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DEGRADATION OF ELSINAN BY ALPHA AMYLASES, AND ELUCIDATION OF ITS FINE STRUCTURE*

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ABSTRACT

Elsinan, a unique fungal polysaccharide consisting of maltotriose and maltotetraose units joined by α-D-(1→3)-glucosidic linkages, was previously found to be degraded by particular types of alpha amylases, *e.g.*, salivary, pancreatic, and bacterial (saccharifying) alpha amylase, to produce *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-D-glucose as the major product. Taka amylase (alpha amylase of *Aspergillus oryzae*) is also capable of hydrolyzing elsinan, by cleaving α-D-(1→4)-glucosidic linkages in the maltotetraose units, but not in the maltotriose units, to produce several novel oligosaccharides. Methylation, and chemical and enzymic fragmentation-analyses, showed that the tetrasaccharide (TG_4), as the major product, is *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-D-glucose, and the heptasaccharide (TG_7) has the sequence α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*. The molar ratios of tetra-, hepta-, deca-, trideca-, and hexadeca-gluco-oligosaccharide released from elsinan by digestion with Taka amylase were estimated by gel-filtration chromatography to be 2.78:1.0:0.61:0.26:0.11. The high-molecular weight saccharide of $\overline{d.p.}$ 30–35 (recovered in 33% yield) in the enzyme digest consisted exclusively of α-D-(1→3)-linked maltotriose units. On the basis of the quantitation of these degradation products, the fine structure of elsinan and the substrate specificities of alpha amylases are discussed.

INTRODUCTION

Elsinan is a new, linear α-D-glucan, elaborated by *Elsinoe leucospila* when grown in sucrose or other carbohydrate-containing media. In previous studies, methylation and chemical-fragmentation analyses proved that this D-glucan consists of maltotriose and maltotetraose (minor) units that are joined by α-D-(1→3)-glucosi-

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dic linkages[1,2]. This polysaccharide was found to be partially hydrolyzed by particular types of amylolytic enzymes, such as human salivary, hog pancreas, and *Bacillus subtilis* saccharifying alpha amylase, but not by bacterial, liquefying alpha amylase, beta amylase, or glucoamylase[3].

The action of these elsinan-degrading alpha amylases results in release of *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-α-D-glucose (4-*O*-α-nigerosyl-D-glucose) in yields of 75–92% (weight basis), and small proportions of D-glucose and *O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-D-glucose (3-*O*-α-maltosylmaltose) in the case of human, salivary alpha amylase. During further enzymic-degradation studies, we became aware that the action of the alpha amylase of *Aspergillus oryzae*, known as Taka amylase, results in the production of novel tetra- and hepta-glucosaccharides. As the substrate specificity of this enzyme appears to differ from those of salivary-type alpha amylases, our attention was drawn to the nature of the degradation products, and the mode of action on elsinan in comparison with those of other enzymes. We now report the isolation and characterization of new tetra- and hepta-glucosaccharides and other degradation products, revealed by using chemical and enzymic techniques, and the arrangements of the α-(1→3)-linked D-glucosyl residues in the elsinan molecule.

MATERIALS AND METHODS

Materials. — Elsinan was prepared[1] from the culture filtrate of *E. leucospila* after growth in sucrose–potato extract medium, and purified as described[1]. Human, salivary alpha amylase was partially purified from the saliva of adult, male volunters[4]. Taka amylase (thrice recrystallized) was purchased from Sankyo Co., Tokyo; this enzyme gave a single, protein band in gel electrophoresis. Saccharifying, alpha amylase of *Bacillus subtilis* (twice recrystallized) was purchased from Seikagaku Kogyo Co., Tokyo. Hog-pancreas alpha amylase, *Saccharomyces cerevisiae* α-D-glucosidase, and glucoamylase (twice recrystallized) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Soluble starch, purchased from Kishida Chemical Co., Osaka, was partially purified by dialysis to remove reducing oligosaccharides.

General methods. — All evaporations were conducted below 40° under diminished pressure. Total carbohydrate content and reducing sugars were respectively determined by the phenol–sulfuric acid method[5] and the Somogyi method[6]. Determination of the degree of polymerization (d.p.) of oligosaccharides was performed by the method of Timell[7], based on measurement of the glucose content before and after reduction with borohydride, and the average degree of polymerization ($\overline{d.p.}$) of saccharides of high molecular weight was estimated by measurement of the amount of D-glucitol, by using D-glucitol dehydrogenase (Boehringer Mannheim GmbH), after hydrolysis of the borohydride-reduced saccharide[8] with acid.

Paper chromatography was performed by using Tōyō Roshi No. 50 paper or Whatman No. 3 MM paper with 6:4:3 1-butanol–pyridine–water as the solvent system. Sugars on paper chromatograms were detected by using alkaline silver nitrate

reagent, and, if necessary, after spraying with 1% sodium periodate in 50% acetone solution, or 1% glucoamylase solution.

Paper electrophoresis was performed on Whatman No. 3 MM paper with 0.1M borate buffer, pH 9.2, at 15 V/cm. The paper was dried, dipped in methanol acidified with acetic acid, and treated with alkaline silver nitrate reagent.

Gel-filtration chromatography of the enzymic-degradation product was performed by using a column (2.1 × 100 cm) of Bio-gel P-4 or a column (1.0 × 200 cm) of P-2, carbohydrates being eluted with water at 55° under a slight pressure. Liquid chromatography (l.c.) was conducted by using a Hitachi Liquid Chromatograph, Model 635, fitted with a refractive-index detector, in a column (2.6 × 250 mm) of LiChrosorb NH_2 (E. Merck, AG), and developed at 25° with 7:3 acetonitrile–water.

Gas-liquid chromatography (g.l.c.) was performed with a Hitachi Gas Chromatograph, Model 163, fitted with a flame-ionization detector. Sugars, and partially methylated sugars, were separated in a column (0.5 × 200 cm) of 3% of ECNSS-M on Gas Chrom Q at 180°, or 175°, after conversion into their corresponding alditol acetates.

Methylation analysis. — Methylation of oligosaccharides isolated from enzyme digests of elsinan was performed by the Hakomori method[9]. A sample of oligosaccharide (3–5 mg) was dissolved in dimethyl sulfoxide (2 mL), and treated with methylsulfinyl carbanion (0.5 mL) for 3 h at room temperature under a nitrogen atmosphere, and then with methyl iodide (1.5 mL) for 1.5 h at 20°. The permethylated oligosaccharide was extracted with chloroform, the extract evaporated, the residue dissolved in a small volume of 2:1 chloroform–methanol and the solution applied to a column (1 × 20 cm) of Sephadex LH-20, the methylated product being eluted with the same solvent. A portion of the purified, methylated oligosaccharide was hydrolyzed by heating with 3M trifluoroacetic acid (0.5 mL) for 5 h at 100°. After evaporation of the acid, the hydrolysis products were converted into their corresponding alditol acetates, and analyzed by g.l.c.

RESULTS

Enzymic degradation of elsinan by alpha amylases. — The time course for the degradation of elsinan by Taka amylase is shown in Fig. 1. The hydrolysis of elsinan reaches a maximum at 2 h of incubation, with an apparent hydrolysis of 11% (expressed as glucose), which is much lower than that of soluble starch (apparent hydrolysis, 38%) under the same conditions. The extent of hydrolysis by Taka amylase was significantly lower than those catalyzed by human-salivary, hog-pancreas, and bacterial saccharifying alpha amylases (*cf.*, Table I). To compare the kinetics of the hydrolysis of elsinan by Taka amylase to that of soluble starch, different concentrations of the two polysaccharides were incubated with the enzyme in 0.05M acetate buffer, pH 5.4, at 37°, and the K_m values were calculated from Lineweaver–Burk plots, in the same way as in a previous study[3]. The results showed that K_m for elsinan is 240mM (expressed in terms of concentration of glucosyl unit), whereas K_m for the hydrolysis of soluble

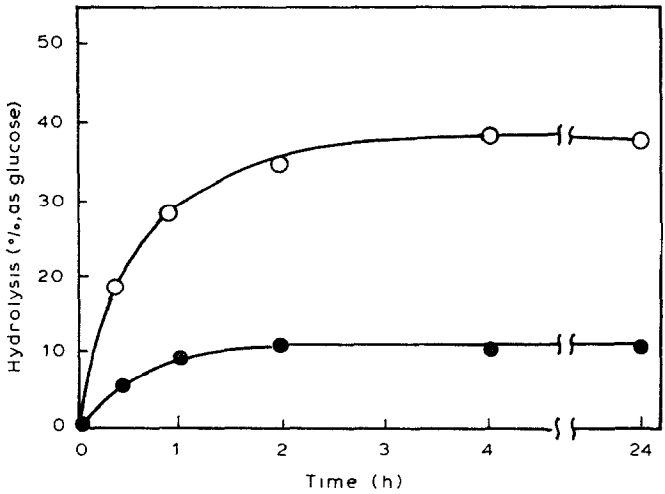

Fig. 1. Time course of Taka amylase digestion of elsinan and soluble starch. [Elsinan (●, 20 mg) and soluble starch (○, 20 mg) were incubated with Taka amylase (0.4 mg) in 0.05M acetate buffer, pH 5.4 (5 mL), at 37°.]

starch under the same conditions was 7.2mM. The maximum velocity (V_{max}, in min per unit of enzyme) for the hydrolysis of elsinan was shown to be approximately one third of that for soluble starch. These results indicated that Taka amylase has a significantly low affinity for the elsinan molecule, compared with that of soluble starch, and, therefore, low hydrolytic activity on elsinan.

In order to examine the degradation products, elsinan (40 mg) was incubated with Taka amylase (0.6 mg) in 0.05M acetate buffer, pH 5.4 (5 mL) for 20 h, and the

TABLE I

COMPARISON OF HYDROLYSIS PRODUCTS FROM ELSINAN BY ACTION OF SEVERAL ALPHA AMYLASES

Alpha amylase	*Extent of hydrolysis, % (as glucose)*	*Molar proportion, %*[a]							
		G_1	G_2	G_3	G_4	G_7	G_{10}	G_{13}	G_{16}
Human salivary[b]	29	29.7	0.7	62.2	7.4	—	—	—	—
Hog pancreas[b]	33	38.5	0.5	70.0	(trace)	—	—	—	—
B. subtilis (saccharifying)[b]	35	19.5	—	81.5	—	—	—	—	—
Asp. oryzae[c] (Taka amylase)	11	—	—	—	58.4	21.0	12.8	5.5	2.3

[a]The sum of mono- and oligo-saccharides corresponds to 100%. [b]The molar proportion of each product was calculated by appropriate correction of the detector response in the analysis by l.c. (column, LiChrosorb NH_2; solvent system, 7:3 acetonitrile–water at 25°). [c]Analyzed by gel filtration on a column of Bio-gel P-4 after removing the high-molecular-weight fraction by ethanol precipitation (see text).

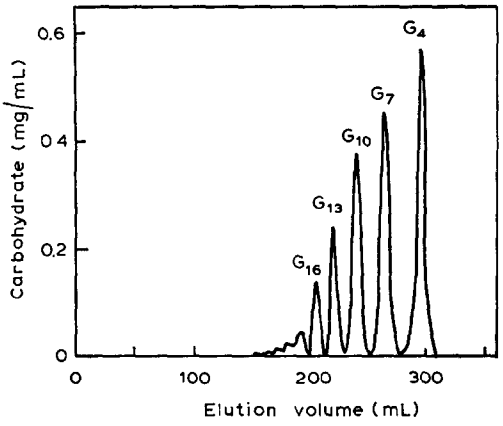

Fig. 2. Gel-filtration profile of reaction products from elsinan after Taka amylase digestion. [After removing the fraction of high molecular weight (33%), the mixture of oligosaccharides was fractionated in a column (2.1 × 100 cm) of Bio-gel P-4 by elution with water, at 55°. Carbohydrate contents were assayed by the phenol–sulfuric acid method.]

digestion product was analyzed by gel-filtration chromatography on Bio-gel P-4. The molar proportions of oligosaccharide products were compared to those obtained by digestion with other elsinan-degrading alpha amylases. As shown in Table I, unlike other enzymes which produce mainly trisaccharide, characterized[3] as *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-D-glucose, the action of Taka amylase resulted in release of tetrasaccharide and a series of higher oligosaccharides, up to hexadecasaccharide. The tetrasaccharide produced by Taka amylase appears to have a structure different from that of that produced by the action of human-salivary alpha amylase.

Isolation and characterization of the products from digestion of elsinan with Taka amylase. — A solution of elsinan (2 g) in 0.05M acetate buffer, pH 5.4 (400 mL) was incubated with Taka amylase (3.6 mg) for 20 h at 37°. After inactivation by heating, ethanol (2 vol.) was gradually added to the mixture, and the precipitate of high-molecular-weight product (800 mg) was removed by centrifugation. A portion of the supernatant solution containing oligosaccharides (976 mg) was fractionated by chromatography on a column of Bio-gel P-4. The gel-filtration profile of the oligosaccharides is shown in Fig. 2, and their molar proportions are given in Table I.

The mixture of oligosaccharides contained tetra-, hepta-, deca-, trideca-, and hexadeca-saccharides, in the molar proportions of 58.4, 21.0, 12.8, 5.46, and 2.34%, or in molar ratios of 2.78 : 1.0 : 0.61 : 0.26 : 0.11. For isolation of the tetra- and hepta-saccharide, which were the major products, the oligosaccharide mixture (900 mg) was applied to a chromatographic column of activated charcoal (Wako Pure Chemical Industry, Osaka). After washing the column with water (500 mL), oligosaccharides were successively eluted with 7.5, 12, and 25% aqueous ethanol, and finally with warm, 50% aqueous ethanol (500 mL of each), in the usual way[2]. The tetra- and hepta-saccharide were respectively present in the 20% and the 25% aqueous ethanol

fractions. Each oligosaccharide was further purified by preparative, paper chromatography, on thick paper (Whatman No. 3 MM) with 6:4:3 1-butanol–pyridine–water as the solvent.

Structure of the tetrasaccharide. — The tetrasaccharide, which was eluted with 20% ethanol, had $[\alpha]_D$ +185° (*c* 1.5, water); d.p. 4.0; R_{Glc} 0.34 in 6:4:3 1-butanol–pyridine–water; and M_{Glc} 0.25 on a paper electrophoretogram. When this tetrasaccharide was methylated, and the product hydrolyzed with acid, 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose, in the molar ratios of 1.0:0.96:1.96, were identified by g.l.c., whereas, after reduction of the terminal residue by treatment with borohydride, acid hydrolysis of the methylated oligosaccharide yielded 2,3,4,6-tetra-, and 2,4,6-tri- and 2,3,6-tri-*O*-methyl-D-glucose, and 1,2,3,5,6-penta-*O*-methyl-D-glucitol, in the molar ratios of 1.0:0.97:1.06:0.83. These results indicated that the original tetrasaccharide comprises one mol of α-(1→3)- and two mol of (1→4)-linked D-glucosyl residues per (nonreducing) terminal group, and that the (reducing) terminal D-glucose residue must be joined by a (1→4) linkage to the adjacent D-glucosyl residue.

The sequence of α-D-(1→4)- and (1→3)-glucosidic sugar residues in the tetrasaccharide was examined by partial, acid hydrolysis and enzymic hydrolysis. The tetrasaccharide (16.7 mg) was heated with 0.8M trifluoroacetic acid (0.75 mL) for 20 min at 95°, and the hydrolyzate was evaporated under diminished pressure. Paper chromatography then revealed the presence of nigerose, maltose, and a mixture of trisaccharides. The trisaccharide fraction, extracted from the paper with water, was shown by l.c. to contain maltotriose and 4-*O*-α-nigerosyl-D-glucose, which was identical to that released from elsinan by the action of salivary alpha amylase (see Fig. 3, **3**). When the tetrasaccharide (10 mg, each) was incubated with α-D-glucosidase or glucoamylase for 3 h, no degradation occurred (paper-chromatographic examination). Under the same conditions, 3-*O*-α-maltosylmaltose, isolated from the salivary-

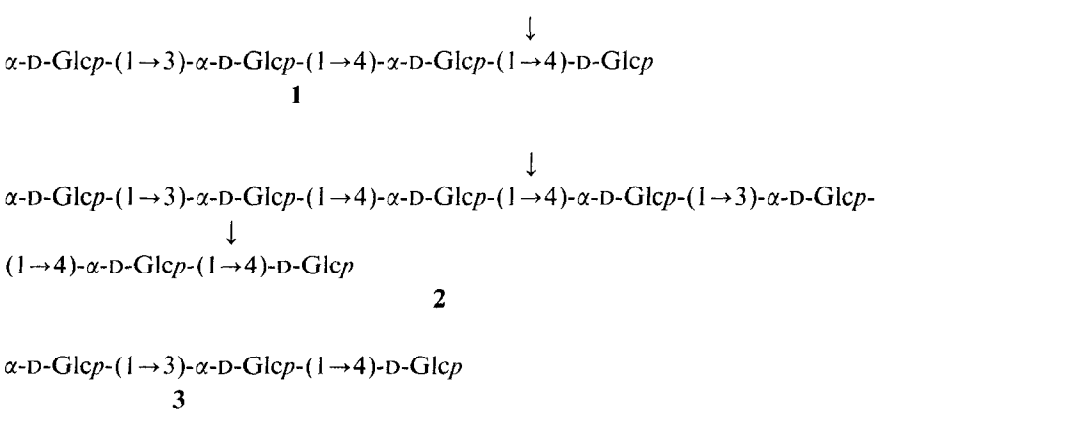

Fig. 3. Structures of tetrasaccharide (TG_4: **1**), heptasaccharide (TG_7: **2**), and trisaccharide (**3**), products of hydrolysis by human-salivary amylase. (↓ represents the site cleaved by the enzyme.)

TABLE II

METHYLATION ANALYSES OF THE HEPTASACCHARIDE AND ITS SALIVARY AMYLASE DEGRADATION-PRODUCTS

O-*Methyl*-D-*glucose*	*Molar ratio*			
	Hepta-saccharide	*R-hepta-saccharide*[a]	*Products from R-heptasaccharide*[b]	
			Trisaccharide	*R-tetrasaccharide*
2,3,4,5,6-Penta-, D-glucitol	—	0.85	—	0.86
2,3,4,6-Tetra-, D-glucose	1.00	1.00	1.00	1.00
2,4,6-Tri-, D-glucose	2.18	1.83	1.04	1.00
2,3,6-Tri-, D-glucose	4.21	3.15	0.97	1.06

[a]Reducing D-glucose residue in the heptasaccharide was converted into a D-glucitol residue; R = reduced. [b]The action of salivary amylase on R-heptasaccharide yielded trisaccharide and reduced tetrasaccharide in the molar ratio of 1.0:1.01 (see text).

amylase digest of elsinan[3], gave glucose and 4-*O*-α-nigerosyl-D-glucose. These results clearly indicated that the tetrasaccharide is *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-D-glucose, *i.e.*, 3-*O*-α-D-glucosyl-maltotriose see Fig. 3, **1**).

Structure of the heptasaccharide. — The heptasaccharide, eluted from the charcoal column with 25% aqueous ethanol, had $[\alpha]_D$ +195° (*c* 1.5, water), d.p. 7.09, R_{Glc} 0.08, and M_{Glc} 0.23. Table II shows the results of methylation analyses of the original heptasaccharide and of its terminally reduced product. Table II also gives the methylation data for the oligosaccharide products from the terminally reduced heptasaccharide. It is clear that the heptasaccharide contains 2 mol of α-D-(1→3)- and 4 mol of α-D-(1→4)-linked D-glucosyl residues per nonreducing (terminal) D-glucosyl group. As one out of 4 mol of 2,3,6-tri-*O*-methyl-D-glucose was eliminated by reduction of the heptasaccharide with borohydride, the (reducing) D-glucose residue must be joined by a (1→4)-bond to the adjacent D-glucosyl residue. When human-salivary alpha amylase or *B. subtilis* (saccharifying) alpha amylase acted on the heptasaccharide, one mol of D-glucose and two mol of the trisaccharide were produced. The trisaccharide was identified, from the methylation study and also its paper-chromatographic migration, as 4-*O*-α-nigerosyl-D-glucose. In another experiment, the terminally reduced heptasaccharide was acted on by human-salivary alpha amylase. Quantitative, paper-chromatographic analysis indicated that the products were the trisaccharide and a reduced tetrasaccharide, in the molar ratio of 1.0:1.1. Methylation analysis indicated that the reduced tetrasaccharide was either *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-D-glucitol or *O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-D-glucitol (see Table II). In order to know the precise structure of the reduced tetrasaccharide, after separation from the trisaccharide by chromatography on thick paper, the tetrasaccharide fraction (15 mg) was subjected to partial, acid hydrolysis with 0.8M

trifluoroacetic acid for 20 min at 95°. Among the hydrolysis products, the trisaccharide fraction, purified by paper chromatography, was analyzed by liquid chromatography, using a column of JEOL LCR-3 (borate form) anion-exchange resin and stepwise elution with 0.13–0.25M borate buffer, in the usual way[2,3]. The result showed that this trisaccharide fraction was a mixture of approximately equal proportions of maltotriitol and 4-*O*-α-nigerosyl-D-glucose, indicating that the parent, reduced tetrasaccharide has the sequence: D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-glucitol. Thus, from the aforementioned, experimental findings provided by chemical and enzymic examination, the heptasaccharide was characterized as *O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→3)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-D-glucose (**2**) (see Fig. 3).

Characterization of the saccharide of high molecular weight. — The high-molecular-weight fraction, obtained in 33% yield from the Taka amylase digest of elsinan by precipitation with 60% ethanol, had $\overline{d.p.}$ 35, as estimated by an enzymic method using D-glucitol dehydrogenase[8]. When this fraction was methylated, and the product hydrolyzed with acid, 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-D-glucose, in the molar ratios of 1.0:9.16:19.70, were obtained. This corresponds to a chain length of ~30, close to the $\overline{d.p.}$ 35 obtained by the enzymic method. The ratio of α-(1→4)- to α-(1→3)-linked D-glucosyl residues, 2.15:1.0, compared to 2.56:1.0 for the native elsinan[2], suggested that the high-molecular-weight saccharide

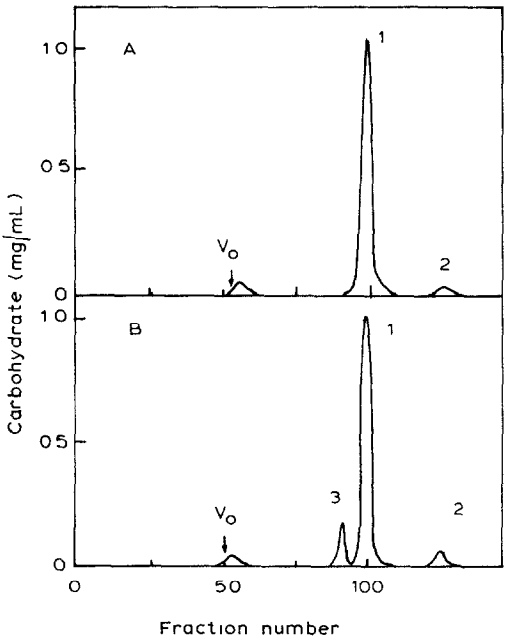

Fig. 4. Comparison of gel-filtration profiles of (A) the fraction of high molecular weight (Taka amylase-degraded elsinan) and (B) native elsinan on a column (1.0 × 200 cm) of Bio-gel P-2 at 25°. (1, 4-*O*-α-nigerosyl-D-glucose; 2, D-glucose; and 3, 3-*O*-α-maltosylmaltose.)

left after digestion with Taka amylase consists mostly of maltotriose units that are flanked by α-D-(1→3)-linked D-glucosyl groups. To confirm this finding, the high-molecular-weight saccharide and native elsinan (200 mg, each) were each incubated with human-salivary alpha amylase (50 units) for 24 h at 37°, and the products were applied to a column (0.8 × 200 cm) of Bio-gel P-2. As shown in Fig. 4 (A and B), the former produced mainly trisaccharide, *i.e.*, 4-*O*-α-nigerosyl-D-glucose, together with a trace of glucose, whereas the native elsinan gave, in addition to the trisaccharide and glucose, a tetrasaccharide, *i.e.*, 3-*O*-maltosylmaltose[3]; a small peak emerging at the void volume may be due to a contaminating, (1→6)-branched, (1→3)-β-D-glucan (unpublished result). These results strongly suggest that this high-molecular-weight saccharide had arisen from the sequence of 10–12 consecutive, maltotriose units, joined by (1→3)-bonds, in the elsinan molecule.

Kinetics of enzymic hydrolysis of the tetra- and the heptasaccharide. — To examine the susceptibilities of the tetrasaccharide and heptasaccharide, designated TG_4 and TG_7, toward elsinan-degrading alpha amylases, the kinetics of the enzymic hydrolysis were studied, in comparison with those of maltotetraose (MG_4) and maltoheptaose (MG_7). Each oligosaccharide, in various concentrations, was incubated with *B. subtilis* saccharifying alpha amylase, human-salivary amylase, and human-pancreatic amylase, at their optimal pH at 37°, and their apparent K_m values (ex-

TABLE III

COMPARISON OF ENZYMIC DEGRADATION OF GLUCO-OLIGOSACCHARIDES

B. subtilis *saccharifying alpha amylase*		*Human-salivary alpha amylase*		*Human-pancreatic alpha amylase*	
Oligosaccharide[a]					
TG_4	*MG_4*	*TG_4*	*MG_4*	*TG_4*	*MG_4*
Initial velocity[b]					
8.0	100	0.5	100	2.1	100
K_m (mM)					
1.1	0.081	2.5	1.7	3.8	1.8
Oligosaccharide[c]					
TG_7	*MG_7*	*TG_7*	*MG_7*	*TG_7*	*MG_7*
Initial velocity					
6.7	100	0.8	100	0.6	100
K_m (mM)					
0.63	0.020	1.3	0.80	1.7	0.72

[a]TG_4 = Glc*p*-(1→3)-Glc*p*-(1→4)-Glc*p*-(1→4)-Glc*p*, MG_4 = maltotetraose. [b]The initial reaction velocity of each enzyme toward TG_4 or TG_7 is expressed as a percentage of that toward the corresponding malto-oligosacchride. [c]TG_7 = Glc*p*-(1→3)-Glc*p*-(1→4)-Glc*p*-(1→4)-Glc*p*-(1→3)-Glc*p*-(1→4)-Glc*p*-(1→4)-Glc*p*, MG_7 = maltoheptaose.

pressed in terms of the concentration of D-glucosyl unit) were calculated from their Lineweaver–Burk plots. Table III shows the K_m value of each enzyme toward various D-gluco-oligosaccharides and the hydrolysis velocity (relative to that of maltotetraose or maltoheptaose). It is evident that TG_4 and TG_7, which contain both α-D-(1→4)- and -(1→3)-glucosidic linkages, are not very susceptible to these enzymes, as shown by higher K_m values and much lower hydrolysis velocities, compared with those for the corresponding maltosaccharides. This is presumably attributable to the presence of α-D-(1→3)-glucosidic linkage(s), which interfere(s) with hydrolytic cleavage of α-D-(1→4)-glucosidic linkages, as proved by examination of the hydrolysis products.

DISCUSSION

As reported previously, methylation and chemical fragmentation analyses indicate that elsinan consists of α-D-(1→4)- and -(1→3)-linked D-glucosyl residues, and its main building-units are maltotriose residues joined by α-D-(1→3)-glucosidic linkages[1,2]. In addition, the presence of maltotetraose residues was suggested by the isolation of a small proportion of maltotetraose from the partial, acid hydrolyzate and acetolyzate of elsinan. Thus, the ratio of (1→4)- to (1→3)-D-glucosidic linkages, *viz.*, 2.3:1.0, suggested that the ratio of maltotriose and maltotetraose units in the elsinan molecule is ~2:1, although their precise locations along the polysaccharide molecule have not yet been elucidated.

As regards enzymic degradation, elsinan can be partially hydrolyzed by particular types of amylolytic enzymes, *e.g.*, human-salivary, hog-pancreas, and *B. subtilis* alpha amylases, which presumably have high activity toward lower maltosaccharides[3]. These salivary-type alpha amylases act on the α-D-(1→4)-glucosidic linkages both in maltotriose and in maltotetraose units, to give 4-*O*-α-nigerosyl-D-glucose [α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-D-Glc*p*] as the final product. The fact that a small but significant proportion of 3-*O*-α-maltosylmaltose [α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-D-Glc*p*] was isolated from the digest with salivary alpha amylase strongly suggests that this particular tetrasaccharide must have arisen from the maltotetraose units[3].

In the present study, elsinan was also found to be hydrolyzed by a particular, fungal alpha amylase, *i.e.*, Taka amylase of *Aspergillus oryzae*. This enzyme showed a considerably lower hydrolytic activity toward elsinan, compared with those of salivary-type alpha amylases, as indicated by the apparent extent of hydrolysis (11% as glucose), compared with that of human-salivary alpha amylase[3] (38%). Comparison of the kinetics of hydrolysis of elsinan by Taka amylase with those for salivary alpha amylase also indicated that the former enzyme has a lower affinity toward elsinan (K_m, 240μM) than the latter enzyme (K_m, 69mM). It is also true that Taka amylase has a conserably lower hydrolytic activity on elsinan than on soluble starch, and such differences must be due to the presence of α-D-(1→3)-glucosidic linkages in the former.

The action of Taka amylase on elsinan resulted in formation of a series of

oligosaccharides, *i.e.*, tetra-, hepta-, deca-, trideca-, and hexadeca-glucosaccharide (tetrasaccharide, TG_4, through hexadecasaccharide, TG_{16}), which contain regularly arranged α-D-(1→3)-glucosidic linkages. Among these oligosaccharide products, the precise structures of the tetra- and hepta-saccharide, which are the major products, were investigated by a combination of methylation, and chemical and enzymic fragmentation techniques. Thus, TG_4 proved to have the sequence α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p* (**1**) whose structure differs from that of the tetrasaccharide 3-*O*-α-maltosyl maltose, isolated from the digest with salivary enzyme; and TG_7 had the sequence α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p* (**2**).

With regard to the substrate specificities of mammalian alpha amylases, there have been some arguments. Alpha amylases from hog and human pancreas, as well as from human saliva, were reported to attack preferentially the second α-D-(1→4)-glucosidic linkage from the reducing (terminal) D-glucose residue of malto-oligosaccharides[10,11]. However, recent work by Saito and Horiuchi[12] indicated that human-salivary alpha amylase may cleave the third α-D-(1→4)-glucosidic linkage from the D-glucitol residue when it acts on maltohexaositol. Our previous study[3] showed that, when it acts on elsinan, human-salivary amylase preferentially cleaves (1→4)-bonds involving O-4 of the D-glucosyl residues whose C-1 atoms are joined to O-3 or O-4 of the adjacent D-glucosyl residues. The present study provides more information on the specificities of the human-salivary enzyme, by using TG_4 (**1**) and TG_7 (**2**). TG_4 yielded one mole of D-glucose and one mol of 4-*O*-α-nigerosyl-D-glucose (**3**), whereas TG_7 gave two mol of **3** together with one mol of D-glucose. The sequences of D-glucosyl residues in these oligosaccharides, and the action pattern of human-salivary amylase are illustrated in Fig. 3. In this connection, it is noteworthy that, when this enzyme acted on terminally reduced TG_7, D-glucitol was not released. Instead, one mol each of **3** and the reduced tetrasaccharide were formed.

Unlike the salivary-type alpha amylase, Taka amylase appears to require somewhat longer maltosaccharide segments. Thus, for the hydrolysis of elsinan, Taka amylase is able to split α-D-(1→4)-glucosidic linkages only in the maltotetraose segments, particularly, the site of O-4-substituted D-glucosyl residues joined to O-3 of the adjacent D-glucosyl residue. Support for such a mode of action was provided by examination of the high-molecular-weight saccharide left after prolonged digestion with Taka amylase (recovery, 33%). This high-molecular-weight saccharide [which consists of α-D-(1→4)- and -(1→3)-linked D-glucosyl residues in the ratio of 2.15:1.0, as revealed by methylation analysis] was degraded by human-salivary alpha amylase, to give 4-*O*-α-nigerosyl-D-glucose and a trace of D-glucose; under the same conditions, the native elsinan yielded a tetrasaccharide (3-*O*-α-maltosylmaltose), in addition to the trisaccharide (see Fig. 4). This strongly suggests that the high-molecular-weight saccharide of d.p. 30–35 contains, exclusively, maltotriose units joined by α-D-(1→3)-glucosidic linkages, and is not susceptible to Taka amylase.

As Taka amylase attacks particular α-D-(1→4)-glucosidic linkages of the

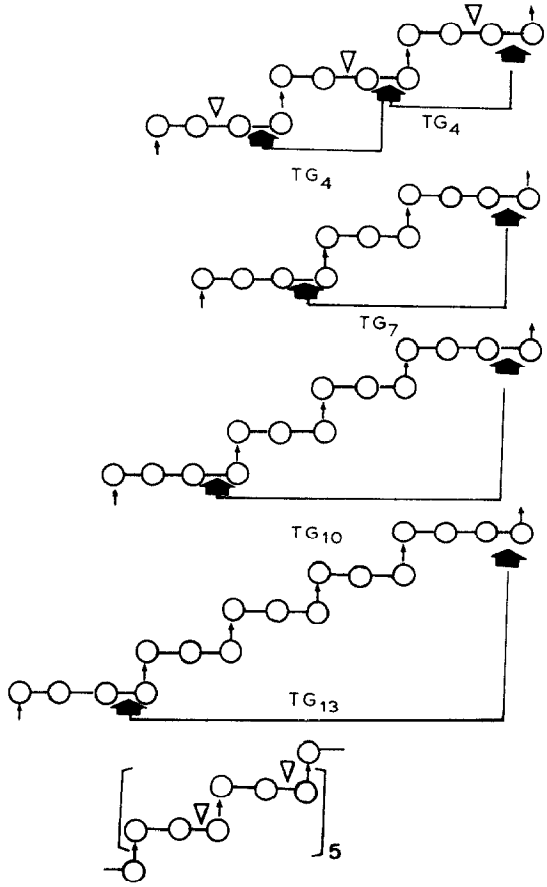

Fig. 5. Distribution of maltotriose and maltotetraose segments in elsinan, and action patterns of amylolytic enzymes. ▲: site of cleavage by Taka amylase, ▽: site of cleavage by human-salivary amylase. (○, 3-*O*-substituted D-glucose; —○, 4-*O*-substituted D-glucose.)

maltotetraose segments in the elsinan molecule, as mentioned previously, TG_4 should have arisen from the segments containing two or more consecutive maltotetraose units, whereas TG_7 must be derived from the segments in which the single maltotriose unit is flanked by maltotetraose units through (1→3) linkages. Similarly, TG_{10}, TG_{13}, and TG_{16}, released by the action of Taka amylase, would originate, respectively, from the segments containing two, three, and four consecutive maltotriose units, flanked by maltotetraose units. Fig. 5 illustrates the distributions of maltotriose and maltotetraose segments in the elsinan molecule, and the action patterns of Taka amylase. In addition, the isolation of the high-molecular-weight saccharide (d.p. 30–35) indicates the presence of 10 to 11 consecutive maltotriose segments. These observations on the action of Taka amylase provide valuable information on the fine-structural features of elsinan, in that the arrangements of maltotriose and maltotetraose units may not have regularity but, rather, heterogeneity.

Salivary-type amylases and Taka amylase are both capable of degrading elsinan; however, there are distinct differences between their action patterns. As previously reported[3], several alpha amylases, including salivary, pancreatic, and bacterial saccharifying amylases, can attack α-D-(1→4)-glucosidic linkages both in maltotriose and maltotetraose segments, to give 4-*O*-α-nigerosyl-D-glucose as the final product. When *B. subtilis* saccharifying alpha amylase acted on elsinan, examination of the intermediate oligosaccharides showed that, at the earlier stage of hydrolysis (90 min), tri-, tetra-, hepta- and octa-saccharides were produced, and at the final stage (24 h), these intermediate oligosaccharides had been further hydrolyzed, to give 4-*O*-α-nigerosyl-D-glucose[13]. As other, common amylolytic enzymes, *e.g.*, bacterial liquefying alpha amylase, beta amylase, and glucoamylase, cannot hydrolyze elsinan, this unique α-D-glucan may be useful in studies on the specificities of various amylolytic enzymes.

With regard to the enzymic degradation of two novel oligosaccharides, TG_4 and TG_7, kinetics for hydrolysis by human amylases (saliva and pancreas) and *B. subtilis* (saccharifying) alpha amylase were examined, in comparison with the hydrolysis of maltotetraose (MG_4) and maltoheptaose (MG_7), with a view to possible clinical, diagnostic applications. As indicated in Table III, these human amylases have considerably lower affinities, and therefore, lower hydrolytic activities toward TG_4 and TG_7, compared with those toward MG_4 and MG_7, probably because of steric hindrance by α-D-(1→3)-glucosidic linkages. Nevertheless, these oligosaccharides (TG_4 and TG_7) may have potential utility for the diagnostic assay of human amylases, as they produce one mol of D-glucose plus one, or two, mol of trisaccharide by the action of these enzymes.

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