→4)-D-Man residues probably located in the side chains with a trace amount of α-(1→3)-L-Rha residue. In biological assays, C. sinensis polysaccharide 1 significantly inhibited proliferation of sarcoma 180 cells and induced apoptosis in a dose-dependent manner. Further studies will elucidate the antitumor mechanism of C. sinensis polysaccharide 1 and promote its utilization for the development of novel, effective anticancer drugs.

Abbreviations

CSPS-1: Cordyceps sinensis polysaccharide 1
FACS: fluorescence activated cell sorter
FTIR: Fourier transform infrared spectroscopy
NSCLC: non-small cell lung cancer
S-180: sarcoma 180

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Natural products have been heavily used in cancer chemotherapy for the past several decades. Mann [1] reported that natural products (including those from plant and microbial sources) and their structural relatives comprise ~50% of the drugs used for cancer chemotherapy. Fungi (particularly mushrooms) have been used by humans as food and as sources of medicinal compounds for thousands of years. A large number of molecules having antitumor properties have been identified from various mushroom species.

Cordyceps sinensis (Berk.) Sacc. has been classified as belonging to the family Clavicipitaceae, clade B, Cordyceps unilateralis subclade [2]. As a famous edible and medicinal mushroom in China, C. sinensis possesses many important biological properties, such as antioxidant [3], immunomodulating [4], and antitumor [5], and has been the subject of many research studies. Various bioactive metabolites with antitumor properties have been extracted from C. sinensis; these include sterols [6], cordycepin [7], and polysaccharides [8]. The practical applicability of C. sinensis compounds depends not only on their biological properties but also on their biotechnological availability [9]. C. sinensis grows slowly and only in high-altitude habitats, and supplies are generally insufficient to meet demand [10]. During the past two decades, culture techniques have been developed to provide abundant mycelia or fruiting bodies as sources of medicinal compounds [11]. Recent attention has focused on polysaccharides as abundant and useful components [12].

Previous studies have succeeded in extracting polysaccharides from mycelia, fruiting bodies, or culture broth of C. sinensis, and the polysaccharides vary in their structural characteristics and biological properties. Wasser [9] showed that polysaccharide structures may depend on the composition of the culture medium as well as on the Cordyceps species.

In the present study, a polysaccharide fraction termed CSPS-1 was extracted, purified, and structurally characterized (molecular weight, mono-
saccharide composition, polymer linkages) from \textit{C. sinensis} mycelia harvested through a solid-state culture. The antiproliferative effect of CSPS-1 against S-180 cells was investigated \textit{in vitro}.

**Table 1** Result of the monosaccharide composition of CSPS-1 using GC-MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>Rha (21.103 min), Ara (21.853 min), Fuc (22.104 min), Xyl (24.396 min), Man (29.063 min), Glc (29.633 min), Gal (30.230 min), and inositol (33.907 min)¹</td>
</tr>
<tr>
<td>CSPS-1</td>
<td>Rha (20.864 min), Xyl (24.305 min), Man (28.981 min), Glc (29.721 min), Gal (30.429 min), and inositol (33.954 min)</td>
</tr>
</tbody>
</table>

¹ Inositol was used as an internal standard.

**Results**

Crude polysaccharides from mycelia of solid-state cultured \textit{C. sinensis} were obtained by a series of procedures, including hot water extraction, degreasing, deproteinization, and lyophilization. The polysaccharide sample was separated on a DEAE cellulose-52 column. Two major fractions were detected by spectrophotometry (490 nm) with a phenol-sulfuric acid method (Fig. 1S, Supporting Information). Fraction 1 was further purified on a Sephadex G-100 column, yielding the single polysaccharide CSPS-1 (Fig. 1S, Supporting Information). The molecular weight of CSPS-1 was estimated as 1.17 × 10^5 Da using the calibration curve described in Materials and Methods.

HPLC revealed CSPS-1 as a single homogeneous peak (excluding the solvent peak; Fig. 2S, Supporting Information). The ultraviolet spectrum of CSPS-1 showed no absorbance at 260 and 280 nm, indicating the absence of nucleic acids and proteins (Fig. 2S, Supporting Information). No sulfate groups were revealed by the turbidimetric method. The FTIR spectrum of CSPS-1 showed no absorbance at 260 and 290 cm⁻¹, indicating the absence of nucleic acids and proteins (Fig. 2S, Supporting Information). No sulfate groups were detected by the turbidimetric method.

The aldonoitrile acetate derivatives of CSPS-1 and monosaccharides were analyzed by GC (Fig. 3S, Supporting Information). Based on the retention times of the standards, five kinds of monosaccharides were identified in the CSPS-1 sample: Rha, Xyl, Man, Glc, and Gal (Fig. 3S). Based on peak areas, the monosaccharide molar ratio of CSPS-1 is Glc: Gal: Xyl: Man: Rha = 30.67: 13.37: 5.40: 2.39: 1.0, indicating that CSPS-1 is a heteroglycan, and is composed mainly of Glc and Gal.

Stable absorption at 233 nm was obtained after treatment for seven days, suggesting that complete periodate oxidation had occurred. The oxidation process consumed 1.43 mol periodate per mol glucose residue and yielded 0.53 mol formic acid (Table 2). The formic acid released that CSPS-1 contained a 1→6 linkage. The excessive consumption of periodate indicated that CSPS-1 also contained a 1→2 and/or 1→4 linkage. Following transformation of the periodate oxidation/Smith degradation product of CSPS-1 into aldonoitrile acetate derivatives, the GC spectrum showed three peaks (excluding the internal standard inositol): glycerol, erythritol, and glucose in a ratio of 10.165: 1.949: 1 (Fig. 4S, Supporting Information). The large amount of glycerol corresponded to 1→2 and/or 1→3 glycosidic linkages, while the trace amounts of erythritol and glucose corresponded to 1→4 and 1→3 glycosidic linkages, respectively. These findings indicate that (1→2) and/or (1→6) should be asccribed to the main chain linkages, and a low content of (1→3) and (1→4) exists in the main chain or branch.

The ¹H NMR spectrum of CSPS-1 (Fig. 5S A, Supporting Information) mainly contained signals for five anomer protons at δ 5.10 ppm, 5.05 ppm, 4.99 ppm, 4.91 ppm, and 4.86 ppm. A chemical shift at δ 4.66 ppm corresponded to residual HDO (25°C), with other protons on the glycosidic ring appearing in the region of δ 3.40–4.40 ppm. In the anomic carbon region, signals at δ 5.10 ppm and 5.05 ppm could be attributed to the α configuration, and the β conformation was confirmed at 4.99 to 4.86 ppm. The ¹³C NMR spectrum (shown in Fig. 5S B) also showed multiple resonances of α- and β-glycosidic bonds. Combined with the aforementioned results, the signals between δ 107.66 and 102.44 ppm were derived from C-1 of β-(1→4)-D-Man, δ 101.47–100.20 ppm assigned to C-1 of β-(1→4)-D-Man, and δ 99.65–97.75 ppm assigned to C-1 of α-(1→6)-D-Glc and α-(1→6)-D-Gal, respectively [13–15]. Thus, CSPS-1 is a branch-type heteroglycan with both α- and β-glycosidic linkages. It was supposed to have a backbone of (1→6)-linked α-D-Glc and α-D-Gal, and also contain minor β-(1→4)-D-Xyl and β-(1→4)-D-Man residues in the branches. The trace amount of α-(1→3)-L-Rha residue is probably located randomly in the side chains. The effect of CSPS-1 on \textit{in vitro} S-180 cell proliferation as a function of time is summarized in Fig. 1. Incubation for 12 h with a high concentration (300 µg/mL) of CSPS-1 caused a highly significant (p < 0.01) reduction of S-180 proliferation in comparison with the control group. Treatment of cells with CSPS-1 at concentrations of 50, 150, and 300 µg/mL for durations of 24, 36, and 48 h also caused highly significant decreases in proliferation. After 48 h treatment, the percentages of inhibition in the 50, 150, and 300 µg/mL groups were 72.74%, 81.22%, and 90.24%, respectively. Proliferation in the positive control group (treated with 25 µg/mL 5-Fu) was significantly lower than that in the nor-

**Table 2** Periodate oxidation/Smith degradation of CSPS-1.

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Consumption of periodate (mol/mol Glc)</th>
<th>Yield of formic acid (mol/mol Glc)</th>
<th>Products of Smith degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1→2</td>
<td>1</td>
<td>nd²</td>
<td>glycerol</td>
</tr>
<tr>
<td>1→3</td>
<td>nd</td>
<td>nd</td>
<td>glucose/mannose</td>
</tr>
<tr>
<td>1→4</td>
<td>1</td>
<td>nd</td>
<td>erythritol</td>
</tr>
<tr>
<td>1→6</td>
<td>2</td>
<td>1</td>
<td>glycerol</td>
</tr>
<tr>
<td>CSPS-1 sample</td>
<td>1.43</td>
<td>0.53</td>
<td>glycerol: erythritol: glucose = 10.165:1.949:1</td>
</tr>
</tbody>
</table>

² nd: not detected
Discussion

The potentially important role of polysaccharides in tumor/cancer treatment was first recognized over 100 years ago. Antitumor effects have been documented for numerous polysaccharides isolated from 22 mushroom species and 50 types of culture media [16]. The genus *Cordyceps* has received considerable research attention because it is a well-known and popular food source in China. Several polysaccharides have been isolated from the fruiting bodies, cultured supernatant, or mycelia of *C. sinensis*, and their structures have been characterized in previous studies. SCP-I (MW 1.84 × 10^5 Da), a homoglucan from *C. sinensis* mycelia, was shown to have a backbone of (1→4)-D-Glc residues and to carry a single (1→6)-linked D-Glc residue [17]. Sheng et al. [4] extracted an exopolysaccharide fraction, EPS (1.04 × 10^5 Da), from the cultured supernatant of *C. sinensis*. EPS contained Man, Glc, and Gal in a molar ratio of ~23:1.0:2.6, according to GC analysis. Nie et al. [18] reported that the structure of a bioactive hydrophilic polysaccharide (CBHP; MW 2.6 × 10^5 Da) from cultured *C. sinensis* consisted mainly of Glc (95.19%) plus trace amounts of Man (0.91%) and Gal (0.61%), with a backbone composed of glucopyranose (Glcp) joined by 1→4 and 1→3 linkages. Wang et al. [19] isolated an acidic exopolysaccharide (AEPS-1; MW 36.3 kDa) from a mycelial culture of *C. sinensis* and found that it was composed of Glcp and pyrano-glucuronic acid (GlcUp) in a molar ratio of 8:1 plus a trace amount of Man, and had a linear backbone of (1→3)-linked α-D-Glcp residues with two branches, α-D-Glcp and α-D-GlcUp.

In the present study, we purified CSPS-1, a heteroglycan of MW ~1.17 × 10^5 Da composed of Glc, Gal, Man, Xyl, and Rha in a molar ratio of 30.67:13.37:5.40:2.39:1.0, from a hot water extract of *C. sinensis* mycelia. The structural properties of CSPS-1 are strikingly different from those of the other polysaccharides mentioned above. Differences in culture medium, culture conditions, and extraction and purification procedures account for the variability of isolated polysaccharides, including differences in MW, monosaccharide composition, and polymer chain linkages [10, 16, 20]. Mycelial cultures in the previous studies were maintained primarily in liquid culture media and conditions. In contrast, a solid-state culture was used in the present study to obtain large amounts of mycelia.

The biological activities of polysaccharides are affected by their structural properties [20, 21]. Ji et al. [22] studied the adjuvant action of a polysaccharide from *C. sinensis* in the treatment of NSCLC. The proliferation of NSCLC cells treated with this polysaccharide for 48 h was not significantly different from that of the control group. In the present study, the proliferation of S-180 cells was significantly reduced by 24, 36, or 48 h incubation with CSPS-1. After 48 h treatment, the percentages of inhibition in the 50, 150, and 300 µg/mL CSPS-1 groups were 72.74%, 81.22%, and 90.24%, respectively. Structural features such as (1→3)-β-1 linkages in the glucan backbone have been proposed as important factors in antitumor activity [23, 24]. However, other types of chemical structures were also found in polysaccharides having antitumor effects. A series of water-soluble polysaccharides (GTM1, GTM2, and GTM3) extracted from *Ganoderma tsugae* mycelia displayed high antitumor activity (inhibition ratio > 50%) against S-180 [25]. Structural analyses showed that GTM1 and GTM2 are heteropolysaccharide-protein complexes whose fraction 2 is composed of Gal, Glc, Man, and bound proteins. In this study, CSPS-1 was supposed to have a backbone of (1→6)-linked α-D-Glc and α-D-Gal, and contain minor β-(1→4)-D-Xyl and β-(1→4)-D-Man residues located in the side chains with a trace amount of α-(1→3)-L-Rha residue. Previous studies showed that polysaccharides display antitumor activity not only by stimulating proliferation of T lymphocytes, cytokine secretion, and immune function in vivo [26, 27], but also have direct effects on tumor cells in vitro [28–30]. In the present study, the antiproliferative effect of CSPS-1 on S-180 cells was exerted through an apoptotic pathway in vitro. The caspase family

Fig. 1  Effect of CSPS-1 on S-180 cell proliferation in vitro. 5-Fu: 5-Fu-treated group; CSPS-1: groups treated with CSPS-1 at three dosages (50, 150, or 300 µg/mL). The values shown are mean ± SD (n = 6). **P < 0.01 compared with the normal control group.
and Bcl protein family play important roles in apoptotic processes [31]. Xu et al. [32] reported that chitooligosaccharides induced apoptosis of human hepatocellular carcinoma cells in a dose-dependent manner in vitro by upregulating the pro-apoptotic protein Bax and triggering the initiation of an apoptosis program in the cells. The growth of breast cancer cells was inhibited by an extract from *Phellinus linteus* through the upregulation of p27, resulting in S-phase cell cycle arrest [33]. The antitumor activity of a polysaccharide from *C. jiangxiensis* was exerted through the up-modulation of caspase-3 and down-modulation of Bcl-2 [28]. Further studies will elucidate the antitumor mechanism of CSPS-1 and promote its utilization for the development of novel, effective anticancer agents.

**Materials and Methods**

**Materials and reagents**

*C. sinensis* was obtained from the Microbial Genetic Stock Center of Huazhong Agricultural University (Wuhan, China). Dried mycelia were harvested through a solid-state culture. DEAE cellulose-52, Sephadex G-100, and T-series dextran molecular weight standards (T-10, T-20, T-40, T-70, T-100) were from Pharmacia Co. D-glucose (Glc), D-galactose (Gal), D-mannose (Man), D-arabinose (Ara), D-xyllose (Xyl), D-fucose (Fuc), L-rhamnose (Rha), inositol, and erythritol (purity of standards ≥ 99%) were from Sigma-Aldrich. 5-Fu (99%) was purchased from Aladdin Industrial Corporation. Other reagents and chemicals were of analytical grade and from Sinopharm Chemical Reagent Co.

**Extraction and purification of Cordyceps sinensis polysaccharide 1**

After solid-state fermentation, the mycelia of *C. sinensis* were harvested, dried to a constant weight at 60°C, and pulverized. The dried mycelia powder was obtained. Subsequently, 100 g of mycelia powder was extracted twice with hot water for 3.5 h at 80°C and treated with sufficient α-amylase to remove starch at 60°C. Ethanol was added to the resulting solution (final concentration 70%), stirred overnight at 4°C, and centrifuged at 5000 × g for 30 min. The crude polysaccharides obtained were deproteinated with Sevag reagent (chloroform/1-butanol 1:4 v/v) and centrifuged at 3000 × g for 15 min. The resulting supernatant so-
olution was loaded onto a DEAE cellulose-52 column (2.6 × 40 cm) pre-equilibrated with double-distilled water, and the column was then eluted with double-distilled water and NaCl (2 mol/L) at a flow rate of 30 mL/h. Eluates were collected with a fraction collector and assayed for carbohydrates by the phenol-sulfuric acid method [34]. The first peak sample (3 mg) was purified further by gel filtration chromatography on a Sephadex G-100 column (2.6 × 60 cm) and eluted with deionized water at a flow rate of 30 mL/h. The resulting purified polysaccharide fraction, termed CSPS-1, was lyophilized and subjected to a series of analyses as described below.

Homogeneity and molecular weight determination
The homogeneity of CSPS-1 was evaluated using an HPLC system (model 2695, Waters Corp.) equipped with an Agilent column (4.6 mm × 250 mm × 5 µm). The presence of proteins and nucleic acids was detected by an ultraviolet spectrophotometer (model 752, Shanghai Optical Instrument Factory Co.). The sulfate content was measured by the turbidimetric method of Dodgson and Price [35]. The molecular weight of CSPS-1 was determined using a calibration curve obtained by plotting the weights of T-series dextran standards against the retention time.

Fourier transform infrared spectroscopy and nuclear magnetic resonance
The FTIR spectrum of a CSPS-1 sample was collected from KBr pellets using a FT-IR spectrophotometer (model Nexus 470, Thermo Scientific Nicolet) over the wavelength range of 400–4000 cm⁻¹ [36]. For NMR analysis, a CSPS-1 sample was lyophilized in 99.96% D2O, according to the method described by Wang et al. [19]. ¹H and ¹³C-NMR were performed with an Agilent 400MR DD2 spectrometer at 25°C.

Monosaccharide composition
The monosaccharide composition of CSPS-1 was analyzed by the GC method of Wang et al. [37], with minor modifications. A solution of 10 mg CSPS-1 powder and 6 mL TFA (2 mol/L) was hydrolyzed at 120°C for 2 h, evaporated, and methanol was added. The dried hydrolysate was mixed with hydroxylamine hydrochloride (10 mg) and pyridine (0.6 mL) and incubated at 90°C for 30 min, with inositol (5 mg) as the internal standard. The mixture was cooled to room temperature, 1 mL acetic anhydride was added, and the resulting aldononitrile acetate derivatives were analyzed by GC (model 6890 N, Agilent Technologies) with a phenyl methyl siloxane capillary chromatographic column (Hp-5, 30.0 µm × 320 µm × 0.25 µm), and detected by a flame ionization detector. The column temperature was maintained at 120°C for 3 min and then 210°C for 4 min. The flow rate was 1.0 mL/min. Seven monosaccharide standards were labeled by the same procedure.

Periodate oxidation and Smith degradation
CSPS-1 was subjected to periodate oxidation and Smith degradation by the method of Linker et al. [38], with minor modifications. A CSPS-1 sample (20 mg) was mixed with 20 mL sodium periodate (15 mM) at 4°C in the dark. The reaction was monitored by spectrophotometry at 223 nm. Ethylen glycol (0.4 mL) was added to deoxidize surplus sodium periodate, and the reaction was stopped after 30 min. The solution was reduced with NaBH₄ overnight, pH adjusted to 5.5–7.0 with 0.1 M acetic acid, dialyzed with deionized water for 48 h, and concentrated to dryness by the addition of 2.0 mL methanol. The product obtained was subjected to GC analysis.

In vitro antitumor assay
The antitumor effect of CSPS-1 was evaluated using S-180 cells (American Type Culture Collection). These cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (Gibco BRL), penicillin (100 U/mL), and streptomycin (100 U/mL) in a humidified incubator (37°C, 5% CO₂). The cells were divided into five groups. The normal control group was treated with the medium only, and 5-fluorouracil (5-Fu) (25 µg/mL) was used as a positive control. The other three groups were treated with sterilized CSPS-1 samples (final concentrations 50, 150, and 300 µg/mL). After incubation for 24 h, S-180 cells (1 × 10⁵ cells/well) containing various drugs were placed on a 96-well plate and cultured for 12, 24, 36, or 48 h. Cell proliferation was estimated by the MTT (Sigma) assay. The inhibition rate was evaluated according to the following formula:

\[
\text{Inhibition rate (\%) = (1 - OD_{exp}/OD_{con}) \times 100%}
\]

where OD_{exp} and OD_{con} are the optical densities of the experimental groups and normal control group, respectively.

Cell apoptosis assay
The apoptotic effect of CSPS-1 on S-180 cells was assayed by flow cytometry. Following 24 h administration of various drugs in the aforementioned five groups, cells were harvested and examined with an annexin V-FITC/PI apoptosis detection kit (Wuhan Shangcheng Biotechnology Co.). The cells were then pelleted and subjected to FACS analysis (Becton-Dickinson).

Statistical analysis
The experimental data are expressed as the mean ± SD. Statistical differences between means were evaluated using the program SPSS for Windows, v. 19.0 (SPSS, Inc.). Differences with p < 0.05 or p < 0.01 were considered significant or highly significant, respectively.

Supporting information
Purification, HPLC profile, Ultraviolet absorption curve, FTIR, ¹H-NMR, and ¹³C-NMR spectra of CSPS-1 as well as the aldononitrile acetate derivative profiles of a mixture of monosaccharide standards and CSPS-1, and their GC spectra following periodate oxidation/Smith degradation are available as Supporting Information.

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Conflict of Interest
It should be understood that none of the authors have any financial or scientific conflicts of interest with regard to the research described in this manuscript.

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