Carbohydrate Research, 66 (1978) 53–65

A NEW FUNGAL α-D-GLUCAN, ELSINAN, ELABORATED BY *Elsinoe leucospila**

YOICHI TSUMURAYA AND AKIRA MISAKI†,

Department of Food and Nutrition, Faculty of Science of Living, Osaka City University, Sugimoto-cho, Sumiyoshi, Osaka 558 (Japan)

SHIGEO TAKAYA,

National Research Institute of Tea, Kanaya-cho, Haibara, Shizuoka 428 (Japan)

AND MITSUO TORII

Department of Immunology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka (Japan)

(Received January 3rd, 1978; accepted for publication, March 24th, 1978)

ABSTRACT

A new α-D-glucan, designated elsinan, has been isolated from the culture filtrate of *Elsinoe leucospila* grown in potato extract–sucrose medium. Acid hydrolysis of the methylated polysaccharide gave 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucose, in the ratio of 2.5:1.0, together with small proportions of 2,3,4,6-tetra- (0.7%) and 2,4-di-*O*-methyl-D-glucose (0.5%), indicating that the glucan is an essentially linear polymer containing (1→4)- and (1→3)-α-D-glucosidic linkages. Periodate oxidation, followed by borohydride reduction and mild hydrolysis with acid (mild Smith degradation) yielded 2-*O*-α-D-glucosyl-D-erythritol and erythritol, in the molar ratio of 1.0:1.4, and a trace of glycerol. Partial acid hydrolysis, and also acetolysis, of elsinan gave nigerose, maltose, *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose, *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose, maltotriose, and a small proportion of maltotetraose. It is concluded that elsinan is composed mainly of maltotriose residues joined by α-(1→3)-linkages, in the sequence →3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→. The unique structural features of elsinan are discussed in comparison with other glucans.

INTRODUCTION

Elsinoe leucospila is a pathogenic fungus responsible for the white scab of tea leaves. During cultivation of *E. leucospila*, isolated from a spot of the white scab, we found that fungal colonies were surrounded with a mucous layer when grown on a potato–sucrose agar plate[1]. Our initial study[2] showed that the mucous material,

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.
†To whom reprint requests should be sent.

designated elsinan, is a new type of α-D-glucan. This paper deals with the detailed structural features of elsinan as revealed by chemical investigations, namely methylation, Smith degradation, and fragmentation analyses by partial, acid hydrolysis and by acetolysis.

RESULTS AND DISCUSSION

Properties of elsinan. — When *E. leucospila* was grown under aerobic conditions in a synthetic medium containing potato extract–sucrose, the medium became viscous because of the production of elsinan. The polysaccharide purified from the culture filtrate was essentially free from nitrogen (N < 0.1%), and was composed solely of D-glucose residues, as shown by paper chromatography and by gas–liquid chromatography (g.l.c.) of the alditol acetate derivatives, after complete hydrolysis by heating for 1 h with 2M sulfuric acid. The homogeneity of elsinan was assessed by ultracentrifugal analysis, which showed $S_{20,w}$ 5.92×10^{-13} (Fig. 1). The high specific rotation, $[\alpha]_D^{25}$ +243° (*c* 0.8, water) and +239° (*c* 0.8, M sodium hydroxide), and the characteristic absorbance at 840 cm^{-1} in the i.r. spectrum indicated α-D-glucosidic linkages. Elsinan did not give a color with iodine. The glucan is readily soluble in water to give a highly viscous solution. Although aqueous solutions are stable at low concentrations, they tend to gradually form a gel at 4° at concentrations >2% (w/v).

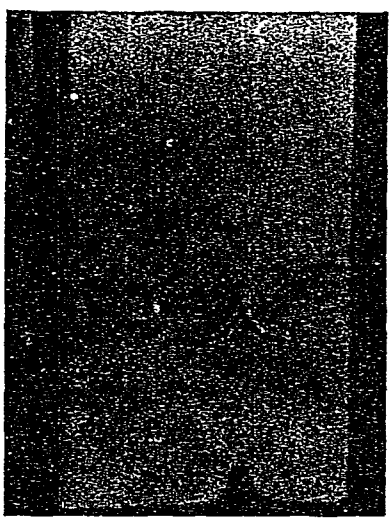

Fig. 1. Sedimentation diagram of elsinan. Conditions; elsinan (5 mg/ml in water) after 44 min at 56,000 r.p.m.

Position of D-*glucosidic linkages.* — Methylated elsinan (5 mg) was hydrolyzed with acid, and the methylated sugar fragments were analyzed by g.l.c.–mass spectrometry as their alditol acetates. The result showed the presence of 2,3,6- (70.8%)

and 2,4,6-tri-*O*-methyl-D-glucose (28.0%), together with small proportions of 2,3,4,6-tetra- (0.7%) and 2,4-di-*O*-methyl-D-glucose (0.5%). Although the retention times of 2,3,6- and 2,3,4-tri-*O*-methyl-D-glucose were very close to each other under the conditions employed, the foregoing methylated sugars were also identified by g.l.c. as the corresponding methyl glycosides.

The glucosidic linkages assigned were also supported by the results of periodate oxidation and Smith degradation. Elsinan (50 mg) was oxidized with 0.03M sodium metaperiodate at 4° and, after complete oxidation (5 days; periodate consumption, 0.80 mol, and formic acid production, 0.07 mol per glucose residue), the oxidized glucan was reduced with sodium borohydride. A portion of the resulting glucan-polyalcohol was subjected to complete Smith degradation[3]. Paper chromatography of the hydrolysis products indicated the presence of erythritol, glucose, and a trace of glycerol. These products were quantitatively analyzed by high-pressure liquid chromatography (h.l.c.), and also (after borohydride reduction) by g.l.c. as the corresponding alditol acetates (Fig. 2). Their molar ratios are listed in Table I. These

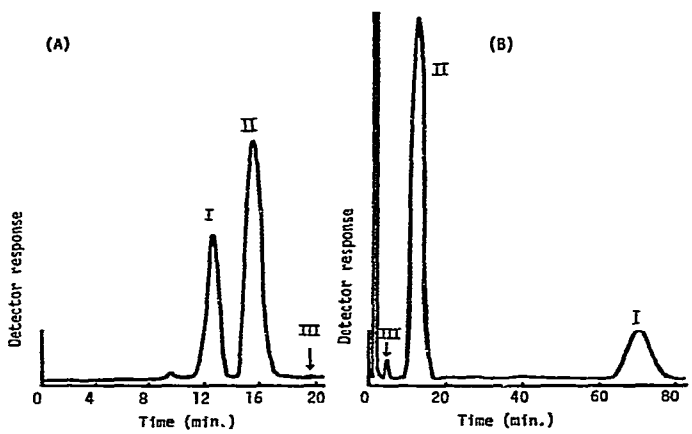

Fig. 2. Identification of the products of complete Smith degradation of elsinan. (A), analyzed with a Yanaco liquid chromatograph Model L-1030 fitted with a refractive-index indicator, on a column of SCX 1001 (6 × 500 mm), with water as carrier, at 25°; (B), analyzed by gas–liquid chromatography, on a column of 3% of ECNSS-M, programmed from 100–190° (6°/min); I, glucose; II, erythritol; III, glycerol.

TABLE I

MOLAR RATIOS OF PRODUCTS FROM THE COMPLETE SMITH DEGRADATION OF ELSINAN

Compound	*Molar proportion* (%)	
	by h.l.c.	*by g.l.c.*
Glycerol	(trace)	1.5
Erythritol	71.0	68.4
Glucose	29.0	30.1

values are in good agreement with those expected from the methylation analysis. The erythritol and glucose presumably arose from α-(1→4)- and α-(1→3)-D-glucosidic linkages, respectively, and the trace of glycerol observed would arise from the non-reducing terminals of the slightly branched glucan.

Both methylation and periodate-oxidation studies clearly indicated that elsinan is an essentially linear molecule consisting mainly of (1→4)-α- and (1→3)-α-D-glucosidic linkages in the molar ratio of 2.3–2.5:1.0. The presence of very small proportions of 2,4-di- and 2,3,4,6-tetra-*O*-methyl-D-glucose may be due to a limited amount of branching through O-6 of the (1→3)-linked D-glucose residues, one branch per ~140 D-glucose residues.

Sequence of the D-*glucosidic linkages in elsinan.* — As elsinan contains both (1→3) and (1→4) linkages, the glucan–polyalcohol was subjected to mild Smith degradation. Paper chromatography showed the presence of erythritol and an unknown component having R_{Glc} 0.88 [6:4:3 (v/v/v) butanol–pyridine–water], together with a trace of glycerol. Complete acid hydrolysis of the unknown component gave approximately equimolar amounts of glucose and erythritol. G.l.c. analysis of the mild-hydrolysis products of the glucan–polyalcohol, separated as their trimethylsilyl ethers, revealed glycerol, erythritol, and an unknown peak. The unknown peak, which corresponded to the compound having R_{Glc} 0.88 extracted from the paper chromatogram, had properties identical with those of 2-*O*-α-D-glucosyl-D-erythritol synthesized by the method of Charlson *et al.*[4]. The molar ratio of glycerol, erythritol, and 2-*O*-α-D-glucosyl-D-erythritol in the hydrolyzate was 0.04:1.44:1.00, as estimated by g.l.c. It is evident from the principle of the Smith degradation[5] that erythritol arises from consecutive α-(1→4)-linked D-glucose residues, such as →4)-D-glucopyranosyl-(1→4)-D-glucopyranosyl-(1→, whereas 2-*O*-α-D-glucosyl-D-erythritol must have arisen from a single, α-(1→3)-linked D-glucose residue flanked by α-(1→4) linkages. Moreover, when the mild hydrolyzate from the glucan–polyalcohol was applied to a column of Bio-Gel P-2, no appreciable peak corresponding to a polysaccharide or oligosaccharide emerged. This result suggests the essential absence of consecutive α-(1→3)-linked D-glucose residues.

Fragmentation of elsinan by partial, acid hydrolysis. — For examination of detailed structural features, elsinan (5 g) was subjected to partial acid hydrolysis with 0.5M sulfuric acid (1.5 liters) for 4 h at 85°. The degradation products were fractionated by charcoal-column chromatography, followed by preparative, paper chromatography. Disaccharide, trisaccharide, and tetrasaccharide components were detected on paper chromatograms, and were isolated and characterized.

Disaccharides. — Two disaccharide components were detected by paper chromatography. One component (22 mg) had R_{Glc} 0.78 [6:4:3, (v/v/v) butanol–pyridine–water], degree of polymerization (d.p.) 2.16, and $[\alpha]_D^{25}$ +135° (*c* 0.7, water). From the results of methylation analysis, this disaccharide was thus identified as nigerose. The other component (35 mg), having R_{Glc} 0.69, d.p. 1.92, and $[\alpha]_D^{25}$ +133° (*c* 0.8, water), was identified, by methylation analysis, as maltose.

Trisaccharides. — Two trisaccharide components were separated by paper chromatography. One (17 mg), having d.p. 3.13 and $[\alpha]_D^{25}$ +152° (*c* 0.6, water), had the same R_{Glc} value (0.49) as that of maltotriose; it was identified, by methylation analysis, as maltotriose.

The other trisaccharide components (trisaccharide A, 20 mg), which gave a single spot in paper chromatography (R_{Glc} 0.53) and had d.p. 2.97, were further fractionated by preparative liquid chromatography according to the method of Torii *et al.*[6]. This procedure clearly separated trisaccharide A into two distinct components, trisaccharide A-1 (3.5 mg) and A-2 (5.5 mg). In paper electrophoresis, the migration values of A-1 (M_{Glc} 0.28) and A-2 (M_{Glc} 0.60) were closely related to those of authentic samples of maltose (M_{Glc} 0.29) and nigerose (M_{Glc} 0.61), respectively. However, acid hydrolysis of methylated trisaccharide A-1 yielded 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1.00:0.92:1.10. The action of yeast α-D-glucosidase on A-1 gave glucose and maltose, as revealed by paper chromatography. From the foregoing results, trisaccharide A-1 was identified as *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose. Trisaccharide A-2, on methylation followed by acid hydrolysis, also gave 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1.00:1.08:1.10. However, the action of α-D-glucosidase, which resulted in the release of glucose and nigerose, differed from that observed with A-1. Therefore, trisaccharide A-2 was identified as *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose.

Tetrasaccharides. — At least, two tetrasaccharides were detected by paper chromatography, one having R_{Glc} 0.30 and the other (R_{Glc} 0.26) corresponding to maltotetraose. They were separated from each other on a filter-paper sheet. The first component (R_{Glc} 0.30; 6 mg) yielded, on methylation followed by acid hydrolysis, 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1.00:1.01:1.90. However, paper electrophoresis of this tetrasaccharide fraction showed two components, one having M_{Glc} 0.22 and the other 0.51. Therefore, the tetrasaccharide fraction appears to be a mixture of two components, both containing one α-(1→3)- and two α-(1→4)-linked D-glucose residues. The first component (M_{Glc} 0.22) was identical to *O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-D-glucose, which has been isolated from a digest of elsinan with human salivary alpha amylase (Y. Tsumuraya and A. Misaki, unpublished result). The other component (M_{Glc} 0.51) may be either *O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→3)-D-glucose or *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-D-glucose. The possibility of the presence of a tetrasaccharide comprising two consecutive α-(1→3)- and one α-(1→4)-linked D-glucose residues may be ruled out, as no 2-*O*-α-D-nigerosyl-D-erythritol was detected in the product of Smith degradation of elsinan.

The other tetrasaccharide component (R_{Glc} 0.26, 7 mg) had the same R_{Glc} value as that of maltotetraose, and M_{Glc} 0.22, together with a trace spot of M_{Glc} 0.51 on paper electrophoresis. Methylation analysis revealed the presence of 2,3,4,6-tetra-,

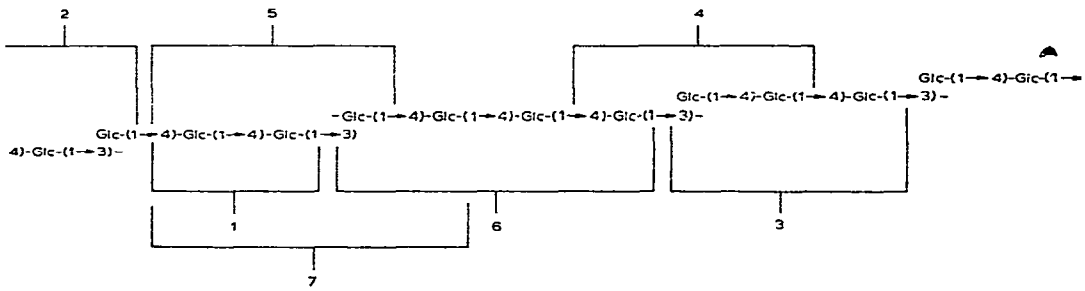

Fig. 3. Proposed structure of elsinan and formation of oligosaccharides by partial, acid hydrolysis. **1**, maltose; **2**, nigerose; **3**, maltotriose; **4**, *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose; **5**, *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose; **6**, maltotetraose; **7**, tetrasaccharide composed of one α-(1→3)- and two α-(1→4)-linked D-glucose residues; and Glc, α-D-glucopyranosyl group.

2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1.00:0.18:2.91. Action of beta amylase on the tetrasaccharide, under conditions for complete hydrolysis of authentic maltotetraose, gave maltose and a trace of an unreacted tetrasaccharide component (paper chromatogram). The borohydride reduced tetrasaccharide, after incubation with the same enzyme, showed maltose, maltitol, and a trace of unreacted tetrasaccharide. These results suggested that the main tetrasaccharide must be maltotetraose. The detection of a trace of unreacted tetrasaccharide after action of beta amylase might to be due to a contamination by a trace of the first tetrasaccharide component.

The formation of oligosaccharides by partial, acid hydrolysis of elsinan is illustrated in Fig. 3.

Fragmentation of elsinan by acetolysis. — Elsinan (3 g) was subjected to acetolysis, and the degradation products were fractionated on a charcoal column, with subsequent paper chromatography. The oligosaccharide fractions thus obtained were compared with those obtained by partial, acid hydrolysis.

Paper chromatography showed nigerose and maltose, as observed upon partial acid hydrolysis. The trisaccharide fraction contained at least two components (R_{Glc} 0.52 and 0.47). Their R_{Glc} values (and M_{Glc} values of 0.27 and 0.56) were in good agreement with those observed after partial, acid hydrolysis. Therefore, the trisaccharide fraction from acetolysis of elsinan appeared to be a mixture of maltotriose, *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose, and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose. The tetrasaccharide fraction appeared to be a mixture of two components, each having the same R_{Glc} and M_{Glc} values as those of the products from partial, acid hydrolysis of elsinan. The tetrasaccharide fraction obtained by acetolysis also appeared to be a mixture of maltotetraose and a tetrasaccharide composed of one α-(1→3)- and two α-(1→4)-linked D-glucose residues.

Effects of various carbon sources on the structure of elsinan. — All glucan

preparations elaborated in shaking culture, utilizing different carbon sources in the presence of an appropriate concentration of corn steep-liquor, were found to be composed solely of D-glucose. These glucan preparations were shown by methylation analysis to have essentially the same structures. Table II shows the identities and molar ratios of the methylated glucose fragments. The glucan produced from a medium of D-glucose appeared to have a slightly higher content of (1→4) linkages than the glucan preparations from other carbohydrate sources. It is apparent from Table II that there is no essential structural difference between the glucan preparation

TABLE II

YIELDS AND METHYLATION ANALYSES OF ELSINAN[a] PRODUCED FROM VARIOUS CARBON SOURCES

	Carbon source						
	Sucrose	D-*Fructose*	D-*Mannose*	D-*Glucose*	*Maltose*	D-*Xylose*	D-*Mannitol*
Yield of elsinan (g dry weight/ 100 ml of broth)	2.6	2.3	1.6	1.5	1.4	1.0	0.9
O-Methyl-D-glucose (%)							
2,3,4,6-tetra-	0.4	0.7	0.4	1.6	0.4	0.4	0.4
2,4,6-tri-	27.9	27.4	27.3	21.4	27.6	28.0	27.6
2,3,6-tri-	71.7	71.9	72.1	76.1	72.0	71.6	72.0
2,4-di-	(trace)	(trace)	0.2	0.9	(trace)	(trace)	(trace)

[a]Elsinan was produced by shaking culture in a medium containing 5% of the carbon source and 0.5% of corn steep-liquor.

synthesized from sucrose plus potato extract and the glucan produced from sucrose in the presence of corn steep-liquor.

The foregoing findings indicate that the main chain of elsinan produced by *E. leucospila* consists most probably of maltotriose residues joined by single α-(1→3)-linkages, as illustrated in Fig. 3. However, the ratio of α-(1→4) to α-(1→3) linkages (2.3–2.5:1.0), is higher than that (2.0:1.0) for such a structure, and suggests the presence of a minor proportion of maltotetraose residues in addition to maltotriose residues. This supposition was confirmed by the detection of maltotriose and maltotetraose in the partial, acid hydrolyzate of elsinan. Acetolysis of elsinan also gave oligosaccharide products similar to those obtained from the partial, acid hydrolyzate.

Although elsinan has an essentially linear structure, there may be a very few branches, one out of ~140 D-glucosyl residues, attached to the α-(1→3)-linked D-glucosyl residues by (1→6) linkages. The essentially linear character, arising from the regularity of repeating glucosyl residues, is evidently related to the gel-forming property of the glucan.

This glucan may be produced extracellularly from such different carbohydrate sources as sucrose, D-fructose, D-mannose, maltose, D-xylose, and D-mannitol, but

not from D-galactose, D-glucitol, ethylene glycol, or sodium acetate. The yields of the polysaccharide vary according to the carbon source; sucrose and D-fructose appeared to be the best sources among those investigated.

The present glucan has some points of structural resemblance with pullulan, an extracellular α-D-glucan of a black yeast, *Aureobasidium* (*Pullularia*) *pullulans*[7], and also with nigeran, the α-D-glucan of *Aspergillus niger*[8]. Pullulan is a water-soluble, linear polymer composed of maltotriose residues joined by α-(1→6) linkages, instead of the α-(1→3) linkages found in the present glucan. Furthermore, the structure of pullulan, which contains about 6% of maltotetraose residues[9], is again reminiscent of the presence of maltotetraose residues in elsinan, although further structural analyses would be necessary to determine the exact ratio of maltotetraose to maltotriose residues in elsinan. Nigeran, obtained by hot-water extraction from the mycelia of *Aspergillus niger*, contains both α-(1→3)- and (1→4)-D-glucosidic linkages, in an alternating sequence. Interestingly, the solubility of elsinan differs from that of nigeran. Nigeran is water-soluble at higher temperatures, but becomes insoluble upon cooling. Elsinan is water-soluble, but at higher concentrations it forms a gel. Both *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose, isolated from partial hydrolyzates and acetolyzates of elsinan, have also been obtained as trisaccharide components from nigeran[10]. However, the isolation of maltotriose from the present glucan clearly indicates its unique arrangement of α-linked D-glucosyl residues. It may be noted that lichenan, a water-soluble β-D-glucan extracted from Iceland moss (*Cetraria islandica*), is a linear polysaccharide having almost the same structural sequences as elsinan, except for the opposite anomeric configurations, namely, cellotriose and cellotetraose residues joined by (1→3)-β-D-glucosidic linkages[11,12]. Isolichenan, another type of D-glucan obtained from Iceland moss, is known[23] to contain both α-(1→3)- and α-(1→4)-D-glucosidic linkages in the relative proportion of 3:2. However, unlike elsinan, it is iodophilic and contains two consecutive, α-D-(1→3)-linked sugar residues, in addition to single sugar residues that are flanked by α-D-(1→4)-linkages[23,24]. It may be of value to compare such physical properties as solubilities and rheological of elsinan with those of lichenan and isolichenan.

The present methylation and fragmentation data establish that elsinan consists of three consecutive α-(1→4)-linked D-glucose residues joined by α-(1→3)-linkages. This type of glucan is new as far as we are aware, and we have named this glucan elsinan[2]. In the previous short communication[2], we reported elsinan as a slightly more highly branched glucan (average repeating unit, ~70 D-glucosyl residues). In this connection, we have occasionally observed that the same fungal strain does not produce the viscous glucan in good yield. This observation prompted us to re-isolate a better strain that produced more mucous product on cultivation on a potato extract–sucrose agar plate, and we have re-examined the structure of elsinan. As reported in this paper, there is little significant difference between the present and previous glucan preparations, except for the length of the average repeating-units.

In relation to chemical taxononomy, it should be pointed out that other plant pathogenic fungi belonging to the *Elsinoe* species, such as *Elsinoe fawcetti*, which is responsible for citrus scab, have been shown to produce an extracellular α-D-glucans similar to the present glucan. Structural comparison of elsinan from several *Elsinoe* species will be reported elsewhere.

EXPERIMENTAL

Materials. — α-D-Glucosidase (from *Saccharomyces cerevisiae*, type I) and beta amylase (from sweet potato, type I-B) were purchased from Sigma Chemical Co. Standard sugar specimens were available in our laboratories.

Production of polysaccharide. — *Elsinoe leucospila*, strain CS-1, was isolated from a spot of the white scab of tea leaf. In a preliminary experiment, the conidia of *E. leucospila* were suspended in sterilized water and inoculated on a cellophane sheet that covered an agar plate containing 2% of sucrose plus potato extract. After growing for 4–5 days at 24°, the slimy colonies were suspended in water, centrifuged, and the polysaccharide in the supernatant precipitated by addition of 60% ethanol. The polysaccharide was dissolved in water, reprecipitated with ethanol, and dried *in vacuo*.

For submerged culture, the fungus was grown by shaking the culture in a medium containing 5% of sucrose, 0.5% of yeast extract (Difco Laboratories), potato extract (300 g of potatoes were sliced and boiled in water, dialyzed against distilled water, and the dialyzable fraction used for preparing one liter of culture medium), 0.042% of $Na_2HPO_4 \cdot 12H_2O$ and 0.018% of KH_2PO_4, pH 6.8. Each 10 ml of preculture grown in the same medium for 24 h at 24° was added to 120 ml of the foregoing medium in a 500-ml shaking flask, and cultured for 5–6 days at 24°. The viscous culture-broth was clarified by centrifugation and the crude polysaccharide precipitated by addition of 2 volumes of ethanol. It was dissolved in water, dialyzed, and precipitated again with ethanol. After repeating the same procedure twice, the polysaccharide was lyophilized; yield ~1.6 g per 100 ml of the broth.

Another experiment, to examine the effects of various carbohydrates as carbon sources on the production and the structures of the glucan, was performed by shaking the culture. The medium contained 0.5% of corn steep-liquor, 0.2% of $NaNO_3$, 0.1% of K_2HPO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.05% of KCl, and 5% of the following carbon sources: sucrose, D-fructose, D-mannose, maltose, D-xylose, D-mannitol, D-galactose, D-glucitol, ethylene glycol, or sodium acetate, pH 6.8. Cultivation was conducted for 7 days at 26° in the same way as with the potato extract–sucrose medium. The glucan so produced was purified by repeated precipitation with methanol, and dialysis.

General analytical methods. — The total carbohydrate content was generally determined colorimetrically by the phenol–sulfuric acid method[13]. The reducing-sugar content was measured by the Nelson–Somogyi method[14]. The degree of polymerization (d.p.) of oligosaccharides was estimated from the ratio of total-sugar content to reducing-group content. The nitrogen content was determined by the method of Kjeldahl[15]. Deionization was conducted with a short column of Amberlite

IR-120B(H^+) resin, or a column of the mixed resins Amberlite IR-120B(H^+) and IRA-400(OH^-). All evaporations were conducted below 40°.

Paper chromatography was performed on Toyo-Roshi No. 50 or Whatman 3MM paper with 6:4:3 (v/v/v) butanol–pyridine–water as solvent. Sugars on the chromatograms were detected by alkaline silver nitrate reagent[16] or *p*-anisidine hydrochloride[17]. In some instances, the paper was treated with glucoamylase solution (Glucozyme, Nagase & Co., Ltd.) before treatment with the silver nitrate reagent.

Gas–liquid chromatography was performed with a Shimadzu gas chromatograph GC-6AM fitted with a flame-ionization detector. Partially methylated sugars were separated on a column (2 m) of 3% of ECNSS-M on Gaschrom Q at 180°, as their corresponding alditol acetates. The methyl glycosides were separated on a column (1 m) of 15% of butanediol succinate polyester on Neosorb N at 175°. Separation of the mild Smith-degradation products was performed as their trimethylsilylated derivatives on a column (1 m) of OV-17 programmed from 80–320° (6°/min).

Preparative liquid chromatography was performed with a JEOL liquid chromatograph Model JLC-3BC by using a anion-exchange resin, JEOL resin LCR-3 (Japan Electronic Optical Laboratory, Tokyo, Japan). The oligosaccharides were separated by stepwise elution with 0.13M (pH 7.5), 0.25M (pH 9.0), and 0.35M (pH 9.6) borate buffers, at 55°, according to the method of Torii *et al.*[6]. Fractions containing oligosaccharides were combined, neutralized with Amberlite IR-120B(H^+) resin, evaporated in the presence of methanol, and dried *in vacuo*.

Paper electrophoresis was performed on Whatmann 3MM paper with 0.1M borate buffer (pH 9.2) at 15 v/cm. After electrophoresis, the paper was dried, dipped in methanol acidified with acetic acid[18], and then the spots of sugars were detected by alkaline silver nitrate reagent.

Methylation of elsinan and oligosaccharides. — Methylation was performed by the method of Hakomori[19]. Elsinan (20 mg) was dissolved in dimethyl sulfoxide (2 ml) with ultrasonication in a nitrogen atmosphere. The solution was treated with methylsulfinyl carbanion (0.5 ml) for 4 h at room temperature, and then with methyl iodide (1.5 ml) for 1.5 h at 20°. The mixture was dialyzed against water and dried *in vacuo*. The methylation procedure was repeated twice, and the fully methylated glucan (5 mg) was then 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 at 100°. The methylated sugar components were reduced with sodium borohydride and the corresponding alditols were acetylated by heating with 1:1 pyridine–acetic anhydride (0.2 ml) for 2 h at 100°, and the products were analyzed by g.l.c. Another portion of the methylated glucan (5 mg) was methanolyzed by heating with 3% methanolic hydrogen chloride (0.5 ml) for 18 h at 100°. The mixture of methyl glycosides was neutralized with silver carbonate, and analyzed by g.l.c.

Oligosaccharides (1–5 mg) were methylated by the same procedure. The methylated product was extracted with chloroform and the extract was washed with distilled water, and evaporated to a syrup. The syrup was dissolved in a small volume of chloroform–methanol (2:1, v/v), and applied to a column (1 × 20 cm) of Sephadex

LH-20 that was eluted with the same solvent. Fractions containing methylated oligosaccharides were combined, and evaporated to dryness. The fully methylated oligosaccharide thus obtained was hydrolyzed by the procedure already described, and then analyzed by g.l.c. after conversion into the corresponding alditol acetates or methyl glycosides.

Periodate oxidation. — The glucan (50 mg) was oxidized with 0.03M sodium metaperiodate (100 ml) at 4° in the dark. The production of formic acid and consumption of periodate were periodically determined by titration with 0.01M sodium hydroxide and by the Fleury–Lange method, respectively[20]. After completion of the oxidation, the reaction was stopped by addition of ethylene glycol (5 ml), the mixture dialyzed, and the resulting oxidized glucan was reduced to the corresponding glucan–polyalcohol by treatment with sodium borohydride. A portion of the glucan–polyalcohol was hydrolyzed with 1.75M sulfuric acid for 1 h at 100°, and neutralized with barium carbonate (complete Smith degradation)[3]. The other portion was hydrolyzed with 0.05M sulfuric acid for 20 h at 25° (mild Smith degradation). The products of both Smith degradations were examined by paper chromatography, g.l.c., and also by h.l.c.

Partial acid hydrolysis of elsinan. — Elsinan (5.0 g) was treated with 0.5M sulfuric acid (1.5 liter) for 4 h at 85°. The hydrolyzate (apparent conversion into D-glucose, 40.4%) was neutralized with barium carbonate, centrifuged, and the supernatant solution passed successively through columns of Amberlite IR-120B(H^+) and IRA-400(OH^-) resins. The eluate was evaporated to a syrup (recovery 3.5 g). A portion of the hydrolyzate (2.3 g) was fractionated on a column of charcoal (Activated Charcoal for Chromatography, Wako Pure Chemical Industries, Ltd., Japan). After washing the column with distilled water, oligosaccharides were successively eluted with 4 liters each of 4, 7, 9, 11, 13, 15, 20, 25, and warm 50% aqueous ethanol, respectively, the eluate being collected in 1-liter fractions. The fractions were monitored by paper chromatography, and those containing oligosaccharides were pooled and evaporated. Table III shows the results of the fractionation. Oligosaccharides in

TABLE III

YIELDS OF OLIGOSACCHARIDE FRACTIONS [a] FROM PARTIAL, ACID HYDROLYSIS OF ELSINAN

Fraction	*Eluent (% ethanol)*	*Weight (mg)*	*Components*
1	water	1160	glucose
2	4, 7, and 9% (2 liters)	400	disaccharides
3	9 (2 liters), 11, and 13% (2 liters)	208	trisaccharides
4	13 (2 liters) and 15% (2 liters)	55	tri- and tetra-saccharides
5	15% (2 liters)	28	tetrasaccharides
6	20%	90	tetra- and penta-saccharides
7	25%	59	penta-, hexa-, and hepta-saccharides
8	warm 50%	22	higher oligosaccharides

[a]Oligosaccharides were separated on a charcoal column (4.2 × 57 cm), with stepwise elution by 4-liter portions of aqueous ethanol.

the fractions were further purified by preparative, paper chromatography, and then characterized.

Action of enzymes on the oligosaccharides. — Each oligosaccharide (2 mg) isolated from the partial, acid hydrolyzate was incubated with α-D-glucosidase (50 μg) in 0.05M phosphate buffer (0.5 ml), pH 6.8, at 37°. At appropriate time-intervals, portions were withdrawn, inactivated by heating, and desalted by treatment with ion-exchange resins. The products were examined by paper chromatography. In other experiments, each oligosaccharide (1.5 mg) was incubated with beta amylase (6 μg) in 0.02M acetate buffer (0.2 ml), pH 5.0, at 28°, and the hydrolysis products were examined as before.

Acetolysis of elsinan. — Acetolysis of elsinan was performed according to the method of Matsuda *et al.*[21]. Elsinan (3.0 g) was added to a mixture of acetic anhydride (14.4 ml), acetic acid (9.6 ml), and sulfuric acid (1.8 ml) at 25°. The mixture was stirred for 7 days at 25°, and then heated for 30 min at 80°. The solution was poured into ice–water, neutralized with solid sodium hydrogencarbonate, and the resulting precipitate was extracted with three 100-ml portions of chloroform. The extracts were combined, dried, and evaporated to a syrup (4.5 g). The syrup was deacetylated by treatment with sodium methoxide in methanol; yield 2.1 g. The deacetylated product was applied to a charcoal column (3.5 × 60 cm) and eluted by stepwise elution with 5-liter portions of 0, 7.5, 12, 20, 25, and warm 50% aqueous ethanol. The recoveries of saccharides were as follows: distilled water (680 mg, glucose), 7.5% ethanol (590 mg, mixture of disaccharides), 12% ethanol (230 mg, mixture of trisaccharides), 20% ethanol (100 mg, mixture of tetra- and penta-saccharides), 25% ethanol (20 mg, higher oligosaccharides), and warm 50% ethanol (10 mg, higher oligosaccharides). Each sugar fraction was further fractionated by preparative paper-chromatography, and characterized.

ACKNOWLEDGMENTS

We thank Dr. H. Kumagai, Food Research Institute, Kyoto University, for the ultracentrifugal analysis, and Professor A. Kobata, Kobe University Medical School, for g.l.c.–mass spectrometry. We are also indebted to Dr. K. Yokobayashi and Mr. T. Sugimoto, Hayashibara Biochemical Laboratories, Inc., for their co-operation in cultivation of the microorganism.

REFERENCES

1 S. TAKAYA, T. FUKUDA, AND Y. OIKE, *Chagyo-gijutsu-kenkyu* (Study of Tea), 49 (1975) 79–88.
2 A. MISAKI, Y. TSUMURAYA, AND S. TAKAYA, *Agric. Biol. Chem.*, 42 (1978) 491–493.
3 M. ABDEL-AKHER, J. K. HAMILTON, R. MONTOGOMERY, AND F. SMITH, *J. Am. Chem. Soc.*, 74 (1952) 4970–4971.
4 A. J. CHARLSON AND A. S. PERLIN, *Can. J. Chem.*, 34 (1956) 1200–1208.
5 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Abstr. Pap. Am. Chem. Soc. Meet.*, 135 (1959) 3D.
6 M. TORII, AND K. SAKAKIBARA, *J. Chromatogr.*, 96 (1974) 255–257.
7 H. BENDER, J. LEHMANN, AND K. WALLENFELS, *Biochim. Biophys. Acta*, 36 (1959) 309–316.

8 S. A. BARKER, E. J. BOURNE, AND M. STACEY, *J. Chem. Soc.*, (1953) 3084–3090.
9 B. J. CATLEY AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 143 (1971) 138–142.
10 S. A. BARKER, E. J. BOURNE, D. M. O'MANT, AND M. STACEY, *J. Chem. Soc.*, (1957) 2448–2454.
11 S. PEAT, W. J. WHELAN, AND J. G. ROBERTS, *J. Chem. Soc.*, (1957) 3916–3924.
12 A. S. PERLIN AND S. SUZUKI, *Can. J. Chem.*, 40 (1962) 50–56.
13 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
14 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380; M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19–23.
15 R. B. BRADSTREET, *Chem. Rev.*, 27 (1940) 331–350.
16 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
17 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702–1706.
18 K. SAKAKIBARA, M. TORII, A. MISAKI, AND H. MIYAJI, *Carbohydr. Res.*, 25 (1972) 443–451.
19 S. HAKOMORI, *J. Biochem. (Tokyo)*, *55* (1964) 205–207.
20 P. F. FLEURY AND J. LANGE, *J. Pharm. Chim.*, 17 (1933) 107, 196.
21 K. MATSUDA, H. WATANABE, K. FUJIMOTO, AND K. ASO, *Nature (London)*, 191 (1961) 278.
22 N. B. CHANDA, E. L. HIRST, AND D. J. MANNERS, *J. Chem. Soc.*, (1957) 1951–1958.
23 S. PEAT, W. J. WHELAN, J. R. TURVEY, AND K. MORGAN, *J. Chem. Soc.*, (1961) 623–629.
24 M. FLEMING AND D. J. MANNERS, *Biochem. J.*, 100 (1966) 24P.