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Antitumor Activity and Structural Characterization of Polysaccharide Fractions Extracted with Cold Alkali from a Fungus, *Peziza vesiculosa*

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Polysaccharides in the fruit body of *Peziza vesiculosa* were extracted with cold aqueous sodium hydroxide. The antitumor effect and structural features of the extracted fractions were examined. The above extraction gave a water-soluble heteroglycan fraction and a water-insoluble glucan fraction. The structural characteristics of these polysaccharides were estimated from the results of methylation analysis and ^{13}C-nuclear magnetic resonance spectroscopy. The heteroglycan fraction was composed of glucose and mannose (20 : 1). The fraction contained a large amount of β-1,3-glucosidic linkages (about 64%) and a small amount of α-1,4-glucosidic linkages (about 30%). Some of the β-1,3-linked glucose residues included branching points. The water-insoluble fraction contained a large amount of β-1,3-linkages (about 90%). Both fractions showed potent antitumor activity against the solid form of sarcoma 180 tumor in ICR mice.

Keywords——polysaccharide; *Peziza vesiculosa*; antitumor agent; vesiculogen; β-1,3-glucan

Introduction

Peziza vesiculosa is a fungus belonging to the Ascomycotina, Pezizales, Pezizaceae. The hot water extract of the fruit body, named "Vesiculogen" was found to possess potent immunomodulating activities such as B cell mitogenic activity,[1] polyclonal B cell activator activity,[2] activity to enhance the function of the reticuloendothelial system and antitumor activity.[3] Vesiculogen is composed of protein (*ca.* 60%), carbohydrate (*ca.* 30%), and a small amount of amino sugar, uronic acid, phosphate, and lipid.[4] However, it is not certain whether all of these activities result from only one active substance or not.

Recently, we obtained antitumor-active glucans from the cultured fruit bodies of *Grifola frondosa*, which belongs to Basidiomycotina, by extraction with hot water and sodium hydroxide. The latter extract possessed stronger antitumor activity than the former.[5] Including the case of the above glucan, typical antitumor materials from fungi include the β-glucans obtained from Basidiomycotina such as lentinan (*Lentinus edodes*)[6] and schizophyllan (*Schizophylum commune*).[7] Antitumor-active glucans have also been obtained from Ascomycotina, *e.g.*, scleroglucan (*Sclerotium glucanicum*)[8] and PS-1426 (*Pseudoplectania nigrella*).[9] To study the immunomodulating activities of *P. vesiculosa* in detail, the cold sodium hydroxide extract of the fruit body was examined.

In this paper, we describe the structural characterization and the antitumor activity of alkali extracts on transplantable sarcoma 180 tumor in ICR mice.

Materials and Methods

Isolation of Each Polysaccharide Fraction——As shown in Chart 1, the dried fruit bodies were boiled in water

and filtered (vesiculogen). The residue was extracted by stirring with 10% sodium hydroxide containing 5% urea for 24 h at 4 °C. The extract was neutralized with acetic acid and dialyzed extensively against tap water and distilled water. The above extraction procedure was repeated 6 times. The precipitates formed during the dialysis were collected by centrifugation (PVA-P). The supernatant was concentrated and lyophilized (PVA-S).

Analytical Methods——Other methods used in this paper, such as those for quantitative analysis, methylation, ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy, and the measurement of antitumor activity, were described in the previous paper.[5]

Results and Discussion

The source of fractions examined in this paper was the hot-water extracted residue of *P. vesiculosa*. The antitumor activity of the hot-water extract, named vesiculogen, was reported in the previous paper.[3] The fractions were prepared by alkaline extraction of the residues as shown in Chart 1. When the alkaline extracts were neutralized with acetic acid and then dialyzed, a brown precipitate was formed. The water-soluble fraction was named PVA-S and the insoluble fraction, PVA-P. As shown in Table I, the yield of PVA-S (22%) was more than four times that of PVA-P (5%). Major constituents of both PVA-S and PVA-P were carbohydrates (about 60%). These fractions also contained protein (about 20%) and small amounts of amino sugar, uronic acid, and phosphate (data not shown). By component sugar analysis using gas liquid chromatography, (GLC), PVA-S was found to be composed of glucose and mannose (20 : 1) (heteroglycan) and PVA-P was found to be composed of only glucose (glucan).

The antitumor effect of each fraction was assayed by comparing the growth of solid-form sarcoma 180 tumor cells in mice (Table II). It was found that PVA-S and PVA-P showed growth inhibition of more than 90% ($p<0.001$) at 500 μg/mouse × 10 d. PVA-S gave a higher complete regression (C.R.) ratio than PVA-P at 500 μg/mouse × 10 d.

The most abundant carbohydrate linkage in PVA-S was found to be β-1,3-linkage (64%), as estimated from the results of methylation analysis (Table I) and ^{13}C-NMR spectroscopy (Fig. 1, b, C-1 at 103 ppm). The other linkage in PVA-S was α-1,4-linkage (30%). The results

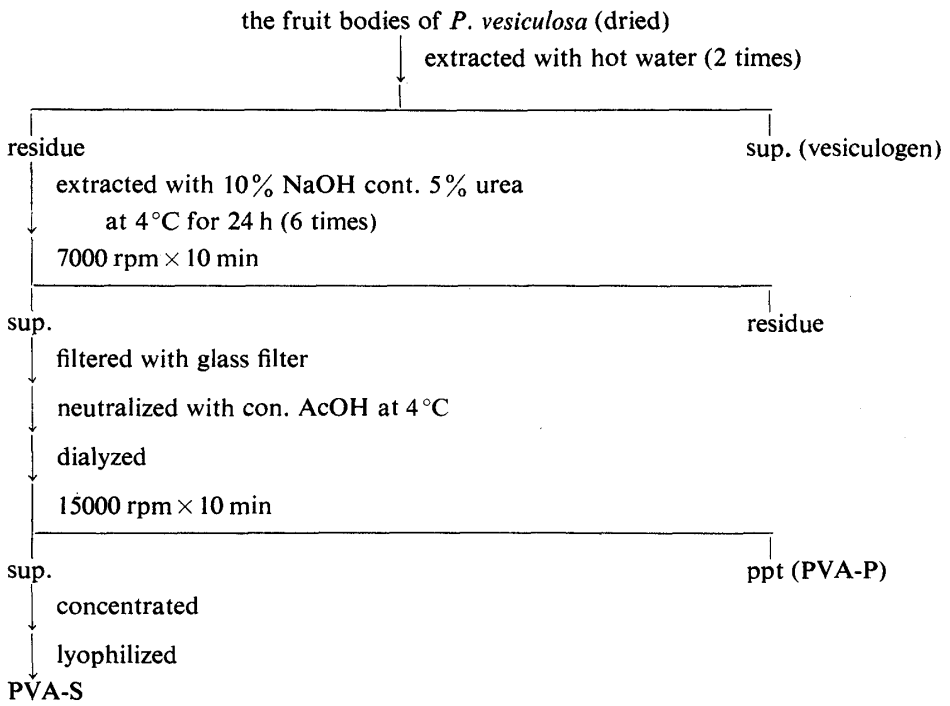

Chart 1. Isolation of the Polysaccharide Fractions from the Fruit Bodies of *P. vesiculosa*

TABLE I. Some Properties of the Polysaccharide Fractions

	PVA-S	PVA-P	PF-5
Yield (%)[a]	22	5	nd[b]
Carbohydrate (%)	56	60	nd
Protein (%)	20	18	nd
Component sugar[c]	Glc, Man (20:1)	Glc	nd
Methylation analysis[d]			
2,3,4,6-Me_4-Glc	1.00	1.00	1.00
2,4,6-Me_3-Glc	3.15	8.40	1.78
2,3,4-Me_3-Glc	0.03	0.20	0.73
2,3,6-Me_3-Glc	1.38	0.20	0.21
2,6-Me_2-Glc	—	—	—
3,4,6-Me_3-Glc	0.35	0.44	—
2,4-Me_2-Glc	1.12	1.42	1.08
2,3-Me_2-Glc	0.19	—	—
3,6-Me_2-Glc	—	—	—

a) From 40 g of the fruit bodies. *b*) Not determined. *c*) Determined as alditol acetate derivatives by GLC. Glc, glucose; Man, mannose. *d*) Only partially *O*-methylated alditol acetates of glucose residues are listed.

TABLE II. Antitumor Effect of Polysaccharide Fractions from *P. vesiculosa*

Sample	Dose ($\mu g \times 10$)	No. of mice	Tumor weight (g, mean ± S.D.)	Inhibition (%)[b]	C.R. (%)[b]	Significance p[c] <
Control	—	18	4.7±2.4	—	0	—
PVA-S	20	9	4.2±2.8	10.6	0	ns[d]
	100	9	2.5±2.4	46.9	0	0.05
	500	9	0.3±0.7	93.6	33	0.001
PVA-P	20	9	5.0±2.8	−6.4	0	ns
	100	11	2.7±2.3	42.1	9	0.05
	500	11	0.4±0.5	91.5	9	0.001

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered for 10 consecutive days as a saline solution or suspension by intraperitoneal injection. *b*) Inhibition and C.R. (complete regression) were determined at 35 d after tumor inoculation. *c*) The significance was evaluated according to Student's *t*-test with $p < 0.05$ as the criterion of a significant difference. *d*) Not significant.

were confirmed by methylation analysis of α-amylase-digested PVA-S (data not shown). Similarly, from the methylation data and ^{13}C-NMR spectrum, the most abundant carbohydrate linkage in PVA-P was found to be β-1,3-linkage. PVA-P contained small amounts of 1,4-, 1,2-, and 1,6-linkage. In general, the ^{13}C-NMR spectra of linear and branched β-1,3-glucan show different patterns.[5] The reference compounds used in this paper were curdlan (Fig. 1, d, linear; *Alcaligenes faecalis* var. *myxogenes* 10C3, purchased from Wako Pure Chemical Industries, Ltd.) and PF-5 (Fig. 1, a, branched). PF-5 is an antitumor glucan obtained from *G. frondosa* and it possesses a 6-branch for every three 3-substituted β-glucosyl units.[5] The NMR spectra of PVA-S and PVA-P were similar to the spectrum of curdlan (Fig. 1). These observations suggest that the numbers of branching points of the β-1,3-glucans in PVA-S and PVA-P were less than in PF-5 (Table I, Fig. 1).

Antitumor polysaccharides have been identified in many kinds of Basidiomycotina and Ascomycotina (*e.g.* lentinan, schizophyllan, PS-1426, scleroglucan, and PS-K). The above polysaccharides can be classified into two types. One type is composed of only carbohydrate, being 6-branched β-1,3-glucans (lentinan, schizophyllan, PS-1426, and scleroglucan). The

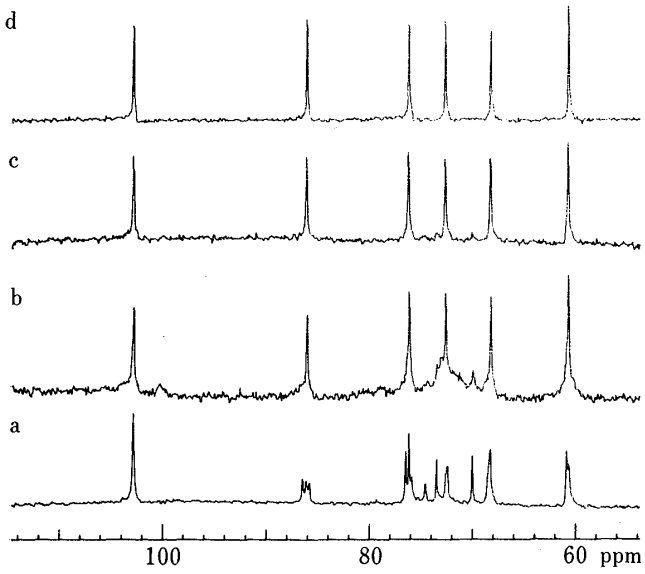

Fig. 1. ^{13}C-NMR Spectra of Polysaccharide Fractions in DMSO-d_6 at 60 °C

a, PF-5; b, PVA-S; c, PVA-P; d, curdlan.

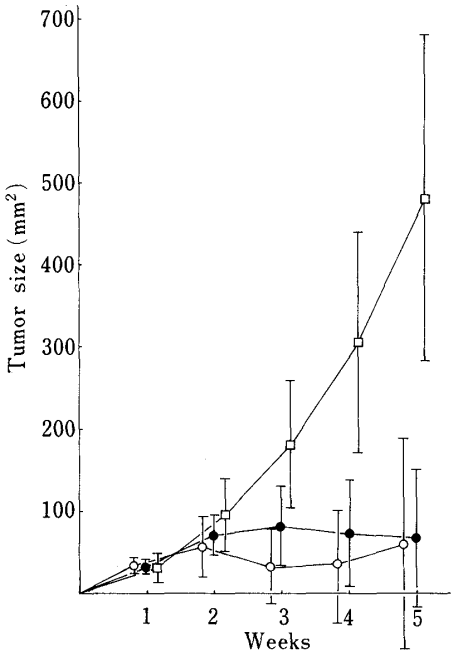

Fig. 2. Kinetics of Antitumor Effect on the Solid Form of Sarcoma 180

Mice were administered *i.p.* with 500 μg of PVA-S and PVA-P for 10 successive days after tumor inoculation.

○—○, PVA-S; ●—●, PVA-P; □—□, control (saline).

other type, such as PS-K,[10] contains both of carbohydrate and protein. As described in this paper, PVA-S and PVA-P contained a small amount of protein (about 20%). It is not certain whether the protein contained in PVA-S and PVA-P is necessary for the antitumor activity. However, by using gel filtration with Sepharose CL-2B, the carbohydrate peak and protein peak in PVA-S and PVA-P were separated (data not shown). This result suggests that the protein part was not bound with the carbohydrate part. Furthermore, in the case of lentinan, tumor weight is decreased from 2 or 3 weeks after inoculation.[11] Similar observations were made in the cases of PVA-S and PVA-P (Fig. 2). Therefore, the antitumor activity of PVA-S and PVA-P may also be due to the presence of β-1,3-glucan.

PVA-S and PVA-P showed potent antitumor effects on sarcoma-180 cells at the same dose (500 μg/mouse × 10), although they contained different numbers of 6-branches (Table I). This observation suggests that, in the case of *P. vesiculosa* the number of 6-branches on the β-1,3 main chain has relatively little effect on antitumor activity.

Vesiculogen, which is a fungal immunomodulator extracted with hot water from *P. vesiculosa*, possesses potent antitumor activity[3] and various immunomodulating activities such as B cell mitogenic activity,[1] polyclonal B cell activator activity,[2] activity for enhancing the function of the reticuloendothelial system, and activation of macrophages *in vitro*.[12] It was not certain which components were responsible for these activities. In our laboratory, the antitumor activity of the extracts from an edible mushroom, *G. frondosa* has been examined.[5] In the case of *G. frondosa*, both the hot water and alkali extracts showed potent antitumor activity. The activity of the alkali extract was more potent, because the alkali extract contained more 6-branched β-1,3-glucan than the hot water extract. These observations and the results described in this paper suggest that the antitumor activity of Vesiculogen is at least partly due to a similar glucan extracted with aqueous sodium hydroxide. Furthermore, it has been clarified that Vesiculogen possesses antitumor effects against both solid and ascites-form

sarcoma 180.[3] Generally, antitumor glucans are not effective against ascites-form tumors. It may be speculated that the antitumor effect of vesiculogen is due to the cooperation of β-1,3-glucan and other immunomodulating substances from *P. vesiculosa*. Detailed studies of the alkali extracts are required to clarify the active components of vesiculogen. Purification and structural characterization of the β-1,3-glucan from PVA-S are in progress.

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