Carbohydrate Research, 148 (1986) 309–319
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ENZYMIC DEGRADATION OF THE MYCOBACTERIAL *O*-METHYL-D-GLUCOSE POLYSACCHARIDE BY A *Rhizopus*-MOLD ALPHA AMYLASE, AN ENZYME ACTIVE ON 6-*O*-METHYL-AMYLO-OLIGOSACCHARIDES

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(Received August 29th, 1985; accepted for publication, November 22nd, 1985)

ABSTRACT

An enzyme activity that catalyzes hydrolysis of an α-(1→4)-linked 6-*O*-methyl-D-glucan was detected in, and purified from, *Rhizopus oryzae* mold. The enzyme acts like an alpha amylase and digests unmodified amylo-oligosaccharides 10 to 15 times as fast as it does the 6-*O*-methyl and 6-deoxy derivatives. When the limit product obtained by digesting the mycobacterial *O*-methyl-D-glucose polysaccharide with pancreatic alpha amylase and *Aspergillus* glucoamylase was further digested with the *Rhizopus* alpha amylase, di-, tri-, and tetra-saccharide fragments composed of α-(1→4)-linked 6-*O*-methyl-D-glucose were released. The rest of the molecule was recovered as oligosaccharides terminated by two, or three, α-(1→4)-linked 6-*O*-methyl-D-glucose residues.

INTRODUCTION

The recycling of natural polymers from dead organisms that accumulate in the soil implies the existence of enzymes capable of degrading the polymers. For the *O*-methyl-D-glucose polysaccharide (MGP) from *Mycobacterium smegmatis*[1,2], however, none of the amylases previously tested[3] was able to remove more than 4 hexose residues from this predominantly α-(1→4)-linked 6-*O*-methyl-D-glucan. The amylases were apparently inhibited by the methyl ether groups in the alpha amylase- and glucoamylase-digested MGP (AGMGP)[3], an inhibition previously observed with an amylose analog in which the primary hydroxyl groups were partially *O*-methylated[4,5].

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0008-6215/86/$ 03.50

We now describe an alpha amylase activity, found in a commercial glucoamylase (amyloglucosidase) preparation from a *Rhizopus* mold, that has the ability to cleave MGP and AGMGP between α-(1→4)-linked 6-*O*-methyl-D-glucose residues[6]. The products obtained confirmed the structure of MGP that had been determined by other procedures[2], and the activity of the enzyme suggests that it could be involved in recycling mycobacterial residues present in the soil.

EXPERIMENTAL

Materials. — DEAE-Trisacryl M, CM-Trisacryl M, and SP-Trisacryl M were obtained from LKB, and Bio-Gel P-4 from Bio-Rad. The *Rhizopus oryzae* glucoamylase (EC 3.2.1.2) was amyloglucosidase (No. A-7255) purchased from Sigma. Amylo-oligosaccharides were obtained by partial acetolysis of amylopectin[7], whereas MGP[8] was isolated from extracts of *Mycobacterium smegmatis* ATCC 356 and was converted into AGMGP as described[3]. Soluble starch was purchased from Mallinckrodt. 6-Deoxyamylo-oligosaccharides, prepared by partial acetolysis of 6-deoxyamylose[9], contained at least 95% of 6-deoxy-D-glucose, and were provided by D.-p. Lu of this laboratory.

Analytical procedures. — Carbohydrate was determined by the phenol–sulfuric acid method[10] with D-glucose as the standard. ^{1}H-N.m.r. spectra of carbohydrates in D_2O were recorded with a Bruker 500-MHz spectrometer in the Department of Chemistry, University of California, Berkeley, CA. Chemical shifts were measured from an internal standard of acetone (δ = 2.217)[11]. The mass spectra were recorded at Imperial College with a VG Analytical ZAB HF mass spectrometer equipped with a fast-atom-bombardment (f.a.b.) source and an M-Scan f.a.b. gun[12].

Enzyme assays. — Glucoamylase activity was determined with starch as the substrate. One mL of 0.4% soluble starch solution in 50mM sodium acetate buffer, pH 5.0, and 0.05 mL of enzyme solution were incubated for 10 min at 37°. The mixture was heated for 2 min at 100°, to stop the reaction, and the D-glucose was determined by the D-glucose oxidase method[10]. Under these conditions, one unit of glucoamylase activity liberates 1 μmol of D-glucose/min.

Alpha amylase activity was determined by incubating 1.0 mL of 0.4% soluble starch in 50mM acetate buffer, pH 5.0, with 0.05 mL of enzyme solution for 2 min at 37°. The solution was then heated for 2 min at 100°, and its iodine-binding capacity was measured at 700 nm by mixing 0.10 mL of the solution with 1.0 mL of 0.005% I_2 in 0.05% KI solution[13]. One unit of alpha amylase activity causes a 10% diminution of the absorbance (A) per minute.

AGMGP-hydrolase activity was determined by incubating 40 μL of 0.025% AGMGP in 50mM acetate buffer, pH 5.0, with 20 μL of enzyme solution for 24 h at 30°. The mixture was heated for 2 min at 100°, and the reducing sugar was determined by the Park–Johnson method[10]. One unit of enzyme activity produces 1 nmol of reducing group in 24 h. These conditions were used for determination of the

substrate specificity of the alpha amylase.

Purification of the Rhizopus alpha amylase. — All operations were conducted at 4°. A solution of 1 g of glucoamylase (Sigma "amyloglucosidase") in 40 mL of 50mM sodium acetate buffer, pH 5.0, was allowed to stand overnight. Insoluble material was removed by centrifugation at 300 r.p.m. for 5 min, sufficient ammonium sulfate (15.6 g) was added to the supernatant solution to give 60% saturation[14] at 25°, the mixture was stirred for 1 h, and the precipitate that formed was removed by centrifugation. To the supernatant solution (40 mL) was added ammonium sulfate (5.7 g) to give 80% saturation at 25°, and the mixture was stirred for 2 h. The precipitate, collected by centrifugation, was dissolved in the minimal volume of 20mM Tris · HCl buffer, pH 7.5, and the solution was desalted on a column (1.5 × 25 cm) of Bio-Gel P-6DG in the same buffer.

This 60–80% ammonium sulfate fraction, which contained most of the glucoamylase and alpha amylase activities, was applied in 5.0-mL portions to a column (2 × 40 cm) of DEAE-Trisacryl M equilibrated with 20mM Tris · HCl buffer, pH 7.5, and the column was eluted with 150 mL of the same buffer, followed by a linear gradient of 0 to 0.4M NaCl in the same buffer. The glucoamylase activity was eluted in the break-through fraction and the alpha amylase and AGMGP-hydrolase activities were eluted[15] at ~0.1M NaCl.

The fractions containing the last two activities were combined, dialyzed against 20mM sodium acetate buffer, pH 5.0, and applied to a column (2 × 10 cm) of CM-Trisacryl M equilibrated with the same buffer, and the column was eluted with a linear gradient of 0 to 0.5M NaCl in the same buffer. The two enzyme activities were coeluted in the break-through fraction, which was dialyzed against 20mM acetate buffer, pH 4.0, applied to a column (2 × 10 cm) of SP-Trisacryl M equilibrated with the same buffer, and the column eluted with a linear gradient of 0 to 0.5M NaCl in acetate buffer, pH 5.0. The alpha amylase and AGMGP-hydrolase activities were eluted together at ~0.2M NaCl. The solution of pure enzymes was dialyzed against 2mM sodium acetate, pH 5.0, and stored at 4°.

Digestion of AGMGP. — AGMGP (~4.7 mg) was dissolved in 0.25 mL of 20mM sodium acetate, pH 5, and 0.5 mL (0.35 mg of protein, 1.4×10^3 units) of the purified *Rhizopus* amylase mixture was added. The resulting mixture was incubated for 72 h at 37°, the pH adjusted to 7 with 0.1M NaOH, and the mixture applied to a column (1 × 5 cm) of DEAE-Sephadex A-25 (HCO_3^-). Neutral carbohydrate was eluted with water (8 mL), and acidic carbohydrate with 0.4M ammonium acetate (8 mL). About equimolar amounts of hexose were obtained in the two fractions. The neutral carbohydrate was applied to a column (1.5 × 185 cm) of Bio-Gel P-4 (400 mesh) equilibrated in 0.1M acetic acid, and 1.0-mL fractions were collected by elution with 0.1M acetic acid. The acidic carbohydrate was dissolved in 0.1M acetic acid, the solution applied to the same Bio-Gel P-4 column, and 1-mL fractions were collected by elution with 0.1M acetic acid. A portion of each fraction was assayed for carbohydrate[10].

RESULTS

Preliminary studies on the digestion of the *O*-methyl-D-glucose polysaccharide (MGP) by a crude extract of *Rhizopus* mold revealed that the molecule was hydrolyzed to yield neutral and acidic oligosaccharides[6]. Among the neutral products were identified *O*-(3-*O*-methylglucosyl)glucose and oligomers of 6-*O*-methylglucose having 2–8 hexose units. The acidic fragments contained ~5 glucose units, variable numbers of 6-*O*-methylglucose units, and a glyceric acid residue.

We have purified the enzyme activity responsible for hydrolysis of the 6-*O*-methylglucosyl bonds, and have separated and characterized the products formed by the action of this enzyme on AGMGP (see Fig. 1), the polysaccharide in which the 3-*O*-methylglucose and 3 neighboring D-glucose units have been removed from the nonreducing end of MGP. This simplified substrate allowed us to quantify a "6-*O*-methylamylase" activity and lessened the complexity of the products that are formed in the reaction.

Purification of the AGMGP-hydrolase activity. — In monitoring the purification, three assays were used: one for the amylase activity, based on a diminution in the iodine-binding capacity of starch; one for glucoamylase activity, based on the formation of D-glucose from starch as the substrate; and one for AGMGP-hydrolase activity, based on the increase in reducing sugar with AGMGP as the substrate. The crude extract of the *Rhizopus*-mold preparation contained all three activities, but the glucoamylase activity was removed by chromatography on DEAE-Trisacryl (see Fig. 2). In all subsequent steps, the amylase and AGMGP-hydrolase activities were copurified (see Table I). A yield of 50%, with a 10-fold increase in specific activity, was obtained.

In gel electrophoresis, the purified enzyme showed a major band having M_r 47,000 and a minor band with M_r 50,000 (see Fig. 3). We considered that these might reflect differences in carbohydrate, as the enzyme contains ~5% of hexose, but digestion with endoglucosaminidase H did not alter the electrophoretic pattern (data not shown).

The relative activities of the purified amylase with various substrates are compared in Table II, wherein all assays were based on the increase in reducing sugar

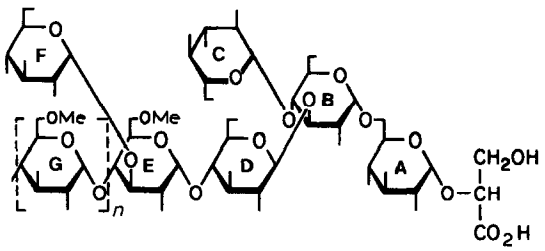

Fig. 1. Structure of AGMGP and homologs. In AGMGP, n = 10, whereas for acidic oligosaccharides A-1 and A-2, n = 2 and 3, respectively.

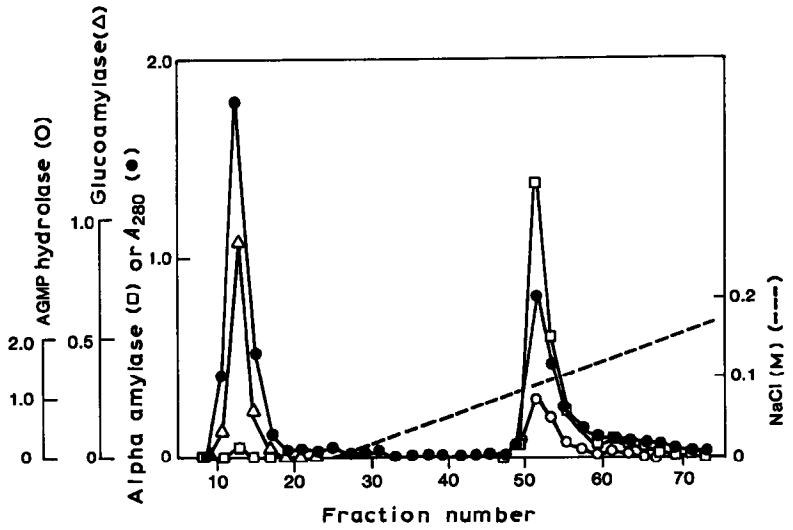

Fig. 2. Separation of the *Rhizopus* amylase activities. The ammonium sulfate precipitate, obtained between 60 and 80% saturation, was passed through a column of DEAE-Trisacryl to which the glucoamylase [△, (units/mL) × 10^{-2}] failed to bind. A gradient of NaCl (– – –) displaced the alpha amylase [□, (units/mL) × 10^{-3}] and the AGMGP hydrolase [○, (units/mL) × 10^{-3}]. The absorbance at 280 nm is also shown (●).

owing to hydrolysis of glycosidic bonds. The results showed that the hydrolysis of amylo-oligosaccharides occurs 10 to 15 times faster than that of the modified analogs.

Isolation of the enzymic-degradation products from AGMGP. — A 72-h

TABLE I

PURIFICATION OF RHIZOPUS AMYLASES

Purification step	*Total protein (mg)*	*Amylase*[a]		*AGMGP hydrolase*[a]		*Glucoamylase*	
		Total units	*Specific activity*	*Total units*	*Specific activity*	*Total units*	*Specific activity*
Ammonium sulfate 60–80% precipitate	202	86,000	426	82,700	408	5,406	27
DEAE-Trisacryl chromatography							
Fraction A	100	0	0	0	0	3,870	39
Fraction B	23	51,500	2,230	48,300	2,090	0	0
CM-Trisacryl chromatography of fraction B	17.4	42,300	2,430	47,500	2,720	0	0
SP-Trisacryl chromatography	9.4	37,400	3,980	41,800	4,440	0	0

[a]The elution patterns for alpha amylase and AGMGP hydrolase activities were concident in all chromatographic steps. These two activities have different units in this table, but a direct comparison with similar units is given in Table II.

Fig. 3. Gel electrophoresis, in sodium dodecyl sulfate, of purified *Rhizopus* amylase. Lane A, molecular-weight standards, from top to bottom, phosphorylase b (92,000), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (31,000); lane B, ammonium sulfate precipitate; lane C, purified amylase.

digest of AGMGP with the purified amylase yielded equimolar amounts of neutral and acidic saccharide (measured as total hexose based on the phenol–sulfuric acid assay), which indicated that ~8 of the 6-*O*-methylglucose units are released from the polymer that contains 16 hexose units. Fractionation of the neutral oligosaccharides by gel filtration (see Fig. 4B) revealed 3 components, respectively having the elution positions expected for a di-, a tri-, and a tetra-saccharide composed of 6-*O*-methylglucose[7]. The molar ratios were ~2:2:1, and this agrees with the conclusion that ~8 hexose units were removed during the digestion; and the accumulation of some tetrasaccharide indicated that the amylase is not very active on this fragment.

TABLE II

SUBSTRATE SPECIFICITY OF THE RHIZOPUS AMYLASE

Substrate	*Relative activity*[a]
AGMGP	1.0
6-Deoxyamylo-oligosaccharide (10–16 hexose units)	1.7
Maltodecaose	16.0

[a]Activities were determined at substrate concentrations of 25 mg/100 mL by measurement of reducing sugar formed after incubation for 24 h at 30° with purified enzyme (46 μg/mL). The products of the reactions were not investigated.

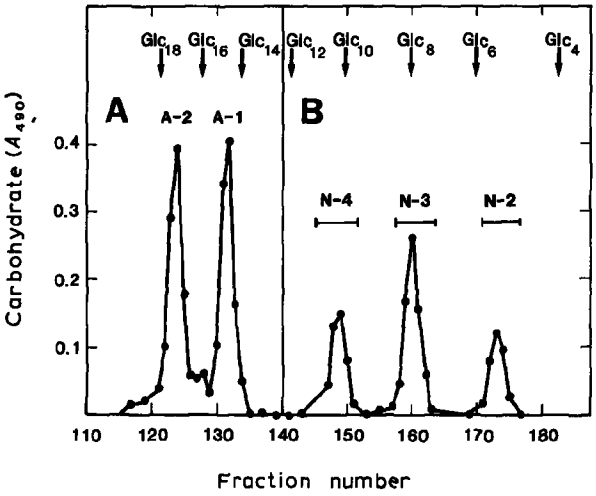

Fig. 4. Fractionation of the enzymic-degradation products from AGMGP. The neutral and acidic fragments were separated from each other, according to charge, on a column of DEAE-Sephadex (data not shown), and each fraction was then resolved, according to size, on a column (1.5 × 185 cm) of Bio-Gel P-4 by elution with M acetic acid: *panel A*, acidic fraction; *panel B*, neutral fraction. Elution positions of standard (1→4)-α-D-glucans are indicated on the Figure.

The acidic fraction was resolved by gel filtration (see Fig. 4A) into two components that differed in size by one 6-*O*-methylglucose unit and had elution positions consistent with a composition of 3–4 *O*-methylhexose units, 5 glucose units, and a glyceric acid unit[7]. Analyses described in the next Sections established the exact structures of all of these fragments.

Characterization of the neutral oligosaccharides. — These fragments were expected to be oligomers of 6-*O*-methylglucose in α-D-(1→4) linkage, and the anomeric-proton n.m.r. spectra confirmed this expectation (see Table III)[2]. The

TABLE III

ANOMERIC-PROTON N.M.R. CHEMICAL SHIFTS FOR NEUTRAL OLIGOSACCHARIDES

Oligosaccharide	*Sugar residue*				*Chemical shift, δ*[a]				
	D	*C*	*B*	*A*	*D*	*C*	*B*	A_α	A_β
N-2			6MeGlc→	6MeGlc			5.426[b]	5.199	4.631
N-3		6MeGlc→	6MeGlc→	6MeGlc		5.397	5.432[b]	5.198	4.630
N-4	6MeGlc→	6MeGlc→	6MeGlc→	6MeGlc	5.396	5.422	5.442[b]	5.199	4.630

[a]All coupling constants were 3.4–3.8 Hz, except for the β anomer of the reducing-end hexose, which was 7.6–7.8 Hz. Relative signal-intensities were $A_\alpha + A_\beta = 1$, B = 1, C = 1, and D = 1. [b]In all spectra, this signal was a triplet formed by two overlapping doublets that resulted from differing influences by the two anomers of the reducing-end hexose. All other signals were simple doublets resulting from coupling to the proton on C-2.

TABLE IV

METHYL ETHER-PROTON N.M.R. DATA

Oligosaccharide	*Chemical shift, δ*[a]	
	Nonreducing methylhexose	*Reducing methylhexose*
N-2	3.387 (3)	3.371 (1.5)
		3.363 (1.5)
N-3	3.394 (3)	3.367 (1.5)
	3.383 (3)	3.359 (1.5)
N-4	3.392 (3)	3.373 (1.5)
	3.382 (6)	3.363 (1.5)
A-1	3.390 (3)	[b]
	3.384 (3)	[b]
	3.369 (3)	[b]
A-2	3.392 (3)	[b]
	3.385 (6)	[b]
	3.370 (3)	[b]

[a]The number of protons is given in parentheses, and the signal for the reducing-end methylhexose is twinned owing to α,β-isomerism. [b]Not applicable.

smallest, a disaccharide (N-2), showed a signal equivalent to 1 proton at δ 5.246 for the nonreducing terminus, and signals at δ 5.199 and 4.631, which together equaled 1 proton and are assignable to the α and β anomers, respectively, of the compound. A signal for the methyl ether protons of a nonreducing hexose was observed at δ 3.387, and two signals for a reducing hexose at δ 3.371 and 3.363 (see Table IV). The size of the fragment was confirmed by the mass spectrum (see Table V), which showed a molecular ion at *m/z* 371 $[(MeGlc)_2 + H]^+$.

The next larger oligosaccharide, N-3, gave a molecular ion at *m/z* 547 ascribable to $[(MeGlc)_3 + H]^+$ (see Table V), and the anomeric-proton n.m.r. spectrum showed signals for 1 proton each, at δ 5.432 and 5.397, with signals of lower intensity for the α and β anomers, at δ 5.198 and 4.630 (see Table III). The expected signals for the methyl ether protons of two nonreducing and one reducing methylhexose were observed (see Table IV). The largest oligosaccharide, N-4, had a molecular ion at *m/z* 723 $[(MeGlc)_4 + H]^+$, and gave anomeric-proton signals at δ 5.442, 5.422, and 5.396 (1 proton each) and at δ 5.199 and 4.630 (each ~0.5 proton), as well as the expected methyl ether signals (see Tables III–V).

Characterization of the acidic oligosaccharides. — Oligosaccharide A-1 (see Fig. 4A) gave 8 anomeric-proton n.m.r. signals (see Fig. 5), 5 of them assignable to the five glucose units previously identified at the glyceric acid end of MGP, and the others to three 6-*O*-methylglucose units (see Table VI)[2]. Two of the glucose units have β linkages (hexoses C and D, Fig. 1) and their anomeric protons show large coupling constants[8], although, in the spectrum shown in Fig. 5, these signals overlap and give the appearance of two protons having small coupling constants.

TABLE V

MASS-SPECTRAL DATA FOR NEUTRAL AND ACIDIC OLIGOSACCHARIDES

Oligosaccharide	*Positive pseudomolecular ions*			*Proposed structures*[a]
	$[M - OH]^+$	$[M + H]^+$	$[M + Na]^+$	
N-2	353	371	[b]	$(6MeGlc)_2$
N-3	529	547	569	$(6MeGlc)_3$
N-4	705	723	745	$(6MeGlc)_4$
A-1	[b]	1445	1467	$(6MeGlc)_3(Glc)_5Ga$
A-2	[b]	1621	1643	$(6MeGlc)_4(Glc)_5Ga$

[a]Me is methyl, and Ga is glyceric acid. [b]Not observed.

The unit ratios of all anomeric-proton signals, and their sharpness, constitute strong evidence that A-1 is a homogeneous oligosaccharide in which $n = 2$ (in Fig. 1), and this is confirmed by the presence of 3 methyl ether signals (see Table IV). Oligosaccharide A-2 differs from A-1 in having an additional anomeric-proton signal in the region of the 6-*O*-methylglucose units, and so it was concluded that A-2 is represented by the structure in Fig. 1 in which $n = 3$. This homolog shows four methyl ether signals (see Table IV).

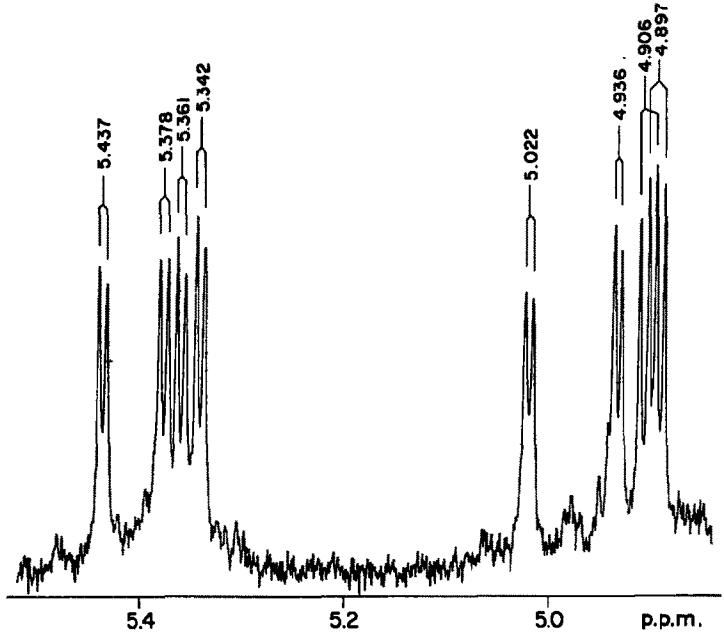

Fig. 5. Anomeric-proton n.m.r. spectrum for acidic oligosaccharide A-1. Signals for three 6-*O*-methylglucoses are shown, at δ 5.342, 5.361, and 5.378; for three α-linked glucoses, at δ 4.936, 5.022, and 5.437; and for two β-D-linked D-glucoses, at δ 4.897 and 4.906. More-complete data are given in Table VI.

TABLE VI

ANOMERIC-PROTON N.M.R. DATA FOR ACIDIC OLIGOSACCHARIDE FRAGMENTS

Hexose unit[a]	*Oligosaccharide A-1* (n = 2)[a]		*Oligosaccharide A-2* (n = 3)[a]	
	δ	$J_{1,2}$ (Hz)	δ	$J_{1,2}$ (Hz)
A	5.022	3.6	5.022	3.6
B	4.936	3.6	4.936	3.6
C	4.906	8.0	4.906	8.0
D	4.897	7.9	4.897	7.8
E	5.342	3.8	5.342	3.6
F	5.437	3.8	5.456	3.8
G-1[b]	5.361	3.9	5.370	[c]
G-2[b]	5.378	3.9	5.387	[c]
G-3[b]			5.378	3.9

[a]Refer to Fig. 1. [b]Assignments for specific hexoses were not defined. [c]Overlapping of signals prevented determination of the coupling constant.

The molecular sizes of the two oligosaccharides were confirmed by f.a.b.-m.s. (see Table V). Oligosaccharide A-1 gave a protonated molecular-ion in the positive mode at *m/z* 1445 $[MeGlc)_3(Glc)_5Ga]^+$, whereas oligosaccharide A-2 gave an ion at *m/z* 1621 $[(MeGlc)_4(Glc)_5Ga]^+$. Both compounds also gave ions for the sodium-cationized species at $[M + 23]^+$.

DISCUSSION

Herein we describe the purification, properties, and mode of action of an alpha amylase having the ability to act on 6-*O*-methylamylose-like polymers. Because this enzyme was found in *Rhizopus oryzae*, a mold that inhabits the soil, a reasonable hypothesis is that it may serve to recycle the 6-*O*-methylglucose lipopolysaccharide that is elaborated by all strains of mycobacteria, many of which are also soil organisms.

Previous studies on the substrate specificity of amylases had revealed that, although some of them catalyze hydrolysis of bonds between D-glucose units in partially methylated amylose, not one has been reported that was able to cleave the glycosidic bond of a 6-*O*-methylglucosyl unit[5]. In our initial investigations on the structure of the 6-*O*-methylglucose polysaccharide, it was found that pancreatic alpha amylase could split between α-(1→4) D-glucosyl units and release di- and tri-saccharide fragments, even though the nonreducing end of the polysaccharide is terminated by a 3-*O*-methylglucosyl group[3]. In that instance, the alpha amylase left attached to the end of the 6-*O*-methylglucosyl chain a single D-glucose unit that could subsequently be removed by digestion with *Aspergillus niger* glucoamylase I. We have now found that this limit 6-*O*-methylglucose-enriched product can be further degraded by the *Rhizopus* alpha amylase, to yield di-, tri-, and tetra-saccharide fragments composed of 6-*O*-methylglucose, along with a branched,

acidic oligosaccharide composed of glucose and 6-*O*-methylglucose attached to a glyceric acid unit.

Although amylases previously studied failed to act on 6-*O*-methylamylose, some of them hydrolyze 6-deoxyamylose[5]; this suggests that the failure of amylases to act on the methyl ether derivative is not attributable to the requirement for a free 6-hydroxyl group to form a hydrogen bond to the enzyme active-site, but to the bulkiness of the methylated polymer. The (smaller) 6-deoxyamylose can fit into the active site and undergo hydrolysis, even though it is unable to form such a hydrogen bond. If these suppositions are correct, it may be concluded that the *Rhizopus* alpha amylase must have a more exposed, or less restricted, active site, such that it can accommodate a 6-*O*-methylamylose oligosaccharide. A direct comparison of the action of the *Rhizopus* enzyme on oligosaccharides prepared from amylose and 6-deoxyamylose, and on AGMGP, showed that the last two analogs are hydrolyzed at 10%, or less, of the rate of the unmodified oligosaccharide. The results are consistent with a single enzyme being involved in all of these reactions, and the chromatographic patterns gave no suggestion that the preparation contained more than one alpha amylase activity, even though gel electrophoresis of the purified enzyme still showed two protein bands.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant AI-12522 and National Science Foundation Grant PCM84-00251 (to C.E.B.). A.D. acknowledges support from grants from the Medical Research Council and the Science and Engineering Research Council (To Howard R. Morris, Imperial College).

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