Note

Effect of Molecular Mass on Antitumor Activity of Heteropolysaccharide from Poria cocos

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A water-soluble heteropolysaccharide ac-PCM0 from Poria cocos was successfully fractionated using a preparative size exclusion chromatography (SEC) column, and their weight-average molecular mass (M_w) was characterized by analytical SEC combined with laser light scattering (SEC-LLS). The results indicate that the fractions having relatively high M_w exhibited higher inhibition ratio in vivo antitumor activity than those having M_w below 3.29 × 10^6. However, the relatively low molecular mass was beneficial to the in vitro antitumor activity. Moreover, ac-PCM0 has a significantly higher enhancement ratio of the body weight than 5-fluorouracil, and its 50% lethal dose is above 1250 mg/kg, indicating a nontoxic nature.

Key words: heteropolysaccharide; molecular mass; antitumor activity

Poria cocos has been long used as a traditional Chinese herb with diuretic and sedative activity. The polysaccharides from sclerotia or mycelia of Poria cocos have been studied from the viewpoint of their antitumor effects.1-3 In previous work,4,5 some water-soluble heteropolysaccharides isolated from the mycelia of Poria cocos have exhibited antitumor activity. The bioactivity of polysaccharides is related to their molecular mass, degree of substitution, degree of branching, conformation in solution, and sugar component.6,7 So fractions of the polysaccharides having different molecular mass are often needed to investigate physical properties and bioactivity. Size exclusion chromatography (SEC) is presently the most popular method for fractionation and characterization of both synthetic and natural polymers.8-11 In our laboratory, a preparative SEC column packed with regenerated cellulose gel particles has been used successfully for fractionation of a dextran in water12 and β-D-glucan PC3 from Poria cocos sclerotium in dimethylsulfoxide (DMSO).13 It has been found that preparative SEC is simple, fast, and suitable for relatively large-scale fractionation for various polysaccharides in aqueous or organic solvents.

In this study, a water-soluble polysaccharide ac-PCM0 was fractionated by preparative SEC column to obtain samples with different molecular masses. The samples were characterized by size exclusion chromatography combined with laser light scattering (SEC-LLS) in 0.2 mol/l NaCl aqueous solution, and their in vivo and in vitro antitumor activities against Sarcoma 180 tumor cells and Hela cells were evaluated.

The monosaccharides of ac-PCM0 were determined by infrared spectroscopy, gas chromatography, elemental analysis, and 13C NMR to be mannose, galactose, glucose, fucose, and arabinose (43.0:27.4:27.2:1.4:1.0 by weight percent), and there was 25.5% protein in the polysaccharide.4 The sample ac-PCM0 was stored for six months and then used in this study. It was fractionated using a preparative SEC column (550 mm × 25 mm) packed with two kinds of regenerated cellulose gel particles. The sample was dissolved in distilled water to prepare 0.005g/ml concentration. For each run, 5 ml polysaccharide solution was injected into the column, and double distilled water was used as eluent at ambient temperature. The flow-rate was adjusted to 1.0 ml/min using a peristaltic pump during the run. The column effluent and fractions were monitored using a UV–Vis spectrophotometer (UV-160, Shimadzu, Japan) at 200 nm, the absorption peak of polysaccharide. Eleven fractions were collected with an amount from 0.15 to 0.25 g of each fraction (coded as ac-PCM0-F1~ac-PCM0-F11).

The polysaccharides were dissolved in 0.2 mol/l NaCl to obtain clear aqueous solutions. SEC-LLS measurements of the samples were carried out on a Dawn® DSP laser light scattering (LLS) instrument (Wyatt Technology) combined with a P100 pump (Thermo Separation Products, San Jose, CA, U.S.A.) equipped with a TSK-GEL G5000 and a G3000 PWXL column (7.8 mm × 300 mm) in aqueous medium at 25 °C. A differential refractive index detector (RI-150) was simultaneously connected. The eluent was a 0.2 mol/l NaCl aqueous solution with a flow rate of 1.0 ml/min.

The polysaccharide solutions with concentrations...

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ranging from $2.0 \times 10^{-3}$ to $4.0 \times 10^{-3}$ g/ml were first filtered with a sand filter, followed by a 0.45 μm filter (NYL, 13 mm Syringe filter, Whatman, England). Astra software was utilized for data acquisition and analysis.

The $M_w$ of the unfractonated ac-PCM0 sample and its fractions were determined by SEC-LLS to be 3.97, and 7.05, 7.03, 5.84, 5.17, 4.34, 2.38, 2.45, 2.75, 2.39, 3.12, and 1.01 × 10^4 respectively. The $M_w$ values decreased with the fractionation progress on the whole, and the polydispersity index ($M_w/M_n$) of the fractions was in the range of 1.2 to 1.8, narrower than that of original ac-PCM0 (3.04), suggesting a basically successful fractionation. The amount of the fractions was so small that it was enough in the antitumor activity test, so the eleven fractions were combined into four fractions labeled ac-PCM0-I to ac-PCM0-IV. SEC-LLS chromatograms of the four fractions are shown in Fig. 1, and the values of $M_w$ are summarized in Table 1. Each fraction exhibited a single peak, indicating that there was no aggregate in the heteropolysaccharide aqueous solution, and that the fraction was pure. The protein contents of the three fractions were determined by using a Kjeltec 1030 self-analyzer (Bern, Switzerland) according to the semimicro Kjeldahl principle. The results are listed in Table 1. Clearly, the protein content of the fractions was less than that of the original sample.

Sarcoma 180 tumor cells $(5 \times 10^6$ cells/mouse), provided by Tongji Medical College of Huazhong University of Science and Technology, were subcutaneously inoculated into 8-week-old female BALB/c mice weighing 17 ± 1 g. 5-Fluorouracil (5-Fu) and ac-PCM0-I to ac-PCM0-IV were dissolved in autoclaved water, and then injected intraperitoneally (i.p.) once daily for 7 d at 24 h after tumor inoculation. The same volume of water was injected i.p. into the control mice. The tumors were allowed to grow on the mice for 7 d before they were removed from the animals and weighed. The inhibition ratio ($\xi$) and enhancement ratio of body weight ($f$) were calculated as follows:

$$
\xi = \left[\frac{W_c - W_t}{W_c}\right] \times 100\%
$$

$$
f = \left[\frac{W_a - W_b}{W_b}\right] \times 100\% \quad (2)
$$

where $W_c$ is the average tumor weight of the control group and $W_t$ is the average tumor weight of the test group. $W_b$ and $W_a$ are the body weight of mice before and after treatment.

The inhibition ratios against Sarcoma 180 solid tumor in vivo of the ac-PCM0-I to ac-PCM0-IV samples are summarized in Table 1, which also shows the results of 5-Fluorouracil (5-Fu) in parallel tests. Clearly, ac-PCM0-I and ac-PCM0-II exhibited higher inhibition ratios than the other two fractions, indicating that relatively high $M_w$ and a certain degree of protein content increase the $in vivo$ antitumor activities of the polysaccharide more than low $M_w$ below $3.29 \times 10^4$. This result is similar to schizophyllan and grifolan, which can induce antitumor activity when they have higher $M_w$ than that from 1 to $4 \times 10^4$. It is worth noting that four fractions showed low inhibition ratios at a low dose level of 20 mg/kg, suggesting that the dose limit is above 40 mg/kg. Interestingly, the enhancement ratios of body weight of the ac-PCM0 fractions were much higher than that of 5-Fu, suggesting that the polysaccharides are not as toxic as 5-Fu, which kills normal cells as well as cancer cells. Furthermore, the heteropolysaccharide solutions with a concentration of 50 mg/ml were injected intravenously (i.v.) into BALB/c mice weighing 20 ± 1 g once daily. Mouse activity was investigated for 7 d. Toxicity and death distribution was recorded, and the 50% lethal dose (LD50) of samples was calculated. The LD50 is the dose that kills half of the animals tested. An $in vivo$ acute toxicity test illustrated that the mice were not dead or abnormal after injection of the samples with a concentration of 50 mg/ml into them for 7 d. The LD50 of polysaccharide

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**Table 1.** *In Vivo* Antitumor Activities of Fractions of ac-PCM0 from Poria Cocos Mycelium against Sarcoma 180 Solid Tumor Grown in BALB/c Mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_w \times 10^4$</th>
<th>Protein content (%)</th>
<th>Dose mg/kg × days</th>
<th>Mice numbers (start/end)</th>
<th>$f$ (%)</th>
<th>$\xi$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>10/10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Fu</td>
<td>6.08</td>
<td>8.97</td>
<td>20 × 7/10/10</td>
<td>43.8 6.3</td>
<td>48.5</td>
<td>50.5</td>
</tr>
<tr>
<td>ac-PCM0-I</td>
<td>4.92</td>
<td>12.28</td>
<td>20 × 7/10/10</td>
<td>41.4 21.3*</td>
<td>46.9</td>
<td>28.2*</td>
</tr>
<tr>
<td>ac-PCM0-II</td>
<td>3.29</td>
<td>11.7</td>
<td>20 × 7/10/10</td>
<td>45.4 11.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac-PCM0-IV</td>
<td>1.01</td>
<td>2.97</td>
<td>20 × 7/10/10</td>
<td>42.4 8.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Compared with control group $p < 0.05$. 

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**Fig. 1.** SEC Chromatograms of Fractions ac-PCM0-I, ac-PCM0-II, ac-PCM0-III, and ac-PCM0-IV.
was above 1250 mg/kg, indicating that polysaccharide shows no toxicity.

The colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to measure the proliferation of adherent tumor cells. Sarcoma 180 tumor cells were inoculated on a 96-well cultivation plate at a concentration of 10^4/ml. Each well was inoculated with 100 µl tumor cell solution and 20 µl samples and incubated for 24 h under 5% CO₂ at 37 °C. The mixture was continuously inoculated for another 4 h after 12 µl MTT (5 g/l) was added. Then the supernatant was removed. DMSO (100 µl) was added to terminate the reaction and the plate was shaken slightly to dissolve the crystals formed. The number of living tumor cells was determined by colorimetric assay based on the tetrazolium salt MTT, as described by Mosmann.15) Optical density was measured with an auto enzyme-labeled meter (CliniBio 128, Austria) at 550 nm. Other Hela tumor cells were used in this investigation. The in vitro experiment method for Hela cells is as same as that for Sarcoma 180 tumor cells. Optical density was measured with a Microplate Reader (GENios VA200, TECAN, Salzburg, Austria). All in vitro results are expressed as the inhibition ratio of tumor cell proliferation calculated as [(A – B)/A] × 100%, where A and B are the average numbers of viable tumor cells of the control and samples respectively. The in vitro inhibition ratios against Sarcoma 180 solid tumor of the four fractions are shown in Fig. 2. In the MTT assay, all of the four fractions had certain effect in inhibiting the proliferation of Sarcoma 180 solid tumor cells. But the samples having relatively low Mₚ exhibited a relatively high inhibition ratio against Sarcoma 180 solid tumor. In addition, we also tested the in vitro antitumor activities against Hela cells (cervical carcinoma cells) by the MTT method. But there was no effect of inhibiting the proliferation of Hela cells. This indicates that ac-PCM0 heteropolysaccharide has selective antitumor activity in vitro.

**References**


13) Zhang, L., Ding, Q., Meng, D., Ren, L., Yang, G., and Liu, Y., Investigation of molecular masses and aggregation of β-D-glucan from *Poria cocos sclerotium* by size-
