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Production of Anti-Complementary Exopolysaccharides from Submerged Culture of *Flammulina velutipes*

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Summary

Seven species of basidiomycetes have been investigated for anti-complementary activity in hot water extracts and ethanol soluble fractions. Since *Flammulina velutipes* had the most potent activity, culture conditions for its mycelial growth were optimized to increase the production efficiency of anti-complementary exopolysaccharides. The optimal medium composition was (in g/L): galactose 15, sodium nitrate 5, glutamic acid 3, KH_2PO_4 2.5 and $MgSO_4 \cdot 7H_2O$ 0.6. Optimal production of anti-complementary activity was achieved at pH=3.5–5.5 and 25 °C. With these optimal medium and culture conditions, mycelial dry mass was maximized at 3.17 mg/mL after 6 days of cultivation in a 5-liter stirred-tank bioreactor, without pH control. The anti-complementary activity of exopolysaccharides increased sharply after 4 days of cultivation, and showed a high level at 5–6 days of cultivation. A periodate-labile carbohydrate moiety played a leading role in the anti-complementary activity exhibited by exopolysaccharide produced from *F. velutipes*. Results of tests on the anti-complementary activity in the absence of Ca^{2+} and immunoelectrophoresis indicated that the mode of complement activation by exopolysaccharide from *F. velutipes* is *via* both the classical and alternative pathways and that the activation degree is almost the same in each pathway.

Key words: anti-complementary, exopolysaccharides, submerged culture, bioreactor, *Flammulina velutipes*

Introduction

There is much interest in biotechnological methods for the production of polysaccharides for application in food, pharmaceutical, cosmetic and other industries (*1*). In fact, the production of exopolysaccharides in submerged cultures offers unique advantages such as high concentration of relatively pure product, process reproducibility and stable costs, and independence from seasonal variations (*2*). Many of the exopolysaccharides produced by macrofungi have physiological activities (*3*,*4*). Some mushroom polysaccharides, such as lentinan

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(from *Lentinus edodes*), schizophyllan (from *Schizophyllum commune*), and krestin (from *Coriolus versicolor*), are currently commercialized by the pharmaceutical industry (*5–7*). Some have potent antitumor and immunomodulating activities (*8,9*). Historically, hot water extracts (decoctions and essences) from medicinal mushrooms, which contain crude polysaccharides as active ingredients, were used as medicine in the Far East, where knowledge and practice of the medical use of mushrooms primarily originated. Mushrooms such as *Ganoderma lucidum* (reishi), *L. edodes* (shiitake), *Inonotus obliquus* (chaga), *Flammulina velutipes* (enokitake) and many others have been collected and used for hundreds of years in Korea, China, Japan, and eastern Russia.

F. velutipes, which belongs to the family *Tricholomataceae* (Hymenomycetes, Basidiomycota), is an edible mushroom frequently consumed fresh in Korea and Japan. Various proteins, polysaccharides and glycoproteins with antitumor and immunomodulatory activities have been isolated from its fruiting body (*10*). Although there have been many studies of exopolysaccharide production by edible and medicinal mushrooms in submerged culture, the nutritional requirements and environmental conditions necessary for many fungi have not been widely reported (*11–13*), and have received almost no attention in the case of *F. velutipes*.

In the present study, the immunomodulating effect of seven species of basidiomycetes was determined as an anti-complementary activity, and, for the one that showed the highest activity, *Flammulina velutipes*, the submerged culture requirements for production of exopolysaccharides as anti-complementary substances were investigated.

Materials and Methods

Microorganisms

Lentinula edodes (KCTC, Korean Collection for Type Culture, 6733), *Coriolus versicolor* (KCTC 16781), *Pleurotus ostreatus* (KCTC 6730), *Armillariella mellea* (KCTC 6742), *Pholiota nameko* (KCTC 26164), *Grifola frondosa* (KCTC 26148) and *Flammulina velutipes* (KCTC 6367) were kindly provided by the National Forestry Cooperative Federation in Seoul (Korea). The strains were maintained on the modified Sabouraud's agar [glucose 4 % (by mass per volume), peptone 1 %, agar 1.8 % and distilled water, initial pH=7.0] slant (*14*) at 5 °C and transferred once every 3 months. Stock cultures were incubated at 25 °C for 15 days, and then stored in a refrigerator before use.

Inoculum preparation and flask culture

The strains of the seven species of basidiomycetes were initially grown on Sabouraud's medium in a Petri dish for 10 days and transferred to a seed culture medium by punching out two 5-mm diameter pieces of the agar plate culture with a sterilized puncher. The seed culture was grown in a 250-mL flask containing 100 mL of basal medium (mannitol 1.5 g, ammonium nitrate 0.5 g, glutamic acid 0.3 g, KH_2PO_4 0.25 g, $MgSO_4{\cdot}7H_2O$ 0.06 g and distilled water, initial pH=6.5) at 25 °C on a rotary shaker incubator at 150 rev/min for 5 days. In flask culture experiments, 500-mL flasks were used containing 150 mL of medium after inoculation with 4 % (by volume) of the seed culture.

Anti-complementary activity

The mycelial-free spent culture broths of the seven basidiomycetes species were screened for the production of exopolysaccharides with anti-complementary activity. The exopolysaccharide (EPS) was precipitated by the addition of four volumes of cold ethanol to culture broths. The precipitate was then dissolved in water and dialyzed using Spectra/Por 2 (MWCO 12 000–14 000, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The non-dialyzable portion was finally lyophilized to give the exopolysaccharide. The activity was measured by the complement fixation test based on complement consumption and degree of red blood cell lysis by the residual complement (*15*). Normal human serum (NHS) was obtained from a healthy adult. A 50-μL aliquot of exopolysaccharide (1000 μg/mL) was mixed with equal volumes of NHS and GVB (gelatin veronal buffered saline, pH=7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+}. 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 erythrocytes (EA cell) at 1×10^8 cell/mL. NHS was incubated with water and GVB to provide a control. The anti-complementary activity of exopolysaccharide was expressed as the percentage inhibition of the control TCH_{50}:

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

Culture conditions for mycelial growth and EPS production

The following factors were investigated using the one-factor-at-a-time method: medium carbon and nitrogen sources, temperature, and initial pH value. The experiments were conducted in 500-mL flasks on a rotary shaker incubator as described before. To select the optimal carbon source, the anti-complementary activity was determined after 5 days of incubation in the basal medium containing 15 g/L of one of the following carbohydrates: glucose, fructose, carboxyl methyl cellulose (CMC), galactose, lactose, mannitol, starch or sucrose. The effect of nitrogen source on the production of anti-complementary EPS was investigated in the basal medium containing 15 g/L of the carbon source that had shown the best result in the previous experiment and 5 g/L of one of the following nitrogen sources: ammonium chloride, ammonium sulfate, ammonium nitrate, ammonium phosphate, sodium nitrate, potassium nitrate, sodium nitrite or potassium nitrite. The effects of pH and temperature were investigated using the best carbon and nitrogen sources.

Time profile in the bioreactor under optimized conditions

The optimal carbon and nitrogen sources, initial pH and temperature were put together to determine the time profile of the mycelial growth and anti-complementary activity of the EPS produced by *F. velutipes*. The experiment was carried out on a 5-liter stirred tank

bioreactor (KoBioTech Co., Seoul, Korea), under the following additional conditions: no pH control, aeration rate of 2 vvm, agitation speed of 150 rev/min and working volume 3 L. The medium was inoculated with seed culture at 4 % (by volume).

Preparation of EPS and measurement of mycelium mass

Samples collected from shake flasks at various intervals were centrifuged at 8000×*g* for 20 min, and the supernatant was filtered through a membrane filter (0.45 μm, Millipore). The resulting culture filtrate was mixed with a four-fold volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated EPS was collected by centrifuging at 8000×*g* for 20 min (*11*) and the crude exopolysaccharide was lyophilized and weighed. The dry mass of the mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying overnight at 70 °C to constant mass.

Chemical and enzymatic treatments of EPS

These procedures were performed according to the method of Yamada *et al.* (*16*). Briefly, for periodate oxidation, 50 mg of exopolysaccharide produced from *F. velutipes* under the optimum culture conditions were stirred in 50 mM acetate buffer (pH=4.5) containing 25 mM $NaIO_4$ (40 mL) at 4 °C for 4 days in the dark. The oxidized products were reduced with $NaBH_4$ and dialyzed to obtain the periodate-oxidized exopolysaccharide. In addition, EPS (20 mg) was digested with pronase (30 mg, Sigma) in 50 mM Tris-HCl buffer (pH=7.5) containing 10 mM $CaCl_2$ (20 mL) at 37 °C for 2 days. The reaction was terminated by heating at 100 °C for 20 min and then the mixture was centrifuged, dialyzed and lyophilized to obtain the pronase-digested EPS.

Crossed immunoelectrophoresis

Normal human serum was incubated with an equal volume of one of the following three solutions: (*i*) EPS and GVB (EPS-GVB), (*ii*) 10 mM EGTA (ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid) solution containing 2 mM $MgCl_2$ in GVB (Mg^{2+}-EGTA-GVB), or (*iii*) 10 mM EDTA solution in GVB (EDTA-GVB). Incubation took place at 37 °C for 30 min. The serum was then subjected to crossed immunoelectrophoresis to observe the C3 cleavage products (*17*). Shortly after the first run in barbital buffer (pH=8.6; ionic strength, 0.025 with 1 % agarose), the second run was performed in a gel plate (layer thickness 1.5 mm) containing 0.5 % of anti-human C3 serum (Sigma) at a potential gradient of 1 mA/cm for 15 h. After electrophoresis, the plate was fixed and stained with bromophenol blue.

Statistical analysis

Experiments were performed in triplicate and the data were obtained by variance analysis. Differences of $p<0.05$ were considered statistically significant.

Results

Anti-complementary activities of seven basidiomycetes species

Anti-complementary activities greater than 40 % were obtained with the hot water extracts of *P. ostreatus* and *F. velutipes* and the ethanol soluble fractions were obtained from these two species and also from *L. edodes*, *C. versicolor* and *P. nameko* (Table 1). The ethanol soluble fraction obtained from *C. versicolor* had 50 % anti-complementary activity, while its hot water extract had about 30 %. Notably, *F. velutipes* had the most potent anti-complementary activity in both the hot water extract and the ethanol soluble fraction. Therefore, this species was chosen for a further study.

Table 1. Anti-complementary activities of hot water and ethanol extracts of exopolysaccharides produced by seven basidiomycetes species

Basidiomycetes	Inhibition of TCH_{50}/%	
	Hot water extract	Ethanol soluble fraction
Lentinula edodes	31.3	44.3
Coriolus versicolor	29.3	50.0
Pleurotus ostreatus	41.4	44.4
Armillariella mellea	37.1	32.3
Pholiota nameko	32.3	43.2
Grifola frondosa	24.7	30.2
Flammulina velutipes	47.1	57.5

Culture conditions for mycelial growth and EPS production by F. velutipes

The performances of the different carbon sources (Fig. 1) were as follows: (*a*) for mycelial growth: fructose > mannitol > starch > sucrose = glucose > galactose > CMC > lactose; and (*b*) for the production of anti-complementary exopolysaccharides: galactose > lactose > fructose > glucose > mannitol > sucrose > starch > CMC. These results are consistent with our previous study, in which monosaccharides were better carbon sources for mycelial growth than disaccharides or polysaccharides (*18*), except for the good growth on starch obtained in the present study. On the other hand, the addition of galactose and lactose led to significantly high levels of anti-complementary activity.

Since galactose gave the EPS preparation with the best anti-complementary activity, it was chosen as the carbon source for the subsequent experiments. In the nitrogen source experiment (Fig. 2), sodium nitrate and potassium nitrate showed similar effect on mycelial growth (2.81 and 2.79 mg/mL, respectively), but differed very much concerning the production of anti-complementary EPS (76.3 and 38.7 %, respectively). In both experiments, glutamic acid (3 g/L) was used as a component of the basal medium because, as previously reported (*18*), this amino acid was the most effective for mycelial growth compared to 14 other amino acids (*18*).

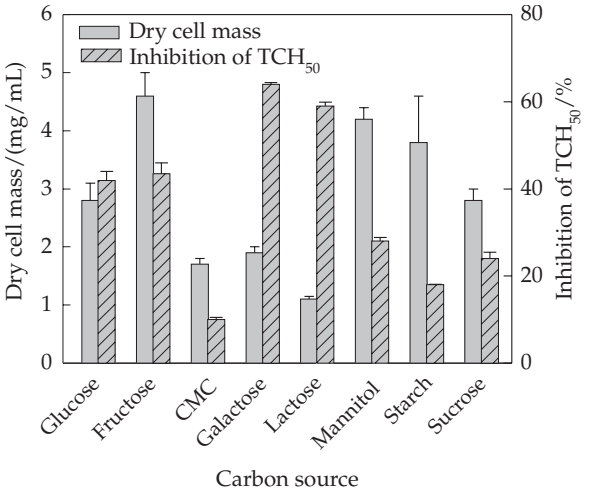

Fig. 1. Effect of carbon sources on the mycelial growth and anti-complementary activity of the EPS produced by *F. velutipes* in 500-mL flasks containing 150 mL of medium (in g/L: carbon source 15, ammonium nitrate 5, glutamic acid 3, KH_2PO_4 2.5, and $MgSO_4{\cdot}7H_2O$ 0.6; with initial pH= 6.5). The experiment was carried out on a rotary shaker incubator at 150 rev/min, 25 °C, for 5 days. All points represent the mean ± S.E. of triplicates

Manipulation of the other components in the basal medium did not increase the anti-complementary activity significantly (data not shown). Therefore, the optimal medium composition for the production of anti-complementary EPS by *F. velutipes* was defined as (in g/L): galactose 15, sodium nitrate 5, glutamic acid 3, KH_2PO_4 2.5 and $MgSO_4{\cdot}7H_2O$ 0.6.

The optimum initial pH for mycelial growth ranged from pH=4.5 to 6.5, while the anti-complementary activities of the EPS produced at the initial pH values from 3.5–5.5 were higher than those at pH=6.0–7.5, with maximum activity of 89.8 % being achieved at pH=5.5 (Fig. 3A). The optimal temperature for mycelial growth was 25–30 °C, whereas the anti-complementary activity of the EPS produced at 25 °C (89.8 %) was significantly higher than that at 30 °C (53.2 %) (Fig. 3B).

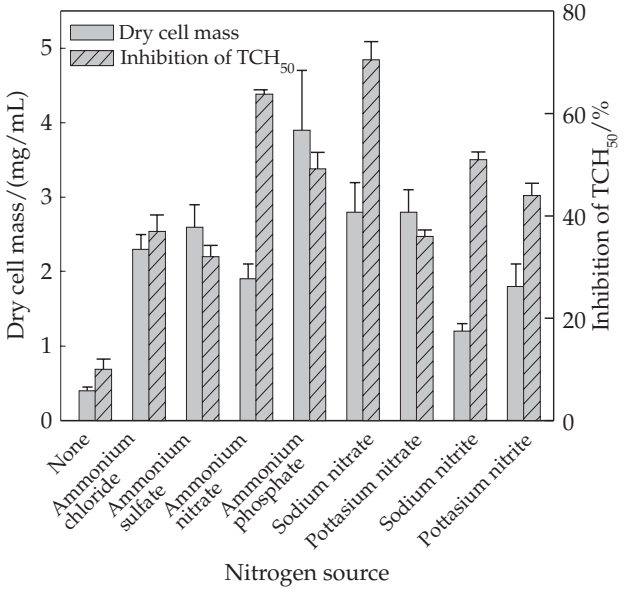

Fig. 2. Effect of nitrogen sources on the mycelial growth and anti-complementary activity of the EPS produced by *F. velutipes* in 500-mL flasks containing 150 mL of medium (in g/L: galactose 15, nitrogen source 5, glutamic acid 3, KH_2PO_4 2.5 and $MgSO_4{\cdot}7H_2O$ 0.6; initial pH=6.5). The experiment was carried out on a rotary shaker incubator at 150 rev/min, 25 °C, for 5 days. All points represent the mean ± S.E. of triplicates

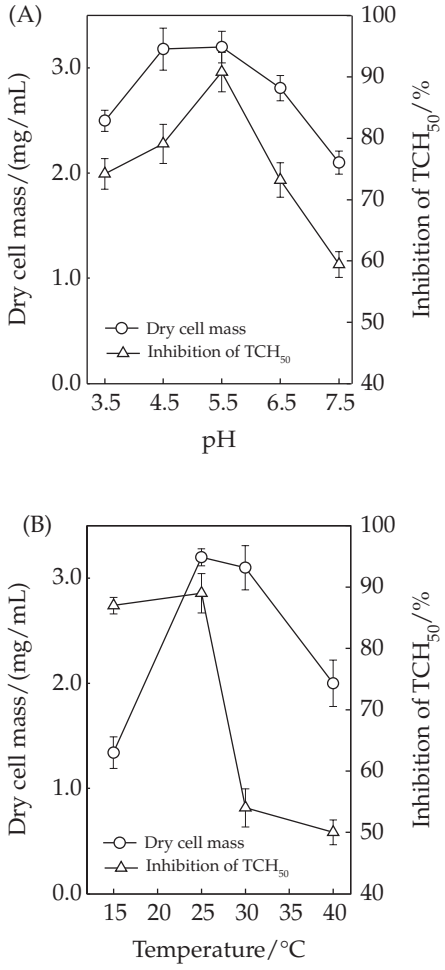

Fig. 3. Effect of initial pH (A) and temperature (B) on the mycelial growth and anti-complementary activity of the EPS produced by *F. velutipes* in 500-mL flasks containing 150 mL of medium with optimal carbon and nitrogen sources (galactose and sodium nitrate, respectively), with initial pH=3.5–7.5, at 25 °C (for the pH effect experiment) and initial pH=6.5, 15–40 °C (for the temperature effect experiment). The experiments were carried out on a rotary shaker incubator at 150 rev/min for 5 days. All points represent the mean ± S.E. of triplicates

Time profile in a 5-liter bioreactor under optimized conditions

A typical time course of mycelial growth and anti-complementary activity of EPS under optimized conditions in a 5-liter stirred-tank bioreactor is shown in Fig. 4. Mycelial dry mass reached a maximum of 3.17 mg/mL after 6 days, although there were no significant differences in mycelial dry mass after day 4 ($p>0.05$). After 4 days of cultivation, the anti-complementary activity of EPS increased sharply, and reached its highest level at

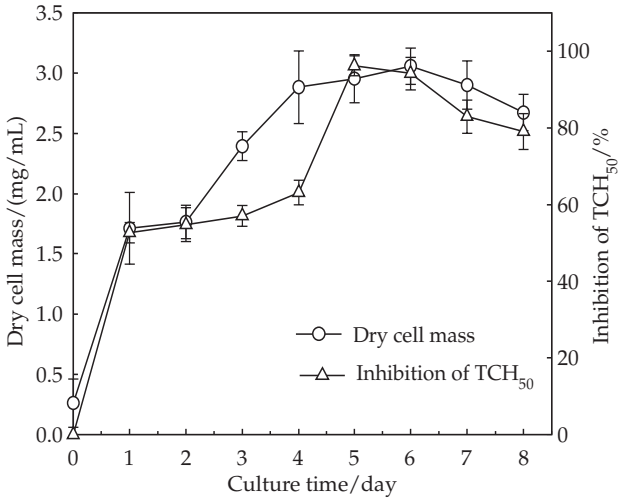

Fig. 4. Time profile of mycelial growth and anti-complementary activity of the EPS produced by *F. velutipes* in a 5-liter stirred--tank bioreactor under the optimized culture conditions (basal medium with galactose as carbon source, sodium nitrate as nitrogen source, pH=5.5, 25 °C). The fermentation medium was inoculated with 4 % (by volume) of the seed culture. Aeration rate 2 vvm, agitation speed 150 rev/min, and working volume 3 L

5–6 days (the difference between days 5 and 6 was not significant, $p>0.05$). Therefore, the maximum anti-complementary activity obtained in submerged culture of *F. velutipes* seems to occur in the late trophophase.

Characterization of EPS

The EPS produced by *F. velutipes* under the optimum culture conditions contained mainly neutral sugar (85.2 %) and protein (4.8 %). Therefore, this EPS was digested with pronase and oxidized with sodium periodate, and its anti-complementary activity was checked to elucidate the active moiety. Whereas pronase digestion had little effect, $NaIO_4$ oxidation of the EPS decreased the anti-complementary activity by about 50 % at 1000 μg/mL (Fig. 5), indicating that the periodate-labile carbohydrate moiety plays a leading role in the anti-complementary activity of exopolysaccharide produced by *F. velutipes*.

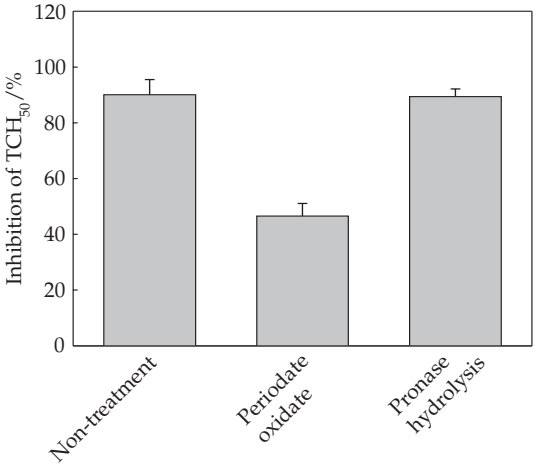

Fig. 5. Changes in anti-complementary activity of the EPS produced by *F. velutipes* after periodate oxidation or pronase digestion. The initial concentration of each sample was 1000 μg/mL. All points represent the mean ± S.E. of triplicates

Identification of C3 activation products

When NHS is incubated with some complement regulators and the remaining complement titer is measured with EA cells, the inhibition of hemolysis by residual complement components represents the anti-complementary activity (*16*). Therefore, the meaning of anti--complementary activity includes both activation and inhibition of the complement system. Crossed immunoelectrophoresis was used to clarify whether or not EPS from *F. velutipes* activates the complement system through C3 activation (C3 activation is the main process of complement activation in sera). When the sera incubated with EPS-GVB were applied to crossed immunoelectrophoresis, two precipitin arcs were observed, with the second arc from the well being higher than the first (Fig. 6). Activation of the complement pathway induces the main complement component C3 to cleave into fragments C3a and C3b. C3 in its inactive state has a β_{1C} electrophoretic mobility (first arc from the well) (Fig. 6), which is converted to a β_{1A} mobility (second arc from the well) after activation. It is, therefore, possible to show the disappearance of the first arc and the appearance of the second (*19*). The C3 activation products, C3a and C3b, were observed as the second arc due to their similar mobility and shared antigenic determinants. These results indicate that exopolysaccharide from *F. velutipes* participated in the complement activation system as an activator. On the other hand, the active polysaccharide incubated in Mg^{2+}-EGTA-GVB (alternative pathway) showed a small second arc in comparison with EPS-GVB (Fig. 6), which indicates that exopolysaccharide from *F. velutipes* could partly activate the complement system through the alternative pathway.

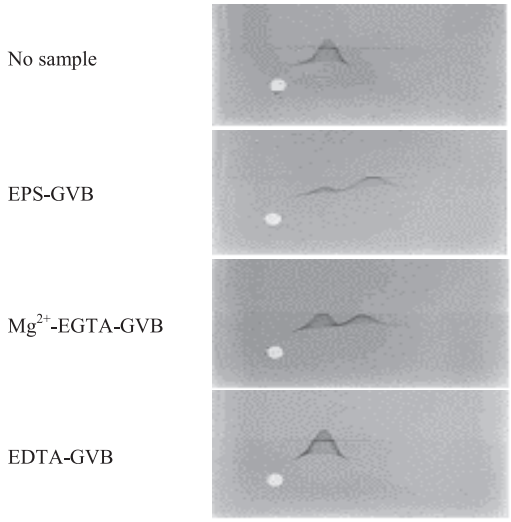

Fig. 6. Crossed immunoelectrophoretic patterns of C3 converted by the EPS produced by *F. velutipes.* Normal human sera were incubated with EPS-GVB, Mg^{2+}-EGTA-GVB or EDTA-GVB at 37 °C for 30 min. The sera were then subjected to immunoelectrophoresis using anti-human C3 antibody to locate the C3 cleavage products. The anode is to the right

Effect of calcium and magnesium ions on complement activation by EPS

Since Ca^{2+} is required for complement activation *via* the classical, but not the alternative, pathway (*20*), the activation through the alternative pathway can be measured using Ca^{2+}-depleted NHS in the presence of EGTA. The complement-activating EPS from *F. velutipes* was incubated with NHS under a Ca^{2+}-free condition (Mg^{2+}-EGTA-GVB) and under a divalent ion-free condition (EDTA-GVB). Under the Ca^{2+}-free condition, the activity decreased by about 50 % compared to the EPS--GVB condition. On the other hand, the activity mostly disappeared in the absence of Mg^{2+} and Ca^{2+} (Fig. 7). The results of these tests on the anti-complementary activity in the absence of Ca^{2+} and of immunoelectrophoresis indicate that the mode of complement activation by EPS from *F. velutipes* is *via* both the classical and alternative pathways, and that the activation degree is almost the same in each pathway.

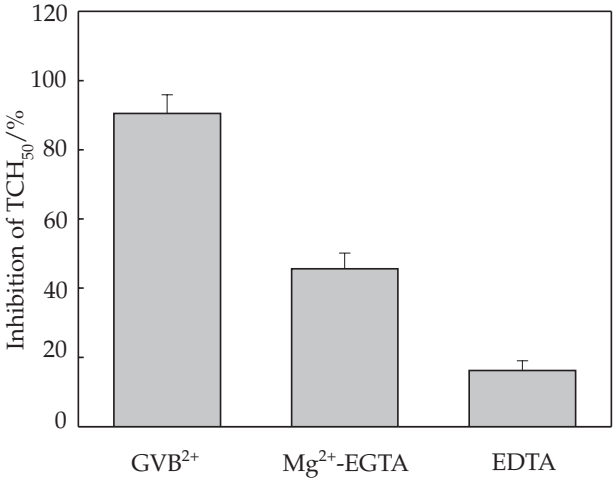

Fig. 7. Effect of calcium and magnesium ions on the anti-complementary activity of the EPS produced by *F. velutipes*. Normal human sera were incubated with EPS-GVB, Mg^{2+}-EGTA-GVB or EDTA-GVB at 37 °C for 30 min. The residual hemolytic complement (TCH_{50}) was determined by using an EA cell. All points represent the mean ± S.E. of triplicates

Discussion

Recent studies on the macrofungus *F. velutipes* have demonstrated many interesting biological activities, including anti-tumor, hypoglycemic, and immunostimulating activities (*21*,*22*). Since the mode of action of mushroom polysaccharides against tumors seems to be through stimulation of the immune system *via* the macrophage and complement systems, the activation of the complement system by such polymers may be closely related to their anti-tumor effects (*23*,*24*). Our results (Table 1) show that EPS produced by *F. velutipes* had a high anti--complementary activity. This contrasts with reports for *Lentinus edodes* (*25*), suggesting the importance of strain selection and culture conditions. *F. velutipes* seems to be a good candidate for the industrial production of substances with anti-complementary activity. With respect to nutrition, inorganic nitrogen sources often lead to relatively low mycelial biomass and EPS yields, in comparison with organic sources, in liquid cultures of higher fungi. In fact, our previous results suggested that proteose peptone was the most useful nitrogen source in submerged cultivation for production of angiotensin-converting enzyme (ACE) inhibitor (*18*). However, for industrial processes, cost considerations mean that inorganic nitrogen sources are often preferred over organic ones. The current work suggests that good production of mycelial biomass and anticomplementary activity can be obtained in a medium containing 5 g/L of sodium nitrate. However, the need for an organic nitrogen source was not completely avoided since it was also necessary to add 3 g/L of glutamic acid. The addition of nitrite led to poor mycelial growth compared to nitrate (Fig. 2). In our previous work, the mycelial growth in the medium containing sucrose and ammonium acetate was not good, but these were the best carbon and nitrogen sources for production of the ACE inhibitor (*18*). These results suggested that good mycelial growth is not necessarily a determining factor for high production yield of polysaccharides.

The morphology of fungal mycelia cultivated with different initial pH values is a critical factor in biomass accumulation and metabolite formation (*26*). The medium pH can affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis (*26*,*27*). In Fig. 3, EPS having the most potent anti-complementary activity was produced under the optimal conditions of initial pH=5.5 and 25 °C. Many kinds of fungi have more acidic optimal pH during submerged culture for EPS production (*11*,*12*). In the case of *Cordyceps sinensis*, the optimal conditions for both mycelial biomass and EPS production were pH=4.0 and 20 °C (*28*). This is comparable with the relatively low optimal temperatures (*e.g.* 20–25 °C) that many kinds of mushrooms have in submerged culture (*11*,*12*).

Submerged cultures of many mushrooms require more than 10 days for maximum formation of biomass and EPS (*29*). In the current work, *F. velutipes* needed only 5 days for maximum production of biomass and anti-complementary activity. Since the maximum level of anti-complementary activity was observed during the late trophophase (Fig. 4), anti-complementary EPS in the submerged culture of *F. velutipes* was the primary product of trophophase metabolism (*30*).

The EPS preparation obtained from *F. velutipes* under the optimum culture conditions contained some protein but was mainly a neutral polysaccharide. The anti--complementary activity resided in the carbohydrate moiety and the activation of the complement system involved both the classical and alternative pathways. This pattern of complement activation by EPS from *F. velutipes* was similar to that of arabinogalactan from *Angelica acutiloba* (*31*), *Zizyphus* arabinan from *Zizyphus jujuba* (*32*), Plantago-mucilage A from *Plantago asiatica* (*33*), and AAFIIb-2 and IIb-3 from *Artemisia princeps* (*34*).

Conclusion

The use of exopolysaccharide from mycelial culture broth as an anti-complementary substance has advantages such as economic mass production, simple extraction procedure, and shorter cultivation time than that required for the production of fruiting bodies. Further study is necessary to develop an effective production process for the anti-complementary agent from *F. velutipes*, to investigate its immunomodulating activity after oral administration, and its use in health-care foods.

Acknowledgments

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References

1. I.W. Sutherland: Extracellular Polysaccharides. In: *Biotechnology, Vol. 6*, H.J. Rhem, G. Reed (Eds.), VCH, Weinheim, Germany (1996) pp. 615–657.
2. I.W. Sutherland, Novel and established application of microbial polysaccharides, *TIBTECH, 16* (1998) 41–46.
3. F. Liu, V.E. Ooi, S.T. Chang, Free radical scavenging activities of mushroom polysaccharide extracts, *Life Sci. 60* (1997) 763–771.
4. H.J. Hwang, S.W Kim, C.P. Xu, J.W. Choi, J.W. Yun, Production and molecular characteristics of four groups of exopolysaccharides from submerged culture of *Phellinus gilvus*, *J. Appl. Microbiol. 94* (2003) 708–719.
5. S. Tsukagoshi, F. Ohashi, Protein-bound polysaccharide preparation, PS-K, effective against mouse sarcoma1 80 and rat ascites hepatoma AH-13 by oral use, *Gann, 65* (1974) 557–558.
6. K. Tabata, W. Itoh, T. Kojima, S. Kawabata, K. Misaki, Ultrasonic degradation of schizophyllan, an antitumor polysaccharide produced by *Schizophyllum commune* FRIES, *Carbohydr. Res. 89* (1981) 121–135.
7. C. Hobbs, Medicinal value of *Lentinus edodes* (Berk.) Sing. (*Agaricomycetideae*). A literature review, *Int. J. Med. Mush. 2* (2000) 287–302.
8. T. Mizuno, Medicinal properties and clinical effects on *Agaricus blazei* Murr., *Int. J. Med. Mush. 4* (2002) 112–125.
9. A.O. Tzianabos, Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biological function, *Clin. Microbiol. Rev. 13* (2000) 523–533.
10. S.P. Wasser, A.L. Weis, Therapeutic effect of substances occurring in higher basidiomycetes mushrooms: A modern perspective, *Crit. Rev. Immunol. 19* (1999) 65–96.
11. J.T. Bae, J. Sinha, J.P. Park, C.H. Song, J.W. Yun, Optimization of submerged culture conditions for exo-biopolymer production by *Paecilomyces japonica*, *J. Microbiol. Biotechnol. 10* (2000) 482–487.
12. J.P. Park, S.W. Kim, H.J. Hwang, J.W. Yun, Optimization of submerged culture conditions for the mycelial growth and exo-biopolymer production by *Cordyceps militaris*, *Lett. Appl. Microbiol. 33* (2001) 76–81.
13. S.W. Kim, H.J. Hwang, C.P. Xu, Y.S. Na, S.K. Song, J.W. Yun, Influence of nutritional conditions on mycelial growth and exopolysaccharide production in *Paecilomyces sinclairii*, *Lett. Appl. Microbiol. 34* (2002) 389–393.
14. C.W. McCoy, R.F. Kanavel, Isolation of *Hirsutella thompsonii* from the citrus rust mite, *Phyllocoptruta oleivora*, and its cultivation on various synthetic media, *J. Invertebr. Pathol. 14* (1969) 386–390.
15. E.A. Kabat, M.M. Mayer: Complement and Complement Fixation. In: *Experimental Immunology*, Charles C. Thomas, Springfield, Illinois, USA (1964) p. 133.
16. H. Yamada, H. Kiyohara, J.C. Cyong, N. Takemoto, Y. Komatsu, H. Kawamura, M. Aburada, E. Hosoya, Fractionation and characterization of mitogenic and anti-complementary active fraction from Kampo (Japanese herbal medicine) Juzen-Taiho-To, *Planta Med. 56* (1990) 386–391.
17. K. Shimura, H. Ito, H. Hibasami, Screening of host-mediated antitumor polysaccharides by crossed immunoelectrophoresis using fresh human serum, *Jpn. J. Pharmacol. 33* (1983) 403–408.
18. J.M. Kim, K.S. Ra, D.O. Noh, H.J. Suh, Optimization of submerged culture conditions for the production of angiotensin converting enzyme inhibitor from *Flammulina velutipes*, *J. Ind. Microbiol. Biotechnol. 29* (2002) 292–295.
19. L. Hudson, F.C. Hay: Two Dimensional or Crossed Immunoelectrophoresis. In: *Practical Immunology*, Blackwell Scientific Publications, Oxford, UK (1989) p. 244.
20. K. Whaley: The Complement System. In: *Complement in Health and Disease*, MTP Press, Lancaster, USA (1986) p. 1.
21. C.H. Song, Y.J. Jeon, B.K. Yang, K.S. Ra, J.M. Sung, The anti-complementary activity of exo-polymers produced from submerged mycelial cultures of higher fungi with particular reference to *Cordyceps militaris*, *J. Microbiol. Biotechnol. 8* (1998) 536–539.
22. D.H. Kim, B.K. Yang, S.C. Jeong, J.B. Park, S.P. Cho, S. Das, J.W. Yun, C.H. Song, Production of a hypoglycemic, extracellular polysaccharide from the submerged culture of the mushroom, *Phellinus linteus, Biotechnol. Lett. 23* (2001) 513–517.
23. V.E. Ooi, F. Liu, Immunomodulation and anti-cancer activity of polysaccharide–protein complexes, *Curr. Med. Chem. 7* (2000) 715–729.
24. K.H. Lee, J.W. Lee, M.D. Han, H. Jeong, Y.I. Kim, D.W. Oh, Correlation between anti-complementary and antitumor activity of the crude polysaccharide from *Ganoderma lucidum* IY009, *Kor. J. Appl. Microbiol. Biotechnol. 22* (1994) 45–51.
25. C.H. Song, Y.J. Jeon, B.K. Yang, K.S. Ra, H.I. Kim, Anti--complementary activity of endo-polymers produced from submerged mycelial culture of higher fungi with particular reference to *Lentinus edodes*, *Biotechnol. Lett. 20* (1998) 741–744.
26. C.H. Shu, M.Y. Lung, Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures, *Process Biochem. 39* (2004) 931–937.
27. S.R. Gerlach, D. Siedenberg, D. Gerlach, K. Schtigerl, M.L.F. Giuseppin, J. Hunik, Influence of reactor systems on the morphology of *Aspergillus awamori*. Application of neural network and cluster analysis for characterization of fungal morphology, *Process Biochem. 33* (1998) 601–615.
28. H.O. Kim, J.W. Yun, A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* in submerged mycelial cultures, *J. Appl. Microbiol. 99* (2005) 728–738.
29. B.C. Lee, J.T. Bae, H.B. Pyo, T.B. Choe, S.W. Kim, H.J. Hwang, J.W. Yun, Biological activities of the polysaccharides produced from submerged culture of the edible Basidiomycete *Grifola frondosa, Enzyme Microb. Technol. 32* (2003) 574–581.
30. J.D. Bu'Lock, D. Hamilton, M.A. Hulme, A.J. Powell, D. Shepherd, H.M. Smalley, G.N. Smith, Metabolic development and secondary biosynthesis in *Penicillium urticae, Can. J. Microbiol. 11* (1965) 765–778.
31. H. Yamada, H. Kiyohara, J.C. Cyong, Y. Otsuka, Studies on polysaccharides from *Angelica acutiloba.* IV. Characterization of an anti-complementary arabinogalactan from the roots of *Angelica acutiloba* KITAGAWA, *Mol. Immunol. 22* (1985) 295–304.

32. H. Yamada, T. Nagai, J.C. Cyong, Y. Otsuka, M. Tomoda, N. Shimizu, K. Shimada, Relationship between chemical structure and anti-complementary activity of plant polysaccharides, *Carbohydr. Res. 144* (1985) 101–112.

33. H. Yamada, T. Nagai, J.C. Cyong, Y. Otsuka, Relationship between chemical structure and activating principle of complement by an acidic polysaccharide, plantago-mucilage A, from the seed of *Plantago asiatica*, *Carbohydr. Res. 156* (1986) 137–145.

34. H. Yamada, K. Ohtani, H. Kiyohara, J.C. Cyong, Y. Otsuka, Y. Ueno, S. Omura, Purification and chemical properties of anti-complementary polysaccharide from the leaves of *Artemisia princeps*, *Planta Med. 52* (1985) 121–125.