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ENZYMIC SYNTHESES OF DOUBLY BRANCHED CYCLOMALTOHEPTAOSES THROUGH THE REVERSE ACTION OF *Pseudomonas* ISOAMYLASE

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ABSTRACT

Two and three new cyclomaltoheptaose (β-cyclodextrin, cG_7) derivatives, respectively, were identified among the products obtained by the action of *Pseudomonas* isoamylase on maltose and maltotriose, and cG_7. They were $6^A,6^D$-di-*O*-α-maltosyl-cG_7 and 6-*O*-α-(6^2-*O*-α-maltosyl)maltosyl-cG_7, and $6^A,6^D$-di-*O*-α-maltotriosyl-cG_7, 6-*O*-α-(6^3-*O*-α-maltotriosyl)maltotriosyl-cG_7, and 6-*O*-α-(6^2-*O*-α-maltotriosyl)maltotriosyl-cG_7. In addition, 6^1- and 6^2-*O*-α-maltosylmaltose were identified as mutual condensation products of maltose. Maltose was the smallest substrate to act as both an acceptor and a donor for the action of *Pseudomonas* isoamylase.

INTRODUCTION

We have reported[1] syntheses of singly branched cyclomalto-oligosaccharides (cG_n) from malto-oligosaccharides and cG_n through the reverse action of *Pseudomonas* isoamylase. During this study, branched tetraoses derived from G_2 were found together with several new products having molecular weights higher than those of singly branched cG_ns. We now describe the identification of 5 new branched derivatives of cG_7 and 2 isomers of maltosylmaltose, and discuss the substrate specificity of *Pseudomonas* isoamylase.

EXPERIMENTAL

Materials. — *Pseudomonas* isoamylase[1] and *Rhizopus delemar* glucoamylase (GIII fraction)[2] were purified as described previously. Maltose (G_2, HHH grade) and maltotriose (G_3) were kindly donated by Hayashibara Biochemical Laboratory

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and used without further purification. cG_7 was a gift from Nihon Shokuhinkako Co., and was purified by recrystallisations from hot water. $6^A,6^D$-Di-*O*-α-D-glucosylcyclomaltoheptaose ($diGcG_7$)[3], 6-*O*-α-D-glucosylcyclomaltoheptaose (GcG_7)[3], 6-*O*-α-maltosylcyclomaltoheptaose (G_2cG_7)[1], and 6-*O*-α-maltotriosylcyclomaltoheptaose (G_3cG_7)[1] were prepared as reported previously.

Analyses. — Total carbohydrate and reducing sugar were determined by the anthrone–sulfuric acid[4] and Somogyi–Nelson[5,6] methods, respectively.

H.p.l.c. was conducted at room temperature with an HLC-803D pump, an RI-8 differential refractometer (Toyo Soda) connected to a Hitachi D-2000 Chromato-integrator, and a column (4.6 × 250 mm) of TSKgel NH_2-60 (Toyo Soda) eluted with acetonitrile–water (55:45) at 0.8 mL/min. A larger column (7.8 × 300 mm), eluted at 1.5 mL/min, was used for preparative purposes. The range of the refractometer was set at 16 or 32 × 10^{-6} RIU/FS. The multi-branched cG_7 isomers were isolated on a column (10 × 250 mm) packed with ODS-Hypersil-5 (Shandon), by elution with methanol–water (8:92) at 1.5 mL/min.

Sugars were methylated by the method of Prehm[7], and the products were hydrolysed, converted into the corresponding alditol acetates, and then analysed[8] with a Hitachi 663-30 gas chromatograph fitted with a flame-ionisation detector on a column (3 mm × 2 m) of 0.3% of OV-275–0.4% of GE XF-1150 on Uniport HP (80–100 mesh).

^{13}C-N.m.r. spectra (50.10 MHz) were recorded at 50° on 2–3% solutions in D_2O, using a JEOL JNM-FX 200 spectrometer. The F.t.-n.m.r. conditions were as follows: spectral width, 3000 Hz; pulse flipping angle, 45°; and number of data points, 16,384. A micro cell was used and chemical shifts are expressed in p.p.m. downfield from the signal of Me_4Si, with reference to internal 1,4-dioxane (67.40 p.p.m.). The signals for C-1,4,6 were assigned by comparison with those[1] of the singly branched cG_7s.

F.a.b.-m.s. was performed with a JEOL JMS-HX 110 mass spectrometer, using xenon atoms with a kinetic energy equivalent to 6 kV, and a data-processing system JMA-DA 5000, with analysis in the negative-ion mode. Glycerol was used as the matrix solution.

RESULTS AND DISCUSSION

The optimum temperature and pH for the condensation reaction with isoamylase were 60° (at pH 4.2) and pH 3.7–4.7 (at 60°), respectively (Fig. 1). The optimum concentration of G_3 for the reaction was 1.6M at near the maximum soluble concentration of cG_7 (65mM); higher concentrations caused a rapid decrease in the yield (Fig. 2). The reaction proceeded rapidly during the first 24 h, then became slower, and reached apparent equilibrium at 36 h (Fig. 3A). A new product (*A*), later identified as a mixture of dimaltotriosyl-cG_7 and two hexaosyl-cG_7s, and several unknown compounds, in addition to branched hexaose (BG_6) and G_3cG_7,

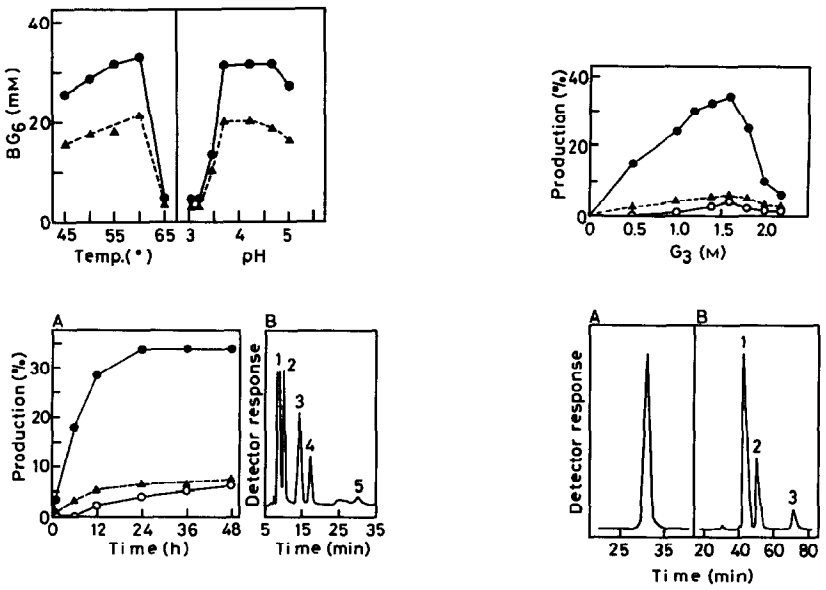


Fig. 1. Optimum temperature and pH for the condensation reaction with *Pseudomonas* isoamylase. A mixture of G_3 (M) and isoamylase (6.2 U) in a final volume of 0.5 mL of 50mM sodium acetate buffer was incubated at various temperatures and pH 4.2 or at 58° and various pHs for 3 h (▲) and 10 h (●). BG_6 was determined by h.p.l.c.

Fig. 2. Optimum concentration of G_3 for the production of G_3cG_7. Reaction mixtures, containing various concentrations (0.5–2.6M) of G_3, 65mM cG_7, and 6.2 U of *Pseudomonas* isoamylase in a final volume of 0.5 mL of 50mM sodium acetate buffer (pH 4.2), were incubated at 58° for 24 h: G_3cG_7 (●) and product *A* (○) are expressed as a % of the initial cG_7, and BG_6 (▲) as a % of the initial G_3.

Fig. 3. A, Time courses of the formation of BG_6, G_3cG_7, and product *A*, and B, h.p.l.c. on TSKgel NH_2-60. A solution of 65mM cG_7 1.6M G_3, and 13.3 U of *Pseudomonas* isoamylase, in a final volume of 1 mL of 50mM sodium acetate buffer (pH 4.2), was incubated at 58°; see Fig. 2 for symbols: 1, G_3; 2, cG_7; 3, BG_6; 4, G_3cG_7; 5, product *A*.

Fig. 4. H.p.l.c. of product *A* on A, TSKgel NH_2-60; and B, ODS-Hypersil-5: 1, A-1; 2, A-2; 3, A-3.

were found among the products from G_3 and cG_7 (Fig. 3B). Similarly, two new products (*B* and *C*), later shown to be maltosylmaltoses (diG_2), and a mixture of dimaltosyl-cG_7 and tetraosyl-cG_7, respectively, were produced in addition to G_2cG_7 from G_2 and cG_7 under the same conditions as above. At the end of the reaction, 6.96 g of G_3cG_7, 15.9 g of BG_6, and 1.14 g of product *A* had been produced from 166 g of G_3 and 17.1 g of cG_7; and 4.9 g of G_2cG_7, 1.9 g of product *B*, and 0.9 g of product *C* from 102 g of G_2 and 17.1 g of cG_7. Each of the products was purified by repeated gel-permeation chromatography on Bio-Gel P-2, Sephadex G-15, and/or Toyopearl HW40S, as previously reported[1], followed by preparative h.p.l.c.

Structure of product A. — Purified product *A* gave a single, symmetrical peak on TSKgel NH_2-60, but 3 peaks (A-1/3) on ODS-silica in the ratio of 20:7:2 (Fig. 4), and the material in each peak was collected. Isoamylolysis of each of these three

TABLE I

ENZYME AND METHYLATION ANALYSES OF A-1/3

	A-1	*A-2*	*A-3*
Products on glucoamylolysis (mol/mol)			
Glucose	3.9	4.8	5.0
GcG_7	0	1.0	1.0
$DiGcG_7$	1.0	0	0
Methylation analysis (molar ratio)[a]			
2,3,4,6-Me_4	2.0	2.0	1.0
2,3,6-Me_3	9.2	9.3	10.0
2,3,4-Me_3	0	0	0.7
2,3-Me_2	1.9	2.0	0.9

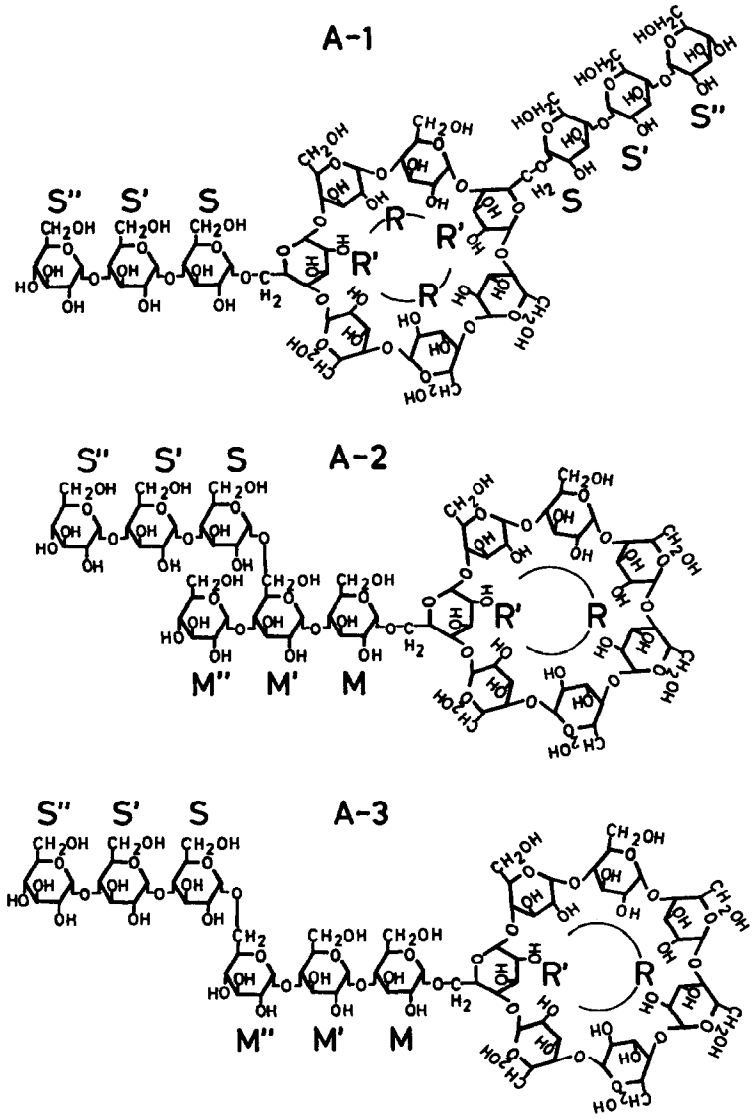
[a]Distribution of methyl group in the derivatives of D-glucitol.

TABLE II

^{13}C-N.M.R. DATA FOR A-1/3

Atom	*A-1*			*A-2*			*A-3*		
	δ (p.p.m.)	*Integral*	*Assignment (residues)*[a]	*δ (p.p.m.)*	*Integral*	*Assignment (residues)*[a]	*δ (p.p.m.)*	*Integral*	*Assignment (residues)*[a]
C-1	99.50	2	S	99.44	2	M, S	98.91	1	S
	100.80	4	S′, S″	100.83	3	M′, S′, S″	99.57	1	M
	102.66	7	R, R′	101.17	1	M″	100.66	1	M′
				102.56	7	R, R′	100.87	2	S′, S″
							100.95		
							101.23	1	M″
							102.64	7	R, R′
							102.98		
C-4	70.38	2	S″	70.39	2	M″, S″	70.41	1	S″
	78.24	2	S′	78.35	1	S′	70.60	1	M″
	79.27	2	S	79.20	2	M, S	78.32	1	S′
	82.15	7	R, R′	80.10	1	M′	78.69	1	M′
	82.35								
				81.99	7	R, R′	79.13	1	S
				82.06					
				82.45			79.43	1	M
							82.03	7	R, R′
							82.34		
							82.42		
C-6	61.31	5	R	61.26	11	R, M, M″, S	61.31	11	R, M, M′, S
				61.36		S′, S″	61.50		S′, S″
	61.52	6	S, S′, S″	61.45			61.58		
				61.53			61.76		
	67.98	2	R′	61.69					
				68.09	2	R′, M′	67.63	1	M″
							68.02	1	R′

[a]See Scheme 1.



Scheme 1. Designation of the glucosyl residues in A-1/3.

materials in dilute aqueous solution gave 2 mol of G_3 and 1 mol of cG_7. The results of glucoamylolysis and methylation analyses (Table I) indicated that A-1 contained two G_3 linked to different glucosyl residues of cG_7, and that A-2 and A-3 contained two α-(1→6)-linked G_3 α-(1→6)-linked to a ring residue.

A-1 was degraded with glucoamylase to give 4 mol of glucose (G) and 1 mol of $diGcG_7$, which was identified by co-chromatography with an authentic specimen[9] on several columns having different modes of separation. The ^{13}C-n.m.r. spectrum

of A-1 (Table II, see Scheme 1) was the same as that of G_3cG_7 (see Fig. 7 in ref. 1), except for the double relative intensities of the peaks for the residues (S″, S′, and S) of the side chain and involved in branching (R′). Moreover, the S-1 (C-1 of the S residue) and S′,S″-1 signals were observed as two single peaks and their intensity ratio was 1:2. These results indicate that two G_3 residues are in the same magnetic environment and are linked to different glucosyl residues in cG_7. The results of methylation analysis are consistent with this structure.

A-2 was expected to be a cG_7 derivative with either 6^1- or 6^2-maltotriosyl-maltotriose as a branch, from the results of enzymic and methylation analysis (Table I). By comparison of the ^{13}C-n.m.r. spectrum of A-2 (Table II) with that of A-1, the peaks at 70.39, 78.35, and 79.20 p.p.m., in the ratios 2:1:2, could be assigned to S″,M″-4, S′-4, and S,M-4, respectively. One C-4 signal that originated from the middle residue (M′) of one G_3 was shifted downfield by ~2 p.p.m. com-

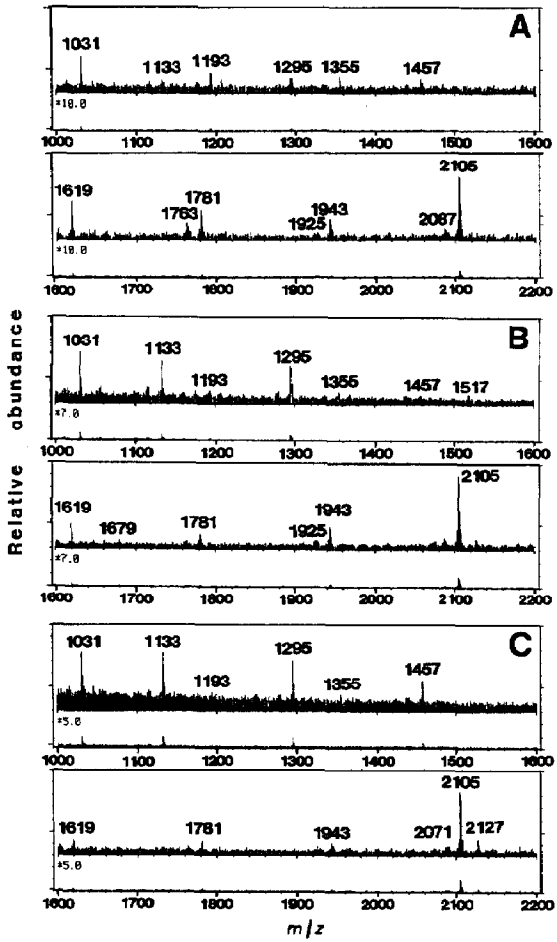


Fig. 5. F.a.b.-m.s. spectra of **A**, A-1; **B**, A-2; and **C**, A-3.

pared to the C-4 signal of the other G_3 (S'-4), suggesting that the other G_3 residue (S) was linked to this middle residue (M') of a G_3 chain which is (1→6)-linked to the ring. Thus, A-2 is concluded to be 6-*O*-α-(6^2-*O*-α-maltotriosyl)maltotriosyl-cG_7. This conclusion was supported by the expanded spectrum of the peak (δ 70.39) which showed two peaks that were closely overlapped, indicating that S''-4 and M''-4 were in slightly different magnetic environments (data not shown). If the side chain of A-2 is 6'-maltotriosylmaltotriose, S''-4 and M''-4 have the same magnetic environment, and therefore they should appear as a single peak.

A-3 was degraded into 5 mol of G and 1 mol of GcG_7, and ~1 mol of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol was detected on methylation analysis. Thus, A-3 should be 6-*O*-α-(6^3-*O*-α-maltotriosyl)maltotriosyl-cG_7. This structure was supported by the ^{13}C-n.m.r. spectrum. Thus, all of the C-4 signals of the side chain were separate peaks with the same intensity; the S-1 and M-1 signals, and also the M''-6 and R'-6 signals of the two branch points, emerged as different peaks, respectively (Table II). These results indicate that one G_3 residue is linked to the non-reducing, terminal residue of another G_3 residue, which is linked to the ring through a (1→6) linkage.

That the magnitude (170 Hz) of the $J_{C\text{-}1,H\text{-}1}$ values of S-1 and M-1 was the same as that of C-1 of the main ring indicates that S-1 and M-1 were α-linked.

The f.a.b.-m.s. spectra of these branched cG_7s in the negative mode were consistent with their structures (Fig. 5). The $[M - H]^-$ peak was clearly observed at *m/z* 2105, and there were two series of fragment ions. The first series appeared at *m/z* 2105 − (162 × *n*) (*n* = 0–6), which were derived by the release of 1–6 glucosyl residue(s) from branch(es). The second series was seen at *m/z* 59 + (162 × *n*) (*n* = 0–10), which were due to cleavage of the cG_7 ring and then a pyranose ring[10], but were less informative for this study.

In the spectrum of A-1, the intensities of the peaks for $[M - 6G - H]^-$ (*m/z* 1133), $[M - 5G - H]^-$ (*m/z* 1295), and $[M - 4G - H]^-$ (*m/z* 1457) were weak, but those for $[M - 3G - H]^-$ (*m/z* 1619), $[M - 2G - H]^-$ (*m/z* 1781), and $[M - G - H]^-$ (*m/z* 1943) were obvious. This situation is because the former ions must be formed through two cleavages (seondary fragments) and the latter through one cleavage of the side chain of an A-1 molecule (primary fragments). The

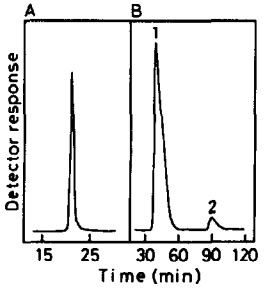


Fig. 6. H.p.l.c. of product *C* on *A*, TSKgel NH_2-60; and *B*, ODS-Hypersil-5: 1, C-1; 2, C-2.

spectrum of A-2 shows that only the intensity of the ion for $[M - 4G - H]^-$ (*m/z* 1457) is weak, because the fragment ion arises through two attacks. If A-2 has a structure such as 6-*O*-α-(6^1-*O*-α-maltotriosyl)maltotriosyl-cG$_7$, the intensity of the ion for $[M - 5G - H]^-$ (*m/z* 1295), in addition to that for $[M - 4G - H]^-$, is expected to be weak because they must be formed through two cleavages. From the A-3 molecule, the six fragment ions were derived on one cleavage of the side chain of the molecule, as shown in Fig. 5C.

The $[\alpha]_D^{21}$ values for A-1–3 in water were 175° (*c* 1), 168° (*c* 1), and 153° (*c* 0.3, water), respectively.

Structure of product B. — Since product *B* gave a single peak on an NH_2-60 column and yielded 4 mol of G and 2 mol of G_2 on complete hydrolysis with glucoamylase and pullulanase, it was concluded to be diG_2. Two isomers, 6^1- and 6^2-*O*-α-maltosylmaltose, were found in the molar ratio of 1.0:1.6 on measurement of the ratio of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol in the methylation analysis. We reported[1] that G_2 could act only as a side-chain donor, but diG_2 was isolated in this experiment. This finding appears to be due to the improved conditions (see *Substrate specificity of* Pseudomonas *isoamylase*).

Structure of product C. — Product *C* was separated into C-1 and C-2, in the ratio of 17:1, on ODS-silica (Fig. 6).

C-1 yielded 2 mol of G and 1 mol of diGcG_7 on glucoamylolysis; thus, C-1 is 6^A,6^D-di-*O*-maltosyl-cG_7. C-2 yielded 3 mol of G and 1 mol of GcG_7 on glucoamylolysis. Methylation analysis gave 2,3,4,6-tetra-, 2,3,6-tri-, 2,3,4-tri-, and 2,3-di-*O*-methyl-D-glucitol in the ratios 1.0:0.8:8.2:1.0. Thus, C-2 was 6-*O*-α-(6^2-*O*-α-maltosyl)maltosyl-cG_7.

Kitahata *et al.*[11] reported the formation of dimaltosyl- and trimaltosyl-cG_n through the condensation of cG_7 or cG_6 and maltosyl fluoride using *Bacillus acidopullulyticus* pullulanase, but did not find cG_n derivatives with diG_2 as a side chain. They used h.p.l.c. on NH_2-bonded silica and confirmed the structures of the multi-branched cG_n by t.l.c. of the products of glucoamylolysis and by methylation analysis. As noted above, diG_2cG_7 and cG_7 with diG_2 as a side chain are not separable on NH_2-bonded silica. Moreover, ECNSS M, which was used by Kitahata *et al.*[11], is inadequate for methylation analysis of starch-related sugars because it is difficult to resolve 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-glucitol[12]. Thus, it is not certain that their specimens were multi-branched.

Substrate specificity of Pseudomonas *isoamylase*. — G_2 was found to be the smallest substrate as a donor and acceptor molecule for isoamylase. Fig. 7 shows that the affinity of isoamylase for G_2 was very low, and diG_2 could be produced in a measurable amount at a high concentration (>0.6M) of G_2. Moreover, only ~1/30 of the products, compared with the present conditions, could be synthesised under the previous conditions[1], even if the concentration of G_2 was raised from 570mM to 1.6M. This was responsible for the misunderstanding in the previous investigation. The enzyme appears not to act on successive branch points because such condensa-

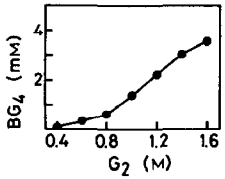


Fig. 7. Formation of diG_2 with *Pseudomonas* isoamylase. Reaction mixtures containing various concentrations (0.4–1.6M) of G_2 and 11.6 U of *Pseudomonas* isoamylase in a final volume of 1 mL of 50mM sodium acetate buffer (pH 4.2) were incubated at 58°.

tion products as 6-*O*-α-(6^1-*O*-α-maltotriosyl)maltotriosyl-cG_7 and 6-*O*-α-(6^1-*O*-α-maltosyl)maltosyl-cG_7 were not found.

According to Kainuma *et al.*[13], 6^1- and 6^2-*O*-maltosylmaltose are resistant to the enzyme, but the present results imply that these tetrasaccharides are hydrolysed. This disagreement is probably due to differences in the concentrations in the reaction mixtures. The hydrolysis reaction is rapid and it is easy to investigate the substrate specificity if the various substrates are available. On the other hand, the substrate specificity can be analysed on the basis of the reversion product by using more readily obtainable substrates.

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