Physicochemical Characteristics and Antitumor Activities of a Highly Branched Fungal (1→3)-β-D-Glucan, OL-2, Isolated from *Omphalia lapidescens*

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Physicochemical properties and antitumor activities of a fungal (1→3)-β-D-glucan, OL-2, isolated from Leiwan (*Omphalia lapidescens*) were examined. OL-2 showed sharp signals on carbon-13 nuclear magnetic resonance spectrum in dimethylsulfoxide-d_6 as a solvent, and these signals were significantly reduced by the addition of distilled water to the concentration of 20%. This phenomenon is consistent with the general property of the gel forming (1→3)-β-D-glucan. Binding of OL-2 to Congo red induced a significant change of λ_{max} to a longer wavelength, and the concentration to induce gel to sol transition was about 0.7 N; in contrast, the concentration was about 0.2 N in the cases of SPG and curdlan. These observations suggested that the gel structure would be significantly stabilized in the case of OL-2. OL-2 showed no or low antitumor activity against the solid form of Sarcoma 180 by intraperitoneal and intralesional administrations; however, it was effective on the ascites form of Sarcoma 180. Of interest, OL-2 also showed significant antitumor activity against the ascites form of MH-134 when administered with 5-fluorouracil. These results indicated that OL-2 showed characteristic features regarding its physicochemical properties and antitumor activity.

Keywords leiwan (*Omphalia lapidescens*); β-glucan; antitumor activity; conformation; gel; OL-2

Introduction

Antitumor (1→3)-β-D-glucan has recently been used clinically for cancer immunotherapy. The mechanisms of antitumor activity of (1→3)-β-D-glucan have been demonstrated to be due to the activation of the host immune system and considerable experimental evidence has supported this concept.[1a)] Chemical modifications of these glucans have demonstrated structure–function relationships.[1b,c)] However, the knowledge about early biochemical events leading from the initial recognition of glucan molecules by the host have been limited, such as activation of the complement[2)] and coagulation systems,[3)] and glucan-specific receptors on phagocytic cells.[4)] Thus, it is still difficult to understand the above structure–activity relationship under the molecular level. Further studies from both chemical and biological approaches would be necessary.

A fungal (1→3)-β-D-glucan, OL-2, extracted from Leiwan (*Omphalia lapidescens*) has two branches in every three main chain units, and showed almost no antitumor activity against the solid form of Sarcoma 180 tumors by intraperitoneal administration.[5)] Recent studies on OL-2 suggested that removal of a part of the side chain glucose by periodate oxidation and borohydride reduction induced significant antitumor activity on the derivatives and that OL-2 showed significant activity on the alternative complement pathway and on limulus factor G.[6)] Precise examinations of the physicochemical characteristics and of immunopharmacological activities and comparison of these data with those of antitumor glucans would help the molecular understanding of glucan-mediated antitumor activity, as well as the critical role of the side chain. In this paper, we have characterized the physicochemical properties and antitumor activities of OL-2.

Materials and Methods

Materials OL-2 (from *Omphalia lapidescens*),[5)] GRN (from *Grifola frondosa*)[7)] and SSG (from *Sclerotinia sclerotiorum*)[1c)] were prepared as described previously. SPG was generously provided by Kaken Pharmaceutical Co., Ltd. Dimethylsulfoxide-d_6 (DMSO-d_6) was purchased from Merck. 5-Fluorouracil (5-FU) was from Sigma Chemical Co. Curdlan, Congo red and another reagents were from Wako Chemical Co.

Antitumor Activity Antitumor activity against the solid and ascites form of Sarcoma 180 tumors was measured by the method described previously using male ICR mice (Japan SLC, Hamamatsu) under specific pathogen-free conditions.[8)] Antitumor activity against the ascites form of MH-134 hepatoma (generously provided by Chugai Pharmaceutical Co., Ltd.) was measured according to life span using male C3H/HeN mice (Japan SLC, Hamamatsu).

Results and Discussion

Physicochemical Properties of OL-2 A fungal (1→3)-β-D-glucan, OL-2 was extracted with 0.5 N sodium hydroxide and was barely solubilized in a neutral aqueous solution, but developed into a turbid gel after extensive sonication. The properties of the gel are thought to be quite important to illustrate various immunopharmacological activities of (1→3)-β-D-glucans; thus we examined the physicochemical properties of OL-2 by two distinct methods, Congo red binding and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.

In order to compare the binding of Congo red to the glucan, λ_{max} was measured under various doses of glucans (OL-2, SPG, and curdlan) and various concentrations of sodium hydroxide. Figure 1a showed the dose response of λ_{max}, and at the saturating dose, λ_{max} was different depending on the glucan, and OL-2 showed the longest wavelength (530 nm). The saturating dose was dependent on the glucan, and the relative binding was SPG : OL-2 : curdlan = 1 : 1 : 4. The value of SPG was also similar in the case of alkaline-treated SPG whose conformation was different from the parent SPG. Figures 1b—d showed the λ_{max} of Congo red in the presence of glucans under various concentrations of sodium hydroxide. In the case of SPG (Fig. 1b), λ_{max} values of both increasing and decreasing sodium hydroxide concentrations were almost identical, and the significant change of λ_{max} was observed around 0.15 to 0.2 N sodium hydroxide. Alkaline-treated SPG showed a similar value. In contrast, in the case of OL-2 (Fig. 1c) and curdlan (Fig. 1d), the values were different from each other. Especially in the case of OL-2, λ_{max} was significantly changed around 0.65 to 0.70 N in the case of

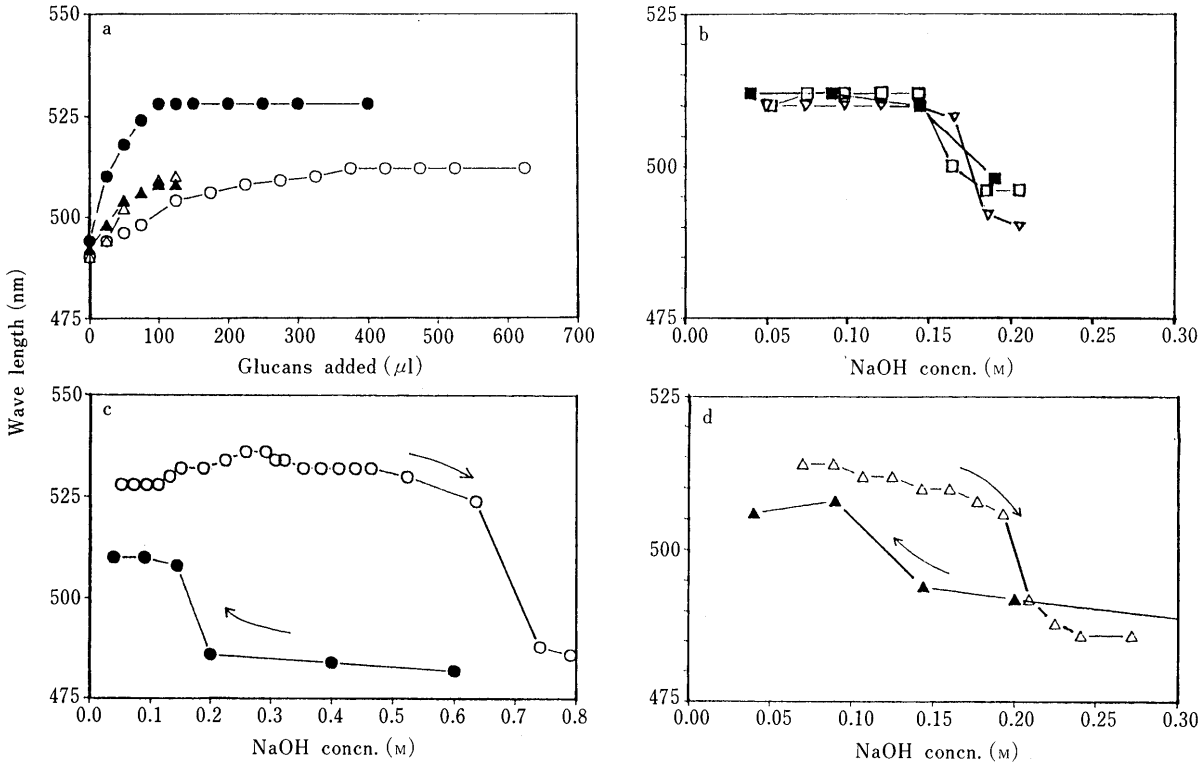

Fig. 1. Characterization of the Gel–Sol Transition of OL-2 by Congo Red Binding

a) Dose response: Each glucan (2 mg/ml) in 0.05 N sodium hydroxide solution was gradually added to the Congo red solution (2 ml, 100 μM) in 0.05 N sodium hydroxide. The λ_{max} value was measured in each addition and plotted. The spectra were measured by a Hitachi 557 instrument. ○, curdlan; ●, OL-2; △, SPG (native); ▲, SPG (alkaline treated). b) Gel–sol transition: SPG (native, 250 μg) was mixed with Congo red solution (2 ml, 100 μM) in 0.05 N sodium hydroxide. Aliquots of 2 N sodium hydroxide were added to the solution and λ_{max} value was measured in each addition and plotted (□). After the λ_{max} value was shifted to the shorter wavelength, aliquots of 4 N sulfuric acid were added to the solution to monitor the λ_{max} value (■). Similarly, alkaline treated SPG was mixed with Congo red solution and aliquots of sodium hydroxide solution was added (▽). c, d) Gel–sol transition of OL-2 (800 μg) or curdlan (1.3 mg) was examined similarly to the procedure shown in b). ○, OL-2 (gel→sol); ●, OL-2 (sol→gel); △, curdlan (gel→sol); ▲, curdlan (sol→gel).

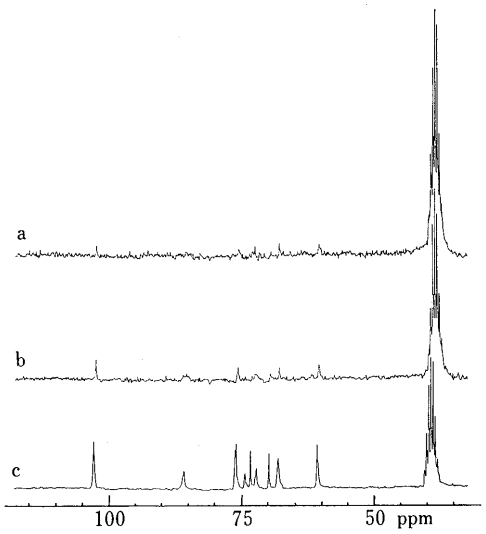

Fig. 2. ^{13}C-NMR Spectra of OL-2 in DMSO-d_6

^{13}C-NMR spectra were measured with JEOL-FX 200 instruments by using a 10 i.d. sampling tube at 60 °C. OL-2 (20 mg) was dissolved in MDSO-d_6 (2 ml) and distilled water was added to the indicated volume. The solvent signal appears at 39.5 ppm as a multiplet. a, 0.6 ml; b, 0.5 ml; c, 0 ml.

increasing sodium hydroxide concentration, and 0.20 to 0.15 in the case of decreasing concentration. The difference of critical concentration was about 0.5 N in the case of OL-2 (0.1 N in the case of curdlan).

To further investigate the physicochemical properties of OL-2, ^{13}C-NMR spectra were measured under several experimental conditions. ^{13}C-NMR spectrum of OL-2 in DMSO-d_6 showed sharp signals, and the addition of distilled water significantly reduced the signal intensities (Fig. 2). These results strongly suggested the sol to gel transition of the conformation by the addition of water.[9] Similarly, a broadening of the ^{13}C-NMR spectrum of OL-2 was also observed in sodium hydroxide: sharp signals in 0.16 N NaOH and broad signals in 0.08 N NaOH. The physicochemical data of OL-2 shown in this section strongly suggested that the conformation of OL-2 would be strongly stabilized under physiological conditions, and would be different from SPG and curdlan.

Antitumor Activity of OL-2 In the previous paper, it is suggested that OL-2 showed negligible antitumor activity by intraperitoneal administration.[5] To further investigate the antitumor activity of OL-2, the activity was measured using solid and ascites forms of tumors. Table I showed the antitumor activity of OL-2 against the solid form of Sarcoma 180. When OL-2 was injected by intraperitoneal (i.p.) or intratumoral (intralesional; i.l.) administration, as expected, i.p. administration of OL-2 showed lower activity than GRN, a well established antitumor glucan obtained from *Grifola frondosa*. Intralesional administration of OL-2 showed higher activity than that of i.p. administration (significant activity at as a low concentration as 20 μg/mouse × 5 times).

We have tested the effectiveness of OL-2 against the ascites form of Sarcoma 180 by using a schedule similar to that described in the previous paper.[10] The single i.p.

TABLE I. Antitumor Activity of OL-2 against Solid Form of Sarcoma 180[a]

Sample	Dose × 5 (μg/mouse)	Route	Tumor weight (g) (mean ± S.D.)[b]	Inhibition (%)	C/R[e]
Saline		i.p.	7.5 ± 3.8	0	0/14
GRN	100	i.p.	0.4 ± 0.9[d]	94	3/7
OL-2	20	i.p.	10.4 ± 9.4	−39	0/7
OL-2	100	i.p.	5.4 ± 5.1	29	0/7
OL-2	500	i.p.	2.9 ± 2.6[c]	62	2/7
OL-2	20	i.l.	2.9 ± 2.6[c]	62	0/7
OL-2	100	i.l.	2.1 ± 2.1[d]	73	0/7
OL-2	500	i.l.	3.3 ± 5.8	57	1/8

a) Sarcoma 180 (5×10^6) were inoculated s.c. into right hind groin of ICR mice on day 0. Glucans were administered in a saline solution by i.p. or i.l. injection 5 times (days 7, 9, 11, 13, and 15). *b*) Tumor weight was measured at day 35. The significance was evaluated according to Student's *t* test [*c*), $p<0.01$, *d*), $p<0.001$]. *e*) Number of cured mice/total mice.

TABLE II. Effect of Combination Therapy of 5-FU and OL-2 on the Ascites Form of MH-134[a]

Treatment	Mean survival time (d, mean ± S.D.)	Median survival time[b] (d)	T/C[f]	58-d survivors/ total[g]
No	23.2 ± 3.0	21	100	0/5
5-FU	39.6 ± 12.7[d]	39	171	1/5
5-FU + OL-2	49.2 ± 8.3[c,e]	46	212	1/5

a) MH-134 cells (1×10^6 cells/mouse) were transplanted intraperitoneally on day 0. OL-2 (250 μg/mouse/d) and/or 5-FU (0.2 mg/mouse/d) were administered at days 2, 3, 4, 5, and 6. *b*) Survival of 59 d was assumed for mice still alive at day 58. The significance of differences was evaluated according to paired *t*-test [*vs.* control; *c*) $p<0.001$, *d*) $p<0.05$: *vs.* 5-FU; *e*) $p<0.05$]. *f*) The mean survival time of treated groups (T) *vs.* the control group (C). *g*) The ratio of 58 d survivors to total mice.

injection of OL-2 at days-1 or -4 significantly elongate the life span of the ascites form of Sarcoma 180 bearing mice [survival day (mean ± S.D.): Nil, 16.2 ± 3.4; OL-2 (-4), 22.8 ± 11.5 ($p<0.05$); OL-2 (-1), 19.4 ± 1.2 ($p<0.01$)]. By contrast, SSG, an antitumor (1→3)-β-glucan obtained from *Sclerotinia sclerotiorum*[11] and the dose (250 μg/mouse) used for this experiment was the optimum for various immunomodulating activities,[12] but was not effective under this experimental condition.

To understand the activity of OL-2 in combination with a chemotherapeutic drug (5-FU), the activity was measured by the ascites form of MH-134 tumor in C3H/HeN mice. In the control group all mice died within 27 d, and while 5-FU prolonged the life span significantly, most of those mice died within 41 d. In contrast, administration of OL-2 significantly prolonged the life span: no mice died within 41 d. These results strongly indicated that OL-2 is also an antitumor glucan, but its mode of action would be significantly distinguished from other antitumor glucans.

The pattern of antitumor action of OL-2 is noteworthy. One major mechanism of antitumor activity against a solid form tumor would be by the induction of a tumor-specific immune response which could be transferred to another host by T cells (Winn type assay).[13] Loss of activity by OL-2 would be dependent on the failure of induction of the tumor-specific immunity. Recently we have found that OL-2 did not exhibit adjuvant activity to sheep red blood cell (manuscript in preparation). This phenomenon would be related to the weak antitumor activity of OL-2. If the β-glucan-mediated antitumor action is only due to the above mechanism, the action of OL-2 shown in this paper could not be explained. In general, (1→3)-β-D-glucan could activate macrophages to release mediators, to present antigens, and to induce cytolytic action. Preliminary investigations suggested that Ll-2 activates the hematopoietic response to induce macrophage (manuscript in preparation). Antitumor action to the ascites tumor would be related to the induction and activation of macrophage.

Previous publications suggested that the antitumor activity is strongly related to the ratio of the side chain group. Of interest, the side chain group significantly modified the physicochemical properties of the (1→3)-β-D-glucans. Indeed, the solubility of OL-2 was quite low. In the case of (1→3)-β-D-glucan, the ratio of the side chain group would be significantly dependent on the physicochemical properties of the glucan. It is quite interesting to clarify whether the primary structure or the accompanying physicochemical properties affect the biological activities of (1→3)-β-D-glucan.

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