Separation of the Hot Water Extracts of Sclerotia of *Sclerotinia sclerotiorum* IFO 9395 into Mitogenic and Antitumor-Active Subfractions

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The hot water extract of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 (TSHW) was divided into representative fractions by ammonium sulfate and ethanol precipitations, and (1→3)-β-D-glucanase treatment. The ammonium sulfate and ethanol precipitations gave a (1→3)-β-D-glucan fraction (TSG) and a mannan fraction (TSM). After the degradation of (1→3)-β-D-glucan in TSHW by (1→3)-β-D-glucanase treatment, a water-insoluble protein fraction (EDP) and supernatant (EDS) were obtained. Among these fractions, the mitogenic and antitumor activities were mainly observed in EDP and TSG, respectively. On the other hand, the stimulatory effect on the reticuloendothelial system was mainly found in EDP and EDS, and a weak effect was observed in TSG. These findings suggest that the mitogenic and antitumor activities of TSHW were mainly due to the protein and (1→3)-β-D-glucan, respectively, and that the mitogenic substance (EDP) is tightly bound to (1→3)-β-D-glucan (TSG) in TSHW, accounting for its solubility in aqueous solution.

Keywords *Sclerotinia sclerotiorum*; sclerotia; immunomodulator; mitogenic activity; (1→3)-β-D-glucanase; reticulostimulating ability; antitumor activity; (1→3)-β-D-glucan

Recently, Oldham proposed the term biological response modifiers (BRM) to describe the many agents whose mechanisms of action involve the individual's own biological responses.[1] One BRM, OK-432, the lyophilized preparation of a penicillin-treated cell suspension of a low-virulent strain Su of *Streptococcus haemolyticus*,[2] has been demonstrated to show significant clinical effects in cancer therapy.[3] However, since OK-432 is the whole lyophilized cell preparation, its physicochemical and biological characteristics are very complicated. Recent studies have attempted to identify the effective material(s) in this preparation.[4] A similar approach has been employed in the field of oriental crude drugs.

Recently, we found that the hot water extracts of sclerotia of *Sclerotinia sclerotiorum* IFO 9395, named TSHW, showed various immunomodulating effects.[5] During the physicochemical characterization of TSHW, it was found that (1) TSHW was composed of carbohydrate (*ca.* 60%) and protein (*ca.* 30%), (2) the (1→3)-β-D-glucan part would participate in antitumor activity, and (3) the mitogenic substance binds very tightly to diethylaminoethyl (DEAE)–Sephadex A-25 equilibrated with 8 M urea. In this paper, to elucidate what material(s) in TSHW show immunomodulating activities, TSHW was fractionated into four fractions and the chemical properties and immunomodulating activities of these fractions were compared.

Materials and Methods

Mice Six- to 12-week-old male ICR and BALB/c mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. Male C3H/HeJ mice were purchased from CLEA, Japan, Inc., Tokyo.

Materials and Microorganisms The mycelia of *Sclerotinia sclerotiorum* IFO 9395 obtained from the Institute for Fermentation, Osaka, Japan (IFO) were cultured at 25 °C. The sclerotia produced on potato-sucrose agar media, which gives the highest yield of sclerotia,[6] were picked up and lyophilized. Lipopolysaccharide (LPS) of *Escherichia coli* 055:B5 prepared by a phenol–water extraction was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Heat-killed cells of *Propionibacterium acnes* C7 were kindly provided by Kowa Co., Tokyo. Sepharose CL-2B, CL-4B and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, Toyopearl TSK-GEL HW-75F from Toyo Soda Mfg. Co., Tokyo, Japan, and Bio-Gel P-2 from Bio-Rad Laboratories. Kitalase K4F 022 used as a source of (1→3)-β-D-glucanase was purchased from Kumiai Chemicals Co., Shizuoka. (1→3)-β-D-Glucanase was partially purified by passing through sulfopropyl (SP)-Sephadex C-50. One unit of (1→3)-β-D-glucanase was defined as the amount of the enzyme which liberated 1 nmol of glucose from laminaran per minute at 37 °C at pH 6.8.

Preparation of the Hot Water Extract of Sclerotia The hot water extract of sclerotia was prepared by the method described previously.[5] Briefly, the lyophilized sclerotia were boiled in water at 121 °C for 60 min. After centrifugation, the supernatant was dialyzed against distilled water and the non-dialyzable fraction was recovered by lyophilization. This preparation is designated as TSHW.

Preparation of the Carbohydrate Moieties of TSHW To prepare the glucan and mannan moieties of TSHW, the precipitate produced at 60% saturation (60 P) and the supernatant remaining after 100% saturation (100 S) with ammonium sulfate were obtained, respectively. Each fraction was made up to 8 M urea and applied to a column of DEAE-Sephadex A-25 (Cl^-) equilibrated with 8 M urea. The pass-through fractions were dialyzed against distilled water, and then ethanol was added to each concentrated fraction to make 2 volumes (v/v). In the case of 60 P, the precipitate was recovered by centrifugation, and dried with acetone and ether (TSG). In the case of 100 S, the supernatant fraction after centrifugation was concentrated, and applied to a column of Bio-Gel P-2 equilibrated with distilled water. The carbohydrate fraction (monitored by the phenol–sulfuric acid method) was collected, concentrated and lyophilized (TSM).

Preparation of the Protein Moieties of TSHW TSHW dissolved in 10 mM phosphate buffer (P.B.), pH 6.8, was digested with one unit of (1→3)-β-D-glucanase per mg TSHW for about 24 h at 37 °C. The insoluble material produced was collected by centrifugation at 15000 rpm for 10 min at 15 °C, dialyzed against distilled water and lyophilized (EDP). Before the assay of immunomodulating activities, EDP was sonicated twice for 30 s at 30 W. The supernatant was dialyzed against 10 mM P.B., pH 5.0, at 4 °C and applied to a column of SP-Sephadex C-50 ion-exchange chromatography equilibrated with the same buffer to remove the (1→3)-β-D-glucanase. The pass-through fraction was dialyzed against distilled water and lyophilized (EDS).

Assay for Immunomodulating Activities Mitogenic activity[7] and antitumor activity[8] were assessed by the methods described previously. Reticulostimulating ability was assessed by the method described previously[9] with the corrected phagocytic index (α) calculated as follows;

$$\alpha = \frac{\text{body weight (g)}}{\text{liver} + \text{spleen weight (g)}} \times \sqrt[3]{K}$$

where K is the phagocytic index of the individual mouse. The value in Table III is the average value. The increase of this value compared with that of the control group indicates the augmentation of individual macrophage function, but the absence of change may reflect the hepatomegaly and splenomegaly.

Results

Physicochemical Properties of Fractionated-TSHW The glucan fraction in TSHW was obtained from the precipitate at 60% saturation with ammonium sulfate (60 P), and the mannan fraction was obtained from the supernatant of 100% saturation (100 S). After passage through a column of DEAE-Sephadex A-25 (Cl^-), the glucan fraction was further purified by the addition of 0.5 volume of ethanol (TSG), and the mannan fraction in TSHW was recovered from the supernatant after addition of 4 volumes of ethanol (TSM). The result of component sugar analysis suggested that TSG was composed solely of glucose (Table I). The molecular weight of TSG was suggested to be more than 2×10^6. In methylation analysis, TSG gave 2,3,4,6-Me_4-Glc, 2,4,6-Me_3-Glc, 2,4-Me_2-Glc in a molar ratio of 1.0 : 1.1 : 0.8. Based on the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of TSHW[5] and the above results, TSG is a (1→3)-β-D-glucan possessing a branch at C-6 of every other main chain glucosyl unit. On the other hand, TSM was composed of glucose, mannose, galactose, and fucose in a molar ratio of 1 : 15 : 3 : 1 (Table I), and the molecular weight was suggested to be about 4×10^4. The ^{13}C-NMR analysis suggested that the major spectrum of TSM was similar to that of yeast mannan, but some differences were observed (Fig. 1), especially in the anomeric region, suggesting differences of linkage and/or anomeric configuration from yeast mannan.

In the preliminary experiments, the mitogenic substance was tightly adsorbed on DEAE-Sephadex A-25 and hardly eluted even by 2 M NaCl containing 8 M urea.[6] In gel filtration, the protein moiety co-migrated with the carbohydrate in TSHW under physiological conditions (Fig. 2),

TABLE I. Physicochemical Properties of TSHW Fractions

	Yield (%)	Carbohydrate (%)[a]	Protein (%)[b]	Component sugars[c]
TSG	38	95	1	Glc
TSM	10	85	1	Glc : Man : Gal : Fuc = 1 : 15 : 3 : 1
EDP	12	15	50	Glc : Man : Gal = 1 : 2 : 3
EDS	20	70	25	Glc : Man : Gal = 1 : 8 : 0.7

a) Phenol–H_2SO_4 method (as glucose). *b*) Lowry–Folin method (as bovine serum albumin). *c*) Determined as the alditol acetate derivatives by gas liquid chromatography.

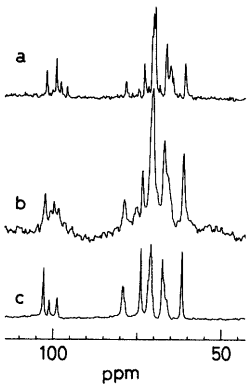

Fig. 1. ^{13}C-NMR Spectra of TSM and EDS

a, TSM; b, EDS; c, yeast mannan.

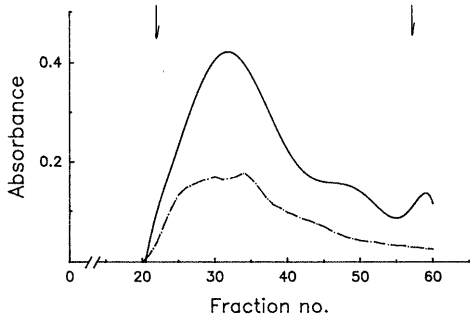

Fig. 2. Elution Profiles of TSHW on a Column of Sepharose CL-4B

TSHW (9 mg) was applied to a column (1.7 × 50 cm) of Sepharose CL-4B equilibrated with distilled water. Fractions of 2 ml were collected at 4 °C and carbohydrate and protein were monitored by the phenol–sulfuric acid method (carbohydrate, 490 nm; ——), and by ultraviolet absorption measurement (protein, 280 nm; —·—). Arrows indicate the void and the bed volumes

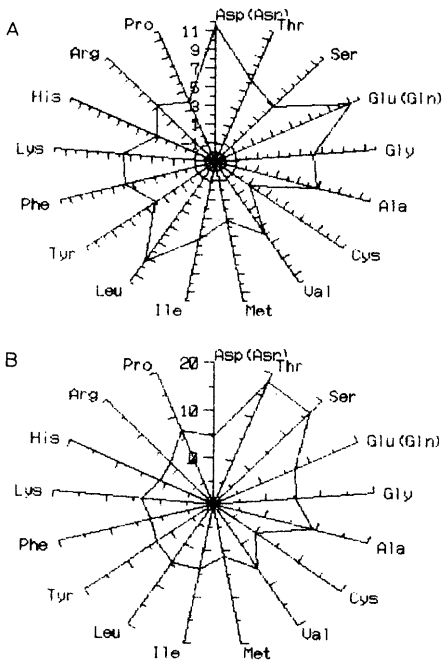

Fig. 3. Amino Acid Compositions of EDP and EDS

Amino acid analyses of EDP (panel A) and EDS (panel B) were performed in a Hitachi L-8500 amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for 24 h. Each value is the percentage by weight with respect to total amino acids.

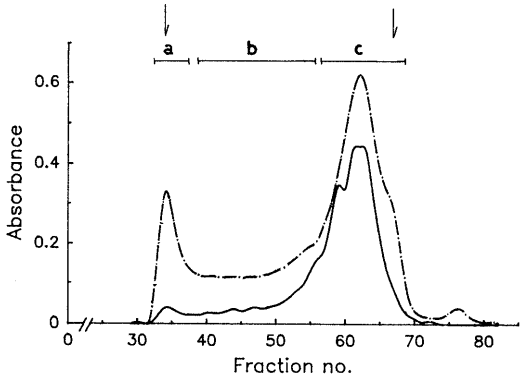

Fig. 4. Elution Profiles of EDP on a Column of TSK-GEL HW-75

A column (1.8 × 98 cm) of TSK-GEL HW-75 was equilibrated with 8 M urea, and 12.3 mg of EDP was applied. Fractions of 3.4 ml were collected at room temperature and carbohydrate and protein were monitored by the phenol–sulfuric acid method (carbohydrate, 490 nm; ——), and by ultraviolet absorption measurement (protein, 254 nm; —·—). Arrows indicate the void and the bed volumes. a, HW-1; b, HW-2; c, HW-3.

suggesting the existence of physicochemical interaction between carbohydrate and protein in TSHW. To obtain the protein moiety of TSHW, the glucan portion, which is the major constituent,[10] was digested by using partially purified (1→3)-β-D-glucanase. During the incubation, insoluble material was produced. After centrifugation, the precipitate fraction was named EDP, and the supernatant fraction was named EDS. EDP was hardly soluble in water and was mainly composed of protein with high contents of aspartic acid and glutamic acid and/or their amide (Fig. 3). From the component sugar analysis, EDP was composed of glucose, mannose, and galactose in a molar ratio of 1:2:3. The result of the gel filtration using a TSK-GEL HW-75 equilibrated with 8 M urea suggested that EDP had a wide molecular weight distribution (Fig. 4). In contrast, EDS was mainly composed of mannose (Table I), and contained neutral and acidic mannans from the result of DEAE-Sephadex A-25 ion-exchange chromatography (data not shown). The neutral mannan would correspond to TSM described above. The result of ^{13}C-NMR analysis indicated that the mannan from EDS was different from yeast mannan (Fig. 1). The amino acid composition of EDS was different from that of EDP and contained relatively high proportions of serine and threonine (Fig. 3). These data suggest that TSHW contains at least two distinct proteins and/or peptides, that is, one rich in acidic amino acids and hardly soluble in water, and the other rich in oxyamino acids and readily soluble in water.

Immunomodulating Activities of Each Fraction As mentioned above, the hot water extract of sclerotia, TSHW, has been demonstrated to show some immunomodulating effects.[5] To identify the component(s) of TSHW with immunomodulating activity, the activity of the fractions obtained in this study was examined. First of all, the mitogenic activity against mouse splenocytes and thymocytes was examined. As shown in Fig. 5, EDP and EDS showed significant mitogenic activity against spleen cells, comparable to that of LPS. In contrast, TSG and TSM showed a little activity at the highest dosage tested, and no mitogenic activity was observed when the thymus cells were cultured with each fraction. EDP also showed mitogenic activity against C3H/HeJ splenocytes (Fig. 6), which are unresponsive to LPS,[11] suggesting that EDP is a B-cell mitogen having a different mechanism of action from that of LPS. Furthermore, all of the fractions of EDP obtained by gel filtration showed mitogenic activity comparable to that of EDP (Fig. 7), suggesting that the mitogenic sub-

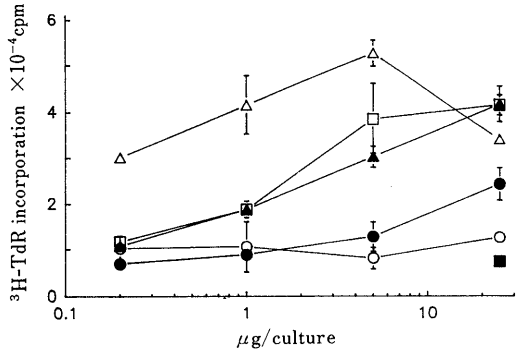

Fig. 5. Mitogenic Activity of Fractionated-TSHW on ICR Spleen Cells

Spleen cells (5×10^5 cells/culture) from ICR mice were cultured with each fraction for 48 h at 37 °C in a CO_2 incubator. Twenty hours before harvesting, 0.5 μCi of tritiated thymidine (^{3}H-TdR) was added to the culture medium in a volume of 20 μl. Values represent mean ± S.D. of triplicate cultures. —○—, TSG; —●—, TSM; —△—, EDP; —▲—, EDS; —□—, TSHW; ■, control.

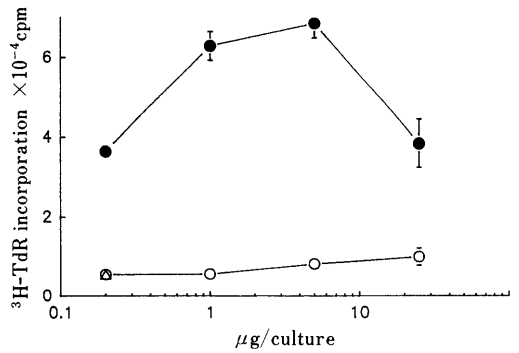

Fig. 6. Mitogenic Activity of EDP on C3H/HeJ Spleen Cells

The spleen cells were cultured for 48 h in the presence of LPS (○) or EDP (●), or in their absence (control) (△). Values represent mean ± S.D. of triplicate cultures.

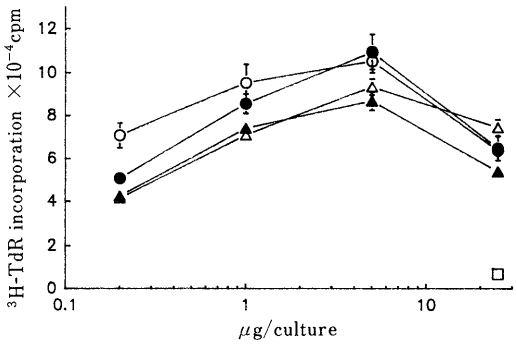

Fig. 7. Mitogenic Activity on BALB/c Spleen Cells of EDP Fractions Obtained by Gel Filtration

The spleen cells were cultured for 48 h in the presence of EDP (○) or its fractions (HW-1, ●; HW-2, △; HW-3, ▲), or in their absence (control) (□). Values represent mean ± S.D. of triplicate cultures.

TABLE II. Antitumor Activity[a] of TSHW Fractions against Sarcoma 180

Sample	Dose (μg/mouse)	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
Exp. 1				
TSG	4	5.89 ± 5.89	−21	0/10
	20	1.42 ± 2.10[b]	71	2/10
	100	0.01 ± 0.04[c]	>99	8/9
	250	0.96 ± 2.47[b]	80	4/10
TSM	4	6.73 ± 6.31	−38	0/10
	20	6.16 ± 4.45	−26	0/10
	100	7.68 ± 6.99	−57	0/10
	250	4.42 ± 5.64	9	1/10
Control	—	4.88 ± 3.12	—	0/10
Exp. 2				
EDP	4	6.30 ± 5.48	−41	0/7
	20	4.11 ± 4.48	8	0/7
	100	2.50 ± 2.15	44	0/7
	250	2.01 ± 1.80	55	0/7
EDS	4	5.31 ± 4.45	−19	0/7
	20	3.46 ± 3.00	23	0/7
	100	3.34 ± 3.46	25	0/7
	250	0.24 ± 0.42[d]	95	4/7
Control	—	4.47 ± 3.57	—	0/7

a) Sarcoma 180 tumor cells (5×10^6 cells/mouse) were inoculated subcutaneously into the right groin of mice. Each sample was administered on days 7, 9, 11, 13 and 15 after the tumor inoculation. Five weeks after tumor inoculation, the mice were sacrificed and tumor weights were measured. b) $p<0.01$. c) $p<0.001$. d) $p<0.05$.

TABLE III. Reticulostimulating Ability of TSHW Fractions on ICR Mice

Sample	Dose × timing (μg/mouse)	Phagocytic index $K^{a)}$ (mean ± S.D.)	Corrected phagocytic index $\alpha^{b)}$ (mean ± S.D.)
Exp. 1			
TSG	4 × 5	0.0593 ± 0.0077	6.39 ± 0.63
	20 × 5	0.0600 ± 0.0027	5.51 ± 0.25
	100 × 5	0.0916 ± 0.0321	6.27 ± 0.53
	250 × 5	0.0964 ± 0.0240	6.40 ± 0.21
P. acnes	350 × 1	0.1593 ± 0.0316$^{c)}$	7.37 ± 0.26$^{c)}$
Saline	—	0.0579 ± 0.0139	5.58 ± 0.58
Exp. 2			
TSM	4 × 5	0.0716 ± 0.0178$^{c)}$	5.45 ± 0.57
	20 × 5	0.1089 ± 0.0243	6.28 ± 0.91
	100 × 5	0.0746 ± 0.0115$^{c)}$	5.52 ± 0.32
	250 × 5	0.0716 ± 0.0255	5.84 ± 0.62
P. acnes	350 × 1	0.1758 ± 0.0373	6.53 ± 0.30
Saline	—	0.1203 ± 0.0091	6.86 ± 0.59
Exp. 3			
EDP	4 × 5	0.0600 ± 0.0163	5.29 ± 0.45$^{c)}$
	20 × 5	0.0884 ± 0.0107$^{c)}$	6.11 ± 0.35
	100 × 5	0.2453 ± 0.0665	7.43 ± 0.11
	250 × 5	0.3364 ± 0.0847$^{c)}$	8.17 ± 0.50$^{c)}$
P. acnes	350 × 1	0.1808 ± 0.0463	7.02 ± 0.53
Saline	—	0.0674 ± 0.0037	6.60 ± 0.65
Exp. 4			
EDS	4 × 5	0.0630 ± 0.0017	5.61 ± 0.61
	20 × 5	0.0710 ± 0.0135	6.16 ± 0.44
	100 × 5	0.1407 ± 0.0377	7.20 ± 0.34$^{c)}$
	250 × 5	0.2646 ± 0.0120$^{d)}$	8.04 ± 0.42$^{e)}$
P. acnes	350 × 1	0.1090 ± 0.0342	6.12 ± 0.22
Saline	—	0.0720 ± 0.0080	6.60 ± 0.65

a) Phagocytic index (K) was calculated by means of the following equation: $K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$ where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. *b*) Corrected phagocytic index (α) was calculated by means of the following equation: $\alpha = (\text{body weight (g)}/(\text{liver} + \text{spleen weight})) \times \sqrt[3]{K}$ where K is the value of the individual mouse. *c*) $p < 0.05$. *d*) $p < 0.001$. *e*) $p < 0.01$.

stances in EDP have a wide molecular weight distribution.

The antitumor effect of each fraction on transplantable sarcoma 180 in ICR mice was examined (Table II). TSG showed a significant antitumor effect and EDS showed antitumor activity at a high dosage, but no activities were observed in other fractions (Table II). These data indicate that the antitumor activity of TSHW is mainly attributable to the (1→3)-β-D-glucan portion (TSG), and that EDS also contributes to the antitumor activity.

The reticulostimulating ability of each fraction is shown in Table III. RES stimulatory ability was assessed in terms of the carbon clearance time from the blood stream, and expressed as the phagocytic index (K). To elucidate whether the increase of phagocytic index is due to only the augmentation of the clearance ability of carbon particles or is accompanied with hepatomegaly and splenomegaly, the corrected phagocytic index (α) was also calculated. Each fraction was administered i.p. (4, 20, 100, or 250 μg/mouse/d) at days −6, −5, −4, −3, −2 as a saline solution. As a positive control, *Propionibacterium acnes*[12] was administered i.p. (350 μg/mouse/d) at day −2. As shown in Table III, the phagocytic index in the cases of EDP and EDS was significantly increased at the doses of 100 and 250 μg/mouse/d × 5. The corrected phagocytic index also increased, indicating that the carbon clearance function was qualitatively changed, and suggesting that EDP and EDS stimulate the reticuloendothelial system. In contrast to EDP and EDS, TSG showed weak reticulostimulating ability, and no activity was observed with TSM.

Discussion

To elucidate what material(s) in TSHW show the immunomodulating activity, we tried to fractionate TSHW, and compared the immunomodulating activity of the fractions. The data presented in this paper suggested that (1) the mitogenic activity was probably attributable to the protein portion of TSHW, (2) the antitumor activity against the solid form of sarcoma 180 on ICR mice was due mainly to the (1→3)-β-D-glucan portion, and (3) the reticulostimulating ability was mainly observed in the protein portion. It seems likely that the immunomodulating activities of TSHW result from additive or cooperative effects of the components.

We were able to separate the mitogenicity and the antitumor activity of TSHW by employing (1→3)-β-D-glucanase to degrade the (1→3)-β-D-glucan moiety because conventional chromatography could not separate the mitogenic substance in TSHW.[6] When TSHW was treated with the (1→3)-β-D-glucanase preparation, a precipitate (EDP) was formed. EDP is the major mitogenic substance in TSHW, being rich in protein and hardly soluble in water. The precipitation during this treatment presumably occurred because the water-insoluble mitogenic proteins in sclerotia had been solubilized in water by forming a complex with (1→3)-β-D-glucan. The physicochemical nature of this complex was not characterized, but the gel-forming ability of (1→3)-β-D-glucan[13] and solubility of the protein in aqueous solution might be important factors.

Many (1→3)-β-D-glucans obtained from fungi show an antitumor effect.[14] In this study, we found that TSG showed antitumor activity against the solid form of sarcoma 180 in ICR mice. As judged from the results of the component sugar, methylation, and ^{13}C-NMR analyses, TSG is a (1→3)-β-D-glucan possessing a branch at C-6 of every other main chain glucosyl unit. Previously, we obtained an antitumor-active (1→3)-β-D-glucan (TS-CAG III) having a different proportion of branches from the cold alkali extracts of the hot water-extracted residue of sclerotia.[15] TS-CAG III has a branch at position 6 of two in every five main chain glucosyl units. These results suggested the presence of a control mechanism for glucan branching points in sclerotia. It is noteworthy that antitumor-active glucans having different branching points were obtained from the same sclerotia. Previously, we found a (1→3)-β-D-glucan similar to TSG in the liquid culture supernatant of *S. sclerotiorum* IFO 9395 (SSG).[16] Since SSG showed antitumor activity not only i.p. but also on oral administration against solid tumor cells,[17] there is a possibility that TSG might also show antitumor activity on oral administration. EDS also showed antitumor activity. Since TSM, the neutral mannan fraction, did not show the activity, the antitumor activity of EDS may be due to the acidic mannan and/or the protein which is rich in serine and threonine. It has also been reported that the acidic mannan from *Saccharomyces cerevisiae* showed antitumor activity.[18]

Mitogenic activity was observed in EDP and EDS. Maximal mitogenicity of EDP was shown at a dose of 5 μg/culture. Considering the yield of EDP, it is suggested

that most of the mitogenic activity in TSHW was accounted for by EDP. EDP also showed mitogenic activity toward C3H/HeJ splenocytes (Fig. 6), and the activity was not significantly inhibited by the addition of polymixin B to the culture (data not shown). These data suggest that the biochemical mechanism of the mitogenic activity of EDP is different from that of LPS. The mitogenic activity was also observed in TSG and TSM at high concentrations. Similar results have been reported by Mikami *et al.* on the mitogenicity of the mannan from *Saccharomyces cerevisiae*.[19] They suggested that mitogenicity of polysaccharides may require relatively high concentrations, and that the mannan possessing a large proportion of branches showed B-cell mitogenic activity. However, the mitogenic activity of TSHW was found to be almost wholly attributable to the protein, which is mainly composed of acidic amino acids and was hardly soluble in water. Identification of the mitogenic substance(s) is in progress.

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