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Production and Characterization of Exopolysaccharides from an Enthomopathogenic Fungus *Cordyceps militaris* NG3

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Optimization of the submerged culture conditions for the mycelial growth and production of exopolysaccharide (EPS) from a newly isolated *Cordyceps* species (*C. militaris* NG3) was studied in flask cultures. The optimal temperature and initial pH for EPS production were 30 °C and 8.0, respectively. Sucrose (30 g/L) and corn steep powder (10 g/L) were the most suitable carbon and nitrogen source for both mycelial growth and EPS production. There was a distinguishable morphological changes in mycelium grown between organic and inorganic nitrogen sources. A smooth pellet growth with heavy hyphal thickness was observed in the medium containing organic nitrogen sources, whereas irregular pellets with less hairy region were formed in the medium containing inorganic nitrogen sources. More highly branched cells appearing in the medium of organic nitrogen sources seemed a favorable morphological form for both EPS production and mycelial growth. Under optimal culture conditions, the maximum concentrations of mycelial growth and EPS were 17.6 and 3.4 g/L in a 5-L stirred-tank fermenter. Four groups of EPSs (designated as Fr-I, Fr-II, Fr-III, and Fr-IV) were obtained from the culture filtrates by size exclusion chromatography (SEC), and their molecular characteristics were examined by a multiangle laser-light scattering (MALLS) and refractive index (RI) detector system. The weight-average molar masses of the Fr-I, Fr-II, Fr-III, and Fr-IV of EPS were determined to be 2.262×10^6, 3.348×10^5, 1.049×10^5, and 5.059×10^4 g/mol, respectively. All four EPSs showed very low polydispersity indices ranging from 1.00 to 1.18. The SEC/MALLS analysis revealed that the molecular shape of the Fr-I was a rigid sphere suspected to be an aggregate of complex polysaccharides, the Fr-II and Fr-III were nearly globular in shape, and the Fr-IV was an almost rodlike structure.

Introduction

Cordyceps militaris, an enthomopathogenic fungus that belongs to the class *Ascomycetes*, adheres to the surface of insects during the winter, followed by penetration of its body to form a fruiting body and sporangium (*1*). The fruiting bodies of several *Cordyceps* species have been used as traditional medicines in China, Japan, Korea, and other oriental countries as a result of their various physiological activities (*1*−*3*). These mushrooms are very difficult to collect because they are very small and their growth is restricted to a specific area. Recently mass production of these strains through artificial cultivation has been successfully established, and these mushrooms will be able to be produced on a large scale soon.

Many types of polysaccharides from higher fungi have been reported to have several physiological activities such as anticomplementary activity and antitumor activity (*4*−*9*). Mass production of these strains subsequently allows several *Cordyceps* species to be supplied for public demand and employed as a target to search for new anticancer and immunomodulating drugs (*10*−*13*).

Although many investigators have attempted to obtain optimal submerged culture conditions for exopolysaccharide (EPS) production from several mushrooms, the nutritional requirements and environmental conditions for submerged cultures have not been extensively demonstrated (*14*−*18*). Moreover, submerged culture of *C. militaris* has scarcely been studied even though it is viewed as a promising alternative for effective production of its valuable metabolites, like other mushrooms (*19*−*22*).

It has been reported that the molecular weight, chemical composition, branching mode, and conformation of the mushroom polysaccharides significantly affect their biological activities (*23*, *24*). In this regard, data of detailed molecular characterization are essentially required for elucidating the correlation between physiochemical properties and bioactivities.

The purpose of this study is first to optimize the submerged culture conditions of *C. militaris* NG3 for the production of EPS in shake flask fermentation. Next, the pure EPSs were isolated by gel filtration chromatography, and their molecular features were investigated by a SEC/MALLS system. A major improvement in the field of molecular weight determination is the on-line combination of SEC and MALLS. This approach has successfully been applied to separation and analysis of a variety

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of biopolymers, by which much valuable characterization data could be obtained, including absolute molecular weight, molar mass distribution, molecular dimension, intrinsic viscosity, polydispersity, and degree of branching of the biopolymers (*25*−*27*).

Materials and Methods

Microorganism and Media. A culture of *Cordyceps militaris* NG3 was kindly provided from NongGong Mushrooms Co., (Kyungbuk, Korea). The stock culture was maintained on potato dextrose agar (PDA) slants, which were incubated at 25 °C for 7 days and then stored at 4 °C. The seed culture was grown in a 250-mL flask containing 50 mL of YM medium containing 10 g/L glucose, 5 g/L peptone (Merck), 3 g/L malt extract (Merck), and 3 g/L yeast extract (Merck) at 25 °C on a rotary shaker incubator at 150 rpm for 4 days.

Inoculum Preparation and Flask Cultures. *Cordyceps militaris* NG3 was initially grown on PDA medium in a petri dish and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized self-designed cutter. The seed culture was grown in a 250-mL flask containing 50 mL of basal medium at 25 °C on a rotary shaker incubator at 150 rpm for 4 days. The flask culture experiments were performed in a 250-mL flask containing 50 mL of the media after inoculating with 4% (v/v) of the seed culture.

Fermentation Conditions. The fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated at 28 °C in a 5-L stirred-tank fermenter (KoBioTech Co., Seoul, Korea). Unless otherwise specified, fermentations were conducted under the conditions of temperature 28 °C, aeration rate 2 vvm, agitation speed 150 rpm, initial pH 8.0, and working volume 3 L. The seed culture was transferred to the fermentation medium and was cultivated for 4 days at 150 rpm and 25 °C. All experiments were performed at least in triplicate.

Analytical Methods. Samples collected at various intervals from shake flasks were centrifuged at 10,000 × *g* for 20 min, and the supernatant was filtered through a Whatman no. 2 filter paper (Whatman International Ltd., Maidstone, England). The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously, and left overnight at 4 °C. The precipitated EPS was centrifuged at 10,000 × *g* for 20 min, and the supernatant was discarded. The precipitate of crude EPS was lyophilized, and the weight was estimated. Dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying overnight at 70 °C to a constant weight. The filtrate from a membrane filtration was analyzed by HPLC (Shimadzu Co., Osaka, Japan) using an Aminex HPX-42C column (Bio-Rad Laboratories, Hercules, CA) equipped with a refractive index detector for quantitative analysis of residual sugar concentration.

Measurements of Morphology. The effect of nitrogen sources on morphological changes of mycelium was carefully examined during the submerged cultures and photographs were taken using an Axiolab microscope (ZEISS, Jena, Germany).

Purification of EPS. The ethanol precipitates of the polysaccharide components were dissolved in 0.2 M NaCl buffer to a concentration of 10 g/L and loaded onto a Sepharose CL-4B column (2.4 cm × 100 cm, Sigma Chemical Co., Louis, MO). The column was eluted with the same buffer at a flow rate of 0.6 mL/min. The protein moiety in EPS was monitored at absorbance of 280 nm, and the carbohydrate moiety was monitored at 480 nm. The active fractions of EPS were pooled and lyophilized until further analysis.

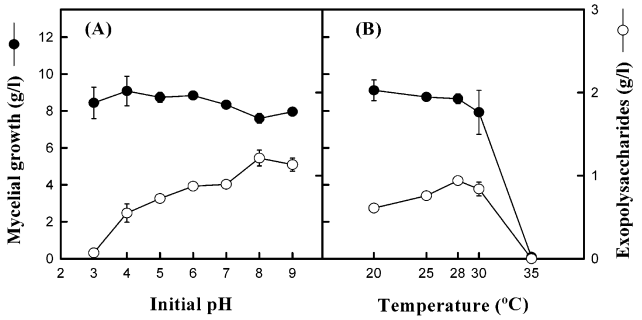

Figure 1. Effect of initial pH (A) and temperature (B) on the mycelial growth (●) and EPS production (○) in *Cordyceps militaris* NG3. All experimental data were mean ± SD of three determinations.

SEC/MALLS Analysis. The molecular weight of the polysaccharides was estimated by SEC coupled with MALLS and RI detectors (Wyatt Technology, Santa Barbara, CA). The EPS samples were dissolved in a phosphate/chloride buffer (ionic strength 0.1, pH 6.8) containing 0.04% diaminotetraacetic acid-disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.22-μm (if necessary, 0.025-μm) filter membranes (Millex HV type, Millipore Corp., Bedford, MA) prior to injection into the SEC/MALLS system (*28*). The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a high performance pump (model 590 Programmable Solvent Delivery Module, Waters Corp., Milford, MA), an injection valve (Rheodyne Inc., Cotati, CA) fitted with a 100-μL loop, and three SEC columns (Shodex PROTEIN KW-802.5, 803, 804, Showa Denko K.K., Tokyo, Japan) were connected in series. The flow rate was 0.8 mL/min, and the injection volume and concentration were 100 μL and 3 mg/mL, respectively. During the calculation of molecular weights of each EPS, the value of d*n*/d*c* (specific refractive index increment) was used according to guide from the Wyatt Technology and data in the literature (*28*) after calibration by reflecting the carbohydrate (67.93%) and protein (32.07%) contents in EPS; accordingly the estimated d*n*/d*c* was 0.1603 mL/g. Data were recorded and processed using Wyatt Technology's ASTRA chromatography software version 4.73.04 for Microsoft windows. Root-mean-squared (RMS) radius of each EPS was determined from the slope by extrapolation of the first-order Debye plot (*29*).

Results and Discussion

Effect of Initial pH and Temperature. The effect of initial pH on mycelial growth and EPS production was studied by growing the fungus on YM medium under different initial pHs (3.0−9.0); the result is shown in Figure 1. The optimal pH for mycelial growth and EPS production was 4.0 and 8.0, respectively. This alkaline pH optimum is quite different compared to other enthomopathogenic fungi such as *Paecilomyces japonica* (pH 5.0) and *C. militaris* (pH 6.0) (*11*, *15*). To find the optimal temperature, *C. militaris* was cultivated at various temperatures ranging from 20 to 35 °C, where the optimum temperatures for mycelial growth and EPS production were found to be 20 and 28 °C, respectively. Park et al. (*19*) have reported that optimal temperature for mycelial growth of another *Cordyceps* species was also at 20 °C.

Table 1. Effect of Carbon Sources on Mycelial Growth and Exopolysaccharide Production in *Cordyceps militaris* NG3[a]

carbon source	mycelial biomass (g/L)	exopolysaccharides (g/L)	final pH
cellobiose	8.8 ± 0.45	0.8 ± 0.11	4.9
dextrose	9.2 ± 0.04	0.7 ± 0.23	5.1
fructose	8.6 ± 0.09	1.1 ± 0.11	5.3
lactose	3.8 ± 0.01	0.8 ± 0.13	7.2
maltose	7.5 ± 0.10	0.6 ± 0.15	5.8
mannitol	9.5 ± 0.31	0.9 ± 0.06	5.2
sorbitol	8.3 ± 0.33	1.1 ± 0.20	5.9
sucrose	9.9 ± 0.20	1.4 ± 0.21	5.3
xylose	6.0 ± 0.38	0.7 ± 0.02	5.4

[a] Fermentations were carried out for 5 d at 28 °C. Values are mean ± SD of three determinations.

Effect of Carbon Source. The influence of carbon sources for EPS production was studied in the medium containing various carbon sources, where each carbon source was added to the basal medium at 10 g/L instead of dextrose (Table 1). When the cells were grown in the sucrose medium, both mycelial growth and EPS production indicated the highest yields. The maximum sucrose concentrations for EPS production and mycelial growth were achieved at 30 and 40 g/L, respectively (Figure 2A). In conjunction with the results reported by other investigators, sucrose is obviously one of good carbon sources for submerged cultures of mushrooms (*19*, *30*).

Effect of Nitrogen Source. To investigate the effect of nitrogen sources on the production of EPS and mycelial growth, 10 different nitrogen sources were examined (Table 2). Although corn steep powder, meat peptone, yeast extract, and soy peptone were favorable for the mycelial growth of *C. militaris* NG3, the maximum EPS production was achieved when corn steep powder (10 g/L) was employed (Figure 2). In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelial growth and EPS production. This result is in good accordance with that obtained by Park et al. (*19*), who observed a high level of mycelial growth in *C. militaris* in media containing corn steep powder in submerged cultures.

Morphological Changes at Different Nitrogen Sources. Medium composition has been recognized as a strong determinant of fungal morphology, where both the type and concentration of the nitrogen source appeared particularly influential (*31*). Figure 3 shows typical morphologies depending on the different nitrogen sources during flask cultures of *C. militaris* NG3. A smooth pellet growth with high hairiness was observed in media with organic nitrogen sources, whereas irregular pellets with less hairy region were formed in media with inorganic nitrogen sources. More highly branched cells appearing in media with organic nitrogen sources seemed a favorable morphological form for both EPS production and mycelial growth. Oh et al. (*32*) reported that the production rate of glucoamylase by *Aspergillus niger* was closely related to the increase in the hyphal thickness. However, Jin et al. (*33*) pointed out that the formation of small compact pellets was preferred under designed cultivation conditions, yielding higher fungal protein synthesis, easier product separation, and better process operation.

Effect of C/N Ratio. Although C/N ratio usually affects the rates of biosynthesis of many metabolites, the influence of C/N ratio on mycelial growth and EPS production in fungi has, however, remained unevaluated until now. The effect of C/N ratio (mass ratio) on EPS production was investigated using the medium with sucrose and corn steep powder. As shown in Table 3, a higher C/N ratio was desired for both mycelial growth and EPS production. Somashekar and Joseph (*34*) also reported that the highest lipid synthesis and biomass of *Rhodotorula gracilis* were observed at high C/N ratio.

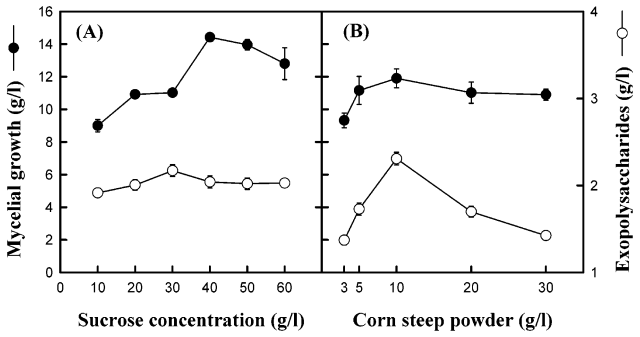

Figure 2. Effect of sucrose concentration (A) and corn steep powder concentration (B) on the mycelial growth (●) and EPSs production (○) in *Cordyceps militaris* NG3. All experimental data were mean ± SD of three determinations.

Table 2. Effect of Nitrogen Sources on Mycelial Growth and Exopolysaccharide Production in *Cordyceps militaris* NG3[a]

nitrogen source	mycelial biomass (g/L)	exopolysaccharides (g/L)	final pH
ammonium citrate	2.2 ± 0.30	0.5 ± 0.02	5.9
ammonium chloride	0.4 ± 0.06	0.3 ± 0.05	7.6
ammonium nitrate	0.5 ± 0.06	0.3 ± 0.04	7.6
ammonium phosphate	2.0 ± 0.14	1.2 ± 0.03	7.1
ammonium sulfate	0.5 ± 0.04	0.3 ± 0.04	5.9
corn steep powder	14.2 ± 1.48	3.1 ± 0.10	6.5
meat peptone	10.2 ± 0.90	2.1 ± 0.44	6.0
polypeptone	7.4 ± 0.68	1.5 ± 0.14	6.2
soypeptone	9.6 ± 0.86	1.7 ± 0.08	5.9
yeast extract	13.0 ± 0.88	2.0 ± 0.10	6.1

[a] Fermentations were carried out for 5 d at 28 °C. Values are mean ± SD of triple determinations.

Effect of Bioelement Supplementation. The effect of different mineral ions on mycelial growth and EPS production in *C. militaris* NG3 is shown in Table 4. The effect of bioelement appeared to have little or no obvious effect on the EPS production. However, maximum mycelial growth was achieved when manganese ion was supplemented into the medium. The importance of bioelements in mushroom fermentation has been demonstrated by Jonathan and Fasidi (*30*). They indicated that calcium, magnesium, and potassium ions also stimulated mycelial growth, whereas copper and zinc (in very low concentration) were required for mycelial propagation of *Psathyerella atroumbonata* cells.

Fermentation Results. Figure 4 shows the time courses of mycelial growth and EPS production by *C. militaris* NG3 in a 5-L stirred-tank fermenter with the basal medium and optimized culture medium. In a basal medium, the EPS concentration reached a maximum level of 1.3 g/L after 7 d, while maximum mycelial concentration was 10.1 g/L after 7 d (Figure 4A). In an optimized culture medium, the EPS production reached 3.4 g/L after 15 d of fermentation, which was 3 times higher than that in a fermentation in basal medium (Figure 4B). However, when the fermentation was carried out with pH control at 8.0, which is an optimal initial pH obtained from flask cultures, both mycelial growth and EPS production indicated lower values than those in uncontrolled pH (data not shown). This probably resulted from the fact that the initial optimal culture pH is not always coincident with actual optimal pH for both

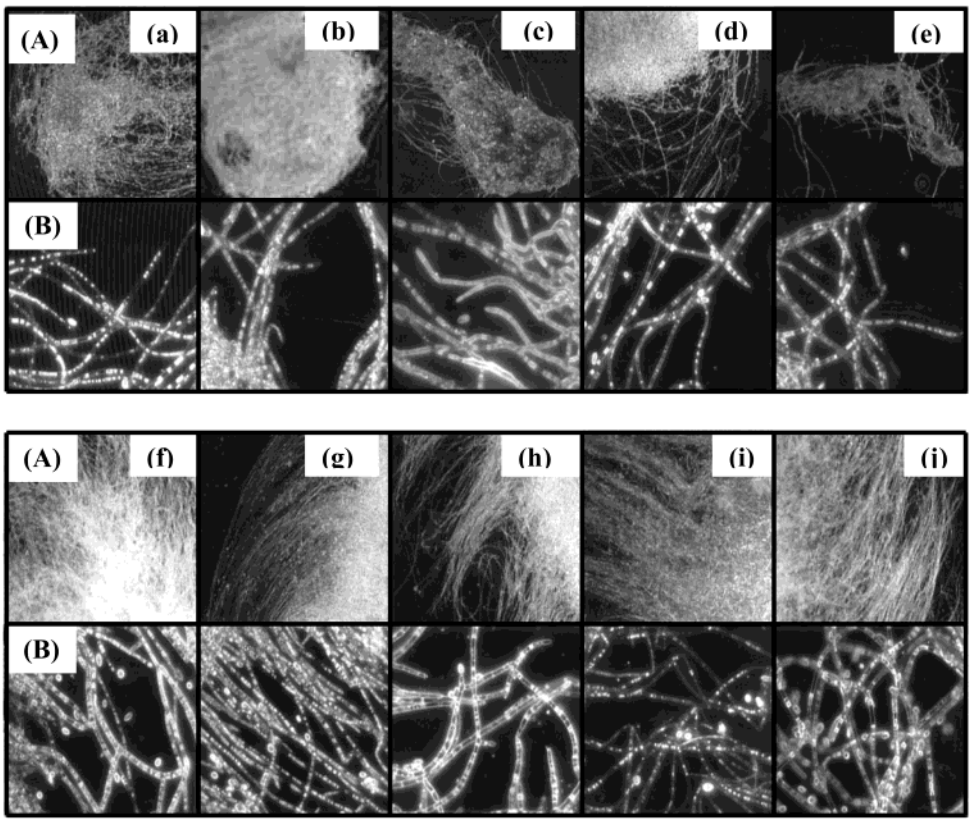

Figure 3. Morpological change of mycelium during submerged culture of *Cordyceps militaris* NG3 under different nitrogen sources: (A) ×100; (B) ×400; (a) ammonium citrate; (b) ammonium chloride; (c) ammonium nitrate; (d) ammonium phosphate; (e) ammonium sulfate; (f) corn steep powder; (g) meat peptone; (h) polypeptone; (i) soypeptone; (j) yeast extract.

Table 3. Effect of C/N Ratio on Mycelial Growth and Exopolysaccharide Production in *Cordyceps militaris* NG3[a]

C/N ratio	mycelial biomass (g/L)	exopolysaccharides (g/L)	final pH
1/1	4.6 ± 0.14	0.9 ± 0.03	7.7
5/1	10.2 ± 0.02	1.4 ± 0.08	6.6
10/1	15.0 ± 1.30	2.3 ± 0.13	6.3
12/1	16.0 ± 1.48	2.4 ± 0.16	6.2
20/1	22.3 ± 1.10	3.8 ± 0.15	6.2

[a] Fermentations were carried out for 5 d at 28 °C. Values are mean ± SD of triple determinations.

Table 4. Effect of Bioelement on Mycelial Growth and Exopolysaccharide Production in *Cordyceps militaris* NG3[a]

bioelement	mycelial biomass (g/L)	exopolysaccharides (g/L)	final pH
control[b]	12.5 ± 0.23	2.0 ± 0.08	6.2
$CaCl_2$	12.3 ± 0.61	2.0 ± 0.17	6.0
$FeSO_4 \cdot 7H_2O$	11.6 ± 1.87	1.1 ± 0.19	6.3
KH_2PO_4	10.5 ± 1.99	2.1 ± 0.21	6.6
K_2HPO_4	11.9 ± 1.16	2.2 ± 0.13	6.7
$MnCl_2 \cdot 6H_2O$	13.9 ± 0.94	1.6 ± 0.10	5.4

[a] Fermentations were carried out for 5 d at 28 °C. Values are mean ± SD of triple determinations. [b] Control means the medium without bioelement supplementation.

mycelial growth and EPS biosynthesis during long-term fermentation.

Absolute Molar Mass of EPSs. A typical chromatogram with MALLS and RI detection is shown in Figure 5A. Four principle peaks appeared at elution volumes between 14 and 29 mL and showed corresponding signals for MALLS and RI. The logarithmic plots of molecular weight of each four EPSs as a function of elution volume were obtained as presented in Figure 5B. The differential refractive index signal, in arbitrary units, is also shown as a solid line in the same figure. For four EPSs, the molecular weights continuously decreased as the elution volume increased in accordance to the SEC mechanism.

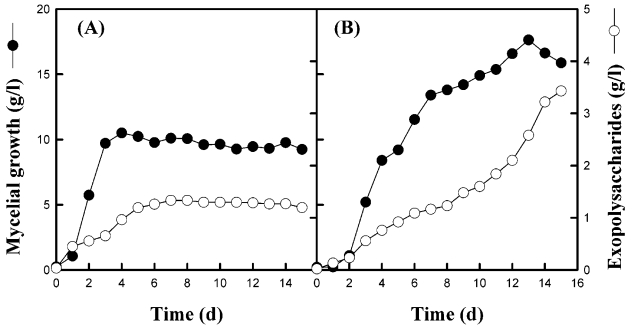

Figure 4. The time profiles of mycelial growth (●) and EPS production (○) during submerged culture of *Cordyceps militaris* NG3 in a 5-L stirred-tank fermenter in basal medium (A) and optimized medium (B) without pH control.

As expected for a broad sample separated by the SEC system, the molecular weight decreased over the elution volume for the larger molecule of Fr-I. On the contrary, the amount of scatter in molecular weight data slightly increased in the later ranges of elution for Fr-II, Fr-III, and Fr-IV. This phenomenon is presumably due to the combination of small sized molecules, decrease in Rayleigh scatter with particle size, and low quantity of material present, making the system too dilute to be measured accurately (*35*). The molecular weights of four EPSs at each retention volume were calculated using the

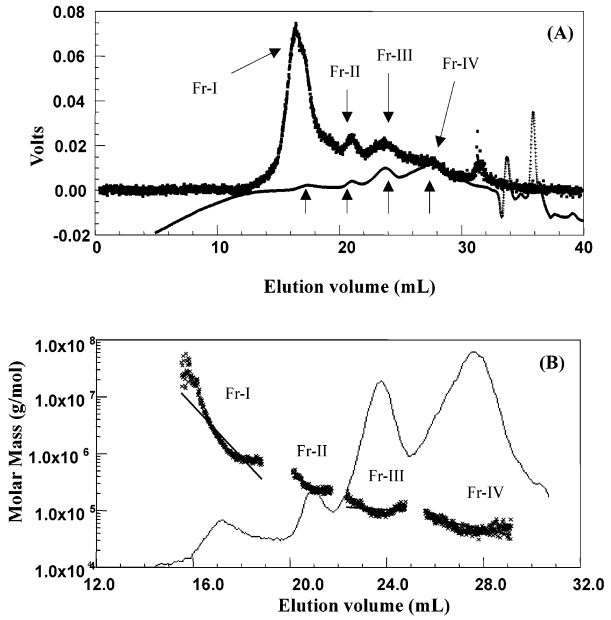

Figure 5. (A) Elution profiles of four groups of exopolysaccharides (Fr-I−Fr-IV) for the determination of molecular mass in SEC/MALLS system. For detailed analysis conditions, see Material and Methods. Thick line (cluster of points) means the elution profile from the MALLS detector, and thin line means the elution profile from the refractive index detector. The dotted curves at elution volumes over 33 mL are baseline noise from the buffer solution. (B) Logarithmic plots of molecular weight of four groups of exopolysaccharides produced from submerged culture of *Cordyceps militaris* NG3 as a function of elution volume. The differential refractive index signal is also shown as a solid line in arbitrary units.

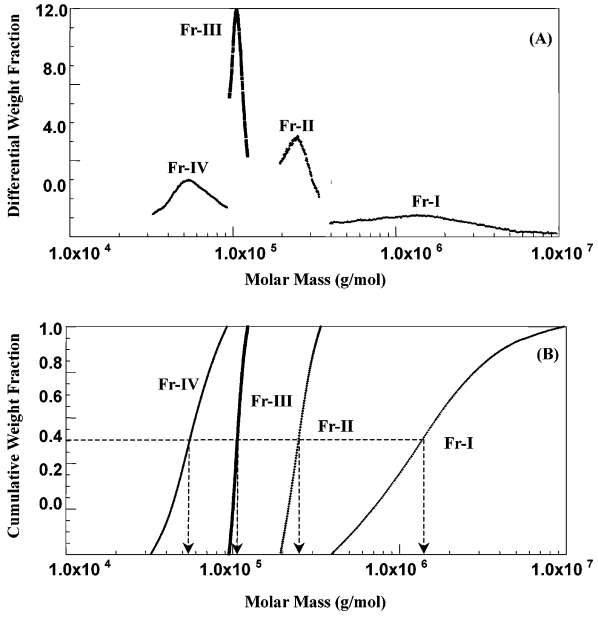

Figure 6. (A) Differential molecular weight distributions of four groups of exopolysaccharides (Fr-I−Fr-IV) produced from submerged culture of *Cordyceps militaris* NG3. (B) Cumulative distribution of molar mass of four groups of exopolysaccharides produced from submerged culture of *Cordyceps militaris* NG3. Dashed lines indicate that 50% of the sample mass is below (and conversely, above) a given molecular weight.

scattering intensities at various angles and the RI values. Eventually, the calculated weight-average molar mass (M_w) in the positions at 17.2 mL (for Fr-I), at 20.5 mL (for Fr-II), at 24.0 mL (for Fr-III), and at 27.3 mL (for Fr-IV) were 2.262×10^6, 3.348×10^5, 1.049×10^5, and 5.059×10^4 g/mol, respectively, within error ranges below 3.0%.

Although there are many reports on polysaccharides extracted from mycelia of *C. militaris*, any useful data about extracellular polysaccharides obtained from culture filtrates of *C. militaris* are scarcely available. The most remarkable difference in the physicochemical factors between EPS and mycelia-extracted polysaccharides from *C. militaris* is the molecular size. Lee et al. (*36*) reported that four groups of polysaccharides from mycelia had molecular weights ranging from 9,400 to 15,000 Da. On the contrary, four fractions of EPSs in the present investigation had much higher molecular weights. Therefore, a comparative study of biological activities between the EPS and mycelia-extracted polysaccharides deserves further investigation.

Although the MALLS system has been widely applied to various biomolecules for their molecular characterization, little literature is currently available concerning application of MALLS to analysis of extracellular polysaccharides produced from liquid-culture of mushrooms (*35*, *37*, *38*). To the best of our knowledge, there is only one report in the literature concerning analysis of polysaccharides of mushroom origin. Zhang et al. (*39*) analyzed many molecular properties of glucan obtained from the fruiting body of *Lentinus edodes* using the MALLS system, including absolute molecular weight, intrinsic viscosity, and molecular conformation.

Molecular Weight Distribution. Figure 6A shows the calculated differential molecular weight distributions for each of the four fractions. This plots show the dependence of the weight fraction versus the molecular mass of EPS. Each step of this distribution plot indicates a fraction of a sample; i.e., the position of the vertical part of the step shows the amount of the material in this fraction. All four EPSs showed highly Gaussian distributions, and the peak molecular weights for the Fr-I, Fr-II, Fr-III, and Fr-IV were 1.7×10^6, 2.6×10^5, 1.0×10^5, and 5.3×10^4 g/mol, respectively. The Fr-I exhibited a wide range of distribution, whereas Fr-III showed the most narrow distribution with a very steep slope. One can speculate from this result that Fr-I is polydisperse, while the other fractions are much less polydisperse, approaching monodisperse behavior, which will be discussed later in terms of polydispersity index.

Figure 6B displays the cumulative molecular weight distribution for the four fractions of EPS. This distribution gives, for each molar mass, the weight fraction of polysaccharides having molecular weight less than the given weight, being approached to zero at low molecular weights and unity at high molecular weights. This plot is particularly useful in determining what molecular weights are contained in the high and low molecular tails of the EPS. The utility of this plot is suggested by the dashed lines in this figure, which indicate that 50% of the EPS mass is below or above a given molecular weight, 1.33×10^6, 2.57×10^5, 1.05×10^5, and 5.37×10^4 g/mol (for Fr-I, Fr-II, Fr-III, and Fr-II, respectively). The combination of the plots of molecular weight and its cumulative distribution as a function of elution volume (Figure 6A and B) provide a complete quantitative characterization of the polysaccharide samples.

Molecular Conformation of EPS. The study of the dependence of RMS radius of gyration on molecular weight can give additional information on the polymer structure. That is, the gross molecular conformations of each EPS in this study can be elucidated from the double

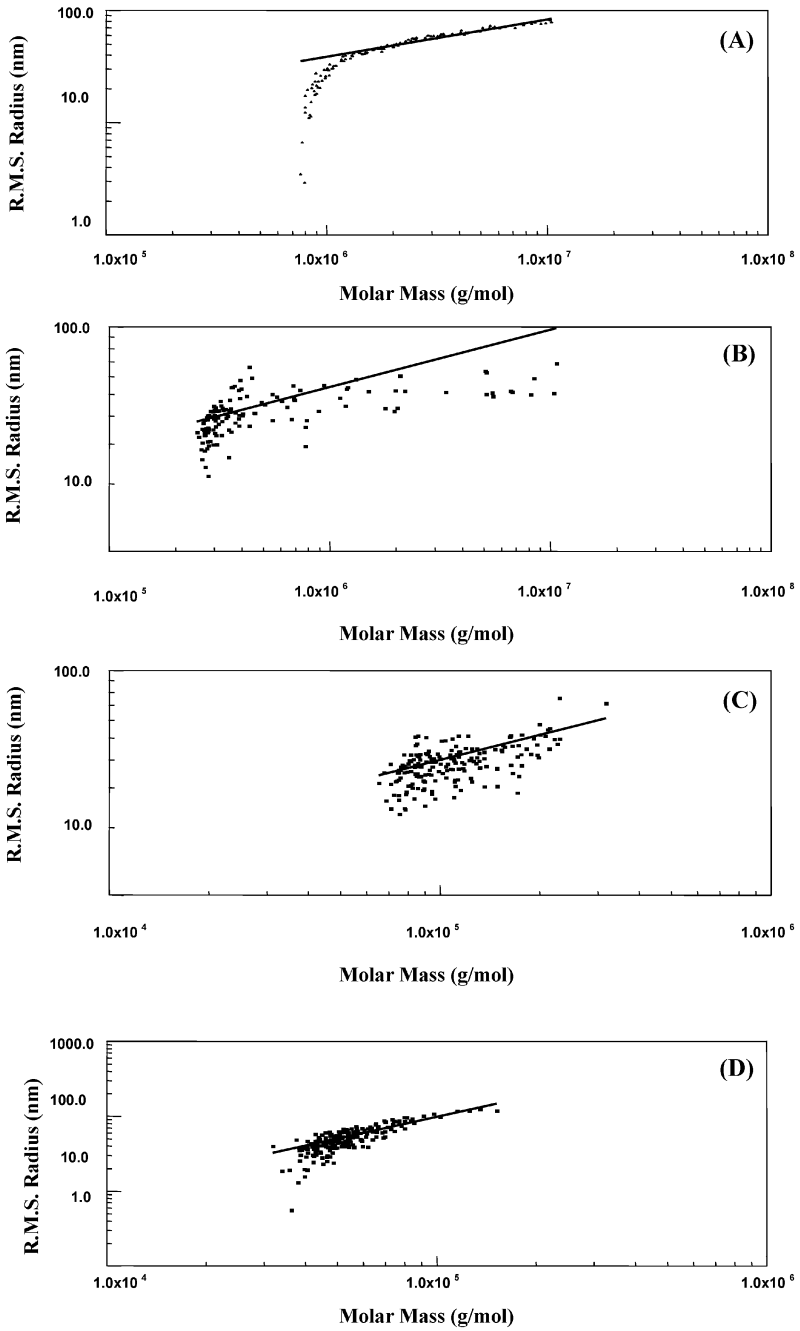

Figure 7. The double logarithmic plots of root-mean-square radius versus molecular mass for four groups of exopolysaccharides (Fr-I−Fr-IV) from culture filtrates of *Cordyceps militaris* NG3. (A) Fr-I, slope = 0.33 (B) Fr-II, slope = 0.26 (C) Fr-III, slope = 0.37 (D) Fr-IV, slope = 0.97.

logarithmic plot of RMS radius of gyration versus molecular mass according to:

$$\log r_i = k + a \log M_i$$

where, r_i is a RMS radius of an EPS molecule, M_i is a molar mass, k is an intercept on the y axis, and a is the slope providing a hint about the conditions of the polymeric chain; values of 0.33 would indicate compact globular structure and 0.5 is obtained for flexible random coil polymers. For rigid rods, their corresponding value of slope is unity (*29*, *40*). It should be noted here that most real coils of biopolymers are frequently slightly more extended, changing the slopes from 0.5 to 0.55−0.6 in a good solvent (*29*, *39*).

Figure 7A−D shows the overall slopes for each EPS in the double logarithmic plots of RMS radius versus molecular weights. The slope of 0.33 obtained from the plot for Fr-I indicates that this EPS exists exactly as a rigid spherical form in an aqueous solution (Figure 7A). Fr-II has the overall slope of 0.26, which implies that the molecule of Fr-II exists in a nearly globular shape (Figure 7B). In contrast, as presented in Figure 7C, the overall slope for Fr-III (0.37) indicates a nearly spherelike structure, which is slightly higher than that was expected for a exact sphere (0.33). In contrast, Fr-IV was almost the same as for a rod, with a slope of 0.97 (Figure 7D). For all fractions of EPSs, the values of RMS radius at the same molecular weight were different. This result indicates that these samples have different branched structures, and thus in EPSs there could be a number of different types of aggregates and complex, which might have very different conformations depending on their M_w.

Table 5. Relevant Molecular Parameters of Four Groups of Exopolysaccharides (Fr-I−Fr-IV) Produced from Submerged Culture of *Cordyceps Militaris* NG3 in MALLS Analysis

parameter[a]	Fr-I (error %)	Fr-II (error %)	Fr-III (error %)	Fr-IV (error %)
M_n (g mol^{-1})	1.914×10^6 (2.8)	3.193×10^5 (3.0)	9.750×10^4 (1.2)	5.004×10^4 (2.0)
M_w(g mol^{-1})	2.262×10^6 (2.6)	3.348×10^5 (3.0)	1.049×10^5 (1.1)	5.008×10^4 (1.9)
M_z (g mol^{-1})	2.561×10^7 (5.0)	3.513×10^5 (7.0)	1.129×10^5 (2.5)	5.011×10^4 (4.0)
M_w/M_n	1.182	1.049	1.076	1.001
R_n (nm)	66.0 (0.4)	42.7 (2.1)	41.1 (1.9)	60.8 (2.3)
R_w (nm)	71.9 (0.4)	43.2 (2.1)	41.8 (1.8)	60.5 (2.3)
R_z (nm)	76.5 (0.3)	43.7 (2.0)	42.6 (1.8)	60.2 (2.3)

[a] M_n, M_w, and M_z refer number-, weight-, and z-average molecular weight, respectively. M_w/M_n represents the polydispersity ratio. R_n, R_w, and R_z refer to number-, weight-, and z-average root-mean-squared radius of gyration, respectively.

It should be noted here that some of the RMS radius data could be close to the lower limit of resolution for the MALLS instrument so that the RMS radius data should be carefully used. This presumably results from the fact that a number of different behaviors present at different parts of the molecular weight distribution spectrum.

There existed a great difference in molecular weights, polydispersity ratio (M_w/M_n), and deviation of three different RMS radii of gyration (R_n, R_w, and R_z) among four EPSs. All polydispersity ratios of EPSs were much closer to unity, suggesting that these EPSs were less polydisperse (mostly monodisperse), unlike other groups of biopolymers (*35*, *38*).

The ratios of M_z/M_w, or more usually M_w/M_n is used by commercial manufacturers of polysaccharides as indices of polydispersity, and these can be related to the standard deviation of the distribution of molecular weights in a preparation, whatever form this takes, either Gaussian or log-normal. This information is important because the functional properties of polysaccharides can be greatly influenced by the molecular weight distribution.

Except that Fr-I had three different RMS radii, the RMS radii for the other three fractions ranged from 41 to 61 nm with similar values but no clear trends (Table 5).

It has been reported that the molecular size, branching ratios, and constituents of EPS were different depending on the growth medium and environments (*40*). Moreover, the structure and molecular mass of EPS has been found to play a critical role in their biological activities (*41*). In this regard, it is worth pointing out that the characterization data for EPS from submerged culture of mushrooms should be obtained for each culture condition.

Conclusions

An enthomopathogenic fungus, *C. militaris* NG3, produced four groups of EPS during submerged cultures, and their molecular characteristics were examined using a SEC/MALLS system. The data presented in this study clearly demonstrate the utility of the SEC/MALLS methodology for determining the absolute molecular weight, molecular weight distribution, polydispersity, and molecular dimensions (shapes) for four groups of exopolysaccharides. In particular, the occurrence of aggregation of the polysaccharides in solution could be recognized by analyzing the information obtained from these characteristic data. In the present investigation, the Fr-I EPS showed evidence of aggregation of complex polysaccharides considering its unusually high molecular weight, broad molar mass distribution, relatively high polydispersity index, and rigid spherelike molecular dimension. It is expected that the MALLS system can be widely applied to analyze the molecular characteristics for polysaccharides produced from either fruiting body or submerged cultures of mushrooms within a markedly short time, thereby obtaining useful relationship between their molecular properties and bioactivities.

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