[Chem. Pharm. Bull. 33(11)5096—5099(1985)]

Purification, Antitumor Activity, and Structural Characterization of β-1,3-Glucan from *Peziza vesiculosa*

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(Received March 20, 1985)

An antitumor β-1,3-glucan (PVG) was purified from PVA-S by sequential use of α-amylase digestion, ethanol precipitation, ion exchange chromatography on diethyl aminoethyl Sephadex A-25, ammonium sulfate fractionation, and gel filtration on Sepharose CL-2B. PVG showed $[\alpha]_D$ +28.4° (water). From the results of methylation analysis and carbon 13 nuclear magnetic resonance spectroscopy, PVG is a β-1,3-glucan branched at position 6 of every fifth 3-substituted β-glucosyl unit. PVG formed a complex with Congo Red in neutral and dilute alkali solution, but this complex was dissociated at more than 0.2 N NaOH. PVG showed potent antitumor activity against the solid form of sarcoma 180 tumor in ICR mice.

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

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.[1] However, it is not certain whether all of these activities result from only one active substance or not.

Typical antitumor materials from fungi, such as lentinan (*Lentinus edodes*),[2] schizophyllan (*Schzophylum commune*),[3] scleroglucan (*Sclerotium glucanicum*),[4] and PS-1426 (*Pseudoplectania nigrella*),[5] are almost always β-1,3-glucans. Recently, we obtained antitumor-active glucan fractions, named PVA-S and PVA-P, from the residue of hot water extract of *P. vesiculosa* by extraction with aqueous sodium hydroxide.[6] PVA-S and PVA-P contained β-1,3-glucan. To confirm the nature of the antitumor-active substance of PVA-S and to estimate the antitumor activity of vesiculogen, β-1,3-glucan was purified from PVA-S.

In this paper, we describe the purification, the structural characterization, and the antitumor activity of the purified β-1,3-glucan.

Materials and Methods

Materials——Sepharose CL-2B and diethyl aminoethyl (DEAE) Sephadex A-25 were obtained from Pharmacia, and α-amylase (No. A-6380) were obtained from Sigma.

Purification of β-1,3-Glucan from *Peziza vesiculosa*——The fruit bodies of *P. vesiculosa* were boiled in water and filtered (vesiculogen). The residue was extracted by aqueous sodium hydroxide as described previously.[6] Briefly the procedure is as follows. 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 non-dialyzable fraction was centrifuged, and the supernatant was concentrated, and lyophilized (PVA-S, 22%).

PVA-S (3.0 g) was dissolved in 50 mM Tris–HCl buffer, (pH 6.9, 2250 ml) and digested with α-amylase (60 mg) at 37 °C, for 24 h. The reaction was terminated by heating at 100 °C for 5 min, and the mixture was dialyzed against tap water and distilled water. The non-dialyzable fraction was concentrated and precipitated with 0.5 vol. of ethanol. The precipitate was collected, and dried with acetone then ether (yield; 49%). The resulting materials were pooled. A

portion (4 g) of the pooled material (4.4 g) was dissolved in 10% sodium hydroxide containing 5% urea and dialyzed against 0.1 M potassium phosphate buffer containing 0.1 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.1). The non-dialyzable fraction was applied to a column (5 × 10 cm) of DEAE Sephadex A-25 (Cl^-) equilibrated with the same buffer. The passed fraction was collected, dialyzed against tap water and distilled water, and precipitated with 4 vol. of ethanol (15%). A portion (425 mg) of the precipitate (600 mg) was dissolved in 10% sodium hydroxide containing 5% urea and dialyzed against distilled water. The non-dialyzable fraction was concentrated and precipitated by 25% saturation of ammonium sulfate. The precipitate was collected, dialyzed against distilled water, and lyophilized (68%; PVA-S-25P). A portion (250 mg) of the precipitate (290 mg) was dissolved in 0.25 N sodium hydroxide and applied to the column (2 × 90 cm) of Sepharose CL-2B equilibrated with 0.25 N NaOH. The fractions containing carbohydrate were collected, and dialyzed against distilled water. The non-dialyzable fraction was concentrated and lyophilized to give PVG (194 mg).

Analytical Methods——Other methods used in this paper, such as those for quantitative analysis, methylation, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, and the measurement of antitumor activity, were described in the previous paper.[7]

Complex Formation with Congo Red——The change of absorption of Congo Red (Wako Pure Chemical Co.) in the presence of glucans was performed by the procedure of Ogawa *et al.*[8] Glucan solutions (1 mg/ml) and 8.64×10^{-5} M Congo Red were mixed in equal volumes, and λ_{max} was measured using a Hitachi 557 spectrophotometer.

Results and Discussion

A β-1,3-glucan (PVG) was purified from PVA-S, which is a water-soluble polysaccharide fraction of the alkaline extracts from *P. vesiculosa* fruit body, by sequential use of α-amylase

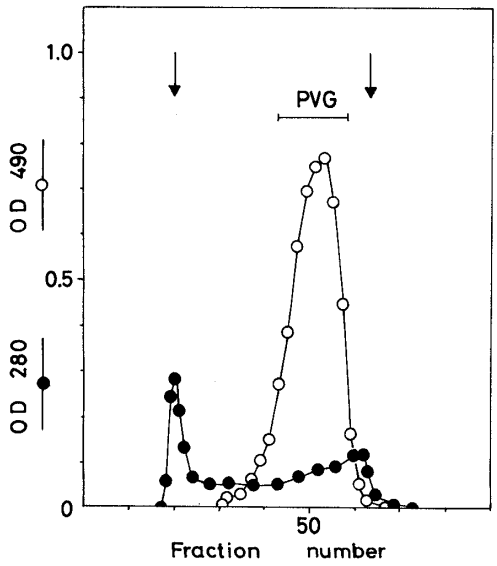

Fig. 1. Elution Profiles of PVA-S-25P on Sepharose CL-2B

PVA-S-25P (50 mg) was dissolved in 0.25 N NaOH and applied to a Sepharose CL-2B column (2 × 90 cm) equilibrated with 0.25 N NaOH. Fractions of 4 ml were collected at 4 °C, and fractions from No. 40 to No. 58 were pooled as PVG. ●, absorbance at 280 nm; ○, absorbance at 490 nm for neutral sugars. Arrows indicate the void and the bed volumes.

TABLE I. Antitumor Effect of PVG[a]

Samples	Dose (μg × 10)	No. of mice	Tumor weight[c] (g, mean ± S.D.)	Inhibition[b] (%)	C.R.[b] (%)
Control	—	19	3.57 ± 2.70	—	—
PVG	18.75	10	0.52 ± 0.83[d]	86	0
	37.5	10	0.52 ± 1.55[d]	85	60
	75	10	0.06 ± 0.08[d]	98	30
	150	10	0.12 ± 0.34[d]	97	40
	300	10	0.34 ± 0.49[d]	91	30

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered for 10 consecutive days as a saline solution 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. Significant difference from the control (*d*) $p < 0.001$).

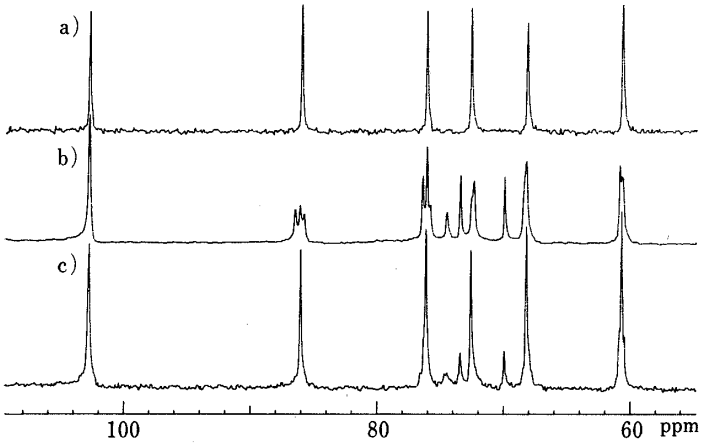

Fig. 2. ^{13}C-NMR Spectra of PVG in DMSO-d_6 at 60 °C
a, curdlan; b, grifolan NMF-5N; c, PVG.

digestion, ethanol precipitation, ion exchange chromatography on DEAE Sephadex A-25, ammonium sulfate fractionation, and gel filtration on Sepharose CL-2B. The glucan fraction showed a single and symmetrical peak on elution from Sepharose CL-2B (0.25 N NaOH, Fig. 1). The yield of PVG was 5%, and PVG was composed of only carbohydrate (glucose 88% as anhydroglucose). Protein, amino sugar, uronic acid, and phosphate were not detected.

The antitumor effect of PVG was assayed against solid form sarcoma 180 tumor cells in mice (Table I). It was found that PVG showed growth inhibition of more than 90% at 75, 150, 300 μg/mouse × 10 d, and a significant difference (Student's t-test; $p<0.001$) from the control was obtained at all doses in the experiment.

PVG had $[\alpha]_D$ +28° (water). Methylation analysis of PVG gave 2,3,4,6-Me_4-Glc, 2,4,6-Me_3-Glc, and 2,4-Me_2-Glc in the molar ratio of 1.00 : 4.20 : 1.15, and 3,4,6-Me_3-Glc (0.04 compared with 2,4,6-Me_3-Glc) was also obtained. The ^{13}C-NMR spectrum (Fig. 2) of PVG in Me_2SO-d_6 showed signals similar to those of curdlan (Fig. 2, a; *Alcaligenes faecalis* var. *myxogenes* 10C3, purchased from Wako Pure Chemical Industries, Ltd.), which is a linear β-1,3-glucan. The ^{13}C-NMR spectrum of PVG also showed 6-branch signals (Fig. 2, c at 70, 73.5, 74.5 ppm) similar to those of grifolan NMF-5N (Fig. 2, b; *Grifola frondosa*), which is a β-1,3-glucan possessing a 6-branch for every three 3-substituted β-glucosyl units.[9] These observations suggest that PVG is a β-1,3-glucan branched at position 6 of every fifth 3-substituted β-glucosyl unit.

Under physiological conditions, β-1,3-glucans are known to form helical conformation.[10] Helix-forming glucans such as curdlan induce metachromasy of Congo Red because of the complex formation between the glucans and Congo Red. PVG also induced metachromasy of Congo Red at below 0.2 N NaOH (498 nm).

These results suggest that the antitumor-active substance of PVA-S is a β-1,3-glucan branched at position 6 of every fifth 3-substituted β-glucosyl unit, and that PVG is also a helix-forming glucan. As described in the previous paper, two glucan fractions were extracted from *P. vesiculosa* with 10% sodium hydroxide containing 5% urea. These fractions showed antitumor effect and contained β-1,3-glucans which possessed different numbers of branches at position 6. It is not yet certain whether the number of branches at position 6 of β-1,3-glucan is an average value or reflects a regular structure.

As described previously, vesiculogen, a hot water extract of *P. vesiculosa*, showed antitumor activity,[1c] but the active substance was not identified. Recently, we isolated an antitumor glucan, grifolan, from *G. frondosa* by extraction with hot water, cold alkali, and hot alkali. Although the content of the active glucan was smallest in the hot water extract, the

primary structures of active glucan contained in each extract were the same.[7,9a)] Thus, the antitumor activity of vesiculogen might be due to the presence of a PVG-like antitumor glucan. To examine this possibility, vesiculogen was bleached with H_2O_2 and metachromasy due to complex formation between β-1,3-glucans and Congo Red were examined. The absorption maximum of Congo Red (488 nm) was shifted by the fraction to λ_{max} 510. The complex between PVG and Congo Red was dissociated at concentration higher than 0.2 N NaOH, but the complex between bleached vesiculogen and Congo Red was not dissociated even when the concentration of sodium hydroxide was increased to 0.5 N (data not shown). These findings suggest that an antitumor β-1,3-glucan is contained in vesiculogen, and is at least partly responsible for the antitumor effects of vesiculogen.

Acknowledgments The authors thank Mr. K. Nunomura for cooperation in collecting the fungus, and Mr. T. Takeyama, Mr. H. Watari, Miss J. Sato, and Miss Y. Yoshii for their technical assistance.

References

1) *a*) T. Yadomae, I. Suzuki, Y. Kumazawa, and T. Miyazaki, *Microbiol. Immunol.*, **23**, 997 (1979); *b*) I. Suzuki, H. Yonekubo, T. Yadomae, K. Nunomura, and T. Miyazaki, *ibid.*, **26**, 275 (1982); *c*) I. Suzuki, T. Yadomae, H. Yonekubo, M. Nishijima, and T. Miyazaki, *Chem. Pharm. Bull.*, **30**, 1066 (1982).
2) G. Chihara, Y. Y. Maeda, J. Hamuro, and F. Fukuoka, *Nature* (London), **222**, 687 (1969); G. Chihara, J. Hamuro, Y. Y. Maeda, Y. Arai, and F. Fukuoka, *Cancer Res.*, **30**, 2776 (1970).
3) N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Sato, and S. Sasaki, *Gann*, **60**, 137 (1969).
4) J. Hamuro and G. Chihara, *Nature* (London), **245**, 40 (1973).
5) Y. Hirata, H. Uchida, K. Nakajima, A. Obayashi, and T. Sasaki, The 40th Annual Meeting of the Japanese Cancer Association, Sapporo, October 1981.
6) N. Ohno, H. Mimura, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, **33**, 2564 (1985).
7) N. Ohno, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **32**, 1142 (1984).
8) K. Ogawa, J. Tsurugi, and T. Watanabe, *Chem. Lett.*, **1972**, 689.
9) *a*) N. Ohno, K. Iino, T. Takeyama, K. Sato, S. Oikawa, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **33**, 3395 (1985); *b*) M. Rinaudo and M. Vincendon, *Carbohydr. Polymer*, **2**, 124 (1982).
10) T. Norisuye, T. Yanaki, and H. Fujita, *J. Polym. Sci.*, **18**, 547 (1980).