J. Microbiol. Biotechnol. (1998), **8**(5), 536 – 539

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

NOTE

The Anti-complementary Activity of Exo-polymers Produced from Submerged Mycelial Cultures of Higher Fungi with Particular Reference to *Cordyceps militaris*

SONG, CHI-HYUN*, YOUNG-JAE JEON, BYUNG-KEUN YANG, KYUNG-SOO RA[1], AND JAE-MO SUNG[2]

Department of Biotechnology, Taegu University, Kyungsan, Kyungbuk 712-714, Korea
[1]Department of Food and Nutrition, Technical Junior College, Taegu 704-350, Korea
[2]Department of Agricultural Biology, Kangwon National University, Chuncheon, Kangwon, 200-701 Korea

Received: May 9, 1998

Abstract The anti-complementary activity (immuno-stimulating activity) was tested for the exo-polymers (extra-cellular polymer) produced from submerged mycelial cultures of 21 types of higher fungi. Anti-complementary activity of the exo-polymer from *Cordyceps militaris* showed the highest (69.0%) followed by *Pleurotus ostreatus* (63.7%) and *Trametes suaveolens* (61.4%). The mycelial growth rate and biomass doubling time of *C. militaris* in a 5 l air-lift fermenter were 0.0255 h^{-1} and 27.2 h, respectively. The yield of the exo-polymer produced from the culture broth of *C. militaris* was 2.95 mg of dry weight/ml of culture broth. Sugar and amino acid compositions of this exo-polymer were analyzed.

Key words: Anti-complementary activity, exo-polymer, *Cordyceps militaris*

Various bio-polymers, such as endotoxic lipopolysaccharide [4], inulin [5], and water-insoluble β-1,3 glucan [22] from bacteria, plants, and fungi, are known to activate the complement system. It is common knowledge that the complement system plays an important role in the host defense system in inflammation and in allergic reactions. In particular, many types of polymers produced from higher fungi have been reported to have immunomodulating activities such as anti-complementary activity [11], mitogenic activity on lymphocytes [8], interferon-inducing activity [13], and anti-tumor activity [6]. The anti-complementary activity of polymers extracted from fruiting bodies [23], mycelium [11], and culture broth [18] of various mushrooms has been previously investigated. It has been reported that activation of the complement system by fungal bio-polymers was closely related with anti-tumor effect [9] and anti-complementary activity was also proportionate to anti-tumor actions [19]. Most polymers extracted from mushrooms were found to be β-1,3-glucans with β-1,6-glucose as a side chain [3] or polysaccharide-protein complexes [20]. Lentinan from *Lentinus edodes* [2], shizophyllan from *Schizophyllum commune* [24], krestin from *Coriolus versicolor* [25], and meshima from *Phellinus linteus* [7] are commercially used as anti-tumor agents. However, those polymers are endo-polymers from mycelia. Therefore, it is worth studying exo-polymers which are produced during submerged mycelial cultures of higher fungi.

In this work, the anti-complementary activities of exo-polymers produced from submerged mycelial cultures of 21 types of higher fungi were tested for screening purpose. Growth rate and production of exo-polymer produced from chosen strain were also examined in a 5 l air-lift fermenter. Sugar and amino acid compositions of this exo-polymer were analyzed.

Twenty-one types of higher fungi were collected mainly from Kyungbuk province in Korea and isolated or donated by K. Y. Cho from the Dept. of Microbiology, the University of Sydney. All organisms were maintained on potato dextrose agar (Difco) slopes, stored at 4°C and subcultured every 3 months.

The composition of the synthetic medium for mycelial growth was as follows (g/l): galactose 1, sucrose 9, xylose 1, glucose 9, yeast extract 0.5, peptone 2, potato dextrose broth 2, $NH_4H_2PO_4$ 0.5, DL-serine 0.5, KH_2PO_4 1, $CaCl_2$ 0.6, $MgSO_4 \cdot 7H_2O$ 2, $FeSO_4 \cdot 7H_2O$ 0.02, $MnSO_4 \cdot 5H_2O$ 0.02 and thiamine HCl 1×10^{-3}. The pH was adjusted to 4.5.

The submerged mycelial culture was carried out in 500 ml flask containing 200 ml of medium on a rotary shaker

*Corresponding author
Phone: 82-53-850-6555; Fax: 82-53-850-6509;
E-mail: chsong@biho.taegu.ac.kr

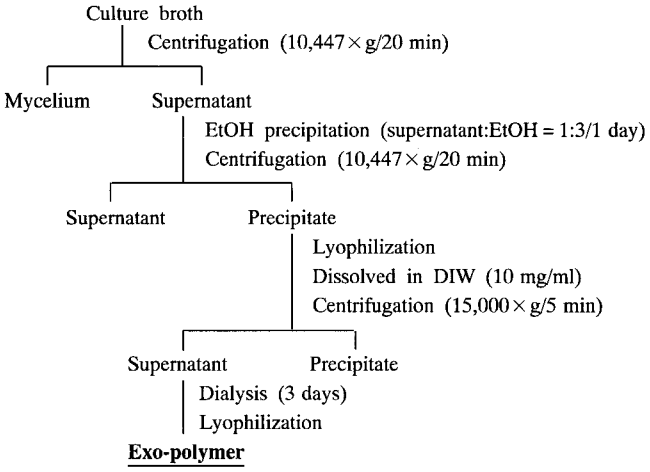

Fig. 1. Preparation of exo-polymers from the submerged mycelial cultures of higher fungi.
EtOH, ethanol; DIW, disfilled water.

(120 rpm, 14~30 days) or in a 5 l air-lift fermenter at 20°C with an air flow rate of 1.0 vvm, and pH of 6.0. Culture broth was harvested by centrifugation (10,447× g/20 min) and supernatant was treated with ethanol to collect the exo-polymers as shown in Fig. 1.

Anti-complementary activity was measured by the complement fixation test based on complement consumption and degree of red blood cell lysis by the residual complement [12]. Normal human serum was obtained from a healthy adult. The fifty microliters of water solution (1,000 μg/ml) of exo-polymer were mixed with equal volumes of normal human serum and GVB^{++} (gelatin veronal-buffered saline, pH 7.4) containing 500 μg Mg^{++} and 150 μg Ca^{++}. The mixtures were incubated at 37°C for 30 min and the residual total hemolytic complement (TCH_{50}) was determined by using IgM-hemolysin-sensitized sheep erythrocyte at 1×10^8 cell/ml. Normal human serum was incubated with water and GVB^{++} to provide a control. The anti-complementary activity of the exo-polymer was expressed as the percentage inhibition of the TCH_{50} of control.

$$ITCH_{50}(\%) = \frac{TCH_{50}\text{ of control} - TCH_{50}\text{ treated with sample}}{TCH_{50}\text{ of control}} \times 100$$

Dried sample was hydrolyzed at 121°C for 24 h with 2 M trifluoroacetic acid (TFA) in a sealed tube. The hydrolysate was evaporated to remove 2 M TFA for analysis of amino acid. The composition of amino acid was analyzed by Pharmacia Biochrom 20 amino acid analyzer and the analytical condition was as follows (Table 1).

The exo-polymer was hydrolyzed and acetylated for analysis of sugar by the method of Jones and Albersheim [10]. Sugar composition was analyzed by HPLC under the following conditions (Table 2).

Table 1. Conditions for the analysis of amino acid by amino acid analyzer.

Apparatus	Pharmacia Biochrom 20 amino acid analyzer
Column	High performance 4.6 mm× 200 mm L
Ion exchange resin	No. 3906
Flow rate	Buffer solution: 0.58 μl/min, Ninhydrin 0.41 μl/min
Analysis cycle time	45 min
Column pressure	56.1 kg_f/cm^2
Ninhydrin pressure	12.24 kg_f/cm^2
Column temperature	48~89°C gradient
Reaction coil temperature	135°C
Wavelength	440 nm, 570 nm

Table 2. Conditions for the analysis of sugar composition by HPLC.

Apparatus	Varian STAR 9040
Detector	Refractive Index
Column	Stainless column (L: 300 mm, ID: 6.5 mm)
Column packing material	IC6020
Column temperature	85°C
Column pressure	575 Psig
Mobile phase	DDI H_2O
Flow rate	0.7 ml/min
Injection volume	20 μl

Anti-complementary Activity of Exo-polymers Produced from Submerged Cultures of 21 Types of Higher Fungi

Twenty-one types of exo-polymer produced from the culture broth of higher fungi were examined for anti-complementary activity by using the complement fixation test and the activities were presented as $ITCH_{50}$ (Fig. 2). Among the exo-polymers produced from submerged mycelial cultures tested, *C. militaris* showed the highest activity (69.0%) followed by *P. ostreatus* (63.7%) and *T. suaveolens* (61.4%). Endo-polymers from mycelia had generally low anti-complementary activity, for example, *Coriolus vesicolor* (29.3%), *Armillariella mellea* (37.1%), and *Lentinus edodes* (31.3%) [17].

Cordyceps sp. is a fungus that is parasitic on the larvae of Lepidoptera and has been used as a Chinese medicine for eternal youth [14]. The polysaccharide fraction obtained from mycelia or ascocarps of *Cordyceps* sp. has been reported to have anti-tumor [1], hypolipidemic, and hypoglycemic activity [15]. However, the biological activity of the exo-polymer produced from a submerged mycelial culture of *Cordyceps* sp. has not been reported.

Our results show that exo-polymer from *C. militaris* presented a high anti-complementary activity which is

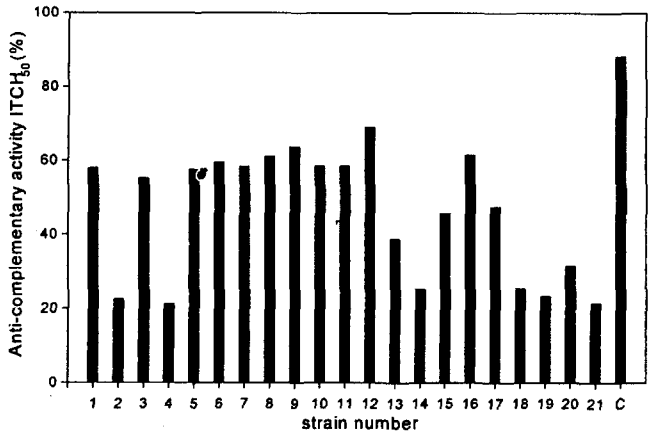

Fig. 2. The anti-complementary activities of exo-polymers produced from the submerged mycelial cultures of 21 types of higher fungi.

1, *Pleurotus sajor-caju*; 2, *Lentinus edodes*; 3, *Flammulina velutipes*; 4, *Pholiota nameko*; 5, *Agrocybe cylindracea*; 6, *Lyophyllum cinerascens*; 7, *Grifola frondosa*; 8, *Ganoderma lucidum*; 9, *Pleurotus ostreatus*; 10, *Auricularia polytricha*; 11, *Coriolus versicolor* TG-1; 12, *Cordyceps militaris*; 13, *Collybia maculata*; 14, *Lycoperdon pyriforme*; 15, *Lycoperdon unbrinum*; 16, *Trametes suaveolens*; 17, *Phellinus linteus* TGP-2; 18, *Sarcodon asparatus*; 19, *Coriolus vesicolor* TG-2; 20, *Phellinus linteus* TGP-3; 21, *Phellinus linteus* TGP-4; C, positive control (CAP-0).

worth studying on exo-polymers produced from submerged mycelial culture of various fungi.

Mycelial Growth Curve and Production of Exo-polymer from *C. militaris*

The mycelial growth rate and biomass doubling time of submerged culture in synthetic medium were 0.0255 h^{-1} and 27.2 h, respectively, in a 5 l air-lift fermenter. The production of exo-polymer from the culture broth of *C. militaris* was 2.95 mg of dry weight/ml of culture broth. Mycelial growth was maximum at 12 days, whereas, maximum exo-polymer production was reached at 14 days cultivation (Fig. 3). It is generally known that the precursor form of microbial exo-polymers accumulate on the cell surface during logarithmic growth and is subsequently released in the stationary phase [21].

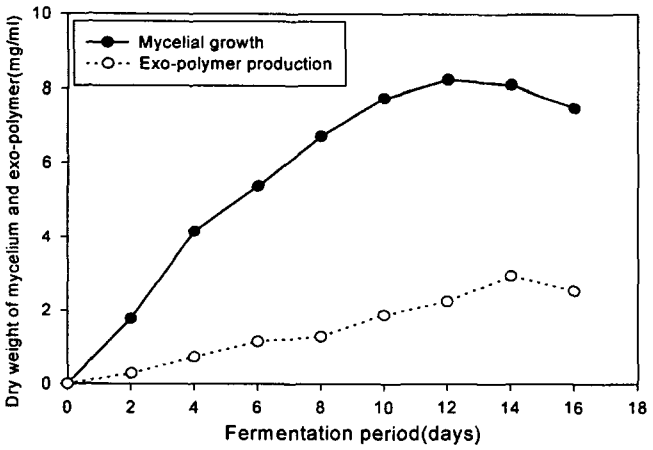

Fig. 3. Growth curve and production of exo-polymer produced from *Cordyceps militaris* in an air-lift fermenter with synthetic medium.

Table 3. Amino acid and sugar composition of exo-polymer produced from *Cordyceps militaris*.

Amino acid	Composition (%)	Sugar	Composition (%)
Aspartic acid	6.566	Glucose	9.283
Threonine	0.804	Galactose	87.255
Serine	2.001	Arabinose	3.463
Glutamic acid	1.994		
Proline	2.373		
Glycine	5.626		
Alanine	1.295		
Cysteine	5.570		
Valine	9.671		
Methionine	4.929		
Isoleucine	5.943		
Leucine	2.691		
Tyrosine	38.269		
Histidine	5.559		
Lysine	6.697		

Amino Acid and Sugar Compositions of Exo-polymer from a Chosen Strain

The exo-polymer produced from the culture broth of *C. militaris* consisted mainly of a large quantity of galactose (87.2%) and a small amount of glucose (9.3%) and arabinose (3.5%). Whereas, Kim *et al.* [16] reported that the polymer from ascocarps of *C. militaris* was composed of glucose (78.6%), galactose (19.1%), and arabinose (2.2%). When the above results are compared, the compositional difference was present according to the method of production of the polymers.

This exo-polymer also contained fifteen kinds of amino acids: aspartic acid, 6.6%; threonine, 0.8%; serine, 2.0%; glutamic acid, 2.0%; proline, 2.4%; glycine, 5.6%; alanine, 1.3%; cysteine, 5.6%; valine, 9.7%; methionine, 5.0%; isoleucine, 5.9%; leucine, 2.7%; tyrosine, 38.3%; histidine, 5.6%; lysine 6.7%. Therefore, it can be concluded that the produced exo-polymer could be a glycopeptide which is composed of sugars and amino acids. Since the anti-complementary activity of a polymer from Kampo (Japanese herbal medicine) was reduced by both pronase digestion and periodate oxidation [26], it can be suggested that carbohydrate and protein moieties in this exo-polymer contribute to the expression of the activity. Further studies on the chemical structure and mechanisms of this exo-polymer are in progress.

Acknowledgements

This work was supported by Korea Science and Engineering Foundation (1996, Grant number: 961-0603-021-2) and in part by the Medicinal Resoures Research Center (98-16-02--05-A-3) sponsored by Korea Science and Engineering Foundation and Chollabuk-Do.

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