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Structural analysis of monocyte activation constituents in cultured mycelia of *Cordyceps sinensis*

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ABSTRACT

It has been reported that mycelia of the *Cordyceps sinensis* (CS) can function as an immunostimulant. However, the active constituents of the mycelia are not well known. In this study, we investigated which components of the mycelia of CS induce monocyte activation and then structurally analyzed the active components.
Assay of the effect of crude-(CS-P), soluble-(CS-Ps) and insoluble-(CS-Pp), polysaccharides extracted from the mycelia of CS, on macrophage production of TNF-α, indicated that CS-Pp enhanced TNF-α production to the highest extent. Furthermore, Structural analyses demonstrated that CS-Pp is a 1,3-β-D-glucan contained some 1,6-branched chains and the mean particle diameter is 1.5 μm.

1. Introduction

The fungus *Cordyceps sinensis* belongs to the Ascomycetes class of fungi that are parasitic in the larvae of Lepidoptera. These fungi live predominately in highlands such as Sichuan in China, or Yunnan in Tibet, at altitudes between 3000 and 4000 m. Natural *C. sinensis* is scarce and is of high value as Chinese medical lore from ancient times suggests that *C. sinensis* is an herbal medicine effective for improvement of several disease symptoms, for recovery from fatigue and for nutritional fortification.

Extensive research on *C. sinensis* indicates that it is pharmacologically active on respiratory, renal, liver and nervous systems and has anti-tumor, anti-aging, hyposexuality, and hyperlipidemia effects [1–5]. Natural *C. sinensis* is difficult to obtain in large quantity for biological studies. However, recently, artificial culture of *C. sinensis* mycelia (CSM) has been developed which makes available a high quality, stable culture of CSM. Reports based on physiological research suggest that CSM has immunomodulatory [6], anti-oxidant [7,8], anti-tumor cytotoxic [9], apoptosis-inducing [10,11], anti-bacterial [12], anti-fatigue and anti-stress [13], hypocholesterolemic [14], hypoglycemic [15–17], steroidgenic [18–22] and anti-tumor [23–25] effects. The use of CSM as a medicinal herb is, therefore, increasing in popularity.

It has been shown that cordycepin, ergosterol, polysaccharides, glycoprotein and peptides [2] are chemical constituents of *C. sinensis*. In CSM, cordycepin, ergosterol and adenosine isolated from CSM were reported to be cytotoxic for tumor cells [9], polysaccharide was reported to have anti-oxidant and hyperglycemic effects [7,16,17] and the exopolysaccharide fraction was reported to have anti-tumor and immunomodulatory effects [24–26].

Other immunologically active mushrooms activate innate immunity by activation of dendritic cells or monocytes and may also induce specific immunity [27]. Similarly, it has been reported that CSM can stimulate the activity and function of monocytes thereby affecting innate immunity [28–30]. However, the structure of the CSM constituents that induce monocyte activation has not yet been studied in detail.

In this study, in order to determine constituents of CSM that induce monocyte activation we assayed the effect of polysaccharides present in cultured CSM on the production of TNF-α in a mouse macrophage cell line RAW264.7 and in mouse splenocyte cells. In addition, the structure of a

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polysaccharide with high cytokine-inducing ability was analyzed.

2. Experimental

2.1. Extraction and analysis of CSM

CSM were cultured in a liquid medium at 15–18 °C for 14 days. Thereafter, the liquid medium was removed by centrifugation, the obtained mycelia were pulverized by freeze drying and then used as cultured CSM (The State Food and Drug Administration approval code: H37023596, Shandong Lukang Pharmaceutical Co., Ltd., China.) The cultured CSM was composed of water (3.3%), protein (36.2%), lipids (19%), sugar (12.4%), food fiber (24.2%) and ash (4.9%).

2.2. Fractionation of cultured CSM

Fats present in 500 g of cultured CSM were removed by two incubations with 1 L of ethanol at 80 °C for 6 h followed by three incubations with 1 L of 70% ethanol at 80 °C for 6 h. The residue was suspended in 1 L of distilled water, treated with hot water at 100 °C for 6 h and centrifuged at 5000 ×*g* for 15 min. The supernatant was concentrated to 1/2 volume under reduced pressure and the equivalent volume of ethanol was added. Thereafter, the mixture was allowed to stand at 4 °C overnight and centrifuged at 5000 ×*g* for 15 min. The precipitate was dried by freezing and used as the crude polysaccharide fraction of CSM (CS-P).

CS-P was dissolved in distilled water at a concentration of 50 mg/mL and the low molecular weight constituents were then removed by dialysis against distilled water at 4 °C for 3 days. Thereafter, the liquid within the dialysis membrane was collected and fractionated by centrifugation at 10,000 ×*g* for 15 min to separate the soluble polysaccharides in the supernatant (CS-Ps) from the insoluble polysaccharides in the precipitate (CS-Pp).

2.3. Cytokine production assay

Prior to assay of the polysaccharides, each polysaccharide was suspended in distilled water to a concentration of 1 mg/mL and dissolved by heating at 80 °C for 30 min.

Mouse splenocyte cells were prepared from 6 week-old male C3H/HeJ mice (Japan SLC). Mouse splenocyte cells, suspended in RPMI1640 medium containing 10%FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, were seeded in a 48-well culture plate at a density of 5×10^5cells/well, and each test polysaccharide was added at a final concentration of 12.5 μg/mL or 25 μg/mL. The mixture of cells and polysaccharide (500 μL) were incubated

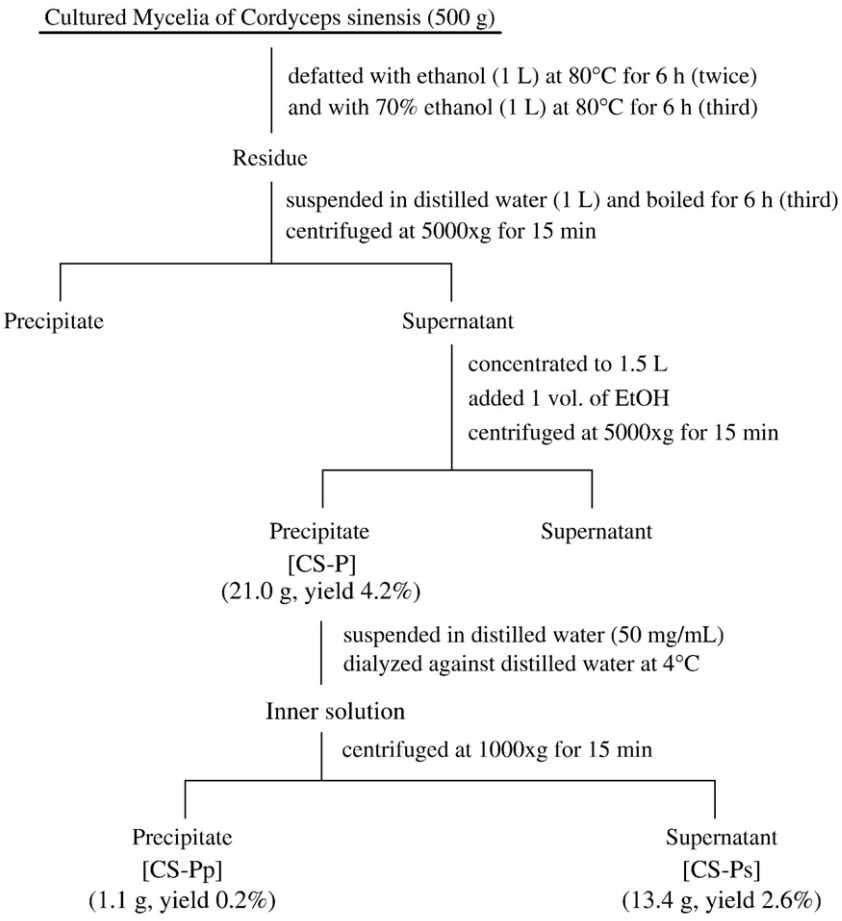

Fig. 1. Extraction and fractionation of the polysaccharides (CS-P, CS-Ps, CS-Pp) from cultured mycelia of *C. sinensis*.