The Correlation between Molecular Weight and Antitumor Activity of Galactosaminoglycan (CO-N) from *Cordyceps ophioglossoides*

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A galactosaminoglycan (CO-N) obtained by ultrasonication from a protein-bound polysaccharide SN-C, which was isolated from *Cordyceps ophioglossoides* culture, has a direct cytotoxicity against tumor cells (Ohmori *et al.*, *Chem. Pharm. Bull.*, 37, 1019 (1989). High peformance liquid chromatographic analysis revealed that CO-N shows a broad molecular weight distribution with an average molecular weight of 33000. A potent antitumor activity of CO-N was observed in the higher-molecular-weight fraction on gel filtration, and the low-molecular-weight fraction below 6600 showed a weak activity. However, the depolymerized CO-N (*ca.* 5500) obtained by further ultrasonication of the original CO-N still retained the antitumor activity of CO-N against Ehrlich ascitic carcinoma or MM46 solid mammary carcinoma.

Keywords *Cordyceps ophioglossoides*; galactosaminoglycan; CO-N; gel filtration; ultrasonication; antitumor activity; Ehrlich carcinoma; MM46 carcinoma

Introduction

There have been many studies on the relationship between the activity and the structure of antitumor polysaccharides from microorganisms.[1,2] Most of these polysaccharides are β(1—3)-D-glucans with β(1—6) monoglucosyl side chains and show a marked host-mediated antitumor activity. These glucans have a molecular weight of 10000 to 2000000, and are less soluble in water. The antitumor activities of the glucans are generally observed in the higher-molecular-weight fraction. There are several reports on the relationship between the activity and structure of β-glucans. However, structure–activity relationships of basic polysaccharides have not been reported except for chitin and related molecules. We have already reported that the protein-bound antitumor polysaccharide, SN-C obtained from *C. ophioglossoides*, can be fractionated into two different types of antitumor polysaccharides, CO-1 and CO-N, after sonic treatment.[3,4] CO-1 showed a host-mediated antitumor effect, whereas CO-N had mainly a direct cytotoxicity. CO-1 is a β(1—3)-D-glucan with (1—6) linked side chains and has a helical higher structure.[5] CO-N is a protein-bound α(1—4)-galactosaminoglycan.[6] In this study, CO-N was further fractionated and examined for direct antitumor activity against Ehrlich carcinoma in order to elucidate the relationship between the structure and the activity of CO-N.

Materials and Methods

Fractionation of CO-N by Gel Filtration CO-N was isolated from SN-C solution as reported previously.[4] CO-N (200 mg) was subjected to gel filtration on Toyopearl HW-60 (column 5 × 100 cm) equilibrated with 2% CH_3COOH–0.5 M NaCl at a flow rate of 4 ml/min, and the eluate was separated into 5 fractions (A, B, C, D, E). Fractionation was based on the peaks of galactosamine determined by the indole–hydrochloric acid method, since amino acid analysis revealed that galactosamine was the only aminosugar component in the CO-N preparation. The purity of each fraction was confirmed by re-chromatography on the Toyopearl HW-60 column. Each fraction could be recovered as a single peak. The average molecular weight of each fraction was determined by high-performance liquid chromatography (HPLC) in comparison with standard pullulan (Seikagaku Kogyo Co., Ltd., Tokyo, Japan).

Depolymerization of CO-N by Ultrasonication CO-N solution was dissolved in 0.05 N acetic acid (100 ml) to 1%, and was exposed for 0.5, 1, 5, 12, 24 or 48 h to ultrasonic waves generated at 200 W output by an ultrasonic generator (Kubota Insonator, Model 200M). Each sample was examined for average molecular weight by HPLC.

HPLC HPLC was performed with a Shimadzu LC-6A (Shimadzu Co., Kyoto, Japan) instrument equipped with an RI (refractive index) detector, a UV 280 nm detector and columns of Shodex OH pak B-800p. B-806. B-804. B-803 (polyhydroxylalkylmethacrylate gel) equilibrated with 0.3 M NaCl/5% CH_3COOH (pH 2.1) at a flow rate of 1.0 ml/min.

Analytical Methods Hexose content was measured by the phenol–sulfuric acid method,[7] aminosugar content by the indole–hydrochloric acid method,[8] and protein content by the Lowry–Folin method.[9]

Animals and Tumors Female ICR mice (7 weeks of age, 20—25 g) were purchased from Clea Japan, Inc., Tokyo. Female C3H/He mice (7 weeks of age, 20—25 g) were purchased from Shizuoka Laboratory Animal Center (SLC, Hamamatsu, Japan). Ehrlich carcinoma and MM46 mammary carcinoma were kindly provided by the National Cancer Center. Ehrlich carcinoma was maintained by several passages in ICR mice and MM46 mammary carcinoma in C3H/He mice.

Antitumor Experiments Each sample was dissolved in 0.01 N acetic acid and neutralized, and the osmolarity was adjusted with glucose. The solution was sterilized by passage through a 0.45 μm Mirex filter and used for animal experiments.

(a) Effect against Ascitic-Type Tumor: Ehrlich carcinoma (1×10^6) was inoculated into the peritoneal cavity of ICR mice, and the sample was administered intraperitoneally at 5 mg/kg or 10 mg/kg for 10 consecutive days from the day after tumor transplantation. The mortality of mice was observed for 60 d and the increase in life span (ILS) was calculated according to the following formula; ILS (%) $= (T/C - 1) \times 100$, where T is the median survival time (MST) of the experimental group and C is the MST of the vehicle-control group.

b) Effect against Solid-Type Tumor: MM46 mammary carcinoma (1×10^6) was transplanted subcutaneously into the right inguinal region of C3H/He mice. On the 6th day, the tumor was palpable. The sample was administered intravenously at doses of 1, 10 and 100 μg/kg on days 6, 8, 10, 12 and 14. The tumor was resected 30 d after the transplantation, and the weight was measured and compared with that of the control group. Inhibition ratio was calculated according to the following formula; inhibition ratio (%) $= (1 - T/C) \times 100$, where T is the average tumor weight of the experimental group and C is that of the vehicle-control group.

Results

Fractionation of CO-N by Gel Filtration CO-N was fractionated by gel filtration on Toyopearl HW-60 into five fractions, fr. A—E as shown in Fig. 1. Each fraction was analyzed for average molecular weight and composition ratio of hexose, hexosamine and protein (Table I). The average molecular weight of CO-N was estimated to be

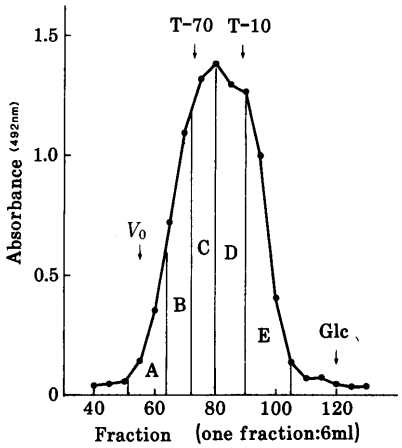

Fig. 1. Gel Filtration of CO-N on Toyopearl HW-60 (Fine)

Aminosugar content (●). Fr. A (fraction number 51—64); fr. B (65—72); fr. C (73—80); fr. D (81—90); fr. E (91—105). T-70 and T-10 are dextran standards (molecular weights, 70000 and 10000, respectively).

TABLE I. Properties of CO-N Fractions Obtained by Gel Filtration

Fraction	Molecular weight[a)]	Component (%)		
		Hexose[b)]	Hexosamine[c)]	Protein[d)]
Fr. A	138000	5.5	72.7	5.3
Fr. B	54700	3.4	75.0	4.4
Fr. C	31600	3.4	78.3	4.0
Fr. D	17400	4.1	77.3	5.7
Fr. E	6600	3.7	74.9	5.1
CO-N	32900	5.5	80.3	5.6

a) Molecular weight was estimated by HPLC. *b*) Hexose content was measured by the phenol–sulfuric acid method. *c*) Hexosamine content was measured by the indole–hydrochloric acid method. *d*) Protein content was measured by the Lowry–Folin method.

32900 by HPLC analysis. This value differed slightly from the previously reported molecular weight, 50000, which was estimated by gel filtration on Toyopearl HW-55.[5)] The average molecular weight of the highest-molecular-weight fraction (fr. A) was 138000, and it was 20 times larger than that of the lowest-molecular-weight fraction (fr. E). The composition ratio of components in each fraction was almost the same.

Depolymerization of CO-N by Ultrasonication CO-N solution was sonicated for 0.5, 1, 5, 12, 24 and 48 h and the molecular weight of each sonication product was estimated by gel filtration. The molecular weight of each sonication products became smaller as the sonication time was increased. The distribution range became even broader when CO-N was sonicated for a longer period, without showing conversion to a specific molecular weight (Fig. 2). The average molecular weight and the relative composition of hexose, hexosamine and protein of the various sonication products were determined (Table II). After 48 h of sonication, the average molecular weight had decreased to 5500, but the composition ratio in each sample was almost the same. Otsuka *et al.* proposed the following formula for the relationship between the ultrasonication time and the resulting molecular weight of high-molecular-weight substances[10)]; $1/(m_0-m)=m_\infty^2/Kk_2(m_0-m_\infty)^2\times t+1/(m_0-m_\infty)$, where m_0 is the average molecular weight before sonication, m is that after t min of treatment and m_∞ is

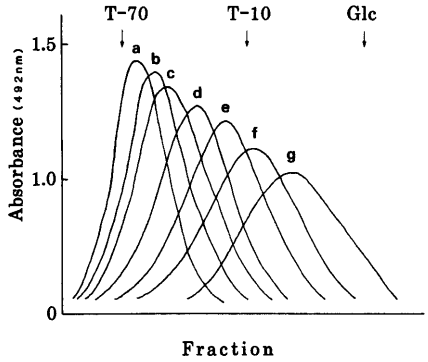

Fig. 2. Gel Filtration of Ultrasonicated CO-N

Sonication time 0 (peak a); 0.5 h (b); 1 h (c); 5 h (d); 12 h (e); 24 h (f); 48 h (g).

TABLE II. Composition of Ultrasonication CO-N

Samples	Time of sonication (h)	Molecular weight[a)]	Relative composition (%)		
			Hexose[b)]	Hexosamine[c)]	Protein[d)]
CO-N-0	0	32900	5.5	80.3	5.6
CO-N-0.5	0.5	30200	5.4	80.3	5.5
CO-N-1	1.0	29100	5.4	80.5	5.5
CO-N-5	5.0	24700	5.3	80.2	5.3
CO-N-12	12.0	21000	5.0	80.1	5.3
CO-N-24	24.0	12900	5.0	80.1	5.2
CO-N-48	48.0	5500	4.8	80.2	5.0

a) Molecular weight was estimated by HPLC. *b*) Hexose content was measured by the phenol–sulfuric acid method. *c*) Hexosamine content was measured by the indole–hydrochloric acid method. *d*) Protein content was measured by the Lowry–Folin method.

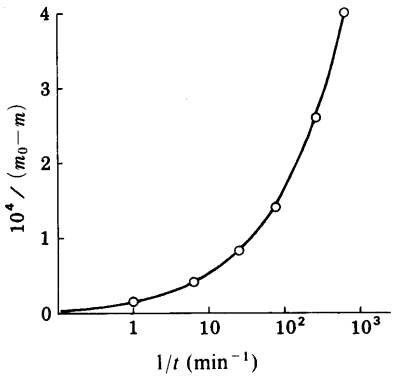

Fig. 3. Effect of Ultrasonication on the Average Molecular Weight of Depolymerized CO-N.

m_0, the average molecular weight before sonication; m, the average molecular weight after t min of sonication.

the final average molecular weight. K is the constant for volume and concentration of the solution, and k_2 is the constant for the breakage speed. The present results are plotted in Fig. 3. The final molecular weight (m_∞), estimated from the intercept at the ordinate approached zero and did not show any specific value.

Antitumor Activity of Each Fraction a) Effect against Ehrlich Ascitic Tumor: The ILS of mice bearing Ehrlich ascitic carcinoma was determined after administration of each fraction separated by gel filtration. Compared to the ILS in the group given 10 mg/kg of CO-N, the ILS in the group given the high-molecular-weight fraction, fr. A, was

Table III. Antitumor Effect of CO-N Fractions Obtained by Gel Filtration against Ehrlich Ascitic Carcinoma

Sample	Dose[a] (mg/kg × d)	MST[b] (d)	ILS (%)
CO-N	10 × 10	36.5	161
	5 × 10	33.5	140
Fr. A	10 × 10	60.0	329
	5 × 10	35.5	154
Fr. B	10 × 10	38.5	175
	5 × 10	30.0	114
Fr. C	10 × 10	37.5	168
	5 × 10	23.5	68
Fr. D	10 × 10	32.5	132
	5 × 10	22.0	57
Fr. E	10 × 10	30.5	118
	5 × 10	14.5	4
	Control	14.0	—

Ehrlich carcinoma cells (1×10^6) were inoculated intraperitoneally into ICR mice on day 0. *a*) Each sample was given intraperitoneally for 10 consecutive days after tumor inoculation. *b*) The mortality of mice was recorded for 60 d after tumor inoculation.

Table IV. Antitumor Effect of Ultrasonicated CO-N against Ehrlich Ascitic Carcinoma

Sample	Dose[a] (mg/kg × d)	MST[b] (d)	ILS (%)
CO-N-0	10 × 10	36.5	148
	5 × 10	32.0	121
CO-N-0.5	10 × 10	35.5	145
	5 × 10	31.5	117
CO-N-1	10 × 10	37.0	155
	5 × 10	31.0	114
CO-N-5	10 × 10	36.0	148
	5 × 10	30.5	110
CO-N-12	10 × 10	35.5	145
	5 × 10	31.5	117
CO-N-24	10 × 10	36.5	152
	5 × 10	30.5	110
CO-N-48	10 × 10	35.5	145
	5 × 10	31.0	114
	Control	14.5	—

Ehrlich carcinoma cells (1×10^6) were inoculated intraperitoneally into ICR mice on day 0. *a*) Each sample was given intraperitoneally for 10 consecutive days after tumor inoculation. *b*) The mortality of mice was recorded for 60 d after tumor inoculation.

significantly higher (Table III). The low-molecular-weight fraction fr. E, showed very little effect. This tendency was more marked when 5 mg/kg of the sample was used, and fr. E did not show any life-prolonging effect. On the other hand, CO-N depolymerized by ultrasonication retained a high level of antitumor activity, comparable to that of non-treated CO-N (CO-N-0) (Table IV).

b) Effect of Ultrasonicated CO-N against MM46 Mammary Solid Carcinoma: In our previous report, CO-N showed a significant inhibitory effect at a low dose on the proliferation of MM46 carcinoma. In this study, the antitumor effect of ultrasonicated CO-N was determined in this MM46 carcinoma system. As shown by the results in Table V, the activity was completely retained in CO-N-1 (1 h of sonication) to CO-N-48 (48 h of sonication). There was no evident relation between the administration dosage and the biological activity. Even with 1 μg/kg of each sample of CO-N sonicated for different periods, over 95% inhibition of tumor growth was observed.

Table V. Antitumor Effect of Ultrasonicated CO-N on MM46 Solid Carcinoma

Sample	Dose[a] (μg/kg × d)	Tumor weight[b] (g) (mean ± S.D.)	Inhibition ratio (%)	Complete cure rate (%)
CO-N-0	1.0 × 5	0.06 ± 0.03	96	80 (8/10)
	10.0 × 5	0.15 ± 0.07	89	70 (7/10)
	100.0 × 5	0.55 ± 0.16	59	50 (5/10)
CO-N-1	1.0 × 5	0.05 ± 0.02	96	90 (9/10)
	10.0 × 5	0.17 ± 0.04	87	80 (8/10)
	100.0 × 5	0.63 ± 0.22	53	40 (4/10)
CO-N-24	1.0 × 5	0.07 ± 0.07	95	70 (7/10)
	10.0 × 5	0.60 ± 0.08	56	70 (7/10)
	100.0 × 5	0.40 ± 0.12	70	50 (5/10)
CO-N-48	1.0 × 5	0.06 ± 0.04	96	70 (7/10)
	10.0 × 5	0.31 ± 0.05	77	70 (7/10)
	100.0 × 5	0.88 ± 0.28	35	40 (4/10)
	Control	1.35 ± 0.33	—	0 (0/10)

MM46 carcinoma cells (1×10^6) were inoculated subcutaneously into the right inguinal region of C3H/He mice on day 0. *a*) Each sample was given intravenously to mice on days 6, 8, 10, 12 and 14 after tumor inoculation. *b*) Tumors were excised from mice and weighed on day 30.

Discussion

We have already reported the antitumor activities of *C. ophioglossoides* polysaccharide SN-C, together with CO-1 [β (1—3) glucan with (1—6)-linked side chains] and CO-N [α(1—4) galactosaminoglycan], both of which were isolated from SN-C. CO-N is a complex polysaccharide, composed mainly of partially *N*-acetylated polygalactosamine. CO-N also contains protein and neutral sugars such as glucose, mannose and galactose.[6] The molecular weight of CO-N was revealed to be 32900 by HPLC analysis, which is relatively small for an antitumor polysaccharide. However, the molecular weight distribution was extremely broad, ranging from 5000 to 2000000. The molecular weight required for the antitumor activity of β (1—3) glucan was reported to be more than 10000 based on an evaluation of partially hydrolyzed products.[2] In order to determine the relationship between the degree of polymerization and the activity of CO-N, it was necessary to obtain depolymerized specimens of CO-N. However, it was difficult to achieve a uniform depolymerization of CO-N by the acid hydrolysis method as used for glucans because of its complicated structure. Therefore, CO-N was fractionated according to molecular weight by gel filtration, and the molecular weight required for antitumor effect was estimated by evaluating the antitumor activity of each fraction. Against the ascitic form of tumor, antitumor activity was observed in the fractions with an average molecular weight of over 17400, but that in the fraction with an average molecular weight of 6600 was markedly decreased. These results suggested that the molecular weight required for the antitumor activity of CO-N was more than 10000. The CO-N solution is very viscous and a long time is required to dissolve CO-N by the use of a stirrer. Therefore, it was preferable to dissolve CO-N rapidly by ultrasonication in order to prepare samples for administration to mice. There was a possibility that the high-molecular-weight compound would be depolymerized by ultrasonication to lower-molecular compounds, resulting in a decrease in antitumor activity. We examined the antitumor activity of depolymerized CO-N fractions ex-

posed to ultrasonic waves for much longer times than the usual period employed for dissolution (15 min). After sonication for 48 h, CO-N was depolymerized to an average molecular weight of about 5500. In spite of the small molecular weight, the activity was well retained. From the fact that the low-molecular-weight fraction (MW 6600) separated by gel filtration did not show antitumor activity whereas the low-molecular-weight fraction obtained (MW 5500) by ultrasonication for a long period retained the activity, it was suggested that the depolymerization by ultrasonication dose not affect the active portion of CO-N. Taking into account that the structural conformations of the high- and low-molecular weight fractions obtained by gel filtration are not necessarily the same, the factor required for the antitumor activity of CO-N may be an active component such as protein, or the linkage of *N*-acetylgalactosamine or neutral sugars, rather than the molecular weight.

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