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Chemical Characterization of a Fungal B-Cell Mitogen Obtained from the Fruit Body of *Peziza vesiculosa*

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The chemical properties of a fungal B-cell mitogen obtained from the fruit body of *Peziza vesiculosa* were investigated. The non-dialyzable fraction (vesiculogen) of the hot water extract of *P. vesiculosa* was separated into mitogenic and non-mitogenic fractions by ammonium sulfate fractionation. The mitogenic fraction (precipitated at 20 to 60% ammonium sulfate) was composed of 75—80% protein and 17—19% carbohydrates. The mitogenic fraction was separated into 4 representative fractions by diethylaminoethyl-Sephadex A-25 and hydroxyapatite chromatographies, sequentially. In Sepharose CL-2B chromatography, all of the fractions showed wide ranges of molecular weight distribution. All of these representative fractions from Sepharose CL-2B were similar in amino acid compositions (rich in Ser, Glu, Gly, and Ala). These findings suggest that the mitogen is a high-molecular-weight, acidic polypeptide, showing charge and molecular weight heterogeneities.

Keywords——B-cell mitogen; fungi; *Peziza vesiculosa*; vesiculogen; heterogeneity

Introduction

Hot water extracts from various fungi including mushroom and yeast show various immunomodulating activities, such as mitogenic activity, polyclonal B-cell activation activity, and antitumor activity.[1] These substances are used as (1) traditional crude drugs for cancer therapy, (2) reagents for stimulating the immune system, and (3) reagents for studying the immune system. Recently, we have reported that the hot water extracts from mushrooms belonging to Ascomycotina also show immunomodulating activities.[2] The hot water extract (vesiculogen) of one of these fungi, *Peziza vesiculosa*, has various immunomodulating activities.[3] Vesiculogen was shown to be a B cell mitogen and to act as a polyclonal B cell activator (PBA), and it also showed *in vivo* and *in vitro* adjuvant activity and antitumor activity. However, the chemical properties of vesiculogen are not well established.[4] The mitogenicity of vesiculogen was found to be due to the presence of anionic groups by means of chemical and enzymic modification studies.

β-1,3-Glucan is one of the antitumor agents obtained from various fungi.[5] *P. vesiculosa* also contains antitumor β-1,3-glucans extractable by alkaline solvent.[6] However, preliminary experiments showed that the mitogenic activity was not owing to the β-1,3-glucan moiety, because the mitogenic activity was sensitive to alkaline solvents and to carboxyl-modifying reagents.[4]

This paper is concerned with the chemical characterization of the mitogenic substance (VGM) of vesiculogen.

Materials and Methods

Preparation of the Hot Water Extract of *Peziza vesiculosa* (Vesiculogen)——*Peziza vesiculosa* was obtained from

local fields and air-dried. Vesiculogen was prepared by the method previously described.[3] Briefly, the dried fruit bodies were boiled in water. After filtration, the filtrate was dialyzed against distilled water and the non-dialyzable fraction was recovered by lyophilization. This preparation is referred to as "vesiculogen."

Ammonium Sulfate Fractionation of Vesiculogen——Vesiculogen was dissolved in water (10 mg/ml), and powdered ammonium sulfate was added in steps of 20% saturation. After centrifugation and dialysis, each precipitated fraction was obtained. Yields and other physicochemical properties are listed in Table I.

Preparation of Representative Fractions——The precipitate obtained at 40% saturation of ammonium sulfate (ppt. 40) was dissolved in 0.1 M potassium phosphate buffer (pH 7.1) containing 0.1 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA), and applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (Cl^-) equilibrated with the same buffer. The column was initially eluted with the same buffer (PASS), and then eluted with the above buffer containing 1 M NaCl (ABS).

After dialysis against water and then 0.01 M phosphate buffer (pH 6.8), PASS fraction was applied to a column of hydroxyapatite equilibrated with the same buffer. The column was eluted with the same buffer (PP), and then with a linear gradient from 0.01 to 0.4 M phosphate buffer (pH 6.8). Fractions eluted at 0.15 to 0.4 M were collected (PA).

The fraction ABS was rechromatographed on a column of DEAE-Sephadex A-25 and eluted with a linear gradient from 0.1 to 1 M NaCl in the above buffer. The fractions eluted at 0.1 to 0.5 M NaCl were collected and dialyzed, and applied to a column of hydroxyapatite under conditions similar to those used for the fraction PASS. The fractions eluted at 0.01 M (AP) and 0.05 to 0.15 M (AA) were collected separately, dialyzed, and then lyophilized.

Four fractions (PP, PA, AP, AA) were applied to Sepharose CL-6B, and the mitogenic fraction (void volume) was fractionated on a column of Sepharose CL-2B eluted with saline, respectively. Fractions 15 to 30 were collected (Fig. 7).

Assay of Mitogenicity——Mice (C3H/HeN) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. Assay of the mitogenicity was performed by the method previously described.[4] Briefly, the fractions (50 μl) in flat-bottomed microtiter plates (Sumitomo Behkuraito Co., Tokyo) were mixed with 100 μl of spleen cell suspension (5×10^6/ml) in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) containing 5% heat-inactivated fetal calf serum (FCS). The mixture was incubated for 48 h in a CO_2 incubator (5% CO_2, 95% air) at 37 °C. Thymidine-^{3}H (0.5 μCi; specific activity, 22.5 Ci/mmol) (NET-355,[6-^{3}H]TdR, New England Nuclear, Boston, Massachusetts, U.S.A.) was added to each well for the last 20 h of the culture. The cultures were harvested with a Labo Mash multiple cell harvester (Labo Science Co., Ltd., Tokyo, Japan) on glass fiber filters. Thymidine incorporated into the cells was determined with a liquid scintillation counter (Aloka 903, Tokyo, Japan) with an Omnifluor/toluene scintillator. Results were expressed as an arithmetic mean cpm ± S.D. of duplicate or triplicate cultures.

Analytical Methods——Amino acid analysis was performed in a Hitachi 835 auto amino acid analyzer after hydrolysis with 6 N HCl at 100 °C for 24 h. Other analytical methods were performed as described previously.[4]

Results

Ammonium Sulfate Fractionation of Vesiculogen

Vesiculogen is a non-dialyzable fraction obtained from the fruit body of *P. vesiculosa* by hot water extraction. For purifying the mitogenic substance(s) (VGM) from vesiculogen, ammonium sulfate fractionation was performed as the first step. An aqueous solution of vesiculogen (10 mg/ml) was precipitated with stepwise increases of 20% saturation of ammonium sulfate. Each fraction was dissolved in water and then dialyzed against water. The recovery and chemical composition of each fraction are listed in Table I and the mitogenic

Table I. Chemical Properties of Ammonium Sulfate-Fractionated Vesiculogen

Fraction	Yield (%)	Protein (%)	Carbohydrate (%)	Phosphate (%)
Vesiculogen	—	50—60	20—30	<4
ppt. 20	10	75	17	1
ppt. 40	13	80	17	1
ppt. 60	13	74	19	2
ppt. 80	12	56	18	7
ppt. 100	7	46	19	7
sup. 100	17	21	57	3

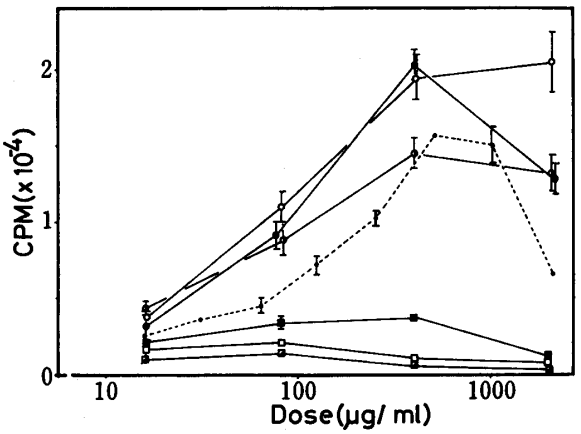

Fig. 1. Mitogenicity of Ammonium Sulfate-Fractionated Vesiculogen

---●---, vesiculogen; —●—, ppt. 20; —○—, ppt. 40; —◑—, ppt. 60; —■—, ppt. 80; —□—, ppt. 100; —◪—, sup. 100.

activity is shown in Fig. 1. Protein and phosphate contents varied in each fraction. Carbohydrate content was highest in the supernatant (sup. 100). Mitogenic activity appeared in the fractions ppt. 20—60. The activities of ppt. 20 and 40 were higher than that of vesiculogen. These results suggest that ammonium sulfate precipitation is effective for purifying the mitogenic substances. The mitogenic fraction was rich in protein and poor in phosphate and carbohydrate. It was previously suggested that the mitogenic activity of vesiculogen was owing to the protein part, because the activity was lost on treatment with carboxymodifying reagents.[4] The data presented in this section are consistent with the previous assumption.

Preparation and Characterization of Representative Fractions

To develop a purification protocol, the elution profiles of the mitogenic substances on various chromatographies were examined. The chromatographic methods examined were as follows. (1) Hydroxyapatite chromatography, (2) Amberlite CG-50 (hydrophobic-ionic) chromatography, (3) phenyl-Sepharose CL-4B (hydrophobic) chromatography, (4) cellulose powder (hydrogen bond) chromatography, and (5) DEAE-Sephadex A-25 (ion-exchange) chromatography (data not shown). All chromatographies separated the mitogenic substances into more than two fractions. These results suggest that the mitogenic substance of vesiculogen is heterogeneous.

To obtain representative fractions, we used, sequentially, DEAE-Sephadex A-25 (Fig. 2) and hydroxyapatite chromatographies. We thus obtained four representative fractions, PP (DEAE pass/hydroxyapatite pass), PA (pass/abs), AP (abs/pass), and AA (abs/abs). These four fractions were further separated by gel filtration on Sepharose CL-2B. Figure 3 shows the elution profiles of these four fractions. All the fractions showed highly dispersed molecular weight distributions. Major mitogenic fractions (fr. 15 to 30, PP-II, PA-II, AP-II, AA-II) were collected, and the mitogenicity of each fraction was investigated. Although the high concentrations could not be used because of the limited yields, all of the fractions showed marked mitogenic activity (Fig. 4). These four fractions were hydrolyzed and their amino acid compositions were compared. All of the fractions showed similar amino acid compositions. The major amino acids were Ser, Glu, Gly, and Ala. The sums of these four amino acids were 60% (PP-II), 43% (PA-II), 64% (AP-II) and 61% (AA-II), respectively. Another acidic amino acid, Asp, was also rich in each fraction (6.7% in PP-II, 9.0% in PA-II, 6.5% in AP-II, 6.7% in AA-II). On the other hand, the sums of the basic amino acids were less than 10% in all fractions (6.4% in PP-II, 8.4% in PA-II, 5.3% in AP-II, 5.3% in AA-II). Thus, the isoelectric points of these molecules should be acidic. Further purification and other quantitative estimations were not performed because of the limited yields of these fractions.

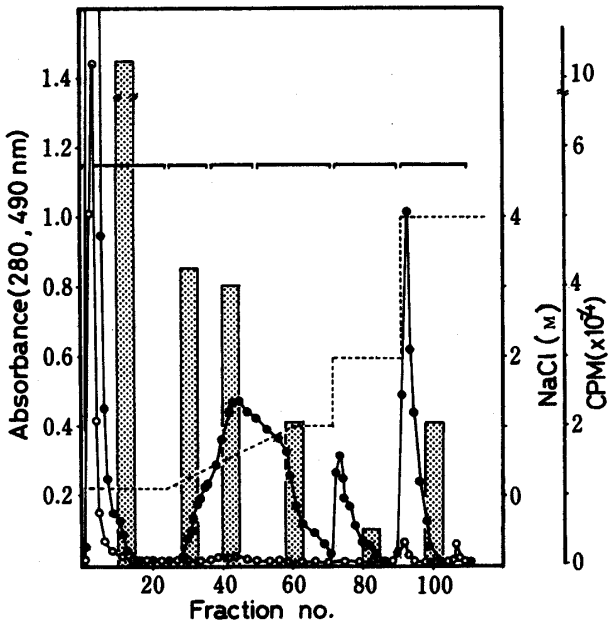

Fig. 2. Elution Profiles of ppt. 40 on a Column of DEAE-Sephadex A-25

Ammonium sulfate-fractionated vesiculogen (ppt. 40) (20 mg) was dissolved in a buffer composed of 0.1 M potassium phosphate and 1 mM EDTA, pH 7.1, containing 0.1 M NaCl, and applied to a column of DEAE-Sephadex A-25 (5 ml) equilibrated with the same buffer. The column was initially eluted with the same buffer, then with a linear gradient from 0.1 to 1 M NaCl containing the buffer, and finally with the buffer containing 1, 2 and 4 M NaCl/8 M urea. Fractions of 3 g were collected at 4 °C, and protein (—●—, 280 nm) and carbohydrate (—○—, 490 nm) contents were monitored. After pooling (indicated by the horizontal bar), dialysis against water and lyophilization, the mitogenicity of each pooled fraction was measured. The shaded bar indicates the mitogenicity of each pooled fraction (500 μg/ml of each fraction was used).

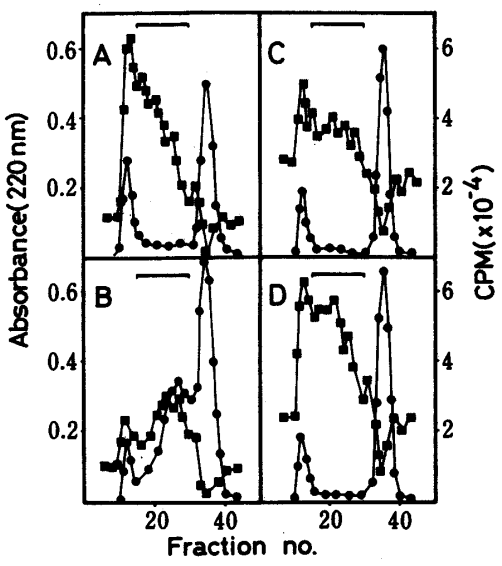

Fig. 3. Elution Profiles of Representative Fractions, PP, PA, AP, and AA, on a Column of Sepharose CL-2B

The representative fractions PP (DEAE pass/hydroxyapatite pass; A), PA (pass/abs; B), AP (abs/pass; C), AA (abs/abs; D) were applied to a column of Sepharose CL-2B (1.2 × 47 cm) equilibrated with 0.15 M NaCl. Fractions of 2.1 ml were collected at 4 °C and aliquots were used for mitogen assay. —●—, absorbance (220 nm); —■—, mitogenicity. Fractions indicated by the horizontal bars were pooled and used for subsequent analysis.

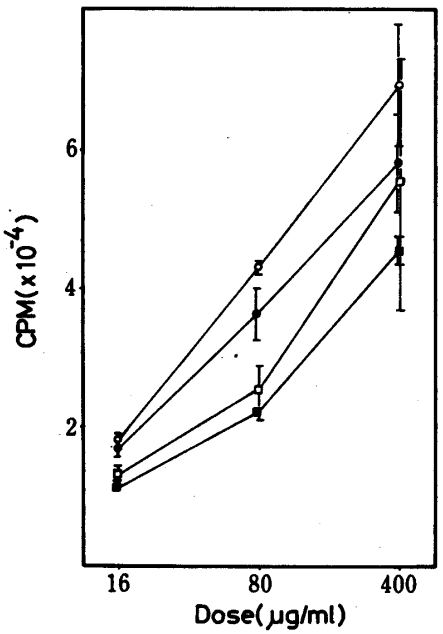

Fig. 4. Mitogenicity of the Representative Fractions, PP-II, PA-II, AP-II, and AA-II, Obtained from Sepharose CL-2B

—○—, PP-II (DEAE pass/hydroxyapatite pass); —□—, PA-II (pass/abs); —■—, AP-II (abs/pass); —●—, AA-II (abs/abs).

Discussion

In this paper, it is suggested that the mitogenic substances (VGM) in the hot water extract (vesiculogen) of *P. vesiculosa* are polypeptides showing marked molecular weight and

charge heterogeneities. Furthermore, VGM was composed of Ser, Glu, Gly and Ala and other minor amino acids. In view of the amino acid composition and molecular weight heterogeneities, the heterogeneity of VGM may result from the presence of amide bonds (Asn, Gln), or differences of minor amino acid constituents or other components such as carbohydrates and phosphate (Table I).

Lai and Maurer reported that synthetic polypeptides $(Tyr–Glu–Ala–Gly)_n$, $(Tyr–Ala–Glu–Gly)_n$, and $(Phe–Glu–Ala–Gly)_n$ showed mitogenic activity on murine B cells.[7] The amino acid compositions of VGM and these synthetic peptides are quite similar in that Glu, Ala, and Gly are contained in both. It is quite interesting that the amino acid compositions of mitogens obtained from fungi and by a synthetic approach are similar. They also reported that the mitogenic activity was induced not by random copolymer but sequential polymer. These results suggest that VGM may also contain some regular structure.

It has been found that in addition to lipopolysaccharides (LPS), polyinosinicpolycytidylic acid and other homonucleotides, as well as polysaccharides (such as dextran sulfate) and thymus-independent antigens (such as polyvinyl pyrrolidone, polymerized flagellin, pneumococcal polysaccharide and levan) are all B cell mitogens.[1,8] From these facts, Coutinho and Muller proposed that all T-independent antigens are also B-cell mitogens.[9] Further, Diamantstein *et al.* suggested that polyanionic charge was an important property of B cell mitogens.[10] In the previous paper, we showed that the mitogenic activity was diminished by treatment with carboxy-modifying reagents.[4] VGM contains a large proportion of acidic amino acids. Further, compared with the amino acid composition of the crude preparation,[4] the sum of the four amino acids was increased. These observations are also consistent with the requirement of anionic groups for mitogenicity.

Many microbial constituents are known to be composed of repeating unit structure, including LPS, pneumococcal polysaccharides, peptidoglycan, and β-1,3-glucan.[5,8] Almost all of these materials show various immunomodulating activities. We suggest that VGM similarly contains regular structure.

The data presented in this paper show that VGM is a polypeptide having marked molecular weight and/or charge heterogeneities. These heterogeneities might be due to the following factors. (1) Because the fungi were obtained from local fields, differences among lots might exist. (2) Posttranslational modifications, such as amino carbonyl reaction or Maillard reaction, might have occurred (for example, glucosylated hemoglobin in diabetes) during the growth of the fungi or during the preparation of VGM.

The crude preparation, vesiculogen, showed various immunomodulating activities, such as mitogenicity, polyclonal B cell activation activity, adjuvanticity, and antitumor activity.[3] However, Lai and Maurer reported that their synthetic polypeptide was not a polyclonal B cell activator.[7] It would be interesting to know whether VGM shows various immunomodulating activities other than mitogenicity.

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