Carbohydrate Research, 129 (1984) 209–227
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF PESTALOTAN, A HIGHLY BRANCHED (1→3)-β-D-GLUCAN ELABORATED BY *Pestalotia* sp. 815, AND THE ENHANCEMENT OF ITS ANTITUMOR ACTIVITY BY POLYOL MODIFICATION OF THE SIDE CHAINS*

AKIRA MISAKI**, KICHITARO KAWAGUCHI,
Faculty of Science of Living, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka 558 (Japan)

HIDEKI MIYAJI, HIDEO NAGAE, SHUSABURO HOKKOKU,
Meito Sangyo Co., Ltd., Nishi-ku, Nagoya 451 (Japan)

MARIKO KAKUTA,
Konan Women's College, Morikita-cho, Higashinada-ku, Kobe 658 (Japan)

AND TAKUMA SASAKI
Chemotherapy Division, National Cancer Center Research Institute, Tsukiji, Chyuo-ku, Tokyo 104 (Japan)

(Received November 10th, 1983; accepted for publication, January 17th, 1984)

ABSTRACT

Pestalotia sp. 815, a newly isolated fungus, produces extracellularly a highly (1→6)-branched (1→3)-β-D-glucan in high yield when grown in a D-glucose-containing medium. This extracellular glucan, designated "Pestalotan", has $[\alpha]_D^{25}$ −0.1° (*c* 0.5, M NaOH) and a molecular weight >2 × 10^6. Chemical and enzymic studies indicated that pestalotan has a very highly branched structure containing a back-bone chain of β-D-(1→3)-linked D-glucosyl residues, and three out of five D-glucosyl residues are substituted at O-6, mostly with single D-glucosyl groups, and a very few with short β-(1→6)-linked oligosaccharide units. This D-glucan becomes water-insoluble after isolation from the culture filtrate followed by dehydration, and shows moderate growth-inhibitory activities against mouse-implanted tumors. However, when the D-glucosyl groups of the side chains were modified by periodate oxidation and borohydride reduction, the resulting, still water-insoluble, D-glucan polyol exhibited potent antitumor activities, confirming that the attachment of many polyhydroxy groups to the β-D-(1→3)-linked D-glucan back-bone gives a remarkable enhancement effect on the antitumor activity of the branched D-glucan. Prolonged treatment of the D-glucan polyol by ultrasonic irradiation afforded

*A part of this work was presented at the Annual Meeting of the Japanese Biochemical Society, Sendai, 1981, and the Second International Conference on Immunopharmacology, Washington, D. C., U.S.A., July, 1982.

**To whom requests for reprints should be addressed.

0008-6215/84/$ 03.00

a low-molecular-weight D-glucan polyol (SD-pestalotan polyol), without alteration of its chemical structure. The water-soluble, SD-pestalotan polyol, having a molecular weight of 4.7×10^5, exhibited remarkable antitumor activities against both allogeneic and syngeneic, mouse-implanted tumors, at small dosages (1–5 mg/kg for 10 days) by intraperitoneal administration. A comparison of values of the molecular weight of SD-pestalotan polyol, estimated by 3-MPa l.c. for the aqueous solution and the dimethyl sulfoxide solution, strongly suggested that the D-glucan polyol must form a triple-chain conformation in aqueous solution.

INTRODUCTION

Fruiting bodies of many *Basidiomycetes* are known to contain (1→6)-branched, (1→3)-β-D-glucans as their major polysaccharide components. Similar D-glucans are also elaborated extracellularly by some fungi, *e.g.*, *Sclerotum glucanicum (Fungi imperfecti)*[1], *Schizophyllum commune*[2,3], and *Aureobasidium pullulans*[4]. Some of these D-glucans, such as schizophyllan *(Schizophyllum commune)*[3] and lentinan *(Lentinus edodes)*[5], have recently received much attention in the field of cancer immunotherapy, because of their remarkable, tumor-inhibitory activity. The results of many physicochemical and pharmacological investigations have suggested that such antitumor actions are closely related to the triple-helix conformation of their β-D-(1→3)-glucosidically linked, back-bone chains[6,7]; however, correlation of their structure to their antitumor effects is not yet fully understood, except for dependence on the molecular weight[8,9].

We had previously reported that a water-insoluble, highly branched (1→3)-β-D-glucan isolated from Kikurage (fruiting body of *Auricularia auricula-judae*) had essentially no antitumor activity, but that modification of the D-glucosyl groups of the side chains to polyhydroxy groups, by controlled, Smith degradation showed significant enhancement of the antitumor activity of the D-glucan[10,11].

In the course of studies on the interrelationship of structures and antitumor activities of polysaccharides, we attempted to obtain new antitumor, fungal polysaccharides Thus, a newly isolated fungus, *Pestalotia* sp. 815, was found to accumulate an abundant amount of an antitumor-active β-D-glucan. This extracellular D-glucan, tentatively designated "pestalotan", having an extraordinarily highly branched structure probably characteristic of *Pestalotia* species, was found to exhibit only moderate antitumor activities. However, its D-glucan polyol, derived by periodate oxidation followed by borohydride reduction, and also the ultrasonically depolymerized D-glucan polyol, showed significantly high, antitumor activities against both allogeneic and syngeneic tumors, implanted to mice.

We now report on the production of pestalotan, the structures of pestalotan, pestalotan polyol, and its depolymerized product, and their antitumor actions.

RESULTS AND DISCUSSION

Production and chemical properties of pestalotan. — In order to ascertain the effect of the carbon source on the extracellular production of the D-glucan, *Pestalotia* sp. 815 was grown by flask-shaking cultivation at 25°, in a medium (100 mL) containing 3.0% of D-glucose or other carbohydrate, 0.3% of yeast extract, 0.3% of peptone, and appropriate inorganic nutrients. After 4-day cultivation, the culture broth was centrifuged to remove fungal cells, the polysaccharide in the supernatant liquor was isolated by precipitation with acetone (2 vol.), and the yields of polysaccharide from different carbon sources were compared. As shown in Table I, D-glucose, sucrose, and maltose were effectively utilized for production of the polysaccharide (yield, 410–450 mg per 100 mL of culture broth), whereas other carbohydrates, such as D-galactose, D-xylose, and D-glucitol were poorly utilized. Therefore, for large-scale preparation of the polysaccharide, *Pestalotia* sp. 815 was cultivated in a 30-L jar-fermenter (15 L of medium, pH 6.5) containing 3.5% of D-glucose, 0.05% of peptone, 0.05% of yeast extract, and inorganic salts. The fermentation was conducted at 25°, with vigorous aeration and agitation. As shown in Fig. 1, D-glucose was rapidly consumed during 3–4 days, with increase in the cell growth and accumulation of extracellular polysaccharide. When the polysaccharide production reached a maximum (4 days), the viscous, cultural broth was heat-sterilized, and diluted with water, and the cells were removed by centrifugation. The polysaccharide in the clarified solution was precipitated by gradual addition of acetone (final concentration, 50%). The crude polysaccharide precipitated was dissolved in water, and reprecipitated with acetone. After one more treatment with water and acetone, the polysaccharide was dehydrated by lyophilization (yield, 39.2 g from 10 L of culture broth).

TABLE I

EFFECTS OF VARIOUS CARBOHYDRATES AS CARBON SOURCES ON THE PRODUCTION[a] OF EXTRACELLULAR POLYSACCHARIDE

Carbohydrate	*Yield (mg per 100 mL of broth)*
D-Glucose	410
D-Galactose	180
D-Mannose	220
D-Fructose	220
D-Xylose	100
D-Glucuronic acid	—
D-Glucitol	30
D-Mannitol	120
Maltose	450
Sucrose	450

[a] *Pestalotia* sp. 815 was grown by the flask-shaking technique in a medium containing 3% of each carbohydrate, for 4 days at 25°.

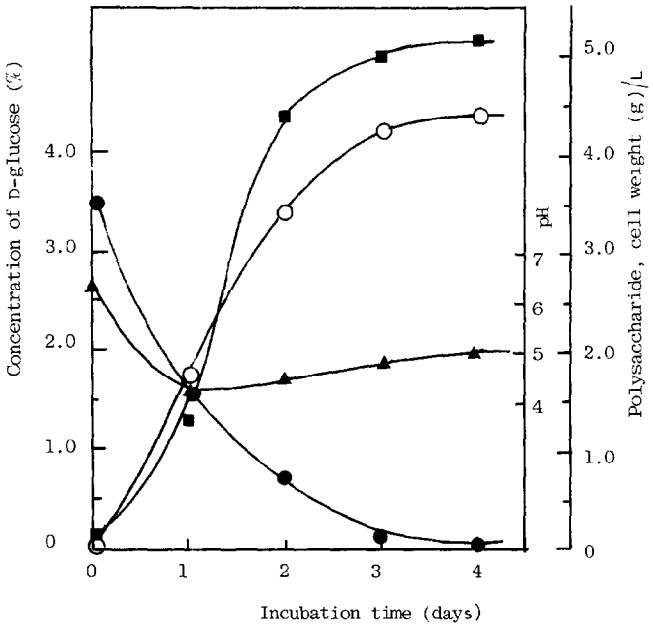


Fig. 1. Time course of production of extracellular polysaccharide. [*Pestalotia* sp. 815 was cultivated in a 30-L jar-fermenter (3.5% of D-glucose medium, 15 L), at 25°, with aeration and agitation. Key: (—■—), dry weight of mycelia; (—●—), consumption of D-glucose; (—○—), weight of polysaccharide; and (—▲—), pH.]

Because the dehydrated polysaccharide so obtained was found to become mostly insoluble in water even on warming, it was separated into a water-soluble (~10%) and an insoluble fraction. The water-soluble polysaccharide had $[\alpha]_D^{25}$ −10.3° (0.5M NaOH) and a molecular weight of 1.6×10^4 (estimated by 3-MPa l.c.). On acid hydrolysis, this water-soluble polysaccharide gave D-glucose (95.6%) and D-mannose (4.4%). The results of methylation analysis indicated that the structure of this polysaccharide resembles that of the water-insoluble D-glucan, except for the presence of a small proportion of D-mannosyl residues; however, it had low antitumor activity on mouse-implanted Sarcoma 180 (inhibition ratio, 35% with dosages of 5 mg/kg of mouse for 10 days). Further chemical and antitumor properties of this polysaccharide fraction were not examined.

The major, water-insoluble polysaccharide, which was free from nitrogen compounds and composed solely of D-glucose, showed $[\alpha]_D^{25}$ −0.1° (M NaOH) and absorption at 890 cm^{-1} in the i.r. spectrum, characteristic of β-D-glucosidic linkages. The D-glucan (not soluble in water) became less soluble in dimethyl sulfoxide when it was dehydrated either by precipitation with acetone or by lyophilization, but it was soluble in M and higher concentrations of alkali, to give a viscous solution. Its molecular weight was $>2 \times 10^6$, as measured by 3-MPa l.c. after being dissolved in alkali followed by dialysis against water (see Table II).

TABLE II

COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF PESTALOTAN, AND OF PESTALOTAN POLYOL AND ITS SONICALLY DEPOLYMERIZED PRODUCT

Polysaccharide	*Solubility*	$[\alpha]_D^{25}$[a]	*Intrinsic viscosity*		*Molecular weight by l.c.*[c]	
			$[\eta]$[b]		*Carrier system*	
			Water	*Me_2SO*	*Water*	*Me_2SO*
Pestalotan	insoluble in water, soluble in alkali	−0.1	—	—	$>2 \times 10^6$	$\sim 2 \times 10^6$
Pestalotan polyol	slightly soluble in water	+19.3	—	5.69	$>2 \times 10^6$	$\sim 2 \times 10^6$
SD-pestalotan polyol-50[d]	soluble in water	+24.8	2.06	0.67	4.7×10^5	1.5×10^5

[a]Degrees (*c* 0.5, NaOH). [b]In g/dL at 25°. [c]Estimated by 3-MPa l.c., using TSK column G-5000 PW, and water or dimethyl sulfoxide (Me_2SO) as the carrier. [d]Ultrasonically depolymerized product of pestalotan polyol (see text).

The very highly branched structure of this β-D-glucan was elucidated by methylation analysis, and Smith degradation, and also by degradation with *exo*-(1→3)-β-D-glucan hydrolase. The D-glucan was methylated by the method of Hakomori[12], and the fully methylated polysaccharide was hydrolyzed with acid. G.l.c. analysis of the hydrolysis products revealed the presence of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose, together with a small proportion of 2,3,4-tri-*O*-methyl-D-glucose (molar ratios, 1.0:0.58:0.99:0.1). This result indicated that the D-glucan consists mainly of (1→3)-linked D-glucosyl residues, three out of five (1→3)-linked D-glucosyl residues being substituted at O-6. In addition, the D-glucan may contain a small proportion of (1→6)-linked D-glucosyl residues, but methylation analysis could not reveal whether they are located in the backbone chain or in the side chains.

On periodate oxidation for 7 days at 10°, the D-glucan consumed 0.79 mol of periodate, with concomitant production of 0.39 mol of formic acid per D-glucosyl residue. The oxidized D-glucan was reduced with sodium borohydride, and the product was completely hydrolyzed by heating with formic acid followed by 2M trifluoroacetic acid; glycerol and D-glucose were liberated. Quantitative analysis by g.l.c., and also by l.c., showed the molar ratio of glycerol to D-glucose to be 0.65:1.0, consistent with the methylation data.

As regards mild Smith-degradation of the branched (1→3)-β-D-glucan, a previous study[10] had indicated that the polyhydroxy groups* of the D-glucan polyol derived from the highly (1→6)-branched, insoluble (1→3)-β-D-glucan of Kikurage by periodate oxidation and borohydride reduction could not be completely hydrolyzed by treatment with 0.05M sulfuric acid at 25°, conditions customarily used in mild Smith-degradation[13]. As the present D-glucan also contains many branches,

*1-(1,3-Dihydroxy-2-propyloxy)-2-hydroxyethyl groups.

TABLE III

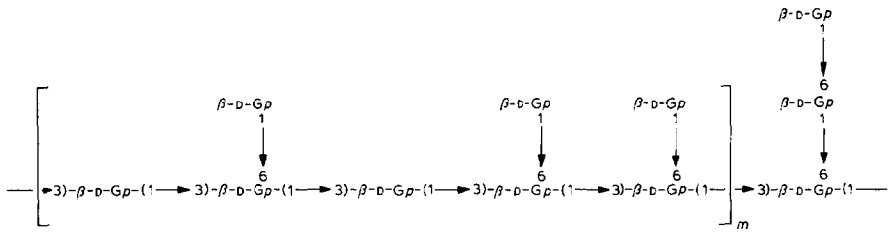
IDENTITIES AND MOLAR PROPORTIONS OF METHYLATED D-GLUCOSES IN ACID HYDROLYZATES OF METHYLATED PESTALOTAN AND ITS D-GLUCAN POLYOL

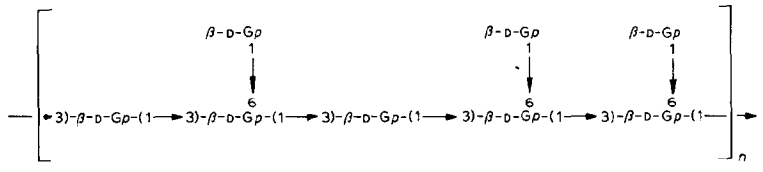
O-Methyl-D-*glucose*	*Linkage indicated*	*Pestalotan*[a]	*Pestalotan polyol*		
			Original	*After hydrolysis with* H_2SO_4	
				0.05M, *24 h, 25°*	*0.1*M, *1 h, 100°*
2,3,4,6-Tetra-	Glc*p*-(1→	37.3	1.7	1.9	0.5
2,4,6-Tri-	→3)-Glc*p*-(1→	21.7	39.1	62.2	97.8
2,3,4-Tri-	→6)-Glc*p*-(1→	3.8	—	—	—
2,4-Di-	→3) →6)-Glc*p*-(1→	37.2	58.2	35.9	1.7

[a]Water-insoluble fraction.

its D-glucan polyol derived by periodate oxidation and reduction was hydrolyzed in two ways: (*1*) with 0.05M sulfuric acid for 16 h at 25°, and (*2*) with 0.1M sulfuric acid for 1 h at 100°, and the mode of D-glucosidic linkages of the degraded polysaccharides, obtained as water-insoluble products, was examined by methylation analysis. In Table III, the proportions of partially methylated D-glucoses in the acid hydrolyzates of methylated, native glucan, glucan polyol, and two Smith-degraded polysaccharides are compared. It is evident that the polyhydroxy groups attached by acetal linkages to the O-6 atoms of β-D-(1→3)-linked D-glucosyl residues are remarkably stable to acid, and the hydrolysis conditions customarily used appear to be insufficient, as indicated by the presence, still, of a high proportion of 2,4-di-*O*-methyl-D-glucose. On the other hand, a controlled, but rather stronger acid hydrolysis resulted in almost complete hydrolysis of the acetal linkages, leaving a linear molecule having only β-D-(1→3)-glucosidic linkages. The resulting, linear (1→3)-β-D-glucan was insoluble, and had a high molecular weight, suggesting that complete deletion of the polyhydroxyl groups under hydrolysis condition *2* was not accompanied by cleavage of (1→3) bonds in the back-bone chain, as observed previously[10]. The foregoing result also suggested that the back-bone chain is built entirely of (1→3)-linked D-glucosyl residues, and that (1→6)-linked D-glucosyl residues must be located in the side chains.

When the D-glucan was incubated with the *exo*-(1→3)-β-D-glucan hydrolase [1,3-β-D-glucan glucohydrolase (EC 3.2.1.58)] of *Basidiomycete* QM 806, it became soluble, and there was an increase in the reducing groups (apparent hydrolysis after 4 days: 59.9%, as D-glucose). The digestion products, analyzed by paper chromatography and l.c., were D-glucose and gentiobiose, in the molar ratio of 1.0:1.47. Other β-D-(1→6)-linked oligosaccharides, such as gentiotriose, could not be detected, probably because of insufficient amounts. Under the same conditions of enzymic hydrolysis, the Smith-degraded D-glucan yielded only D-glucose, confirming that the back-bone chain of the native D-glucan consists entirely of β-D-(1→3)-linked D-glucosyl residues.





1

Possible structure of pestalotan

Glp = glucopyranose

$m + n = 3$

The aforementioned results, obtained by chemical analyses and enzymic degradation, indicate that the extracellular D-glucan of *Pestalotia* sp. 815 contains a back-bone chain of β-D-(1→3)-linked D-glucosyl residues, and that three out of every five D-glucosyl units are attached at O-6 atoms, mostly as single D-glucosyl groups. In addition, there may be a few short side-chains of two (or three) β-D-(1→6)-linked D-glucosyl residues. Such an extraordinarily highly branched (1→3)-β-D-glucan appears to be rare among fungal β-D-glucans, and to be characteristic of *Pestalotia* species. Therefore, we have tentatively designated this highly branched β-D-glucan of *Pestalotia* sp. 815 as pestalotan. A possible repeating-unit of pestalotan is shown in **1**.

As already mentioned, pestalotan is in a water-soluble state during production by the fungus, but when this D-glucan is separated from the culture filtrate by using a water-miscible organic solvent, such as acetone, and then dehydrated, most of the D-glucan becomes insoluble in water, and less soluble in dimethyl sulfoxide. Such an alteration in solubility may be attributed to molecular aggregation, most probably through intermolecular hydrogen-bonds to form rigid frameworks. The water-soluble polysaccharide fraction, which constitutes ~10% of the total extracellular polysaccharide, may have a structure similar to that of the insoluble D-glucan, as indicated by methylation analysis and digestion with (1→3)-β-D-glucan hydrolase, although it contains a small proportion of D-mannose. However, the water-soluble polysaccharide has a smaller molecular weight (1.6×10^4), and this may affect its water-dispersibility.

TABLE IV

ANTITUMOR ACTIVITIES OF PESTALOTAN AND ITS POLYALCOHOL ON SARCOMA 180 AND EHRLICH CARCINOMA SOLID TUMORS

Polysaccharide	*Dose (mg/kg × 10 days)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*
Sarcoma in 180 ICR mice				
Native D-glucan	1	8.23	16.0	0/6
	5	4.18	57.3	0/6
	50	2.39	75.6	2/6
D-Glucan polyol	1	1.17	88.1	4/6
	5	0.75	92.3	4/6
	50	0.45	95.4	5/6
Control	—	9.80	—	0/6
SD-glucan polyol-50	1	0	100	6/6
	5	0	100	6/6
Control	—	10.23	—	0/6
Sarcoma 180 in C3H/He mice				
Native glucan	5	12.42	−13.1	0/6
D-Glucan polyol	5	2.20	80.0	3/5
SD-glucan polyol	5	1.05	90.4	4/6
Control	—	10.98	—	0/6
Ehrlich carcinoma in ICR mice				
Native D-glucan	1	14.85	0.4	0/6
	5	7.90	47.0	0/6
	20	3.78	74 7	2/6
D-Glucan polyol	1	0	100	6/6
	5	0	100	6/6
Control	—	14.91	—	0/6
SD-glucan polyol-50	1	0	100	6/6
	5	0	100	6/6
	20	0	100	6/6
Control	—	11.58	—	0/6

TABLE V

CHANGES IN VISCOSITY AND REDUCING GROUPS DURING ULTRASONIC IRRADIATION OF PESTALOTAN POLYOL

Irradiation time[a] *(min)*	*Relative viscosity, η_{rel} (c 0.5%, at 25°)*	*Reducing power (weight %, as glucose)*
0	suspension	0
40	9.15	0.09
80	5.14	0.17
130	3.16	0.22
150	2.75	0.28
180	2.58	0.28
210	2.26	0.33

[a]An aqueous suspension (1.5%, 300 mL) was exposed to ultrasonic irradiation (25 kHz) at 40°.

Ultrasonic depolymerization and antitumor effect of the glucan polyol. — Our previous study showed that the chemical modification of the branched (1→3)-β-D-glucan of Kikurage to the corresponding D-glucan polyol remarkably enhanced the antitumor activity of the glucan[10,11]. Because a similar enhancement effect on the water-insoluble pestalotan had been anticipated, the present D-glucan was treated with periodate, and the product, with borohydride. The resulting D-glucan polyol certainly showed higher antitumor activities than the original pestalotan (see Table IV); however, it was still water-insoluble, and this might present problems in the tumor-immunotherapic application. This prompted us to depolymerize the insoluble pestalotan polyol without alteration of its molecular structure. For this purpose, the ultrasonic irradiation technique that was successfully applied to schizophyllan, an antitumor (1→3)-β-D-glucan of *Schizophyllum commune*[3], was used.

An aqueous suspension of pestalotan polyol at a concentration of 1.5% was exposed to 25-kHz ultrasonic irradiation at 40°. Although the rate of depolymerization appeared to be changeable, depending on the shape and size of the irradiation vessel, when the aqueous suspension of pestalotan polyol (300 mL) in a glass beaker was irradiated, at the early stage (40–50 min), the glucan polyol gradually acquired reducing power and became soluble, to give a viscous solution, and the viscosity decreased with increase in the reducing power, as shown in Table V. As the D-glucan polyol had originally contained no reducing group, the increase in reducing power presumably arose in part, if not entirely, from newly formed reducing end-groups by cleavage of β-D-(1→3)-glucosidic linkages of the back-bone chains, as already shown in the case of ultrasonic degradation of schizophyllan[3]. When the relative viscosity of the irradiated pestalotan polyol reached ~2.2 (220-min irradiation), the depolymerized product was precipitated by addition of 75–85% acetone, and then lyophilized (recovery, 75–82%).

Some physicochemical properties of pestalotan, pestalotan polyol, and its depolymerized product are compared in Table II.

The stability toward ultrasonic irradiation of polyhydroxy groups attached by acetal linkages to the β-D-(1→3)-linked D-glucan back-bone was examined by determination of D-glucose and glycerol in the acid-hydrolysis products of the original and depolymerized D-glucan polyol. The former gave the molar ratio of D-glucose to glycerol of 1.52:1.0, and the latter gave 1.65:1.0, indicating that the polyhydroxy groups are relatively stable. Structural similarity of the original D-glucan polyol, which was insoluble in water, to its depolymerized product was supported by their identical ^{13}C-n.m.r. spectra, measured in dimethyl sulfoxide-d_6 at 55°. Fig. 2 shows the spectrum of the depolymerized D-glucan polyol (SD-pestalotan polyol-50; see Table II), possible assignments of peaks being made by using our previous data[10] and those reported for the 6-*O*-substituted D-glucose isolated from the D-glucan polyol of *Ganoderma applanatum*[14].

Neither pestalotan nor the corresponding D-glucan polyol was soluble in water, unless first dissolved in aqueous alkali. They had average molecular weights

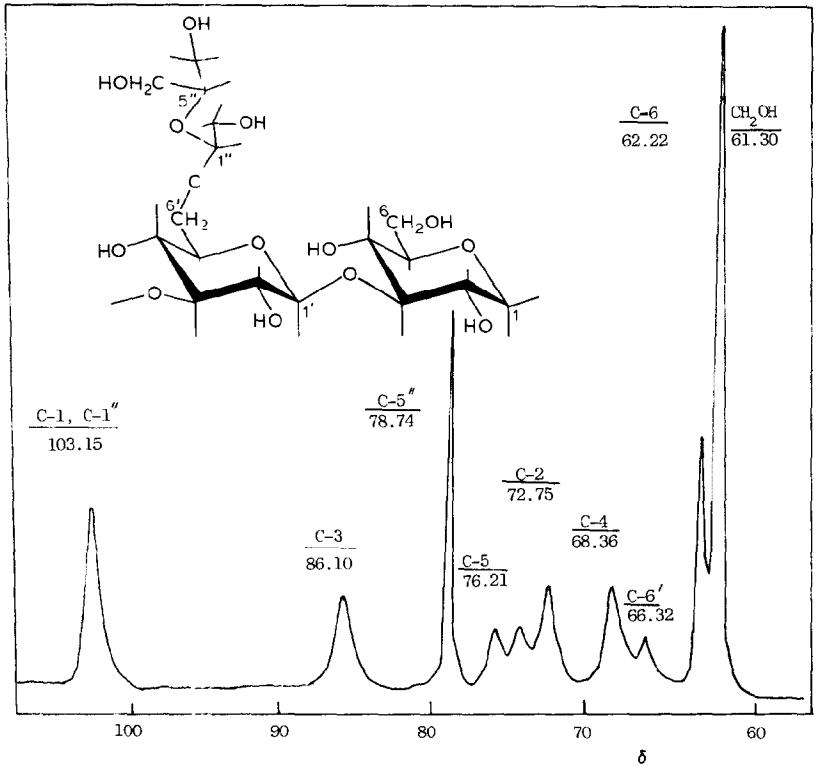


Fig. 2. ^{13}C-N.m.r. spectrum of SD-pestalotan polyol-50, measured for 7% solution in Me_2SO-d_6, at 25°.

$>2 \times 10^6$, as estimated by 3-MPa l.c., using a column of TSK G5000PW, and 0.1M Tris · HCl buffer, pH 8.0, as the carrier solution. On the other hand, the depolymerized, pestalotan polyol preparations were readily soluble in water, to give solutions having a low viscosity. A preparation of the sonically depolymerized D-glucan polyol having molecular weight 4.7×10^5, designated SD-pestalotan polyol-50, was used for studies of the antitumor activities. Most (1→3)-β-D-glucans in their natural state are known to have a triple-helix conformation. From the interrelation of viscosities and molecular weights of ultrasonically degraded schizopyllan preparations, Norisue *et al.* concluded that, in aqueous solution, the D-glucan forms a rigid, rod-like, triple-helix chain, whereas, in the dimethyl sulfoxide solution, the triple chains are dissociated, to form single chains[7]. The triple-helix conformation of the D-glucan polyol in aqueous solution was elucidated by comparison of the apparent molecular weight in water and in dimethyl sulfoxide. SD-pestalotan polyol-50 gave a molecular weight of 4.7×10^5 as measured by 3-MPa l.c. on a TSK G5000PW column, eluted with 0.1M Tris HCl, pH 8.0, whereas it gave a molecular weight of 1.5×10^5 when measured on the same type of column, modified for use of dimethyl sulfoxide as the carrier, as shown in Table II. This result strongly

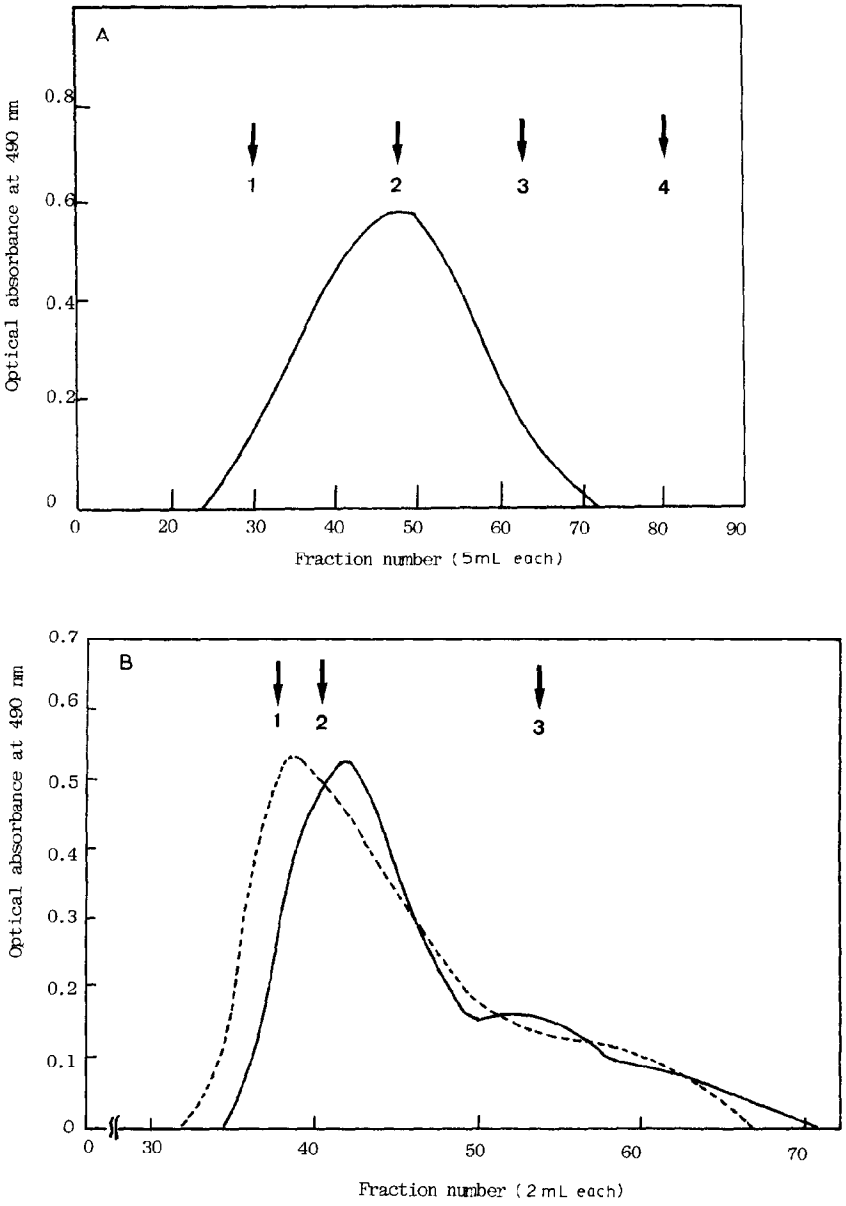


Fig. 3. A. Gel-filtration profile of SD-pestalotan polyol-50 on a column of Sepharoses 2B and 4B, and Sephadex G-100, eluted with 50mM sodium acetate buffer (pH 7.5). [Arrows indicate the elution position of (1) dextran T-2000, (2) T-500, (3) T-40, and (4) D-glucose.] B. Gel-filtration profiles of original pestalotan polyol and SD-pestalotan polyol-50 on a column of Toyo Pearl HW-65-F, eluted with dimethyl sulfoxide. [(-------), pestalotan polyol; and (——), SD-pestalotan polyol-50. Arrows indicate the elution positions of (1) dextran T-2000, (2) T-500, and (3) T-40.]

suggests that the D-glucan polyol also has a triple-helix conformation in aqueous solution.

The molecular-weight distribution of SD-pestalotan polyol-50 was examined by gel filtration using water or dimethyl sulfoxide as the carrier solution. When the depolymerized D-glucan polyol was applied to a column[3] consisting of Sepharose 2B (top layer), Sepharose 4B (middle layer), and Sephadex G-100 (bottom layer), and eluted with 50mM sodium acetate buffer, pH 7.5, it gave a somewhat broad, but homogeneous, distribution, with a peak corresponding to that of dextran T-500, as shown in Fig. 3A. When the same depolymerized D-glucan and the original D-glucan polyol (before ultrasonication) were applied to a column of Toyo Pearl HW-65-F and eluted with dimethyl sulfoxide, the D-glucan polyol gave a peak close to that of dextran T-2000, whereas the depolymerized D-glucan polyol (SD-pestalotan polyol-50) gave a lower molecular-weight distribution, as shown in Fig. 3B; there are two peaks, a main peak corresponding to a molecular weight lower than that of T-500, and a minor peak having a molecular weight corresponding to that of T-40.

The aforementioned results indicate that ultrasonic irradiation of the D-glucan polyol results in profound depolymerization of the molecule without any appreciable structural alteration. It is also apparent that the depolymerized D-glucan polyol should form a triple-helix conformation, like schizophyllan[7], in aqueous solution.

Antitumor activities of pestalotan, pestalotan polyol, and SD-pestalotan polyol-50 were tested on allogeneic and syngeneic tumors in appropriate strains of mice. In Table IV are compared the antitumor activities of the native and modified D-glucans against Sarcoma 180 solid tumor in ICR-JCL and C3H/He mice, and also Ehrlich carcinoma in ICR-JCL mice. These test-polysaccharides were dissolved or suspended in water, and injected intraperitoneally at dosages of 1–50 mg/kg for 10 days, starting one day after tumor implantation. Pestalotan, insoluble in water, showed only a moderate inhibitory activity in the Sarcoma 180–ICR-JCL mouse system. For instance, it gave an inhibition ratio of 57% at 5 mg/kg dose, but pestalotan polyol, which was still water-insoluble, exhibited a higher activity, and gave an inhibition ratio of 92.3%. At the same dosage, SD-pestalotan polyol-50 which was water-soluble, exhibited a very high antitumor activity (an inhibition ratio of 100% at a dosage of 1 mg/kg). The antitumor activities of polysaccharides appear to differ among different strains of mice. Pestalotan showed essentially no activity against Sarcoma 180 in C3H/He mice, which are known to have a low immunoresponse. On the other hand, its polyols, *i.e.*, the original and depolymerized pestalotan polyol, showed inhibition ratios of 80 and 90.4%, respectively, with high rates of complete regression. Against Ehrlich carcinoma solid tumor, pestalotan again showed only a moderate inhibitory activity, but both pestalotan polyol and its depolymerization product showed potent activities; they gave complete tumor regression in all mice tested at 1 mg/kg dose for 10 days.

As regards the antitumor action on a syngeneic tumor system (Meth-A in

TABLE VI

ANTITUMOR ACTIVITIES OF PESTALOTAN AND ITS SONICALLY DEPOLYMERIZED D-GLUCAN POLYOL ON METH-A FIBROSARCOMA

Polysaccharide	*Dose (mg/kg × 10 days)*	*Average tumor weight (g)*	*Inhibition ratio (%)*	*Complete regression*
Native D-glucan	1	1.93	27.9	0/6
	5	1.83	31.6	0/6
SD-glucan polyol-50[a]	1	1.23	54.1	1/6
	5	0.33	88.0	1/6
Control	—	2.68	—	0/6

[a]Sonically depolymerized pestalotan polyol having a molecular weight of 4.7×10^5. Ascites Meth-A fibrosarcoma tumor cells (1×10^4) were implanted subcutaneously into BALB/C mice, and the polysaccharide was intraperitoneally injected daily for 10 days, starting 24 h after the tumor implantation. After 5 weeks, the inhibition ratio and the complete regression of tumor were estimated.

BALB/C mice), which is an especially weak responder system to antitumor polysaccharides, SD-pestalotan polyol-50 was found to inhibit the growth of Meth-A fibrosarcoma in BALB/c mice remarkably. As shown in Table VI, it gave the high inhibition ratio of 88% at a dosage of 5 mg/kg. Under the same conditions, the native D-glucan gave an inhibition ratio of only 31.6%.

Because the polyol groups attached to the (1→3)-β-D-glucan may play an important role in enhancement of the antitumor activity, the effect of the content of polyol groups was examined by using D-glucan polyol preparations containing different proportions of polyol groups. These D-glucan polyols were obtained by borohydride reduction of partially oxidized pestalotans (prepared by using limited ratios of periodate to D-glucan), as employed in our previous study[10]. These D-glucan polyols, and the polyol-free D-glucan obtained by hydrolysis of the original pestalotan polyol with 0.1M sulfuric acid for 1 h at 100°, were tested for antitumor activity in the Sarcoma 180–ICR-JCL mice system. As shown in Table VII, the antitumor activity decreased with decrease in the polyol content, and its complete re-

TABLE VII

EFFECT OF CONTENT OF POLYOL GROUP OF PESTALOTAN POLYOL ON ANTITUMOR ACTIVITY AGAINST EHRLICH CARCINOMA SOLID TUMOR

Modified pestalotan	*Molar ratio of periodate to D-glycosyl residue*	*Content of polyol group (molar proportion)*	*Dose[a] (mg/kg × 10 days)*	*Average tumor weight*	*Inhibition ratio (%)*	*Complete regression*
Pestalotan polyol I[b]	1.0	37.7	5	0	100	6/6
Pestalotan polyol II	0.6	29.1	5	0.10	98.4	4/6
Pestalotan polyol III	0.2	15.4	5	2.70	56.6	1/6
Polyol-free D-glucan		(trace)	5	5.73	8.0	0/6

[a]Test polysaccharide was injected intraperitoneally into ICR-JCL mice, starting 24 h after the tumor implantation. [b]Original pestalotan preparation (see Table I).

moval resulted in elimination of the activity when tested at a dosage of 5 mg/kg for 10 days. These results were consistent with those obtained in our previous study[10].

It has been well established that the antitumor actions of (1→3)-β-D-glucans are closely related to their triple-helix conformation. Inoki *et al.* recently reported[9] that depolymerized schizophyllan preparations having molecular weights $>1 \times 10^5$ can hold the triple-helix chains and exhibit good antitumor activity[9]. As to pestalotan polyol, we tested antitumor activities of several depolymerized products, having molecular weights of 2×10^5 to 7×10^5, against Sarcoma 180 solid tumor at the dosage of 5 mg/kg for 10 days. All preparations tested exhibited high rates of tumor-inhibitory activity, similar to that obtained with SD-pestalotan polyol.

From extensive immunopharmacological studies, it is conceivable that the antitumor actions of (1→3)-β-D-glucans are involved in stimulation of the immunoresponse mechanisms of hosts, most probably through cellular immunity. Also, the effectiveness of pretreatment of the animals used is characteristic to the ordinary antitumor polysaccharides. In this connection, it is interesting that the antitumor effect of pestalotan polyol, administered before transplantation of Sarcoma 180 tumor cell, was less effective (inhibition ratio, 65%), when it was given at a dosage of 1 mg/kg for 10 days, and tumor cells were transplanted 24 h after the last injection, compared with the inhibition ratio of 100% when it was injected daily at a dosage of 1 mg/kg for 10 days, starting 24 h after tumor implantation. This suggests that, unlike ordinary (1→3)-β-D-glucans, the depolymerized D-glucan polyol is not retained in the tissues, but is rather rapidly excreted through the circulation. It was also observed that combined administration of the depolymerized pestalotan polyol with such chemotherapeutic, anticancer agents as mitomycin C and 5-fluorouracil gave significant prolongation of life spans of mice, implanted either with allogeneic or syngeneic ascites tumor cells[15].

EXPERIMENTAL

Micro-organism. — A new fungus, used in the present study, was isolated from soil in a forest area of Kyoto Prefecture, Japan, during collection of mushrooms. This fungus, belonging to *Fungi imperfecti,* was identified as a strain of *Pestalotia* species by courtesy of Professor K. Tsubaki, Tsukuba University, and named *Pestalotia* sp. 815. The micro-organism was preserved on an agar slant containing 0.3% of peptone, 0.3% of yeast extract (Difco), 0.1% of KH_2PO_4, and 0.05% of $MgSO_4 \cdot 7\,H_2O$ (pH 6.5).

Materials. — Sepharoses 2B and 4B, and dextrans T-2000, T-500, and T-40 were purchased from Pharmacia Fine Chemicals, Sweden. Partially degraded pullulan preparations having definite molecular weights were provided by Hayashibara Biochemical Laboratory Co., Ltd., Okayama, Japan. L.c. columns of TSK G5000PW and G2000PW were obtained from Toyo Soda Industry Co., Ltd, and were used at a pressure of 3 MPa. A G5000PW column modified for use of dimethyl sulfoxide as the carrier in 3-MPa l.c., was provided by Toyo Soda Industry Co., Ltd.

A purified preparation of (1→3)-β-D-glucanase of *Basidiomycete* QM 806 was supplied by Professor S. Kirkwood, University of Minnesota.

General methods. — Evaporations were conducted under diminished pressure at temperatures not exceeding 37°, unless otherwise stated. Paper chromatography was performed with Toyo-Roshi No. 50 paper, usually developed with 6:4:3 (v/v) 1-butanol–pyridine–water. Sugars and alditols on paper chromatograms were detected with alkaline silver nitrate reagent.

L.c. was performed at 3 MPa with a Hitachi Liquid Chromatograph Model 635, and the sugars were monitored with a refractive-index indicator.

Complete hydrolysis of a polysaccharide or its polysaccharide polyalcohol was achieved by heating with 90% formic acid for 8 h at 100°, and after evaporation, heating the residue with 2M trifluoroacetic acid for 6 h at 100°. After evaporation of the hydrolyzate, the residue was analyzed by paper chromatography (p.c.), 3-MPa l.c., or g.l.c.

G.l.c. of sugars or methylated sugars, after conversion into the corresponding alditol acetates, was usually performed with a Hitachi Gas Chromatograph Model 663 (fitted with a flame-ionization detector), in a glass column (0.4 × 200 cm) packed with 3% of ECNSS-M on Gas Chrom Q, at 190° (neutral sugars) or 175° (methylated sugars).

^{13}C-N.m.r. spectra of a polysaccharide and its polyalcohol derivative were recorded with a JEOL FX-100 spectrometer operated at 25 MHz in the pulsed, Fourier-transform mode. The spectra were recorded at 25° for solutions in dimethyl sulfoxide-d_6 at concentrations of 7%. ^{13}C-Chemical shifts are expressed in p.p.m. downfield from external tetramethylsilane.

Methylation analysis. — Methylation of a polysaccharide was performed by the method of Hakomori[12]. The sample (10–20 mg) was dissolved in dimethyl sulfoxide (2 mL) by the aid of ultrasonic irradiation or by heating for a short time at 60° in a nitrogen atmosphere. The solution was treated with methylsulfinyl carbanion (0.5 mL) for 1.5 h at 25°, and then with methyl iodide (1.5 mL). The methylation product was dialyzed against water, the solution evaporated, and the methylated polysaccharide extracted into chloroform. The methylation procedure was repeated until no i.r. absorption band for hydroxyl group (3400–3200 cm^{-1}) was observed. The permethylated polysaccharide was first hydrolyzed with 90% formic acid (0.5 mL) for 8 h at 100°, and then with 2M trifluoroacetic acid (0.5 mL) for 3 h. The methylated sugars in the hydrolyzate were reduced with sodium borohydride, the resulting alditols were acetylated by heating with 1:1 acetic anhydride–pyridine (0.2 mL) for 2 h at 100°, and the products were analyzed by g.l.c.

Determination of molecular weight. — Average molecular weights of water-soluble, sonic-depolymerized D-glucan polyols were estimated by 3-MPa l.c. in a column (0.75 × 60 cm) of TSK G5000PW with 0.1M Tris · HCl buffer (pH 8.0) as the carrier, at a flow rate 0.8 mL/min. The water-insoluble D-glucan and D-glucan polyol were dissolved in 4M sodium hydroxide under a nitrogen atmosphere, and the solutions dialyzed against water until they became neutral. The neutral solu-

tions, at concentrations of 0.5%, were applied to the same l.c. column, without dehydration. The degraded pullulan fractions having definite molecular weights (4×10^4 to 9×10^5) and also purified dextran fractions (mol. wt., 2×10^4 to 2×10^6), provided by Meito Sangyo Co., Ltd., were used as standards. Alternatively, the polysaccharides were dissolved in dimethyl sulfoxide at concentrations of 0.5%, and their molecular weights were estimated by using a column (0.75×60 cm) of TSK G5000PW (modified for use of dimethyl sulfoxide), and elution with the same solvent.

The molecular-weight distributions of the depolymerized D-glucan polyols were examined by gel-filtration chromatography using a column (diameter, 1.5 cm) packed with Sepharose 2B (top layer, 35.5 cm of the length), Sepharose 4B (middle layer, 22.0 cm), and Sephadex G-100 (bottom layer, 14.8 cm), and the polysaccharide, eluted with 50mM sodium acetate buffer (pH 7.5), was monitored with phenol–sulfuric acid[16] (each fraction, 5 mL). Gel filtration of the original and the depolymerized D-glucan polyol was also performed by using a column (1.5×92 cm) of Toyo Pearl HW-65-F, and dimethyl sulfoxide as the carrier. Fractions (2 mL) were collected, and elution of the polysaccharides was monitored with phenol–sulfuric acid. The polysaccharide peaks were compared with those of dextrans T-2000, T-500, and T-40.

Viscosities of the polysaccharides were measured in water or dimethyl sulfoxide at 25 $\pm 0.05°$, by using a capillary viscometer of the Ubbelohde type, and the intrinsic viscosities $[\eta]$ were calculated.

Assay of antitumor activity. — All tumor cells used in the present study were those maintained in the National Cancer Center Research Institute, Tokyo. Two different allogeneic tumors, *i.e.*, ascites Sarcoma 180, and Ehrlich carcinoma ($5–6 \times 10^6$ cells) were respectively implanted, subcutaneously, into the right groin of female mice weighing 23 g. The strains of mice used were ICR-JCL and C3H/He for Sarcoma 180, and ICR-JCL for Ehrlich carcinoma.

Meth-A fibrosarcoma tumor cells ($1O^4$) were implanted subcutaneously into BALB/C mice. This tumor is syngeneic in the relationship of tumor to host.

The test polysaccharide was dissolved or suspended in distilled water, and injected intraperitoneally, daily for 10 days, starting one day after the tumor implantation. The mice were kept under observation, the size of solid tumors being measured for 5 weeks, and then killed. The weights of solid tumors were compared with those in the control mice.

The inhibition of tumor growth was calculated as: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor-weight of the control mice, and B is that of the polysaccharide-treated mice.

Complete regression is expressed as the ratio of the number of tumor-free mice to the number of mice tested, after 5 weeks.

Preparation of pestalotan. — *Pestalotan* sp. 815 was cultivated by the flask-shaking technique, in a medium (100 mL) containing 3.0% of D-glucose, 0.3% of yeast extract (Difco), 0.3% of peptone (Difco), 0.1% of KH_2PO_4, and 0.05% of

$MgSO_4 \cdot 7\ H_2O$, pH 6.5, at 25°. After 4 days of cultivation, the culture broth was heat-sterilized, and centrifuged at 10,000*g* for 30 min to remove the fungal cells, and the crude polysaccharide in the clarified solution was precipitated with an equal volume of acetone, and then dried *in vacuo* (yield, 410 mg). To compare the effect of the carbon source on production of the polysaccharide, D-glucose was replaced with various other carbohydrates. Table I shows the yields from the different carbon sources.

For submerged cultivation in a jar fermenter (volume, 30 L), a seed culture (500 mL) grown for 24 h in the aforementioned D-glucose medium was inoculated to the medium (15 L) containing 3.5% of D-glucose, 0.05% of peptone, 0.05% of yeast extract, 0.1% of K_2HPO_4, and 0.05% of $MgSO_4 \cdot 7\ H_2O$, pH 6.5. The cultivation was allowed to proceed for 4 days at 25°, with vigorous agitation (400 r.p.m.) and aeration (10 L of sterilized air per min). The culture broth was then sterilized by heating for 15 min at 120°, and cooled, diluted with an equal volume of water, and centrifuged for 30 min at 10,000*g*. The crude polysaccharide in the clarified, supernatant liquor was precipitated with 50% acetone. The precipitated polysaccharide was dissolved in water, centrifuged to remove insoluble impurities, and re-precipitated with acetone. After one more treatment with water and acetone, the polysaccharide was lyophilized (yield, 58.8 g). This polysaccharide preparation contained 0.05% of N, and, on acid hydrolysis, gave mostly D-glucose and a trace of D-mannose. A portion (5.5 g) of the lyophilized polysaccharide was heated in 0.85% aqueous sodium chloride (1.0 L) for 3 h at 60° with vigorous stirring. After cooling, the insoluble polysaccharide was collected by centrifugation, washed with water, and then with acetone, and dried *in vacuo* (4.95 g). It contained 0.03% of N, and gave only D-glucose on acid hydrolysis.

Preparation of pestalotan polyol. — The water-insoluble pestalotan (100 mg) was oxidized with 0.05M sodium periodate (200 mL) at 10° in the dark, and, at suitable time-intervals, periodate consumption and production of formic acid were determined by the usual titration methods. The oxidation was complete in 7 days, with consumption of 0.79 mol of periodate and liberation of 0.39 mol of formic acid per D-glucosyl residue.

Pestalotan (15 g) was suspended in 0.05M sodium periodate (2.5 L), and oxidized at 10°, with stirring. After 8 days, the oxidation was terminated by addition of an excess of ethylene glycol, and the insoluble, oxidized D-glucan was collected by centrifugation, and treated with a 2% aqueous solution of sodium borohydride (200 mL), the mixture being stirred for 16 h at 25°. The excess of sodium borohydride was decomposed by addition of acetic acid, and the still-turbid solution was dialyzed against water. The resulting D-glucan polyol in the nondialyzable solution was precipitated with two volumes of acetone, and then lyophilized (yield, 11.8 g). The dehydrated pestalotan polyol was not readily soluble in water, but was soluble in dilute alkali and in dimethyl sulfoxide.

Ultrasonic irradiation of pestalotan polyol. — Pestalotan polyol (4.5 g) was suspended in water (300 mL) in a glass beaker, and exposed to 25-kHz, ultrasonic

irradiation at 40°, with a 200-W ultrasonic apparatus (Tomy Seiko, Co., Ltd., Tokyo). At suitable time-intervals, aliquots were taken, and changes in viscosity were measured at 25 ±0.05°. Also, the increase in reducing power of the solution was estimated by determination with the Somogyi–Nelson reagent[17]. A series of these irradiation experiments showed that, under these conditions, ultrasonic irradiation of pestalotan polyol for 210 min may be the most suitable for depolymerization of the polysaccharide. The ultrasonically degraded pestalotan polyol in the clear solution was collected by addition of 75–85% acetone.

Determination of polyhydroxy groups in pestalotan polyol. — A sample (10–20 mg) of D-glucan polyol preparation was hydrolyzed in a screw-capped vial by heating with 90% formic acid (1 mL) for 8 h at 100°, and then with M sulfuric acid for 3 h. The acidic solution was made neutral with barium hydroxide to pH 7.0, the suspension filtered, and the filtrate treated with sodium borohydride for reduction of the reducing sugars. After removing cations and boric acid in the usual way, glycerol and D-glucitol in the mixture were quantitatively analyzed by g.l.c. in a column of 3% of ECNSS-M on Gas Chrom Q at 90–200°, after conversion into the acetates. Alternatively, the hydrolysis products were analyzed by 3-MPa l.c. in a column (0.75 × 60 cm) of TSK G2000PW.

ACKNOWLEDGMENTS

We are grateful to Dr. K. Tsubaki, Tsukuba University, for identification of the micro-organism, and Dr. S. Kirkwood, of the University of Minnesota, for the gift of the purified (1→3)-β-D-glucan hydrolase. We also thank the Research Laboratory, Toyo Soda Industry, Co., Ltd., for providing gel-permeation columns, and Hayashibara Biochemical Research Laboratories for providing standard pullulan preparations. We are indebted to Miss M. Tsunoda and Mrs. S. Oka for technical assistance.

This work was supported, in part, by a Grant-in-Aid to one of the authors (A.M.) from the Ministry of Education, Science, and Culture, Japan (1983).

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