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# Chemical characteristics and immuno-modulating activities of exo-biopolymers produced by *Grifola frondosa* during submerged fermentation process

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**Abstract**

The immuno-modulating activities and chemical characteristics of exo-biopolymer (EX-GF) produced by a submerged mycelial culture of *Grifola frondosa* were studied. The EX-GF was fractionated into EX-GF-Fr.I, II, and III by Sephadex G-100 gel chromatography. Anti-complementary activity of EX-GF-Fr.III was highest (71.1%) among them, and its activation system occurred through both classical and alternative pathways, where the classical pathway found to be major one. Lysosomal enzyme activity and nitric oxide production ability of macrophage were also found to be mediated by EX-GF-Fr.III. The molecular weight of the EX-GF-Fr.I, II, and III was estimated to be about 163, 40, and 2.8 kDa, respectively. Total sugar and protein contents of the three fractions were 80.3, 61.9 and 89.3%, and 17.3, 35.2, and 10.7%, respectively. The sugar and amino acid compositions of the EX-GF-Fr.I, II, and III were also analyzed in detail.


*Keywords:* Exo-biopolymer; *Grifola frondosa*; Immuno-modulating activity; Submerged mycelial culture

## 1. Introduction

Recently, many research workers are becoming interested in finding new functional compounds in mushrooms. Various biologically active substances isolated from mushrooms have been recorded in the literature, which include, polysaccharides, terpenoids, polysaccharide–peptide complexes and proteins [1,2], as they have been found to exhibit a wide range of chemical characteristics and show various biological effects on immune cells [3,4]. Immuno-active polysaccharide-protein has recently been isolated from various mushroom species [5].

*Grifola frondosa*, which belongs to the family of Aphyllopherales and Polyporaceae, is widely used in Japan, China, and Korea as traditional food additives. Recently, various bioactive properties of this mushroom have been explored, thus attracted considerable attention around the globe. Polysaccharides obtained from the liquid-cultured mycelium and fruiting body of *G. frondosa* have demonstrated many interesting biological activities, including anti-tumor [6], anti-hypertensive [7], anti-diabetic [8], anti-oxidant [9], and anti-hyperliposis effects [10]. Our preliminary study showed that the exo-biopolymer produced during submerged mycelial culture of *G. frondosa* had high anti-complementary activity.

In the present investigation, several immunomodulating studies, which include lysosomal enzyme activity and macrophage activation by purified exo-biopolymer of *G. frondosa* have been carried out along with its chemical analysis.

*Abbreviations:* EX, exo-biopolymer; GF, *Grifola frondosa*; Fr, fractionation; MCM, mushroom complete medium; NHS, normal human serum; GVB, gelatin veronal buffer; TCH, total complement hemolysis; EGTA, ethylenglycol-bis(*b*-aminiethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylendiaminate tetraacetic acid; CIEP, crossed immunoelectrophoresis; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; IFN, interferon; LPS, lipopolysaccharide; PNPP, *p*-nitrophenyl phosphate; NO, nitric oxide; BSA, bovine serum albumin; iNOS, inducible nitric oxide systhase

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## 2. Materials and methods

### 2.1. Microorganism

The culture of *G. frondosa* was obtained from the Korean Agricultural Culture Collection. The seed culture was grown in 250-ml Erlenmeyer flask, containing 100 ml of potato dextrose broth (pH 5.0), and incubated at 25 °C/150 rpm for approximately 10 days. One hundred milliliters medium containing mycelial pellets was homogenized aseptically in a Sorvall omnimixer for 3 min in an ice bath and inoculated (2%, v/v) into the liquid media for submerged cultivation.

### 2.2. Production and purification of exo-biopolymers

The mushroom complete medium (MCM) of the following composition (g/l): glucose 20, $MgSO_4$ 0.5, $KH_2PO_4$ 0.46, $K_2HPO_4$ 1.0, yeast extract 2, and peptone 2 with pH 5.0 was used as a submerged culture media for the production of exo-biopolymer. The submerged cultivation carried out in 500-ml Erlenmeyer flask, containing 200 ml of the media and kept at 25 °C/150 rpm/20 days. Culture broth was harvested by centrifugation (10,000 × *g*/20 min), and the supernatant was treated with ethanol. Ethanol precipitate was dissolved in water, dialyzed, and lyophilized to obtain an exo-biopolymer. Detailed preparation process of water soluble exo-biopolymer is shown in Fig. 1.

### 2.3. Assay of anti-complementary activity

The anti-complementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement [11]. Fifty microliters of exo-biopolymer solution in water was mixed with equal volumes of normal human serum (NHS) and GVB (gelatin veronal buffered saline, pH 7.4) containing 500 μg $Mg^{2+}$ and 150 μg $Ca^{2+}$. The mixtures were incubated at 37 °C/30 min and the residual total complement hemolysis ($TCH_{50}$) was determined by using IgM hemolysin sensitized sheep erythrocytes ($1 \times 10^8$ cells/ml). At the same time, the NHS was incubated with deionized water and $GVB^{2+}$ (GVB containing 500 μg $Mg^{2+}$ and 150 μg $Ca^{2+}$) to provide a control. The anti-complementary activity of crude polymers was expressed as the percentage inhibition of the $TCH_{50}$ of control:

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

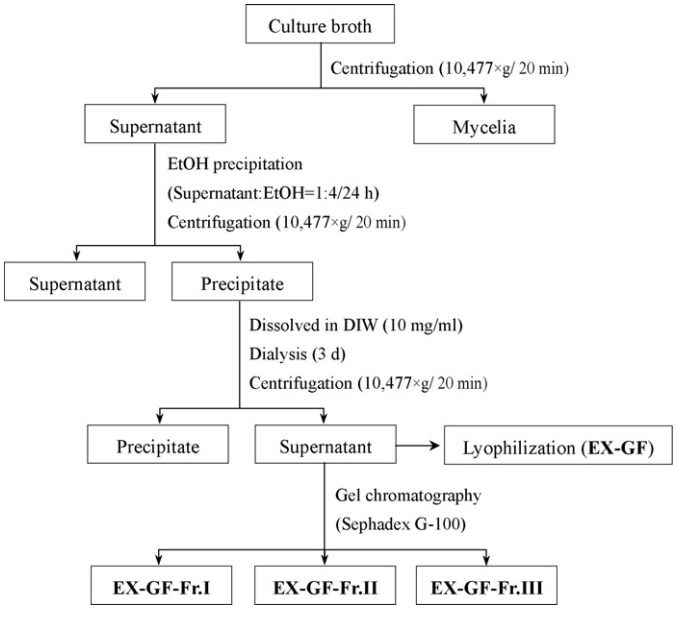


Fig. 1. A schematic diagram depicting the recovery process of exo-biopolymers from submerged mycelial culture of *Grifola frondosa*.

### 2.4. Determination of the complement activated pathway

Normally, the $Ca^{2+}$ ion is required for the activation of complement *via* the classical pathway, but not for the alternative pathway, and the activation through the alternative complement pathway was measured in the $Ca^{2+}$ free condition. The alternative complement pathway determined in 10 mM EGTA containing 2 mM $MgCl_2$ in $GVB^{2-}$ ($Mg^{2+}$-EGTA-$GVB^{2-}$) following a modified method of Platts-Mills and Ishizaka [12]. A sample was incubated with $Mg^{2+}$-EGTA-$GVB^{2-}$ and NHS at 37 °C for 30 min, and the residual complement mixtures were measured by hemolysis of rabbit erythrocytes ($5 \times 10^7$ cells/ml) incubated with $Mg^{2+}$-EGTA-$GVB^{2-}$.

### 2.5. Assay of crossed immunoelectrophoresis

The specific activation of C3 complement component by exo-biopolymers in NHS was assessed by comparative measurements of C3 cleavage. The NHS was incubated with an equal volume of exo-biopolymers solution in $GVB^{2+}$, $GVB^{2-}$ containing 10 mM EDTA (EDTA-$GVB^{2-}$) or $Mg^{2+}$-EGTA-$GVB^{2-}$ at 37 °C/30 min. The mixture was then subjected to crossed immunoelectrophoresis (CIEP) to locate the C3 cleavage product. All samples (10 μl) were subjected to isoelectric focusing on 1% agarose gel. Two hours after, the first run was done in barbital buffer (pH 8.6), ion strength 0.025 with 1% agarose, while the second run was carried out in a gel plate containing an anti-human complement C3, which recognizes both C3 and C3b (complement C3 antiserum raised in goat, Sigma Co., USA), at a potential gradient of 15 mA/plate for 15 h. After the electrophoresis, the plate was fixed and stained with 0.2% bromophenol blue. The ratio between the heights of the C3 and C3b peaks was then calculated [13].

### 2.6. Experimental animals and breeding condition

Male 6-week-old C57BL/6 mice weighing approximately 25 g, purchased from Daehan Biolink Co., Ltd., were housed in plastic cages. The mice were maintained in a room with constant temperature (22 ± 2 °C), humidity (55 ± 5%) with a 12 h light–dark cycle. The mice were fed a commercial pellet diet (Sam Yang Co., Korea) throughout the experimental period.