Antitumor β-Glucan from the Cultured Fruit Body of *Agaricus blazei*

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***Agaricus blazei* is a medically important mushroom widely eaten and prescribed in Japan. Polysaccharide fractions were prepared from cultured *A. blazei* by repeated extraction with hot water (AgHWE), cold NaOH (AgCA), and then hot NaOH (AgHA). By chemical, enzymic, and NMR analyses, the primary structures of AgHWE, AgCA, and AgHA were mainly composed of 1,6-β-glucan. Among these fractions, the NaOH extracts showed antitumor activity against the solid form of Sarcoma 180 in ICR mice. To demonstrate the active component in these fractions, several chemical and enzymic treatments were applied. These fractions were found to be i) neutral β-glucan passing DEAE-Sephadex A-25, ii) resistant to periodate oxidation (I/B) and subsequent partial acid hydrolysis (I/B/H), iii) resistant to a 1,3-β-glucanase, zymolyase, before I/B, but sensitive after I/B/H. In addition, after I/B/H treatment of the neutral fraction of AgCAE, a signal around 86 ppm attributable to 1,3-β-glucosidic linkage was detectable in the ^{13}C-NMR spectrum. These facts strongly suggest that a highly branched 1,3-β-glucan segment forms the active center of the antitumor activity.**

Key words β-glucan; *Agaricus blazei*; immunomodulation; antitumor activity

An immunomodulating substance, a biological response modifier (BRM) or biotherapy is important for the treatment of cancer and infectious diseases. β-Glucan is a well-known BRM which is widely distributed in nature and used as a medicine and food.[1—5] The effects of "Lentinan" from *Lentinus edodes* and "Sonifilan" (SPG) from *Schizophyllum commune* in cancer therapy have been clinically proven.[6,7] We have already developed β-glucans, GRN from *Grifola frondosa*,[8—10] SSG and TSG from *Sclerotinia sclerotiorum*,[11—13] OL-2 from *Omphalia lapidescens*,[14] PVS and PVG from *Peziza vesiculosa*,[15] CSBG from *Candida* spp.,[16] OX-ZYM from a yeast cell preparation, zymosan,[17,18] and SCG from *Sparaccis crispa*.[19] We have also prepared several carboxymethyl, hydroxyethyl, sulfate, and polyol derivatives of the above β-glucans.[20] In addition, we have analyzed the mechanism of β-glucan mediated immunopharmacological activity and identified the conformation dependent and independent activity.[21—25] These rather complicated relationships between structure and activity suggest the contribution of multiple receptor-ligand interactions in β-glucan mediated immunopotentiation.

The incidence of cancer is gradually increasing and the spectrum of cancer-prone organs is changing each year. In addition to surgery, irradiation, and chemotherapy, immunotherapy is believed to be an important cancer therapy.[26] Immunotherapy and biotherapy include various approaches, such as BRMs, cytokines, lymphocyte transplantation, gene therapy, and herbal and alternative medicines. Clinical trials of these therapies are being widely carried out, and clinical evidence suggests their efficacy, although the precise mechanisms are still difficult to understand. The development of additional β-glucans is still needed for better biotherapy and to understand the molecular mechanisms.

Agaricus blazei is a medical mushroom widely eaten and prescribed in Japan. There are various kind of health foods containing *A. blazei* and/or its extracts, but the molecular mechanisms of its action is far beyond complete understanding. The most anticipated pharmacological effect of *A. blazei* is modulation of the immune system against cancer. In the last decade, there have been several studies which focused on an antitumor polysaccharide from *A. blazei*, *i.e.*, 1,6-β-glucan, glucan–protein complex, α-glucan, and heteroglycan.[27—34] Antitumor polysaccharides were screened not only in the fruitbody but also the liquid cultured medium and mycelium. One of the best characterized components, FIII-2-b, was prepared from the sodium hydroxide extract of the fruit body, and the structure was analyzed by methylation and NMR analyses. Contribution of a protein moiety to the activity of FIII-2-b was speculated by loss of the activity by partial formolysis. In the preliminary investigation, we have also determined that the major carbohydrate component of the fruit body of *A. blazei* is 1,6-β-glucan; however, we have already shown that a linear 1,6-β-glucan, islandican, was inactive in terms of antitumor activity in mice.[35] To clearly understand the mechanism of immunomodulation by β-glucans at the molecular level, it is critically important to identify the fine structure of the active component. In the present study, polysaccharide fractions were prepared from cultured *A. blazei*, and the active components of the extracts were examined.

MATERIALS AND METHODS

Materials Fruit bodies of *Agaricus blazei* were cultured, harvested, and air-dried in Brazil and imported by Toei Pharmaceutical Co., Ltd., Tokyo, Japan. Grifolan LE (GRN) obtained from *Grifola frondosa* was prepared as described previously. Sonifilan was generously provided by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. Toyopearl HW-65F was from Tosoh Co., Ltd., Tokyo, Japan.

Carbohydrate Analyses Carbohydrate content was determined by the phenol–sulfuric acid method. Component sugars were determined by capillary gas-liquid chromatography (Ohkura Riken Co., Ltd., Tokyo, Japan) of alditol acetate derivatives after complete hydrolysis by 2 M trifluoroacetic acid. A capillary column of fused silica (J & W Scientific, Inc., CA, U.S.A., 30 m×0.262 mm, liquid phase; DB-225,

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0.25 mM) was used at 220 °C. The molar ratio was calculated from the peak area of each component (glucose as 100).

Preparation of Sodium Hypochlorite Treated Fractions AgCA1 or GRN (250 mg) dissolved in 40 ml of 0.5 N NaOH was mixed with 5 ml of sodium hypochlorite (Wako), and the mixture was kept at 4 °C overnight. The resulting products were dialyzed extensively against tap water and then distilled water, then treated at room temperature with 100 mg of sodium borohydride for 2 h. The resulting solution was again dialyzed and lyophilized (yield; AgCA1 NaClO; 120 mg, GRN NaClO; 200 mg).

Zymolyase Digestion of Polysaccharide Fractions Each fraction (20 mg) dissolved in 10 ml of acetate buffer (50 mM, pH 6.0) was mixed with 1 mg of zymolyase 100T (Seikagaku Corp., Tokyo, Japan). After overnight incubation at 45 °C, the reaction mixture was boiled for 3 min to inactivate the enzyme. After centrifugation, the resulting supernatant was concentrated and applied to a Toyopearl HW-65 column equilibrated with 0.3 N NaOH. The elution profile of each fraction was monitored by the phenol–H_2SO_4 method.

NMR Analysis Fractions and authentic materials were dissolved in D_2O or DMSO-d_6 and the ^{1}H- and ^{13}C-NMR spectra were determined at 70 °C. Bruker DPX400 instruments equipped with the software "XWIN-NMR" were used.

Antitumor Activity Male ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were 5 weeks of age and maintained under specific pathogen-free conditions. Antitumor activity against the solid form of Sarcoma 180 tumor was measured by the method described previously.[8)]

Monitoring Vasculitis Vascular reaction of lentinan, the vascular dilation and hemorrhage (VDH) reaction, was reported by Maeda *et al*.[36—38)] Vasculitis induced by *A. blazei* extracts was monitored by visual inspection of the ear showing a flare, in accordance with Maeda's procedure, with slight modification. The VDH reaction was evaluated as i) a strong (++) reaction: strong and widespread flare in both ears, ii) partial (+) reaction: partial flare in either ear, and iii) no (+/−—−) reaction: no or only faint flare in one ear.

Measurement of 1,3-β-D-glucan by Fungitec G Test MK The activation of factor G (limulus reactivity) by 1,3-β-D-glucans was measured by a chromogenic method using a 1,3-β-D-glucan-specific reagent (Fungitec G-test MK, Seikagaku Corp.), which eliminates factor C.[39,40)] Each 1,3-β-D-glucan was dissolved in 0.3 N NaOH (1 mg/ml) and diluted with 0.01 N NaOH. Usually, enough dilution was done by 0.01 N NaOH, and the sample solution was used directly for the limulus reaction without neutralization. Diluted NaOH was confirmed to be usable for the limulus reaction because of the high buffer action of the reagent. Reactions were performed in a flat-bottomed 96-well Toxipet plate 96F (Seikagaku Corp.) as follows. Samples (50 μl) were placed in the wells, and the Fungitec G test MK reagent (50 μl) was added to each well. The plate was incubated at 37 °C, and during incubation the absorbance at 405 nm (reference: 492 nm) was measured kinetically using a microplate reader (Wellreader SK601, Seikagaku Corp.). Disposable plastic materials for tissue culture or clinical use were employed, and all glassware was sterilized at 260 °C for 3 h. All operations were performed in triplicate under aseptic conditions.

Preparation of Anti-GRN Ab GRN was conjugated with bovine serum albumin (BSA), and the resulting complex was immunized into rabbits as described previously.[41)] Briefly, GRN-BSA dissolved in phosphate buffered saline was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into Japanese white rabbits (500 μg/injection) every other week. Serum was collected at day 42 after the first immunization. The GRN-specific Ab was purified by passing from a column of GRN-conjugated aminocellulofine affinity gel (Seikagaku Corp.). The biotinylation of anti-GRN Ab was performed by mixing the Ab with NHS-LC-biotin (Pierce, Rockford, IL, U.S.A.) in bicarbonate buffer (pH 8.4).

ELISA for Anti-GRN Antibody An immune plate (Nunc 442404, F96 Maxisorp) was used for all ELISA experiments in this study. Phosphate buffered saline containing 0.05% Tween 20 (Wako Pure Chemical Ind.) (PBST) was used to wash the plate (150 μl/each well). Carbonate buffer (pH 9.6, 0.1 M) was used to bind the antigen or antibody. The plate was blocked by 0.5% bovine serum albumin containing PBST (150 μl/each well). Soluble antigen was added with mouse sera.

Statistics Results are expressed as the arithmetic mean ± standard deviation (S.D.). Statistical evaluations were performed by the Student's *t*-test. A value of $p<0.05$ was considered significant.

RESULTS

Preparation of Polysaccharide Fractions Polysaccharide fractions of *A. blazei* were prepared by repeated extraction with distilled water, cold sodium hydroxide, hot sodium hydroxide, and then sodium hypochlorite (Chart 1). Briefly, air-dried and powdered *A. blazei* (25—50 g) was extracted with hot water (121 °C, 2 h). The polysaccharide fraction was prepared from the extract by precipitation with 4 vol. of EtOH (AgHWE-1). The residue was again extracted with hot water and the extracts were prepared as described above (AgHWE-2, AgHWE-3). The resulting residue was then extracted with cold alkali (10% NaOH/5% urea, 4 °C, 1 d, twice; AgCA-1 and AgCA-2), and hot alkali (10% NaOH/5% urea, 65 °C, 1 h; AgHA). Polysaccharide fractions of alkali extracts were collected after extensive dialysis. The residue of the alkaline extract was then treated with sodium hypochlorite (NaClO) to extract skeletal components. The resulting extract was dialyzed extensively (AgPPS). The residue was also dialyzed extensively (AgPPR). By NaClO treatment, about 5% of the polysaccharide fraction was further solubilized, and less than 15% of the total component remained after the final extraction step. The protein and carbohydrate of these fractions were measured by a phenol–sulfuric acid method with glucose as a reference, and by the BCA method with bovine serum albumin as a reference (Table 1). All of the extracts were mainly composed of carbohydrate and contained significant amounts of anionic substances. The clear sodium hydroxide extracts became a milky solution after dialysis, thus, in some experiments, the resulting solution was centrifuged before ethanol precipitation to give the water soluble fraction (AgCAS, AgHAS) and the insoluble fraction (AgCAR, AgHAR).

Component sugars of major fractions were analyzed by an alditol acetate derivative. As shown in Table 2, major component sugar of all the water soluble fractions was glucose. In

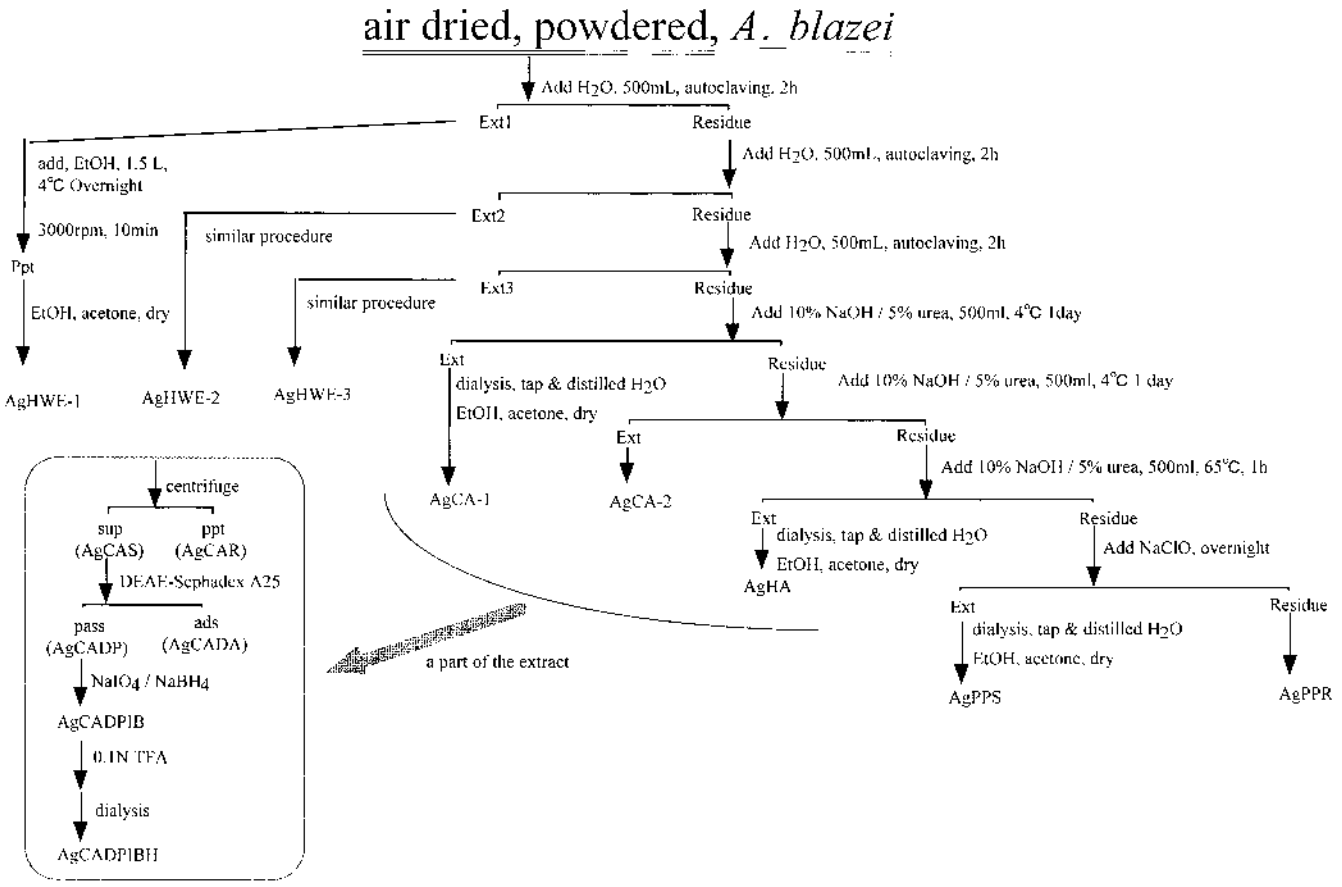

Chart 1. Preparation of *A. blazei* Extract and Derivatives

Table 1. Yield, Carbohydrate and Protein Content of *A. blazei* Extracts

Fraction	Yield	Carbohydrate	Protein	% Acidic component	
AgHWE-1	3.2	59.7	15.6	24.2	53.0
AgHWE-2	0.5	65.3	14.3	36.3	56.8
AgHWE-3	0.4	58.5	23.1	55.0	52.1
AgCA-1[a)]	1.8	57.0	30.9	51.9	56.9
AgCA-2[a)]	1.1	34.3	42.5	51.7	68.9
AgHA[a)]	0.7	56.6	13.4	40.6	71.1
AgPPS	1.2	60.5	14.5	n.d.	n.d.
AgPPR	3.0	n.d.	n.d.	n.d.	n.d.
	from 25 g	by Phenol–H_2SO_4	by BCA	by Phenol–H_2SO_4	by BCA

% acidic component was measured by a DEAE-Sephadex A25(Cl^-/8 M urea) column. *a)* Ratio of water soluble part (---S) was 64% (AgCA-1), 71% (AgCA-2), 94% (AgHA). n.d.; not done.

Table 2. Component Sugar Analysis of *A. blazei* Extract

Sample	Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc
HWE 1	—	2.2	3.6	—	—	8.0	10.1	100
HWE 2	1.6	2.1	4.6	1.1	0.8	32.9	14.3	100
HWE 3	0.9	1.4	5.2	—	—	8.0	9.7	100
CA1S	1.2	1.6	—	—	—	—	9.2	100
CA2S	1.0	1.3	—	—	—	3.1	9.6	100
CA1R	3.1	3.1	—	—	13.6	120.7	11.0	100
CA2R	—	—	—	—	13.7	23.6	—	100
HAS	3.2	—	—	—	—	—	7.7	100
HAR	—	10.1	—	—	—	13.1	75.9	100
PPS	1.9	—	—	1.7	—	2.4	3.0	100
PPR	1.0	0.9	—	2.0	—	7.8	7.1	100

Assessed by GLC using alditol acetate derivatives. Ratio of component sugar was shown using Glc as 100. —; not detected.

contrast, insoluble fractions (AgCAR, AgHAR) contained a significant proportion of xylose, mannose and galactose in addition to glucose. Because of the negligible antitumor activity (shown below, Table 4), only the soluble fractions were analyzed in more detail.

Characterization of Polysaccharide Fractions Carbon-13 NMR spectra of polysaccharide fractions are shown in Fig. 1. Assignment of the spectra was made by comparison with previously published spectra.[8—11)] The anomeric carbon signal apparent around 103 ppm was assignable as a β-configuration. Among the spectra of polysaccharide fractions, AgHWE3 and AgHA show clear and finely resolved signals. These fractions show only 6 major signals on the spectra, attributable to 1,6-β-glucosidic linkage. Major signals of other fractions also involved these 6 signals. AgHWE1 and AgCA1 show a signal around 100 ppm attributable to α-glucan. Of interest, the signal around 86 ppm assignable as the 1,3-β-linkage was not clearly detected. These facts strongly suggest that the major polysaccharide component of *A. blazei* is 1,6-β-glucan.

The antitumor activity of these fractions was examined by the solid form of Sarcoma 180 in ICR mice. As shown in Table 3, sodium hydroxide extracts showed significant activity; however, the hot water extracts and NaClO extract did not. Comparing the optimum dose, the strong activity only

appeared by high dose administration (2000 μg/mouse×5). Compared with the reference polysaccharide, sonifilan, it was 20 times less active. Multiple administration of AgHWE showed activity by peroral (*p.o.*) administration.

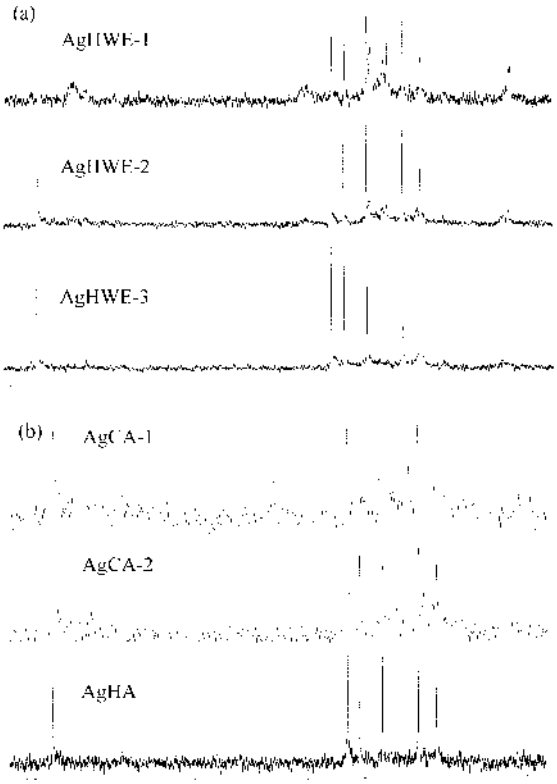

Fig. 1. ^{13}C-NMR Spectra of *A. blazei* Extracts

Twenty milligrams of a polysaccharide fraction was dissolved in 0.6ml of DMSO-d_6. ^{13}C-NMR spectrum was scanned at 70 °C overnight as described in Materials and Methods. (a) Hot water extracts, (b) sodium hydroxide extracts.

Previously we have screened the antitumor activity of various polysaccharides and found that 1,6-β-glucan, islandican from *Penicillium islandicum*, did not show antitumor activity against the solid form of Sarcoma 180. It is important to clarify the discrepancy between islandian and *A. blazei*. It is also difficult to understand why only sodium hydroxide extracts showed significant antitumor activity, because the major linkage of both HWE and sodium hydroxide extracts were 1,6-β-glucosidic linkage, and the average molecular weight of both fractions were similar (data not shown). Considering the relative activity of sodium hydroxide extracts and sonifilan, the content of the active material would be less than 5%, and might not be visible in NMR spectra. The limulus G-test is a specific reagent used to detect 1,3-β-glucan; it was thus applied to the extracts. As shown in Fig. 2, all of the extracts reacted with the limulus G-test, but the relative reactivity was low. If the G-test reactivity is responsible for 1,3-β-glucan, it might be degraded by 1,3-β-glucanase, zymolyase, and result in the loss of G-test reactivity. Figure 3 shows the reactivity of the extracts after zymolyase digestion, and the reactivity still remained. From these findings, the active components in the extracts are suggested to be complicated linkages of β-glucan.

Purification of Active Component from Sodium Hydroxide Extract To demonstrate the active component in the sodium hydroxide extract, it was fractionated, chemically modified, and the activity was examined. The sodium hydroxide extract contained water soluble and insoluble materials in a neutral aqueous condition. After separating the soluble and insoluble fractions, the activity was recovered from the water soluble fraction (Table 4, Exp-2, AgCA-1R, -1S). By sodium hypochlorite treatment, to degrade any proteinous moiety, the nitrogen content was significantly reduced to 0.51% by elemental analysis, and the activity remained (Table 4, Exp-3), similar to other β-glucans. By DEAE-

Table 3. Antitumor Activity of *A. blazei* Extracts against Solid Form of Sarcoma 180 in ICR Mice

Name	Dose (μg)	Times	Route	CR/n	Tumor weight mean±S.D. (g)	% Inhibition	*t*-Test to corresponding control
Control				0/12	8.6±4.3	0.0	
SPG	100	3	i.p.	7/11	0.4±1.1	95.3	<0.001
AgHWE-1	500	5	i.p.	0/10	*	−9.8	ns
	2000	5	i.p.	0/10	10.1±6.7	−18.5	ns
AgHWE-2	500	5	i.p.	0/10	*	5.0	ns
	2000	5	i.p.	0/10	9.2±5.5	−7.6	ns
AgHWE-3	500	5	i.p.	0/10	*	5.2	ns
	2000	5	i.p.	1/10	8.9±9.5	−4.5	ns
AgCA-1	500	5	i.p.	0/10	*	12.1	ns
	2000	5	i.p.	6/10	1.9±4.7	77.7	0.001
AgCA-2	500	5	i.p.	0/10	*	7.6	ns
	2000	5	i.p.	2/10	4.1±6.3	52.6	0.04
AgHA	500	5	i.p.	3/10	3.1±5.1	63.7	0.008
	2000	5	i.p.	8/10	0.1±0.2	99.3	<0.001
Control				0/10	15.0±6.5	0	
AgCWE	2000	35	*p.o.*	0/10	9.6±6.5	36	0.04
AgHWE	2000	35	*p.o.*	0/10	7.9±2.5	47	0.005
Control			i.p.	0/10	12.3±8.8	0	
AgPPS	1000	3	i.p.	0/9	11.7±11.2	5.0	ns

Dose: /mouse, times: day 7, 9, 11, or 7, 9, 11, 13, 15, CR/n: number of tumor free mice/total mouse, *: Measured diameter and calculated inhibition ratio against control group. ns: not significant.

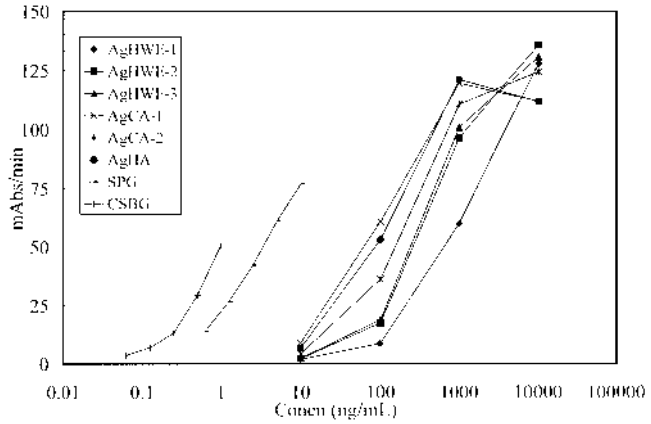

Fig. 2. Limulus G-Test Reactivity of *A. blazei* Extracts

Limulus G-test reactivity of extracts and reference compounds were examined and shown by specific activity (milliabsorbance/min).

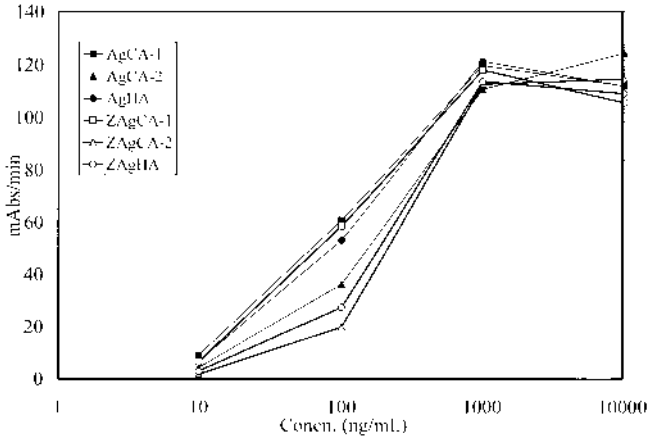

Fig. 3. Limulus G-Test Reactivity of *A. blazei* Extracts and Zymolyase Digests

Limulus G-test reactivity of extracts and zymolyase digests were examined and shown by specific activity (milliabsorbance/min). Z; zymolyase digests

Sephadex A25 chromatography, the activity was recovered from the passed through fraction (Table 4, Exp-4). By zymolyase treatment, the activity also remained (Table 4, Exp-5). By periodate oxidation, borohydride reduction, and subsequent partial acid hydrolysis, the activity remained (Table 4, Exp-6). These facts strongly suggest that the active substance is not a protein component, but β-glucan.

To examine the active substance more precisely, sodium hydroxide extract was fractionated in large scale. AgCA was dissolved once in sodium hydroxide and then neutralized to collect the water soluble fraction. After dialysis of the soluble fraction against distilled water, the retaintate was applied to a column of DEAE-Sephadex A-25. The passed through fraction (AgCADP) was oxidized with sodium metaperiodate, dialyzed, and then reduced with borohydride (AgCADPIB). AgCADPIB was dissolved in 0.1 N TFA and partially hydrolyzed at room temperature overnight, the resulting fraction was dialyzed against distilled water with an MWCO 3500 and MWCO 1000 dialysis membrane. The retaintate (MWCO 3500 NDF) was designated as AgCADPIBH. The ratio of MWCO3500 NDF : MWCO1000NDF : MWCO1000DF was 13 : 1 : 3.

Figures 4—6 show ^{13}C-NMR spectra of various fractions. Figure 4 shows the spectra of neutral fractions prepared by DEAE-Sephadex. In AgCADP, the signal intensity around 86 ppm, attributable to 1,3-β-glucosidic linkage, was increased. AgCADP contained minor signals around 100 ppm, attributable to α-glucan, and thus, ethanol precipitation (50% ethanol) was performed. After the treatment, α-glucan was selectively recovered from the soluble fraction and the precipitate was enriched into β-glucan (Fig. 5). These facts suggested that α- and β-glucans might not be linked to each other. Figure 6 shows spectra of periodate oxidation products. The signal around 86 ppm was more significant in the spectrum of AgCADPIB, and AgCADPIBH. These facts strongly suggested that the sodium hydroxide extract of *A. blazei* contained small but significant amounts of 1,3-β-glucosidic linkage.

To examine the 1,3-β-glucosidic linkage more precisely, zymolyzse digestion of chemically modified fractions were performed, and the resulting fractions were applied to the gel filtration chromatography using Toyopearl HW65. Of interest, the average molecular weight of only AgCADPIBH was significantly reduced by zymolyase digestion (Fig. 7). These facts strongly suggest that the 1,3-β-glucan segment present in the extract of *A. blazei* is highly branched, and resistant to zymolyase digestion.

The presence of a branched 1,3-β-linkage was also examined by an immunochemical method. We have previously established antisera against 6-branched 1,3-β-glucan, grifolan (anti-GRN). We tested the reactivity of AgCA against anti-GRN. Several polysaccharides were adsorbed onto the ELISA plate, and the reactivity was tested against serially diluted anti-GRN. As shown in Fig. 8, AgCA-1 reacted strongly with anti-GRN. These facts also suggested that *A. blazei* contained a small but significant amount of 1,3-β-glucosidic segment.

DISCUSSION

We have long been working on the relationship between the structure and immunomodulating activity of β-glucans, using *G. frondosa*,[8—10] *P. vesiculosa*,[15] *S. sclerotiorum*,[11—13] *S. commune*,[21—23] *O. lapidescens*,[14] *G. lucidum*,[42] *S. cerevisiae*,[17] *C. albicans*,[16] *M. furfur*,[43,44] and *S. crispa*.[19] During this period, in a preclinical animal study, we found that the activity was significantly dependent on the molecular weight, degree of branching, and conformation. The most abundant antitumor substance is a branched 1,3-β-glucan, and the optimum administration schedule for the solid form of Sarcoma 180 is around 20 μg/mouse for three times. In the case of *G. frondosa*, the optimum dose of the crude hot water extract was 2000 μg/mouse, and after purification of the branched 1,3-β-glucan, grifolan, it was reduced to 20 μg/mouse. The concentration of the active component was only 1%, thus, it could not be detected by physicochemical methodologies. In contrast, the concentration of the branched 1,3-β-glucan was significantly high in sodium hydroxide extracts. The solubility of the branched 1,3-β-glucan to physiological saline is usually very low, and it is a skeletal component of the cell wall; thus, partial alkaline hydrolysis would be important to solubilize the branched 1,3-β-glucan.

A. blazei is a mushroom widely used as a health food, with the expectancy of certain pharmacological activity in Japan. Bioactive substances of *A. blazei* have been reported from

Table 4. Antitumor Activity of Various Fractions of *A. blazei* against Solid Form of Sarcoma 180

Name	Dose (μg)	Times	Route	CR/n	Tumor weight mean±S.D. (g)	% Inhibition	*t*-Test to corresponding control
Exp2: solubility							
2-1							
control				0/11	9.3±4.9		
ScCA1	100	3	i.p.	2/10	0.9±1.9	90.6	0
AgCA-1	2000	5	i.p.	3/11	2.8±3.3	70.1	0.001
AgCA-1R	1000	5	i.p.	0/11	9.4±5.3	−0.8	0.486
AgCA-1S	1000	5	i.p.	8/11	0.1±0.4	98.4	0
2-2							
control				0/9	9.4±6.9		
ScCA1	100	3	i.p.	6/10	1.4±2.6	84.8	0.004
AgCA-1R	2000	3	i.p.	0/10	9.4±7.7	−0.4	0.495
AgCA-1R	500	3	i.p.	0/10	10.5±10.6	−12.2	0.390
Exp3: deproteinization							
control				0/11	9.3±4.9		
ScCA1	100	3	i.p.	2/10	0.9±1.9	90.6	0
GRN NaClO	1000	5	i.p.	1/11	4.1±4.2	56	0.007
AgCA-1 NaClO	1000	5	i.p.	1/11	3.6±4.5	61.6	0.005
Exp4: DEAE-Sephadex							
4-1							
control				0/10	7.0±5.9		
AgHA DP	1000	5	i.p.	9/10	0.0±0.0	99.9	0.002
AgHA DA	1000	5	i.p.	0/10	3.8±2.5	36.5	0.071
4-2							
control				0/9	9.4±6.9		
ScCA1	100	3	i.p.	6/10	1.4±2.6	84.8	0.004
AgHWE DP	500	3	i.p.	0/10	8.6±8.5	8.4	0.411
AgHWE DP	2000	3	i.p.	0/10	5.0±5.0	47.2	0.067
AgCA DP	100	3	i.p.	1/10	9.1±9.7	3.2	0.469
AgCA DP	500	3	i.p.	5/10	5.9±8.2	37.1	0.161
AgHA DP	100	3	i.p.	1/10	4.4±4.6	53.1	0.045
AgHA DP	500	3	i.p.	3/9	2.5±4.6	73.5	0.013
Exp5: zymolyase							
Control				0/10	7.0±5.9		
AgCA1	2000	5	i.p.	8/10	0.0±0.1	99.4	0.003
ZAgCA1	2000	5	i.p.	1/9	0.9±1.0	84.8	0.005
ZAgCA2	2000	5	i.p.	2/10	2.4±2.9	60.2	0.023
ZAgHA	2000	5	i.p.	4/10	1.0±2.1	83.2	0.006
Exp6: periodate oxidation							
6-1							
Control				0/10	12.4±8.8		
AgCA-1S IB	200	3	i.p.	8/10	0.04±0.09	99.7	0.001
AgCA-1S IB	1000	3	i.p.	3/10	5.8±11.3	52.8	0.05
6-2							
Control				0/9	9.4±6.9		
ScCA1	100	3	i.p.	6/10	1.4±2.6	84.8	0.004
AgCA DP IB	500	3	i.p.	5/10	1.6±5.2	82.4	0.007
AgCA DP IB	100	3	i.p.	8/10	0.2±0.4	98.0	0.002
AgCA DP IBH	500	3	i.p.	6/10	0.0±0.1	99.5	0.002
AgCA DP IBH	100	3	i.p.	9/10	0.0±0.0	99.9	0.002

Exp1 was shown in Table 3. Abbreviations: ScCA1: branched 1,3-β-glucan of *Sparaccis crispa*, --R: water insoluble fraction, --S: water soluble fraction, --DP: pass through fraction of DEAE-Sephadex, --DA: adsorbed fraction of DEAE-Sephadex, Z---: zymolyase digested fraction, ---IB: $NaIO_4/NaBH_4$ treated fraction, IBH: $NaIO_4/NaBH_4$/TFA treated fraction. Also see footnotes of Table 3.

several laboratories, such as polysaccharides, cytotoxic steroids, lectin, and antimutagens.[45—47] In spite of the fact that the most precisely examined antitumor substance in fungi is branched 1,3-β-glucan, however, it is well known that the major carbohydrate of *A. blazei* is a 1,6-β-glucan-protein complex, FIII-2-b. Kawagishi *et al*. precisely examined the relationship between the structure and antitumor activity of FIII-2-b, purified from the sodium hydroxide extract of the fruit body of *A. blazei*.[27,28] A significant contribution of the protein moiety of FIII-2-b was also speculated by the loss of antitumor activity by formolysis. In this study, we also confirmed the high content of 1,6-β-glucan in hot water, as well as sodium hydroxide extracts. However, the antitumor activity was significantly stronger in the sodium hydroxide extract. From our previous understanding using various fungal extracts, a linear 1,6-β-glucan segment might not con-

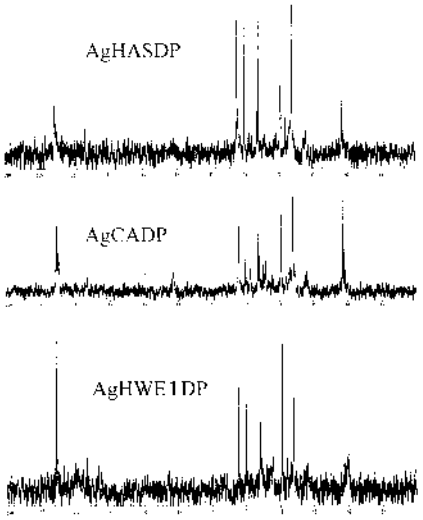

Fig. 4. ^{13}C-NMR Spectra of Neutral Fractions of *A. blazei* Extracts

Twenty milligrams of a polysaccharide fraction was dissolved in 0.6 ml of DMSO-d_6. ^{13}C-NMR spectrum was scanned at 70 °C overnight as described in Materials and Methods.

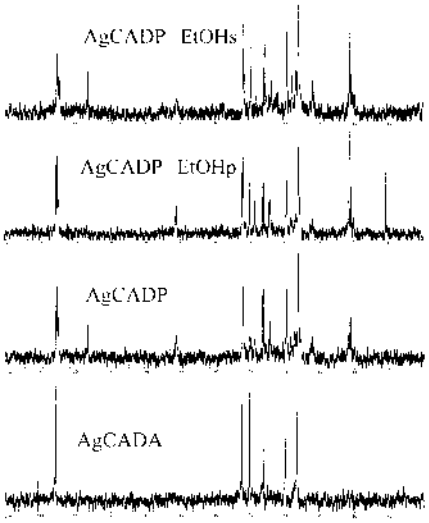

Fig. 5. ^{13}C-NMR Spectra of AgCA and Subfractions

Twenty milligrams of a polysaccharide fraction was dissolved in 0.6 ml of DMSO-d_6. ^{13}C-NMR spectrum was scanned at 70 °C overnight as described in Materials and Methods. DA: DEAE-Sephadex adsorbed fraction, DP: DEAE-Sephadex passed fraction, EtOHp: 1 vol. EtOH precipitate, EtOHs: 1 vol. EtOH soluble fraction.

tribute to the antitumor activity,[35] thus we tried to reevaluate the active structure in *A. blazei*. Comparing the optimum dose between extracts of *G. frondosa* and *A. blazei*, the relative activity of the cold sodium hydroxide extract of *A. blazei* was comparable with the hot water extract of *G. frondosa*. From the results of chemical and enzymic degradation, the active structure of the sodium hydroxide extract was resistant to periodate oxidation and 1,3-β-glucanase, zymolyase, digestion. However, after the periodate oxidation, borohydride reduction, and partial hydrolysis, the resulting product was sensitive to zymolyase digestion. We have previously examined the sensitivity of various 1,3-β-glucans to zymolyase digestion, and found that sonifilan and grifolan, those ratio of branch is 1/3, was sensitive, but SSG, the ratio of branch is 1/2, was resistant. In contrast to these findings, we have previously shown that OL-2, a highly branched 1,3-β-glucan obtained from *O. lapidescense* having a 2/3 branch, did not show significant activity. The contents of 1,3-β-glucan in the hot water extract and in the sodium hydroxide extracts were also examined by the limulus G-test, which is a diagnostic

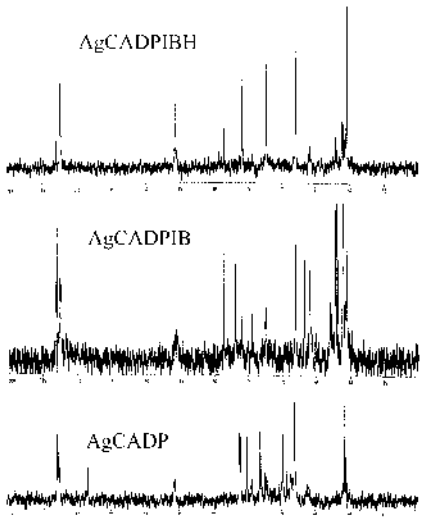

Fig. 6. ^{13}C-NMR Spectra of Periodate Oxidized Fractions

Twenty milligrams of a polysaccharide fraction was dissolved in 0.6 ml of DMSO-d_6. The ^{13}C-NMR spectrum was scanned at 70 °C overnight as described in Materials and Methods. IB: periodate oxidized and borohydride reduced, IBH: periodate oxidized, borohydride reduced, and partially hydrolized.

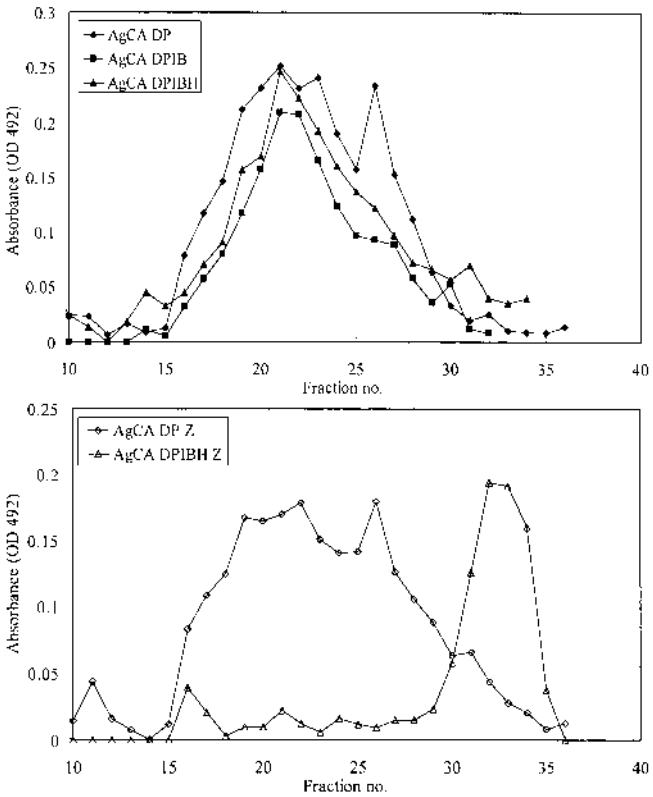

Fig. 7. Gel Filtration Chromatography of AgCA and Derivatives

An aliquot of a polysaccharide fraction dissolved in 0.3 N NaOH was applied to a column of Toyopearl HW-65F (1×45 cm) equilibrated with 0.3 N NaOH then fractionated. Eluted fractions were collected and monitored by the phenol–H_2SO_4 method. The elution volume of glucose as a reference was Fr. 34. IB: periodate oxidized and borohydride reduced, IBH: periodate oxidized, borohydride reduced, and partially hydrolyzed, Z: zymolyase digest.

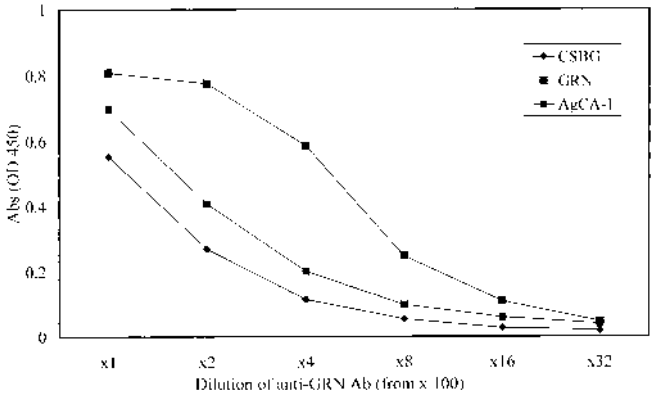

Fig. 8. Reactivity of *A. blazei* Extract to Anti GRN Antibody

Each 25 μg/ml solution of AgCA-1, GRN, and CSBG was coated on an ELISA plate and blocked. Serially diluted anti-GRN (biotinylated) was added, following by incubation. After extensive washing, antibody bound to the plate was determined by peroxidase conjugated with streptoavidine.

test specific for 1,3-β-glucan, and the higher content in the sodium hydroxide extract was confirmed. The presence of a branched 1,3-β-glucan segment in the sodium hydroxide extract was also supported by the reactivity to the anti-GRN antibody. Considering these results, it is strongly suggested that the linear 1,6-β-glucan segment, sensitive to periodate oxidation, did not contribute significantly to the activity, and that the ratio of the branch in 1,3-β-glucan segment of *A. blazei* is strongly suggested to be close to 1/2. Unfortunately, the content of the 1,3-β-glucan segment was low, thus we could not clarify the precise structure without periodate oxidation. Further study should be planned to demonstrate the interaction and/or linkage between 1,3-β-glucan and 1,6-β-glucans. It is already clarified in the yeast cell wall that the 1,3-β-glucan and 1,6-β-glucan were covalently linked in the cell wall.[16—18]

It is of note that we applied sodium hypochlorite degradation to AgCA1 and prepared a deproteinized fraction AgCA1 NaClO. The protein content of the native AgCA was 30—40%, and the resulting deproteinized fraction was assumed to be about 4%, as deduced from the nitrogen content (0.51%) of the elemental analysis. Even after this treatment, it showed significant antitumor activity by thrice i.p. administration (Table 4, experiment-3). Natural products usually co-exist with prokaryotes, and contamination of these metabolites might influence the activity. One such component is bacterial endotoxin. Endotoxin is well known to show various biological activity, beneficial as well as toxic. Endotoxin is known to contain an ester-bond essential for the biological activity, and is sensitive to sodium hydroxide treatment. These facts strongly suggest that a 1,3-β-glucan segment would be the most important part of AgCA, and the contribution of a protein component, as well as contaminated lipopolysaccharide, might be low.

The sodium hydroxide extract showed a VDH reaction. The VDH reaction reported by Maeda *et al*. was a T-cell-mediated vascular reaction which appeared following the administration of a neutral polysaccharide possessing antitumor activity, such as lentinan.[36—38] Dominant genes for VDH induction were recently analyzed by the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) technique, and one major gene (lentinan responsive gene, *Ltnr3*) and three minor genes were identified by Maeda *et al*.[38] Enhancement of the vascular permeability, especially around the tumor tissue, is an important property in establishing and rejecting tumor cells, because of the increase in leukocyte traffic to the tissue. A VDH reaction was induced not only in the tumor tissue but also in a variety of blood vessels. Although metastasis is one big problem in cancer therapy, it is usually difficult to identify the site of micrometastasis. The VDH reaction might help to transport leukocytes to the tumor tissue for micrometastasis, even in an unidentified location.

In this study we are mainly using the sodium hydroxide extract to demonstrate its antitumor substance, because relatively higher activity was shown by the crude extract (Table 3). Kawagishi *et al*. also showed that the most significant antitumor β-glucan–protein complex was prepared from the sodium hydroxide extract in higher yield than the hot water extract. In our study, each fraction was administered only 3 or 5 times to screen the activity. In this experimental condition, the hot water extract, as well as its partially purified fraction, AgHWE DP, did not show statistically significant activity. Additionally, peroral administration of AgHWE for 35 times show significant antitumor activity. These facts strongly suggest that structurally similar β-glucan would be extracted in the hot water extract, but to a smaller extent.

Cancer immunotherapy has been applied for many patients, and enhancement of the quality of life of the patients is also an important goal of cancer treatment. Establishment of a molecular mechanism for use in biotherapy is still needed. We have long been working on the structure and activity of branched 1,3-β-glucans. From the data shown in this paper, the polysaccharide fraction extracted in hot water was a less active compared with the sodium hydroxide extract. Considering our experiences, the activity of the hot water extract was weaker than that of *G. frondosa*, at least in this experimental model. But it might not indicate the clinical disadvantage of *A. blazei*. There are many differences in the animal study and human cancer, such as i) transplantation of the stable established tumor cell line, ii) strong antigenicity of the tumor, iii) rather normal immune responsibility, iv) the experimental period is only several weeks. We have also examined the efficacy of a cold water extract of *A. blazei* in the animal model, and determined that it is not due to the carbohydrate component (manuscript in preparation). Further study is required to extrapolate the results of experimental animals to human cancer.

Acknowledgments The authors wish to thank Mr. H. Fujiwara and Mr. J. Fujimoto for excellent technical assistance.

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