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Characterization of Exopolysaccharides Produced by Submerged Culture of an Entomopathogenic Fungus *Paecilomyces sinclairii* by Using a Multi-Angle Laser Light Scattering System

KIM, SANG-WOO[1], HYE-JIN HWANG[1], JANG-WON CHOI[2], AND JONG-WON YUN[1]*

[1]*Department of Biotechnology &* [2]*Department of Bioindustry, Daegu University, Kyungsan, Kyungbuk 712-714, Korea*

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Abstract Three groups of exopolysaccharides (EPSs) (designated as Fr-I, Fr-II, and Fr-III) were isolated from the culture filtrates of *Paecilomyces sinclairii* by gel filtration chromatography on Sepharose CL-4B. Their molecular characteristics were examined by multi-angle laser light scattering (MALLS) connected online to a size exclusion chromatography (SEC) and refractive index (RI) detector system. The weight-average molar mass of Fr-I, Fr-II, and Fr-III of EPSs were determined to be 1.540×10^6, 6.302×10^4, and 9.389×10^4 g/mol, respectively. All three EPSs showed a fairly low polydispersity indice, ranging from 1.008 to 1.059 (nearly monodisperse behavior), and showed different carbohydrates and amino acids compositions; all fractions of EPSs consisted of mainly cystine, valine, and arginine in the protein moiety, and mainly ribose, galactose, and glucose in the carbohydrate moiety. The determination of gyration radii of the EPSs in SEC/MALLS analysis revealed the molecular shape of the Fr-I to be a rod-like structure, whereas the Fr-II and Fr-III had a random-coil structure in an aqueous solution.

Key words: Exopolysaccharides, MALLS, fungus, *Paecilomyces sinclairii*

The existing and potential importance of microbial polysaccharides has been focused on fungal polysaccharides as a functional food and a source for pharmaceuticals [6, 12, 25, 27]. Recent studies demonstrated many interesting biological activities of EPSs from fungi, including antitumor, immunostimulating, and hypoglycemic activities [11, 14, 20, 24, 27, 30, 33]. More recently, the nutritional requirement and environmental condition for submerged culture of edible or medicinal mushrooms have been extensively demonstrated [15, 16, 19, 29].

**Corresponding author*
Phone: 82-53-850-6556; Fax: 82-53-850-6559;
E-mail: jwyun@daegu.ac.kr

It has been reported that the molecular weight, conformation, chemical modification, and solubility of the polysaccharides significantly affect their antitumor and immunomodulatory activities [2, 23, 27]. Therefore, it is important to elucidate a correlation between the physicochemical properties and bioactivities of polysaccharides [9, 18]. Knowledge of the molecular properties of bioactive polysaccharides is fundamentally important for further study on their applications.

Paecilomyces species are parasitic on the larvae of various lepidopteran insects (entomopathogenic) and forms characteristic fruiting bodies or synnemata. In our previous paper [16], submerged culture conditions to produce the EPSs from *P. sinclarii* were studied. The aim of the present study was to investigate molecular features of three different polysaccharides produced by *P. sinclairii*.

Materials and Methods

Preparation of Crude EPSs

P. sinclairii, a culture collection of our laboratory, was initially grown on PDA medium in a petri dish, and then transferred into the seed medium by punching out 5 mm of the agar plate culture with a house-developed cutter. The seed culture was conducted in a 250-ml flask containing 50 ml yeast medium (YM) (3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose in 1-l of distilled water) at 25°C on a rotary shaker at 150 rpm for 4 days. The fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated in a medium containing 60 g l^{-1} sucrose and 10 g l^{-1} corn steep powder in a 5-l stirred-tank fermenter (KoBioTech Co., Seoul, Korea). The fermentation was performed under the following conditions: temperature 30°C, aeration rate 2 vvm, agitation speed 150 rpm, initial pH 6.0, working volume 3-l. Fermentation broths were centrifuged at 10,000 ×*g* for

20 min, and the supernatant was filtered through a Whatman filter paper No. 2 (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 EPSs were centrifuged at 10,000 ×*g* for 20 min, discarding the supernatant. The precipitates of crude EPSs were lyophilized before further studies.

Purification of the EPS

The ethanol precipitates of the polysaccharide components were dissolved in 0.2 M NaCl to 10 g/l concentration, followed by loading onto a Sepharose CL-4B column (2.4×100 cm, Sigma Chemical Co., Louis, MO, U.S.A.). The column was eluted with the same solution at a flow rate of 0.6 ml/min. The protein moiety in the EPSs was monitored by absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 480 nm. The active EPSs fractions were pooled and lyophilized for further analysis.

Compositional Analysis of the EPSs

The total carbohydrate content of EPS was determined by the phenol sulfuric acid method using glucose as the standard [8]. The sugar composition was analyzed by gas chromatography (Varian Co., Model: Star 3600CX, Lexington, MA, U.S.A.) with a fused silica capillary column (30 m×0.25 mm, Supelco Inc., Bellefonte, PA, U.S.A.) and flame ionization detector. The total protein was determined by the Bradford method with bovine serum albumin as the standard [3]. The composition of amino acid was analyzed by an amino acid analyzer (Pharmacia Biochrom Ltd., Model: Biochrom 20, Cambridge, U.K.) with a high performance ion-exchange column (4.6×200 mm).

MALLS Instrumentation

The molecular weight of the EPSs was estimated by SEC in conjunction with MALLS and RI detectors (Wyatt Technology, Santa Babara, CA, U.S.A.). The EPS samples were dissolved in 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, U.S.A.) prior to injection into the SEC/MALLS system [13]. The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, U.K.), a high performance pump (Model 590 Programmable Solvent Delivery Module, Waters Corp., Milford, MA, U.S.A.), an injection valve (Rheodyne Inc., Cotati, CA, U.S.A.) fitted with a 150-μl loop, and three SEC columns (Shodex PROTEIN KW-802.5, 803, 804, Showa Denko K.K., Tokyo, Japan) 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 *dn/dc* (specific refractive index increment) was used according to a guide from the Wyatt Technology and data in the literature [13]. After calibration by assuming the carbohydrate (65.67%) and protein contents (34.33%) in crude EPSs, the estimated *dn/dc* was found to be 0.1448 ml/g.

Data Treatment

Data obtained from MALLS and RI detectors were recorded and processed using Wyatt Technology's ASTRA chromatography software version 4.73.04 for Microsoft windows. M_w was calculated by using the following equation (1):

$$K^*c/R(\theta)=1/M_wP(\theta)+2A_2c \quad (1)$$

where $R(\theta)$ is the excess intensity of scattered light at angle θ, c is the sample concentration, M_w is the weight-average molecular weight, and A_2 is the second viral coefficient, which describes the interaction between the solvent and the polymer chain (positive value for a good solvent, whereas negative value for a poor solvent). K* is the optical parameter equal to $4\pi^2(dn/dc)^2n_o^2/(N_A\lambda_o^4)$, where n_o is the solvent RI and dn/dc is the RI increment, N_A is Avogadro's number, and λ_o is the wavelength of the scattered light in a vacuum. Function $P(\theta)$ describes the angular dependence of scattered light. Expansion of $1/P(\theta)$ to first order gives:

$$1/P(\theta)=1+(16\pi^2/3\lambda^2)\langle r_g^2\rangle\sin^2(\theta/2)+f_4\sin^4(\theta/2)+\ldots \quad (2)$$

The curve fitting method in this study was based on a second-order Berry method [4] [$\sqrt{(K^*c/R(\theta)}$ vs. $\sin^2(\theta/2)$], and a second viral coefficient, A_2, was set at zero. M_w and R_z [equal to r_g in Eq. (2)] values were calculated from the intercept and slope of the Eq. (1) by extrapolating multi-angle signals to zero angle, respectively [32].

The study of the dependence of root mean square (RMS) radius of gyration on molecular weight can give additional information on the polymer structure [5, 22, 28]. That is, the gross molecular conformations of each EPS in this study can be elucidated from the double logarithmic plot of RMS radius of gyration *versus* molecular mass according to the following Eq. (3):

$$\text{Log } r_i=k+a \log M_i \quad (3)$$

where r_i is the RMS radius of the EPS molecule, M_i is the molar mass of EPS, k is the intercept in the axis of the RMS radius, and a is the slope for providing a hint about the conditions of the polymeric chain, since 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 known as unity [22, 28].

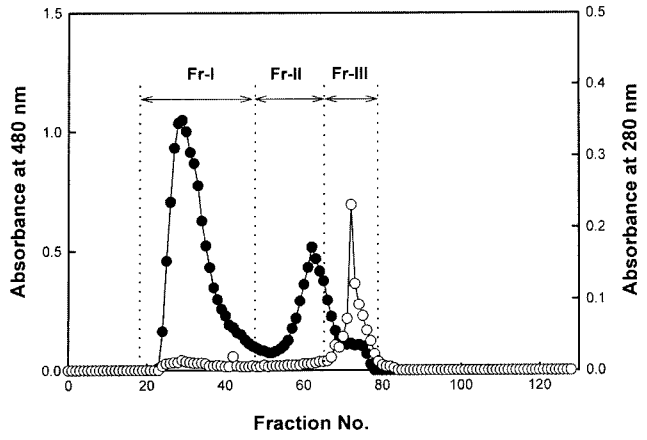

Fig. 1. Elution profiles of the EPSs (Fr-I~III), produced by submerged culture of *Paecilomyces sinclairii*, on Sepharose CL 4B chromatography. Eluates were analyzed by measuring absorbance at 480 nm for carbohydrate (●) and absorbance at 280 nm for protein (○).

RESULTS AND DISCUSSION

Purification of the EPSs

A shown in Fig. 1 on gel filtration chromatography of the culture filtrate on Sepharose CL-4B, three fractions of EPSs (Fr-I, Fr-II, and Fr-III), which consisted of polysaccharides and proteins, were coeluted. Fr-I and Fr-II were found to be polysaccharides containing very little protein, whereas Fr-III was a typical glycoprotein.

Table 1. Amino acid and sugar compositions in three groups of EPSs (Fr-I~III) produced from submerged culture of *Paecilomyces sinclairii*.

	Composition (%)		
	Fr-I	Fr-II	Fr-III
Amino acid			
Threonine	6.8	12.3	nd
Serine	8.5	14.6	nd
Glutamic acid	12.3	nd	nd
Glycine	11.3	13.9	nd
Alanine	9.4	13.1	nd
Cystine	8.7	9.8	28.8
Valine	12.1	5.1	15.6
Methionine	5.2	nd	10.9
Isoleucine	2.6	nd	nd
Tyrosine	3.8	4.9	nd
Lysine	6.8	15.5	nd
Arginine	12.5	10.8	44.7
Sugar			
Rhamnose	nd	nd	13.5
Ribose	77.0	63.0	23.2
Arabinose	nd	nd	15.6
Mannose	8.2	7.5	nd
Galactose	8.3	15.2	19.1
Glucose	6.5	14.3	28.6

[a]nd means not detected.

Sugar and Amino Acid Compositions in the EPSs

The detailed compositions of sugar and amino acid in each purified EPS are shown in Table 1. All fractions of EPSs consisted of mainly cystine, valine, and arginine in the protein moiety, and mainly ribose, galactose, and glucose in the carbohydrate moiety. Kim *et al.* [17] reported that the polymer from ascocaps of *Cordyceps militaris* was composed of glucose (78.6%), galactose (19.1%), and arabinose (2.2%). Adachi *et al.* [2] also suggested that the EPSs isolated from the growing culture of mycelia consisted of mainly glucose and small amounts of galactose and mannose. However, Song *et al.* [24] reported that exopolymer produced from the culture broth of *Cordyceps militaris* consisted of mainly galactose (87.2%) and small amounts of glucose (9.3%) and arabinose (3.5%), while major amino acids were tyrosine (38.3%), valine (9.7%), and lysine (6.7%). Collectively, the constituent carbohydrates and amino acids in polysaccharides of mycelial biomass and/or submerged culture products in basidiomycetes or ascomycetes significantly differ, depending on strains and their culture conditions. This, together with their diversified biological activities, is one of the attractive features of microbial polysaccharides.

Analysis of the EPSs by MALLS

Determination of Absolute Molecular Weight. In order to compare the results of fractionation from Sepharose CL-4B chromatography with those of the MALLS detector, SEC coupled with MALLS and RI detectors was performed, and absolute molecular weights were eventually determined for each EPS. A typical chromatogram with MALLS and RI detection is depicted in Fig. 2, in which the RI

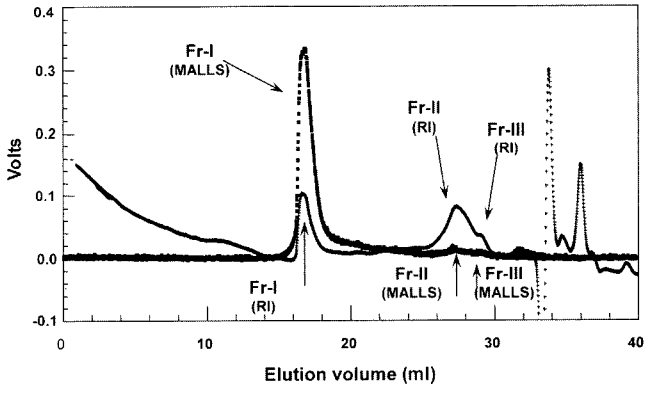

Fig. 2. Elution profiles of EPSs (Fr-I~III) for the determination of molecular mass in a SEC/MALLS system.

For detailed analytical conditions, see Materials and Methods section. The thick line represents the elution profile from the MALLS detector, and the thin line for the elution profile from the refractive index detector. The dotted curves at elution volumes over 33 ml mean baseline noise by buffer solution.

chromatogram was in good accordance with that of gel filtration chromatography on Sepharose CL-4B. Three major peaks were observed between the elution volume of 16–19 ml and 25–30 ml, and showed corresponding signals for MALLS and RI. The molecular mass values for the three fractions eluted were calculated for the portions of peaks that lie within the peak ranges. These ranges were defined by the common detection limit for the MALLS and RI chromatograms in the peak regions. The logarithmic plots of molecular weight of each EPS as a function of elution volume are depicted in Fig. 3. The differential refractive index signal (in arbitrary unit) is also shown in the same figure as a solid line. For all three EPSs, the molecular weights were continuously decreased as the elution volume increased according to the SEC mechanism, even though there were significant amounts of scatter in the molar mass data. The increased scatter in the molar

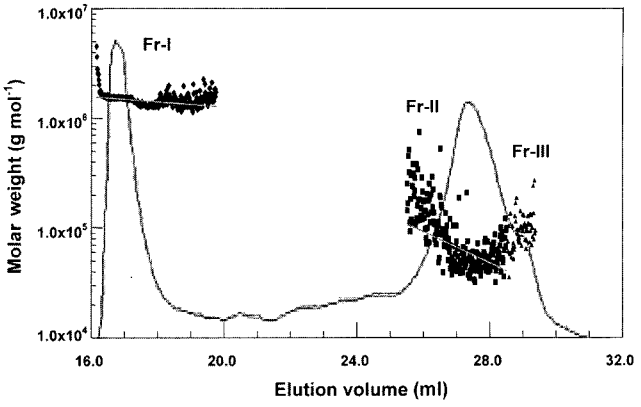

Fig. 3. Logarithmic plots of molecular weight of three groups of EPSs (Fr-I~III) produced from submerged culture of *Paecilomyces sinclairii* as a function of elution volume.
The differential refractive index signal, in arbitrary unit, is also shown as a solid line.

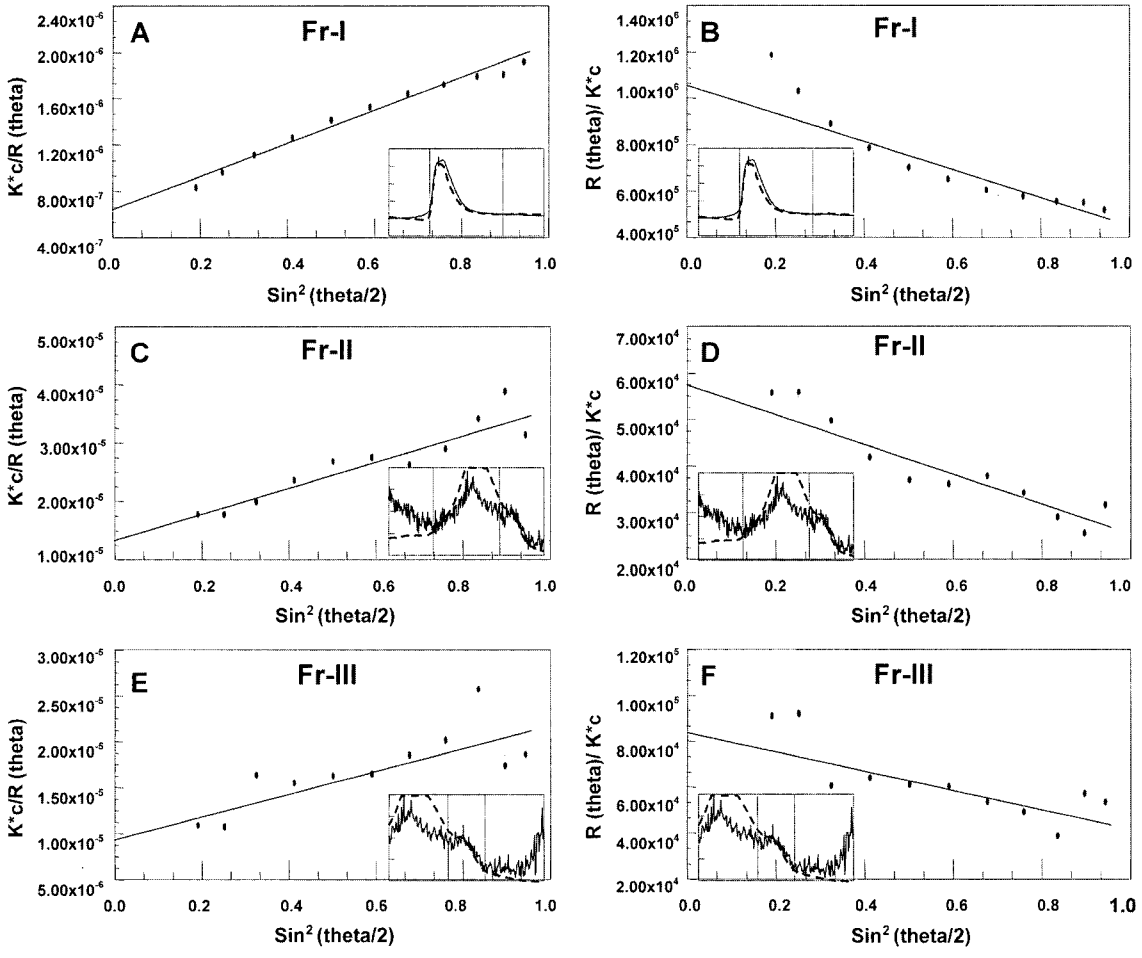

Fig. 4. Angular dependency of light scattered of three groups of EPSs (Fr-I~III) for the same slice of concentration in the Zimm method (panels A, C, and E) and Debye method (panels B, D, and F).
Panels A and B: peak slice: 1,1245; volume: 16.6 ml; concentration: 1.354×10^{-5} g/ml. Panels C and D: peak slice: 1,2049; volume: 27.3 ml; concentration: 1.067×10^{-5} g/ml. Panels E and F: peak slice: 1,2179; volume: 29.0 ml; concentration: 4.563×10^{-6} g/ml. The peak molar mass was obtained by placing the marker on the MALLS trace at a point that coincides with the peak of the RI trace (inner panels for each Figure), where a thick line means the elution profile from the MALLS detector, and a thin line means the elution profile from the refractive index detector.

mass data in the range of elution volume between 28 and 30 ml most likely resulted from the small values of light scattering intensity in the tail of the chromatogram for Fr-III, low quantity of material, and decrease in Rayleigh scatter [26]. Eventually, the weight average molar masses (M_w) of Fr-I, Fr-II, and Fr-III were determined to be 1,540,000 (±13,860), 63,020 (±693), and 93,890 (±1,784) g/mol, respectively. The Fr-I of EPS has an unexpectedly high molecular weight.

Another important factor affecting molecular weight calculation is the data fitting method. There are three different fitting methods, namely Berry [4], Debye [7], and Zimm [34] methods. Yokoyama *et al.* [31] applied these three methods to comparatively analyze M_w and R_z of starch molecules, and reported that the molecular weight calculated from the Zimm method was significantly larger than those calculated by the Debye and Berry methods. Other investigators also suggested that the Zimm method could yield unreasonable results [1], whereas the Berry method was recommended as a more accurate method to determine molecular weight for particularly larger polymer [10]. In the present work, these three fitting methods were compared to determine the M_w and RMS of the three EPSs. As shown in Fig. 4, both the Zimm and Debye plots did not show good curve fittings with rough molecular weight determination for all EPSs. The Debye method uses a fit of $R(\theta)/Kc^*$ vs. $\sin^2(\theta/2)$ for each slice, whereas the Zimm method uses a fit of $Kc^*/R(\theta)$ vs. $\sin^2(\theta/2)$. Then, the desired polynomial order can be adjusted according to the angular dependency of the studied polymer. This is particularly important for large molecular sizes [21]. Figure 4 shows that all fractions were adjusted to determine M_w by these methods. There was a remarkable difference in M_w and R_z between the two methods: For example, M_w and R_z of Fr-I by the Zimm method were 1.558×10^6 g/mol and 106.3 nm, respectively, whereas 1.055×10^6 g/mol and 54.1 nm from the Debye plot. Since the Berry method [plot of $\sqrt{Kc^*/R(\theta)}$ vs. $\sin^2(\theta/2)$] has been demonstrated to determine M_w of larger molecules with greater accuracy [10], the second-order Berry method was applied with laser signals obtained at different angles, as shown in the larger EPS at the peak of the RI signal. As seen clearly in Fig. 5, more accurate curve fittings were obtained for all fractions of EPSs. These results suggest that the Berry method could successfully be applied to determine M_w of all EPSs in liquid solutions.

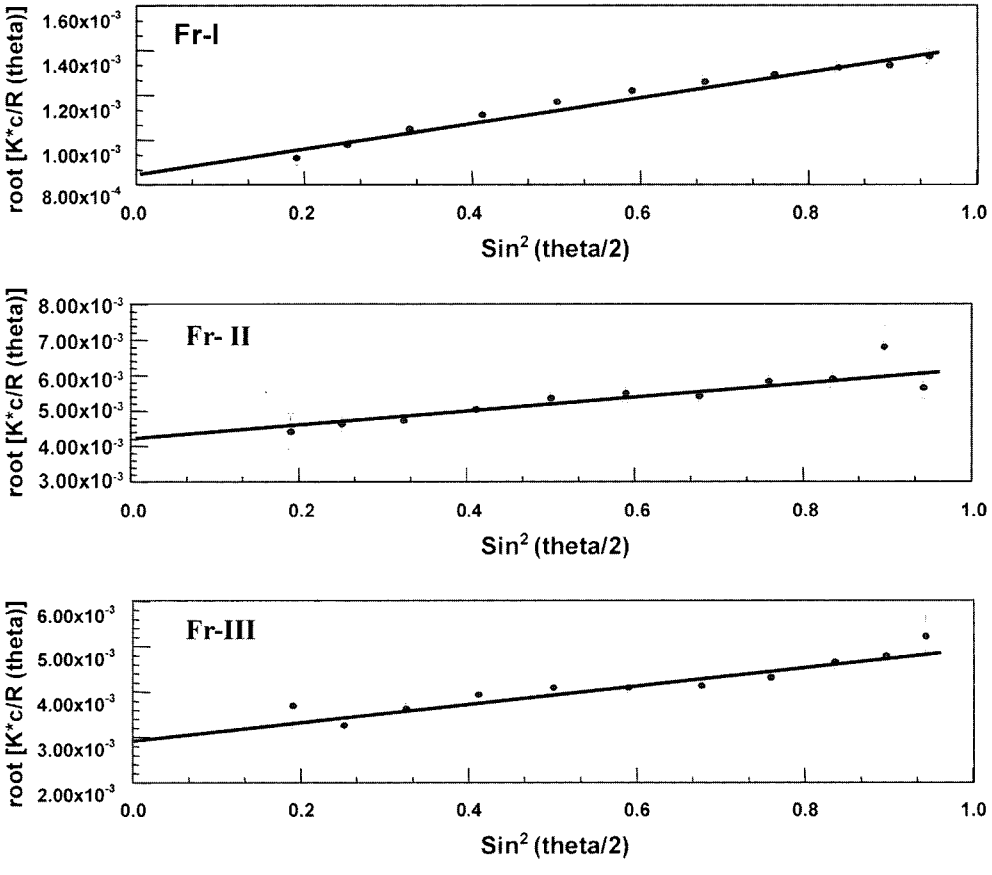

Fig. 5. Light scattering data plots [$\sqrt{(K^*c/R(\theta)}$ vs. $\sin^2(\theta/2)$] of three groups of EPSs (Fr-I~III) at the peak concentration based on the second-order Berry method.

Molecular Conformation of the EPSs. One of the important advantages of MALLS detection lies in an additional possibility to determine dimensions of dissolved polymer in terms of gyration radius (often denoted as either R_g or RMS) from the angular dependence of scattered light. The radius of gyration is directly determined from the angular dependence of the scattered light (see Fig. 4).

Figures 6A-6C show the overall slopes for each EPS in the double logarithmic plots of RMS radius *versus* molecular mass. The slope of 0.91 obtained from the plot for the Fr-I indicates that EPS exists as a nearly linear (rod-like) form in aqueous solution (Fig. 6A). Fr-II and Fr-III have the overall slope of 0.57 and 0.53, respectively (Figs. 6B and 6C). These slopes are in agreement with what is expected for a random-coil polymer. It should be noted here that most real coils of biopolymers are frequently slightly more extended, altering the slopes from 0.5 to 0.55–0.6 in a good solvent [21, 28]. For all fractions of EPS except Fr-I, the values of RMS at the same molecular weight were different from each other. This result indicates that these samples have different branched structures, and therefore, there could be a number of different types of aggregates and complexes in EPSs, having very different conformations depending on their M_w. It is noteworthy that the three RMS radii (*e.g.*, R_n, R_w, and R_z) of Fr-I were exactly the same, whereas Fr-II and Fr-III had different RMS radii, which is probably closely linked to the difference between the molecular dimensions of each EPS (Table 2). Owing to the fairly high molecular mass of the EPSs, the gyration radii of all EPSs indicated high values, ranging 89–105 nm.

Although the biological activities of the EPSs from *P. sinclairii* have not yet been extensively demonstrated, they are also expected to have some beneficial activities, similar to other entomopathogenic fungi. To elucidate this, the hypoglycemic activity of the EPSs is currently under investigation in our laboratory.

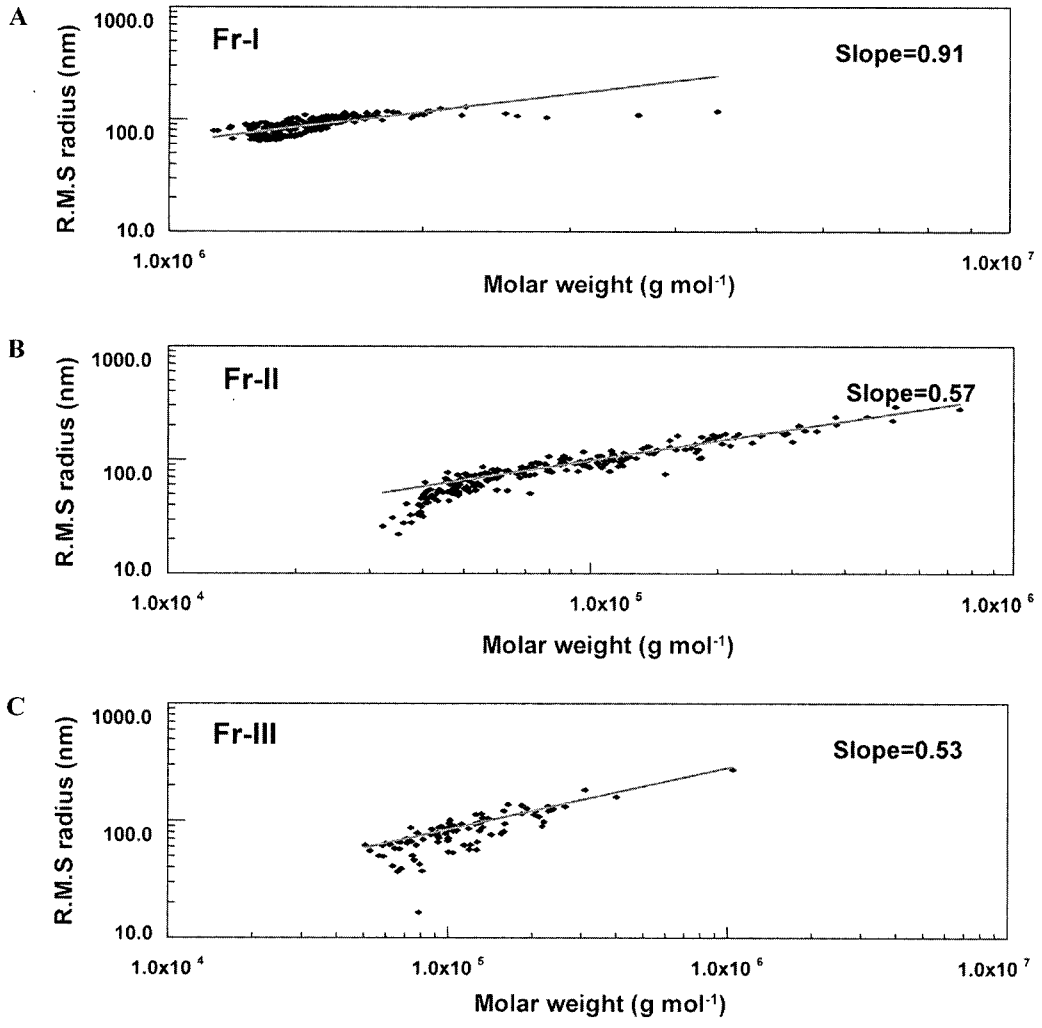

Fig. 6. SEC-MALLS: Conformation plots for three groups of EPSs (Fr-I~III) from culture filtrates of *Paecilomyces sinclairii*.

Table 2. SEC-MALLS analyses of the three groups of EPSs (Fr-I~III) produced from submerged culture of *Paecilomyces sinclairii.*

Parameters[a]	Fr-I (error %)	Fr-II (error %)	Fr-III (error %)
M_n (g/mol)	1.528×10^6 (0.9%)	5.950×10^4 (1.2%)	9.108×10^4 (2.0%)
M_w (g/mol)	1.540×10^6 (0.9%)	6.302×10^4 (1.1%)	9.389×10^4 (1.9%)
M_z (g/mol)	1.551×10^6 (2.0%)	6.699×10^4 (2.5%)	9.695×10^4 (4.0%)
M_w/M_n	1.008	1.059	1.031
R_n (nm)	90.7 (1.3%)	93.5 (2.9%)	98.1 (4.0%)
R_w (nm)	91.7 (1.3%)	98.9 (2.9%)	99.9 (4.0%)
R_z (nm)	91.2 (1.3%)	104.9 (2.9%)	101.8 (4.0%)

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

The SEC-MALLS system is proven to be an efficient technique for characterization of complex polysaccharides and other biopolymers containing mixtures of solvated polymer chains as well as micelle-like aggregates. The SEC/MALLS approach, therefore, could be useful in providing greater insight into the characteristics of the fungal polysaccharides without carrying out elaborate fractionation procedures prior to analysis. Many valuable characterization data could be obtained, including absolute molecular weight, molar mass distribution, molecular dimension, intrinsic viscosity, and so on. The molecular data in the present work can be comparatively studied with other groups of polysaccharides and provide an impetus to investigate the correlation between molecular properties and biological activities.

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