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Anti-complementary Activities of Exo- and Endo-biopolymer Produced by Submerged Mycelial Culture of Eight Different Mushrooms

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The *Elfvingia applanata* (EA), *Hericium erinaceum* (HE), *Grifola frondosa* (GF), *Pholiota nameko* (PN), *Pleurotus eryngii* (PE), *Trametes suaveolens* (TS), *Fomes fomentarius* (FF), and *Inonotus obliquus* (IO) could produce the endo- (EN) and exo-biopolymer (EX) in submerged culture. The highest anti-complementary activity of the EN was exhibited by PN (49.1%), followed by HE (38.6%), TS (37.0%), and FF (33.0%), whereas the high activity of the EX was found with GF (59.8%), followed by HE (36.3%), TS (30.8%), and IO (28.8%). The EN of *P. nameko* (EN-PN) and EX of *G. frondosa* (EX-GF) were found to contain 78.6% and 41.2% carbohydrates, while 21.4% and 58.8% protein, respectively. The sugar and amino acid compositions of EN-PN and EX-GF were also analyzed in detail.

KEYWORDS: Anti-complementary activity, Endo-biopolymer, Exo-biopolymer, Mushroom, Submerged mycelial culture

Among various immune systems, the complement system consisting of a series of enzymes in blood serum which plays an important role in host resistance as a primary humoral mediated antigen-antibody reaction. The complement system is major effectors of the humoral and innate immunity involved in host defense. A number of substances from chemicals, plant or microbial origins have been reported to modulate the complement cascade (Hildebert and Jordan, 1988). The function of the complement system in innate and specific humoral immunity is to promote phagocytosis of microbes on which complement is activated, to stimulate inflammation, and to induce the lysis of these microbes. The biological importance of complement is emphasized by severe symptoms that cause complement deficiencies. The regulation of complement activity affects an important in controlling immune responses (Walport, 1993).

Polysaccharides are essential constituents for all living organisms as they are associated with a variety of vital functions. The polysaccharides of natural origin have emerged as an important class of bioactive materials. Polysaccharides possessing anti-complementary activity have been reported from bacteria, fungi and higher plants. Mushrooms have been proved as an interesting source for new secondary metabolites with a variety of different biological activities. Active substances of various mushrooms can act as biological response modifiers (Maeda and Chihara, 1971) and also can have many pharmacological effects, i.e. immuno-modulating, hypoglycemic, hypolipidemic, and anti-tumor actions. In addition to above biological activities, some anti-complementary activities have also been reported from *Ganoderma lucidum* (Lee *et al.*, 1994), *Lentinus edodes* (Song *et al.*, 1998), and *Pleurotus ostreatus* (Kweon *et al.*, 1990). Recently, immunoactive polysaccharide-protein has been isolated from many kinds of mushroom species. The activation of the complement system by fungal biopolymers is found to be closely related with anti-tumor effect and anti-complementary activity was also proportionate to anti-tumor actions (Lee *et al.*, 1994). Wang *et al.* (1996) investigated immunomodulating activity of lectins from *Tricholoma mongolicum*. The immuno-modulating activity of β-glucan obtained from liquid-culture of *G. frondosa* has also been documented by Suzuki *et al.* (1989).

In this study, the anti-complementary activities of EN and EX produced from submerged culture of EA, HE, GF, PN, PE, TS, FF, and IO were investigated. Chemical compositions of EN and EX produced from chosen strain were also analyzed.

Materials and Methods

Organism and culture medium. The cultures of EA (KACC 50174), HE (KACC 42140), GF (KACC 50027), PN (KACC 50453), PE (KACC 50037), TS (KCTC 26205), FF (KCTC 6363), and IO (KACC 50069) were obtained from the Korean Agricultural Culture Collection (KACC) and Korean Collection of Type Cultures (KCTC). The seed cultures were grown in 250-*ml* flask, containing 100 *ml* of potato dextrose broth (pH 5.0) and incubated on a rotary shaker (120 rpm) at 25°C for approximately 7 d. One hundred *ml* of the medium with mycelial pellets was

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homogenized aseptically in a Sorvall omni-mixer for 3 min in an ice bath and inoculated in the liquid media at the rate of 2% (v/v) for submerged cultivation. The mushroom complete medium (MCM) with the following composition (g/*l*): glucose 20, yeast extract 2, peptone 2, KH_2PO_4 0.46, K_2HPO_4 1.0, and $MgSO_4 \cdot 7H_2O$ 0.5, with pH 5.0 was used to perform submerged mycelial culture for the production of biopolymers. The submerged mycelial cultures were carried out in 500-*ml* flask, containing 200 *ml* of the medium at 120 rpm/25°C/7~15 d.

Recovery of EX and EN. The supernatant and the hot water extract of mycelia were treated with ethanol. The ethanol precipitate was dissolved in water, dialyzed, and lyophilized to obtain an EN and EX. The recovery procedure for EX and EN from submerged mycelial culture of mushrooms is shown in Fig. 1.

Assay of anti-complementary activity. 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. Fifty microliter of water solution of biopolymers was mixed with equal volumes of normal human serum (NHS) and GVB^{++} (gelatin veronal buffered saline, pH 7.4) containing Mg^{++} (500 μg) and Ca^{++} (150 μg). The mixtures were incubated at 37°C for 30 min and the residual total complement hemolysis (TCH_{50}) was determined by using IgM hemolysin sensitized sheep erythrocytes at 1×10^8 cell/*ml*. At the same time, the NHS was incubated with deionized water (DIW) and GVB^{++} (GVB containing 500 μg Mg^{++} and 150 μg Ca^{++}) to provide a control. The anti-complementary activity of biopolymers was expressed as the percentage inhibition of the TCH_{50} of control.

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

Chemical analysis of EX and EN. Total protein content of the EN and EX was determined with bovine serum albumin (BSA) as a standard (Lowry *et al.*, 1951). The amino acid composition was analyzed by a Biochrom 20 (Pharmacia Biotech. Ltd., U.S.A.) amino acid autoanalyzer with a Na-form column after hydrolysis of the protein. The total sugar content was determined by the phenol sulfuric acid method, using a mannose and galactose mixture (1 : 1) as the standard (Dubios *et al.*, 1964). The sugar composition was analyzed by a GC 3600 gas chromatography (Varian Co., U.S.A.) based on the hydrolysis and acethylation method (Jones and Albersheim, 1972).

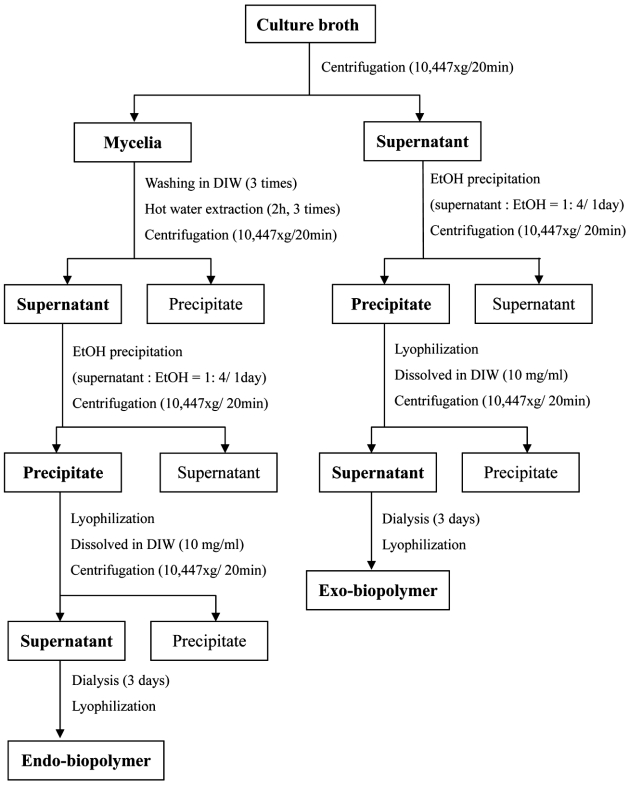

Fig. 1. Schematic diagram depicting recovery process of exo- and endo-biopolymer from submerged mycelial culture of mushrooms.

Results and Discussion

Production of EX and EN. Eight different mushrooms were cultivated for production of EX and EN. Favourable growth was achieved with EA, GF, PN, and PE while high yield of EX and EN were obtained from GF and PN, respectively (Table 1).

Anti-complementary activities of EX and EN. The water soluble EXs and ENs obtained through the submerged mycelial cultures of eight kinds of mushrooms were analyzed for anti-complementary activity (Figs. 2 and 3) by total hemolytic complement assay (TCH_{50}). Their activities were compared at the concentration of 1000 μg/*ml*. The highest activity (49.1%) was recorded with EN-PN followed by EN-HE (38.6%) and EN-TS (37.0%).

Among the EXs (Fig. 3), a highest activity (59.8%) was observed EX-GF, followed by EX-HE (36.3%), EX-TS (30.8%), and EX-IO (28.8%).

In earlier studies, the anti-complementary activity has been reported from fruiting bodies (Kweon *et al.*, 1990), mycelia (Song *et al.*, 1998), and culture broth (Jeong *et al.*, 2004) of various mushrooms. An activation of the complement system by some mushroom polysaccharides is reported to enhance macrophage activation, cytolysis,

Table 1. Mycelial growth, exo- and endo-biopolymer yields by submerged mycelial culture of eight different mushrooms

Strain	Culture time (day)[a]	Mycelial dry weight (g/*l*)	Exo-biopolymer (g/*l*)	Endo-biopolymer (g/*l*)
Elfvingia applanata (EA)	7	8.69 ± 0.21	1.03 ± 0.05	0.43 ± 0.07
Hericium erinaceum (HE)	12	5.65 ± 0.17	1.06 ± 0.03	0.31 ± 0.05
Grifola frondosa (GF)	10	7.88 ± 0.22	1.14 ± 0.07	0.35 ± 0.03
Pholiota nameko (PN)	10	9.02 ± 0.41	0.74 ± 0.04	0.47 ± 0.07
Pleurotus eryngii (PE)	7	8.52 ± 0.28	1.02 ± 0.06	0.45 ± 0.09
Trametes suaveolens (TS)	15	4.32 ± 0.19	0.68 ± 0.10	0.23 ± 0.03
Fomes fomentarius (FF)	15	3.95 ± 0.25	0.71 ± 0.09	0.26 ± 0.03
Inonotus obliquus (IO)	10	5.51 ± 0.33	0.85 ± 0.11	0.30 ± 0.04

[a]Culture time at which the maximum mycelial mass was reached. All the values are given as the mean based on results of triplicate experiments.

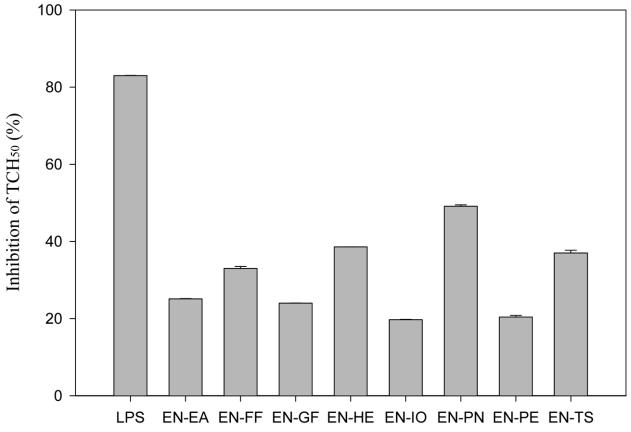

Fig. 2. The anti-complementary activities of the endo-biopolymers obtained from the submerged mycelial cultures of eight different types of mushrooms. LPS: Lipopolysaccharide was used for the positive control. The concentration of each sample and LPS was 1000 $\mu g/ml$. Each value is the mean ± S.D. of triplicate. EN: endo-biopolymer, EA: *Elfvingia applanata*, FF: *Fomes fomentarius*, GF: *Grifola frondosa*, HE: *Hericium erinaceum*, IO: *Inonotus obliquus*, PN: *Pholiota nameko*, PE: *Pleurotus eryngii*, TS: *Trametes suaveolens*.

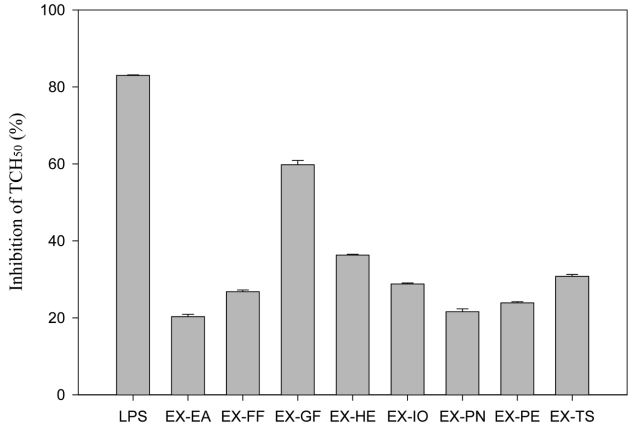

Fig. 3. The anti-complementary activities of exo-biopolymers obtained from the submerged mycelial cultures of eight different types of mushrooms. LPS: Lipopolysaccharide was used for the positive control. The concentration of each sample and LPS was 1000 $\mu g/ml$. Each value is the mean ± S.D. of triplicate. EX: exo-biopolymer, EA: *Elfvingia applanata*, FF: *Fomes fomentarius*, GF: *Grifola frondosa*, HE: *Hericium erinaceum*, IO: *Inonotus obliquus*, PN: *Pholiota nameko*, PE: *Pleurotus eryngii*, TS: *Trametes suaveolens*.

and anti-tumor activity (Suzuki *et al.*, 1989; Wang *et al.*, 1996). The anti-complementary activity of lentinan (Chihara *et al.*, 1970) isolated from *Lentinus edodes* and Schizophyllan (Tabata *et al.*, 1981) from *Schzophyllum commune* has been demonstrated earlier and these polysaccharides, which found responsible for it, are now in clinical use as anti-cancer drugs. Similarly, many workers (Lee *et al.*, 1994; Suzuki *et al.*, 1989) have shown a correlation between the activation of the complement system and anti-tumor effect of polysaccharides. Therefore, the results obtained from this study can suggest that EN-PN and EX-GF may have a potential as an anti-tumor substance.

Amino acid and sugar composition of EN-PN and EX-GF. The EN-PN was found to contain 21.4% and 78.6% of total sugar and protein contents, respectively. Out of seventeen different kinds of amino acids which constitute the protein moiety, the major amino acids were appeared to be glutamic acid (12.6%), glysine (12.2%), lysine (11.9%), and aspartic acid (10.9%), while glucose (81.1%) appeared to be the major carbohydrate of the sugar moiety (Table 2).

The EX-GF seems to be a glycoprotein, which contains 62.2% carbohydrate and 37.8% protein (Table 3). Serine (12.3%), threonine (11.6%), aspartic acid (11.6%), glycine (9.8%), and glutamic acid (9.4%) were found as the major amino acids in the EX-GF, while mannose (49.0%), glucose (15.7%), and fucose (18.4%) were the major sugars present in the carbohydrate moiety.

Yang *et al.* (1987) reported that the polysaccharide-peptide isolated from deep-layer cultured mycelia of *Coriolus versicolor* is composed of glucose (74.6%) with the remainder being galactose, mannose, xylose and fucose. Kim *et al.* (1994) also noticed that the polymer from ascocarp of *Cordyceps militaris* was mainly composed of

Table 2. Sugar and amino acid compositions of the endo-biopolymers obtained from the mycelia of *Pholiota nameko*

Sugar	Composition (%)*	Amino acid	Composition (%)*
Ribose	1.2	Aspartic acid	10.9
Arabinose	2.7	Threonine	6.7
Mannose	6.9	Serine	8.1
Galactose	8.1	Glutamic acid	12.6
Glucose	81.1	Proline	0.6
		Glycine	12.2
		Alanine	9.3
		Cysteine	0.3
		Valine	5.5
		Methionine	0.4
		Isoleucine	3.3
		Leucine	5.6
		Tyrosine	0.7
		Phenylalanine	4.3
		Histidine	2.7
		Lysine	11.9
		Arginine	5.2
Total sugar content	78.6	Total protein content	21.4

*Calculated on the basis of total sugar or protein.

Table 3. Sugar and amino acid compositions of the exo-biopolymers obtained from the submerged mycelial culture of *Grifola frondosa*

Sugar	Composition (%)*	Amino acid	Composition (%)*
Fucose	13.4	Aspartic acid	11.6
Xylose	8.8	Threonine	11.6
Mannose	49.0	Serine	12.3
Galactose	8.1	Glutamic acid	9.4
Glucose	15.7	Proline	0.5
		Glycine	9.8
		Alanine	8.1
		Cysteine	0.7
		Valine	6.5
		Methionine	0.7
		Isoleucine	4.1
		Leucine	7.0
		Tyrosine	1.2
		Phenylalanine	8.1
		Histidine	1.8
		Lysine	4.3
		Arginine	2.3
Total sugar content	62.2	Total protein content	37.8

*Calculated on the basis of total sugar and protein.

glucose (78.6%) as observed in the present studies (endo-polymer). Most anti-complementary polysaccharides generally obtained from mushrooms are composed of glycopeptide or proteoglycan (Moon *et al.*, 2002), and contain arabinose and galactose as a component sugar together with a significant amount of glucose (Yamada *et al.*, 1990). Our results are complementary with the findings of Yamada *et al.* (1985), who also showed the involvement of the carbohydrate moiety in executing anti-complementary activity.

Song *et al.* (1998) while working with endo-polymers of various mushrooms reported that the polymer could be of glycopeptides nature, consisting of sugar and amino acids. Many mushroom proteins have been reported to activate lymphocytes and to stimulate cell proliferation and cytokine secretion *in vitro* (Wang *et al.*, 1998), while some mushroom proteins are known to activate immune cells and inhibit the growth of implanted tumor cell *in vivo* (Wang *et al.*, 1996). Song *et al.* (1998) reported a polysaccharide from the mycelia of *L edodes*, which stimulated the polyclonal antibody production *in vitro*. The protein-bound polysaccharide produced from mycelia of *C. versicolor* is characterized as an agent capable of modifying the host biological response by stimulating the immune system and thereby augmenting various therapeutic effects (Yang *et al.*, 1987). Accordingly, mushroom proteins may affect the host's immune system, and are therefore thought to have potential in anti-complementary activity. Therefore, it can be suggested that carbohydrate and protein moieties in polymer contribute to the expression of anti-complementary activity. In this context, anti-complementary biopolymers from PN and GF are required further purification and a more detailed study on the structure and its property.

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