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Production of Exo-Polymers by Submerged Mycelial Culture of *Cordyceps militaris* and Its Hypolipidemic Effect

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Abstract Hypolipidemic effect of exo-polymers (EPs) from submerged mycelial culture of *Cordyceps militaris* was investigated in male Sprague-Dawley rats. For a dose-dependent study, EPs were administered at the level of 50–100 mg/kg body weight (BW) and the data was compared with the saline administered control group. A significant reduction of both the plasma total cholesterol (TC) and triglyceride (TG) was registered under the influence of EPs. It reduced the low density lipoprotein (LDL) cholesterol level as much as 40.5%. Levels of high density lipoprotein (HDL) cholesterol did not vary significantly within the different experimental groups, but the HDL: TC ratio showed consistently a high value with the increasing dose. The effects of cultural conditions (pH and temperature) in mycelial growth and EPs production were studied. Both the biomass and EPs were produced in a wide range of pH and temperature.

Key words: Hypolipidemic effect, exo-polymers, *Cordyceps militaris*, submerged mycelial culture

Cordyceps militaris, a fungus parasitic on the larvae of Lepidoptera, has long been used as a traditional oriental medicine for eternal youth. Cordycepin, a nucleoside derivative, isolated from the fruiting body of *C. militaris* has drawn a considerable attention [7]. The biological activities of Cordycepin include anti-tumor activity on bladder, colon and lung carcinoma and on fibrosarcoma [10], inhibition of the infection and reverse transcriptase activity of human immunodeficiency virus type I [20, 21], and inhibition of methylation of nucleic acid [23]. Our laboratory demonstrated the stimulatory effect of EPs from submerged mycelial culture of *C. militaris* on classical pathway in the complement system [28].

Recently, considerable attentions are being paid to combat hyperlipidemia. It is a well documented fact that lowering of circulating cholesterol (especially LDL cholesterol) levels can prevent, arrest and even reverse coronary atherosclerosis [2, 16, 25, 29]. Attention is also made now to the natural substances capable of lowering plasma cholesterol level. Various edible mushrooms have already been proven themselves to be an important natural regimen for controlling of hyperlipidemia. It is due to their high content of fiber, proteins, microelements and low fat content [6, 14, 15]. The hypolipidemic effect of a few mushrooms has been studied [3, 5, 13], but no reports on the hypolipidemic effect that was elaborated by the fungus *C. militaris* are available at this time.

Moreover, most of the research on hypolipidemic effect was carried out either with the fruiting bodies or the mycelia. In the submerged mycelial culture, some components are released into the culture medium, which were documented to have various biological properties. Recently, these EPs are being investigated extensively for some possible advantages [4, 14, 35]. The production of EPs from the culture broth requires relatively simple purification steps and thus reduces the cost of downstream processing [1, 4]. There are number of reports available on the isolation of various bioactive components from the culture precipitate of *Lentinus edodes* [19, 30, 31, 33], *Ganoderma lucidum* [17], and etc.

The present study was designed to examine the hypolipidemic effect of the EPs produced from submerged mycelial culture of *C. militaris*. The main concern of this study was with the 1) isolation of EPs from culture broth, 2) dose-dependent study of the EPs by oral administration in normolipidemic rats, and 3) optimization of fermentation condition for the production of EPs and mycelia in a 5-l jar fermenter culture.

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MATERIALS AND METHODS

Strains and Medium

Culture of *C. militaris* was obtained from Rural Development Administration in Korea. For the production of EPs, PMP medium was used. The composition of PMP medium is as follows (g/l): potato dextrose broth (Difco) 24, malt extract (Merck) 10, and peptone (Merck) 1. The pH was adjusted to 5.0 before sterilization.

Preparation of Inoculum

Inoculum was prepared by the method as described by Song *et al.* [27]. The seed culture of *C. militaris* was inoculated into a 500 ml flask containing 200 ml of the medium (pH 5.0) and incubated on a rotary shaker (120 rpm, 25°C). After 7 days of cultivation, 100 ml of the medium with mycelial pellets were homogenized aseptically in Sorvall omni-mixer for 3 min in an ice bath. One percent of mycelial suspensions were used as inoculum for submerged culture in a 5-l jar fermenter.

Submerged Mycelial Culture for the Production of Exo-Polymers

The submerged mycelial culture was carried out in a 5-l jar fermenter (working volume: 3 l, pH: 5, temperature: 25°C, agitation speed: 100 rpm, and aeration rate: 1 vvm) for 25 days. Culture broth was harvested by centrifugation (10,447 ×g for 20 min) and supernatant was treated with four times of ethanol (v/v) to collect the EPs. Recovery process of crude EPs from the culture broth was shown in Fig. 1.

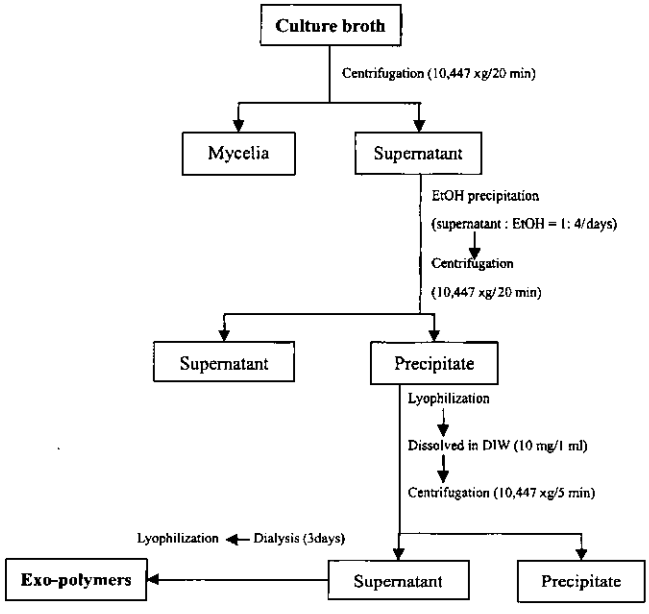

Fig. 1. Schematic diagram for the production of exo-polymers from submerged mycelial culture of *C. militaris*.

Animal Experiment

Sprague-Dawley male rats obtained from the Korean Research Institute of Chemical Technology, weighing approximately 120 g were housed individually in a stainless steel cage in a room with controlled temperature (22 ± 2), humidity (55 ± 5%) and a 12 h cycle of light and dark. The rats were fed with modified AIN-76 [32] diet for 4 weeks. The diet consisted of 55.5% carbohydrate (including 40.5% sucrose), 14.5% fat (30% of total energy) and 20% casein by weight. Rats of each group were administered saline (Control) and EPs at a level of 50 mg- 100 mg/kg body weight by using an oral zonde daily for 2 weeks. Food intake and body weight gains were recorded everyday. At the end of oral administration, the animals were fasted for 9 h and then sacrificed.

Analysis of Plasma and Liver Lipids

Blood samples were collected in heparinized tubes and plasma was separated by centrifugation (1,110 ×g for 20 min). Livers were perfused with cold saline, excised, and kept frozen at -20°C. Liver lipid was extracted by following the method of Folch *et al.* [8]. Plasma levels of TC, HDL cholesterol and TG were measured by using enzymatic kits (Sigma Chemical Company). LDL cholesterol was calculated by the equation of TC - HDL cholesterol - TG/5 [24]. Liver TC was assayed by using the same method as for the plasma TC after the treatment with triton X-100 [26].

Optimization of Culture Condition

For the determination of optimum temperature, the submerged mycelial culture of *C. militaris* was carried out in 500 ml flask containing 200 ml of PMP medium on a rotary shaker (120 rpm, pH 5, 15 days) at the temperature range of 15- 35°C. In order to determine the optimum pH value, pH of the media was varied 3- 10 before sterilization.

Statistical Analysis

Data were expressed as mean ± S.E. Group means were compared by one-way analysis of variance and Duncan's multiple-range test. The statistical differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Body Weight and Food Intake

The final body weight, liver weight and food intake of the rats after the seven days of administration of EPs were presented in Table 1.

The final body and liver weights of the experimental animals did not vary significantly from that of the control diet. EPs also influenced the food intake less significantly. In the present investigation, the body weight of all groups

Table 1. Effects of exo-polymers of *C. militaris* on body weight, liver weight and food intake in dietary induced hyperlipidemic rats.

Diet Groups (Exo-polymers mg/kg body weight)	Body weight (g/2 wk)	Liver weight (g/2 wk)	Food intake (g/2 wk)
Control	43.14±4.17^{NS}	5.85±0.20^{NS}	13.45±0.31^{NS}
50	39.79±1.62	5.40±0.18	13.22±0.33
75	41.49±1.17	5.88±0.16	13.51±0.29
100	41.14±5.05	5.75±0.19	13.28±0.41

Mean±SE (n=6~8).
^{NS}Not significant.

did not vary significantly and oral administration of EPs caused no changes in the gross behavior where none of the animals died. Thus, it can be stated that there were no side effects in rats following oral administration of EPs of *C. militaris*.

Levels of Lipids in Plasma and Liver

The effects in the levels of TG, TC, LDL and HDL cholesterol in plasma, and liver TC under the influence of *C. militaris* EPs were summarized in Table 2.

Maximum reduction of TC (21.6%) and TG (31.8%) was achieved at 75 mg/kg BW EPs dose. Increasing the dose further (100 mg/kg) did not show any improvement either in the plasma TC or TG level. Although the EPs did not influence the plasma HDL cholesterol level, the ratio of HDL cholesterol to TC increased significantly with the increasing concentration of EPs and attained the value above 0.6. A substantial reduction in LDL cholesterol level was noticed even at a dose of 50 mg/kg BW. Maximum LDL depletion was made possible at 100 mg/kg BW dose which could lower the LDL level as much as 40.6%. On the other hand, the EPs did not influence the liver TC level at all and kept the level relatively unchanged in different experimental groups.

The hypolipidemic effect was steadily increased with the increasing the dose of EPs. This is consistent with the increased viscosity in the intestinal content with the increasing dose of the EPs. An increased viscosity of the intestinal contents resulted in a reduced nutrient movement towards the villi network for an efficient absorption, and thereby reduced the levels of plasma TG and TC. The hypolipidemic effect exerted by the EPs of *C. militaris* may result from the high viscous nature of the EPs. Its effects can be compared with a high viscosity water-soluble dietary fiber such as guar gum or pectin [11, 12, 22]. However, this fact cannot exclude the other mechanisms involved in exhibiting hypolipidemic effect by the EPs.

A substantial reduction of LDL and TC in plasma could also be obtained by a reduced production of TC by liver tissue and/or efficient removal of the LDL cholesterol by various tissues without subsequent renewal [9].

The present investigation demonstrated the potential of *C. militaris* EPs in reducing the level of cholesterol rich LDL (which are quantitatively the most significant lipoprotein class in the control of serum cholesterol levels) and preserving the HDL at a relatively high level. All of the above effects would help to reduce the risk of atherosclerosis [18]. Although the exact mechanism of the *C. militaris* EPs in exhibiting hypolipidemic effect is not clear, the possibility of combined effect of the EPs in exerting hyperlipidemia can not be ruled out. These are: 1) the inhibition of cholesterol absorption and/or biosynthesis, 2) inhibition of biosynthesis of VLDL, the precursor of LDL and acceleration of fractional turnover of LDL [34], and 3) increased excretion of bile acids [36].

Effects of Temperature and pH on Mycelial Growth and EPs Production

The effects of temperature was investigated for mycelial growth and EPs production. The temperature varied between 15–35°C. The data obtained was presented in Fig. 2. It was found that mycelial growth and EPs production did not vary significantly within the temperature range of 15–25°C. A significant drop in mycelial yield was evidenced at a temperature above 30°C. In fact, the temperature above 35°C proved detrimental for both the mycelial growth and EPs production. Very little or no EPs production and

Table 2. Hypolipidemic effect of exo-polymers produced from submerged mycelial culture of *C. militaris* in normolipidemic rats.

Diet Groups (Exo-polymers mg/kg body weight)	Blood Plasma (mg/dl)					Liver (mg/dl)
	Triglyceride	Total cholesterol	LDL cholesterol	HDL cholesterol	HDL/Total cholesterol	Total cholesterol
Control	42.84±1.82^{b}	63.92±1.51^{a}	27.61±0.94^{a}	27.74±0.89^{NS}	43.77±1.70^{a}	16.10±0.87^{NS}
50	43.22±0.97^{a}	55.39±2.93^{ab}	20.65±1.85^{ab}	28.01±0.91	45.36±1.41^{ab}	14.90±1.36
75	29.22±1.44^{c}	50.12±3.40^{b}	19.48±2.97^{b}	27.44±0.90	52.66±1.94^{b}	14.76±0.79
100	29.43±1.03^{c}	50.60±1.56^{b}	16.44±2.66^{b}	30.08±1.58	60.94±4.73^{b}	14.05±0.96

Mean±SE (n=6~8).
^{NS}Not significant.
Means in the same column with different superscript are significantly different ($p<0.05$).

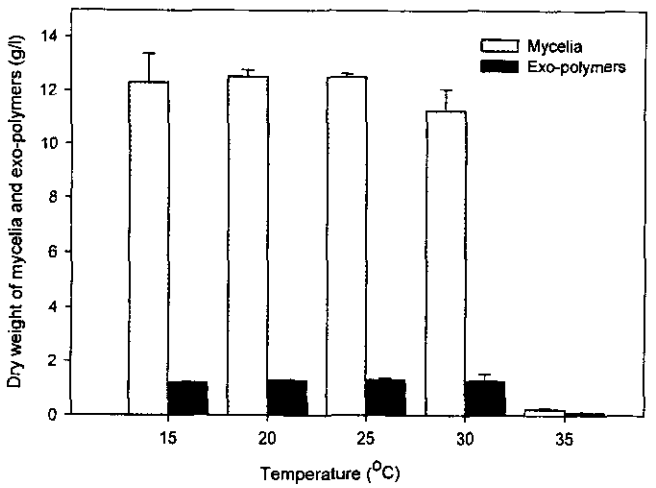

Fig. 2. Optimum temperature for the production of exo-polymers from submerged mycelial culture of *C. militaris*.
Mean±SD (triplication), Culture condition: pH 5, 120 rpm, 15 days.

mycelial growth were achieved at 35°C. Maximum EPs production was achieved at the temperature range of 20–25°C.

The effects of pH for biomass and EPs production were investigated at the range of 3.0–10.0. The data obtained was presented in Fig. 3. A negative correlation between EPs production and biomass yield was noticed under the influence of different pH values. EPs production favored at the alkaline pH and the production increased with the increase in pH. It reached its maximum value at the pH 8 (1.55 g DW/l culture broth), whereas biomass production was favored at the acidic pH and exhibited its maximum yield at pH 4. Our results are significant due to the fact that for EPs production, the submerged mycelial culture of the *C. militaris* can be performed in a wide range of temperature and pH values.

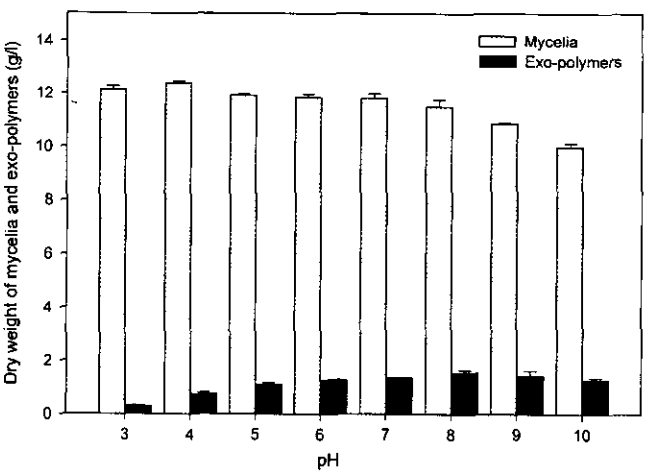

Fig. 3. Optimum pH for the production of exo-polymers from submerged mycelial culture of *C. militaris*.
Mean±SD (triplication), Culture condition: 25°C, 120 rpm, 15 days.

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