Carbohydrate Research, 159 (1987) 303–313
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

FORMATION OF 6-*O*-α-MALTOSYLCYCLOMALTO-OLIGOSACCHARIDES FROM α-MALTOSYL FLUORIDE AND CYCLOMALTO-OLIGOSACCHARIDES BY PULLULANASE

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(Received June 9th, 1986; accepted for publication in revised form, August 1st, 1986)

ABSTRACT

Branched cyclomalto-oligosaccharides are formed when pullulanase from *Bacillus acidopullulyticus* is incubated with α-maltosyl fluoride (α-G_2F) as substrate in the presence of cyclomalto-oligosaccharides as acceptor. It was shown by enzymic and chemical methods that the structures of these branched cyclomalto-oligosaccharides are 6-*O*-α-maltosylcyclomaltohexaose (G_2-cG_6), 6,6′-di-*O*-α-maltosylcyclomaltohexaose [$(G_2)_2$-cG_6], 6-*O*-α-maltosylcyclomaltoheptaose (G_2-cG_7), 6,6′-di-*O*-α-maltosylcyclomaltoheptaose [$(G_2)_2$-cG_7], and 6,6′,6″-tri-*O*-α-maltosylcyclomaltoheptaose [$(G_2)_3$-cG_7]. The optimal conditions for formation of G_2-cG_6 were studied. About 12mM G_2-cG_6 was produced when pullulanase (2.8 U/mL) was incubated with 40mM α-G_2F as substrate and 90mM cyclomaltohexaose (cG_6) as acceptor for 1 h at 60°. The yield of G_2-cG_6, the main transfer-product, was twenty times that by the condensation of cG_6 with α-maltose in the presence of pullulanase.

INTRODUCTION

Cyclomalto-oligosaccharides (cyclomaltopolyoses, cyclodextrins, cycloamyloses) are the homogeneous, cyclic oligosaccharides produced by the action of cyclodextrin glucanotransferase (EC 2.4.1.19) on α-glucans. Among these oligosaccharides, cyclomaltohexaose (cG_6), cyclomaltoheptaose (cG_7), and cyclomaltooctaose (cG_8) are respectively composed of 6, 7, and 8 α-D-glucopyranosyl residues linked by α-(1→4) linkages. These products have the ability to form inclusion compounds with various kinds of inorganic and organic compounds[1], and consequently have become widely used for stabilizing labile materials, emulsifying oils, masking odors, increasing solubility, and changing viscous or oily compounds into powders. On proceeding with an investigation of the potential applications, a few disadvantages have become apparent, especially as regards their utilization in drugs or cosmetics. One of these is that cyclomalto-oligosaccharides and their inclusion

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0008-6215/87/$ 03.50

compounds show poor solubility in water. To improve the solubility, such chemical modifications thereof as methylation have been studied[2].

Recently, Kobayashi *et al.*[3] and Abe *et al.*[4] reported that two 6-*O*-α-D-glucopyranosylcyclomalto-oligosaccharides (G_1-cG_6 and G_1-cG_7) have extremely high solubility compared with that of the original cyclomalto-oligosaccharides, and solubilize various kinds of oily substances. G_1-cyclomalto-oligosaccharides are produced by the action of cyclodextrin glucanotransferase from *Bacillus macerans* on starch[5,6] or amylopectin in the presence of SDS[7], following digestion by glucoamylase (EC 3.2.1.3).

G_2-cG_6 is formed by the condensation[8,9] of maltose with cG_6 in the presence of pullulanase (EC 3.2.1.41).

It is already well known that α-maltosyl fluoride (α-G_2F) is a good substrate for transfer action of such amylases as alpha amylase[10,11] and cyclodextrin glucanotransferase[12].

Recently, we found that branched cyclomalto-oligosaccharides are formed from a mixture of α-G_2F and cyclomalto-oligosaccharides by the transfer action of pullulanase. We now describe confirmation of the structures of the branched products and the reaction conditions necessary to produce them.

EXPERIMENTAL

Materials. — A partially purified preparation of pullulanase from *Bacillus acidopullulyticus*[13] was donated by Novo Industri Japan Ltd. A crystalline glucoamylase (25 U per mg) from *Rhizopus niveus* was supplied by Ueda Chemical Co., Ltd. Cyclomaltohexaose, cyclomaltoheptaose, and pullulan were supplied by Hayashibara Co., Ltd.

Hepta-*O*-acetyl-α-maltosyl fluoride, m.p. 173.5–177°, $[\alpha]_D^{25}$ +111.8° (*c* 0.8, chloroform), was synthesized by treating maltose octaacetate with cold, anhydrous hydrogen fluoride, according to procedures previously described[14,15]. α-G_2F was generated by deacetylating the heptaacetate at 0° with fresh sodium methoxide in dry methanol. Solutions, of known concentration in dry methanol, were kept in a desiccator at −20°.

G_1-cG_6 and G_1-cG_7 were synthesized as described by Kobayashi *et al.*[7].

Assay of pullulanase activity. — A reaction mixture containing 0.3 mL of 4% pullulan in 0.1M acetate buffer (pH 5.0) and 0.3 mL of enzyme solution was incubated at 40°. After an appropriate time, the reaction was stopped by adding 0.9 mL of 0.5M carbonate buffer (pH 10.0), and the reducing sugars released were measured, in 0.5-mL aliquots by the Somogyi–Nelson method[16,17], with D-glucose as the standard. One unit of the enzyme activity was defined as the amount which liberated 1 μmol of reducing sugars as D-glucose per minute under the aforementioned conditions.

Thin-layer chromatography. — Thin-layer chromatography (t.l.c.) of the reaction products was conducted on HPTLC NH_2F_{254s} plates (Merck Co., Ltd.; L

= 10 cm), using 13:7 (v/v) acetonitrile–water as the solvent, with two developments. The carbohydrates on t.l.c. plates were revealed by heating at 110–120° after spraying with sulfuric acid–methanol.

Liquid chromatography (l.c.). — Liquid chromatography was performed under the following conditions: column, Polygosyl 10-NH_2 (4 × 300 mm); solvent system, 13:7 (v/v) acetonitrile–water; flow rate, 2 mL/min; and detection, Shodex RE-11 refractometer.

Fluoride determination. — Fluoride anion concentrations were measured in the presence of TISAB buffer [M acetate buffer, pH 5.2; M sodium chloride; 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate] with a specific fluoride-ion probe (Iwaki specific ion meter, Model 225, and Orion combination fluoride electrode, Model 96-09).

Methylation analysis of branched cyclomalto-oligosaccharides. — In order to elucidate the structures of these branched compounds, samples (each 5 mg) were subjected to microscale methylation by a modification of the Hakomori method[18], and the products hydrolyzed with acid. The methylated monosaccharides were reduced with sodium borohydride and the alditols acetylated by heating with 1:1 pyridine–acetic anhydride for 2 h at 100°. The mixture of alditol acetates so obtained was applied to a gas-liquid chromatograph having a column (2 m long) of 3% of ECNSS-M on Gas-Chrom Q and examined at a gas flow-rate of 30 mL/min at[19] 180°. The methylated alditols were identified by comparison of their retention times with those of authentic specimens.

RESULTS

Action of pullulanase on α-G_2F in the presence of cG_6 or cG_7. — A mixture (0.5 mL) containing α-G_2F (40mM) and cG_6 (90mM), or cG_7 (10% suspension), was incubated with pullulanase (2.8 U/mL) for 15 h at 40°. As shown in Fig. 1, three new spots (Aa, a-1, a-2) from the reaction mixture of α-G_2F and cG_6, and four new spots (Bb, b-1, b-2, and b-3) from that of α-G_2F and cG_7, had appeared. The oligosaccharides corresponding to the spots (Aa, a-1, a-2, Bb, b-1, b-2, and b-3) were isolated by l.c. The oligosaccharides Aa and Bb were identified as maltose on the basis of their R_F values and the results of hydrolysis by glucoamylase and by hydrochloric acid.

In a mixture containing α-G_2F and cG_6 or cG_7 without pullulanase, the product was only maltose (~5mM), formed by nonenzymic cleavage of the C–F bond of α-G_2F.

Structures of a-1 and a-2. — Compounds a-1 and a-2 were respectively stained to brown-purple and brownish-yellow on a t.l.c. plate by spraying with 1% iodine (methanolic solution). This result suggests that a-1 and a-2 have a cG_6 ring in their structure. Compound a-1 was completely hydrolyzed by glucoamylase to glucose and saccharide C, and completely hydrolyzed by pullulanase to maltose and cG_6 (see Fig. 2). Compound a-2 was also completely hydrolyzed by glucoamylase, to

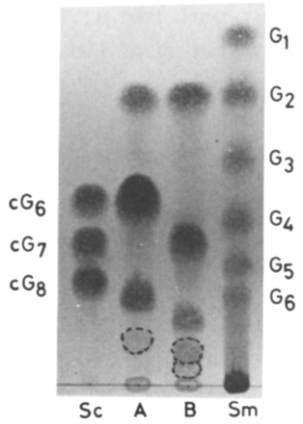

Fig. 1. Thin-layer chromatogram of the reaction products of the action of pullulanase on α-G_2F and cyclomalto-oligosaccharide. [Pullulanase (2.8 U/mL) was incubated with 40mM α-G_2F and 90mM cG_6 (A), or cG_7 (10% suspension) (B) in 0.5 mL of 0.1M acetate buffer (pH 5.0) for 15 h at 40°. Spots for reaction mixture A are, from the top downwards, Aa, cG_6, a-1, and a-2. Spots for reaction mixture B are, from the top downwards: Bb, cG_7, b-1, b-2, and b-3. Sc, standards of cyclomaltohexaose (cG_6), cyclomaltoheptaose (cG_7), and cyclomalto-octaose (cG_8). Sm: standards of malto-oligosaccharides; G_1, G_2, *etc.*, are D-glucose, maltose, *etc.*]

give glucose and saccharide D, and by pullulanase, to maltose and cG_6. Saccharide C was identified as 6-*O*-α-D-glucosylcyclomaltohexaose (G_1-cG_6) by comparing it with an authentic sample prepared by the method of Kobayashi *et al.*[7] on the basis of its R_F value and the staining on a t.l.c. plate with methanolic iodine solution. Also, saccharide D was presumed to be 6,6′-di-*O*-α-glucosylcyclomalto-

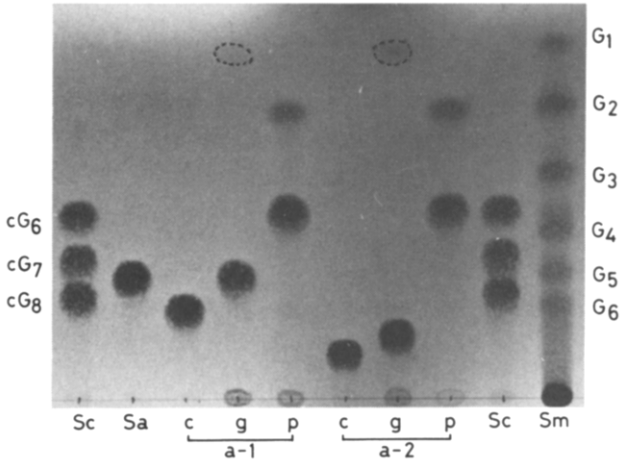

Fig. 2. Enzymic digestion of a-1 and a-2. [a-1 and a-2 in 50mM acetate buffer (pH 4.7) were digested with glucoamylase (0.2 mg/mL) and pullulanase (4.3 U/mL) for 5 h at 40°. Sa, standards of 6-*O*-α-D-glucopyranosylcyclomaltohexaose (G_1-cG_6); c, control; g, digestion with glucoamylase; and p, digestion with pullulanase. Other abbreviations are the same as in Fig. 1.]

TABLE I

ENZYMIC DIGESTION[a] OF a-1 AND a-2

Substrate	*Time (h)*	*Glucoamylase digests*		*Pullulanase digests*	
		Branched-cG_6 (mM)	*Glucose (mM)*	*cG_6 (mM)*	*Maltose (mM)*
a-1	1	9.4[b]	9.3	8.8	8.5
	3	9.4[b]	9.2	—	—
a-2	1	6.5[c]	13.2	6.9	13.1
	3	6.8[c]	13.8	7.3	14.2

[a]Compounds a-1 (9.5mM) and a-2 (7.0mM) were respectively digested by glucoamylase (0.2 mg/mL) and pullulanase (4.3 U/mL) for 3 h at 40°, and the amounts of products were determined by l.c. [b]Calculated from the peak area as G_1-cG_6. [c]Calculated as $(G_1)_2$-cG_6.

hexaose [$(G_1)_2$-cG_6] on the basis of its R_F value, the staining on a t.l.c. plate with methanolic iodine solution, and its resistance to glucoamylase action.

These results show that a-1 is 6-*O*-α-maltosylcyclomaltohexaose (G_2-cG_6) and a-2 is 6,6′-di-*O*-α-maltosylcyclomaltohexaose [$(G_2)_2$-cG_6], not *O*-α-maltosyl-(1→6)-*O*-α-maltosyl-(1→6)-cyclomaltohexaose.

molar ratio of 2:1. By pullulanase action, maltose and cG_6 were produced in the molar ratio of 1:1 from a-1, and in the ratio of 2:1 from a-2.

These results show that a-1 is 6-*O*-α-maltosylcyclomaltohexaose (G_2-cG_6) and a-2 is 6,6′-di-*O*-α-maltosylcyclomaltohexaose [$(G_2)_2$-cG_6], not *O*-α-maltosyl-(1→6)-*O*-α-maltosyl-(1→6)-cyclomaltohexaose.

In order to confirm the structures of a-1 and a-2, these oligosaccharides were subjected to methylation analysis. G_1-cG_6 was used as the standard sugar. G.l.c. analysis of the alditol acetates of the methylated-sugar fragments from G_1-cG_6, a-1, and a-2 revealed the presence of 2,3,4,6-tetra-*O*-methylglucitol, 2,3,6-tri-*O*-methylglucitol, and 2,3-di-*O*-methylglucitol, and the molar ratios were 1.0:4.8:0.8, 1.0:5.9:0.8, and 1.0:3.2:0.8, respectively (see Table II). The results of the methylation analysis support the finding that a-1 is 6-*O*-α-maltosylcyclomaltohexaose, and a-2 is 6,6′-di-*O*-α-maltosylcyclomaltohexaose. However, it is not yet known whether the two maltosyl groups in a-2 are symmetrically bound to the cG_6 ring or not.

Structures of b-1, b-2, and b-3. — The structures of b-1, b-2, and b-3 were determined by enzymic and chemical methods similar to those used for a-1 and a-2. Compound b-1 was completely hydrolyzed by glucoamylase to glucose and 6-*O*-α-glucosylcyclomaltoheptaose (G_1-cG_7) in the ratio of 1:1, and by pullulanase to maltose and cG_7 in the ratio of 1:1. Compounds b-2 and b-3 were also completely hydrolyzed by pullulanase to maltose and cG_7 in the molar ratios of 2:1 and 3:1, respectively. By glucoamylase action, glucose and 6,6′-di-*O*-α-glucosylcyclomaltoheptaose were produced from b-2, and 6,6′,6″-tri-*O*-α-glucosylcyclomalto-

TABLE II

METHYLATION ANALYSIS[a] OF BRANCHED CYCLOMALTOHEXAOSES

Branched cyclomaltohexaoses	*Ratio of alditol acetates*			
		2,3,4,6-tetra O-methyl-glucitol	*2,3,6-tri-O-methyl-glucitol*	*2,3-di-O-methyl-glucitol*
G_1-cG_6	*Calc.*	1.0	5.0	1.0
	Found	1.0	4.8	0.8
a-1	*Calc.*[b]	1.0	6.0	1.0
	Found	1.0	5.9	0.8
a-2	*Calc.*[c]	2.0	6.0	2.0
	Found	1.0	3.2	0.8

[a]Compounds a-1 and a-2 (5 mg each) were methylated, with G_1-cG_6 as the standard sugar. Alditol acetates of the methylated sugar fragments from G_1-cG_6, a-1, and a-2 were identified, and the ratios of the acetates were determined, by g.l.c. [b]Calculated as 6-*O*-α-maltosylcyclomaltohexaose (G_2-cG_6). [c]Calculated as 6,6′-di-*O*-α-maltosylcyclomaltohexaose [$(G_2)_2$-cG_6].

heptaose from b-3 (see Fig. 3). These results show that b-1 is 6-*O*-α-maltosylcyclomaltoheptaose (G_2-cG_7), b-2 is 6,6′-di-*O*-α-maltosylcyclomaltoheptaose [$(G_2)_2$-cG_7], and b-3 is 6,6′,6″-tri-*O*-α-maltosylcyclomaltoheptaose [$(G_2)_3$-cG_7]. Also, the results of methylation analysis supported these structures.

Time course of action of pullulanase on α-G_2F in the presence of cG_6. — A mixture (2.0 mL) of 40mM α-G_2F and 90mM cG_6 was incubated with pullulanase

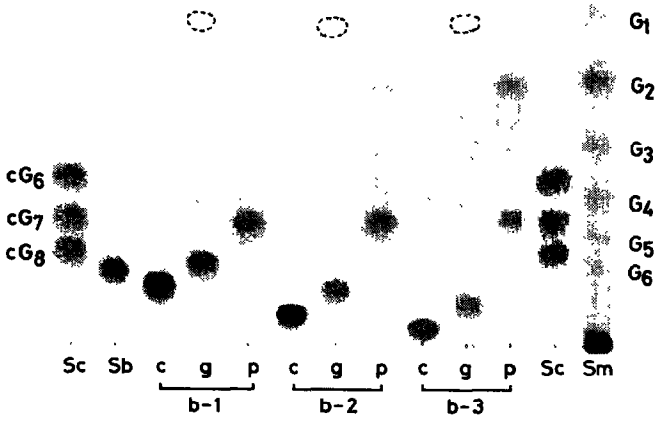

Fig. 3. Enzymic digestion of b-1 and b-2, and b-3. [b-1, b-2, and b-3 in 50mM acetate buffer (pH 4.7) were digested with glucoamylase (0.2 mg/mL) and pullulanase (4.3 U/mL) for 5 h at 40°. Sb, standard of 6-*O*-α-D-glucopyranosylcyclomaltoheptaose (G_1-cG_7); c, control; g, digestion with glucoamylase; and p, digestion with pullulanase. Other abbreviations are the same as in Fig. 1.]

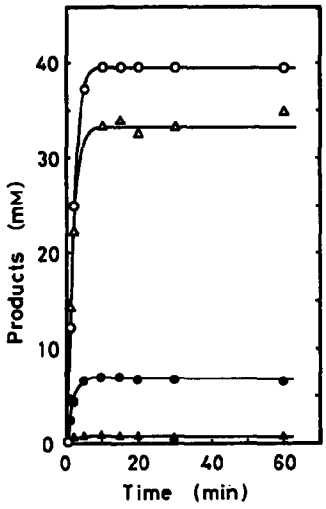

Fig. 4. Time course of formation of branched cyclomaltohexaoses. [α-G_2F (40mM) and cG_6 (90mM) were incubated with pullulanase (2.8 U/mL) at 40°: ○, fluoride anion released; △, ●, and ▲, maltose, G_2-cG_6, and $(G_2)_2$-cG_6 produced, respectively.]

(2.8 U/mL) at 40°. After 2, 5, 10, 15, 20, 30, and 60 min, appropriate aliquots (0.05–0.20 mL) of the reaction mixture were removed, in order to examine the amount of fluoride anion released, and the amounts of maltose, G_2-cG_6, and $(G_2)_2$-cG_6 produced. As shown in Fig. 4, the amounts of fluoride anion released and of maltose, G_2-cG_6, and $(G_2)_2$-cG_6 produced, attained constant values after 10 min, and remained constant for 60 min.

Effect of concentration of cG_6 and α-G_2F on formation of G_2-cG_6. — For the purpose of investigation of the effect of cG_6 concentration on G_2-cG_6 formation, pullulanase (2.8 U/mL) was incubated with 40 mM α-G_2F in the presence of various concentrations of cG_6 at 40°. After 1 h, the amounts of G_2-cG_6 produced in the reaction mixtures (0.2 mL) were determined by l.c. As shown in Fig. 5a, the amounts of G_2-cG_6 produced increase linearly with increase of cG_6 concentration, up to 50mM, and attain a constant value at 90mM cG_6. About 90mM cG_6 is the optimal concentration for G_2-cG_6 formation when α-G_2F concentration is 40mM.

In order to investigate the effect of α-G_2F concentration on G_2-cG_6 formation, pullulanase (2.8 U/mL) was incubated with various concentrations of α-G_2F in the presence of 90mM cG_6 for 60 min at 40°. The amounts of G_2-cG_6 produced in the reaction mixtures (0.2 mL) were measured by l.c. The amounts of G_2-cG_6 increased with increase of α-G_2F concentration up to 120mM (see Fig. 5b). The ratio of conversion (G_2-cG_6 produced to α-G_2F used) decreased, however, with increase of α-G_2F concentration. Therefore, in order to use α-G_2F effectively, 40mM α-G_2F was used in the following experiments.

Effect of pH on formation of G_2-cG_6. — The optimum pH of pullulanase for hydrolysis of pullulan[13] is 5.0. To check the effect of pH on G_2-cG_6 formation,

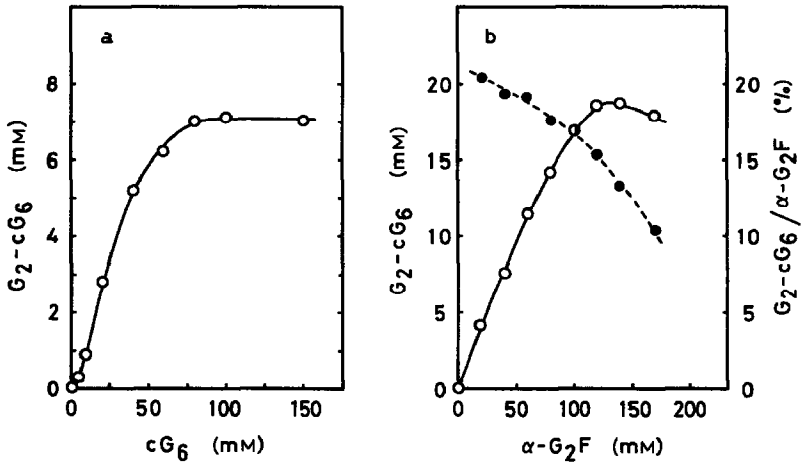

Fig. 5. Effects of concentration of cG_6 and α-G_2F on formation of G_2-cG_6. [Pullulanase (2.8 U/mL) was incubated with (a) α-G_2F (40mM) in the presence of various concentrations of cG_6, and (b) cG_6 (90mM) in the presence of various concentrations of α-G_2F for 1 h at 40°: ○, G_2-cG_6 produced; ●, conversion ratio (G_2-cG_6 produced/α-G_2F used).]

pullulanase (2.8 U/mL) was incubated with a mixture of 40mM α-G_2F and 90mM cG_6 in 60mM acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–7.5), and Tris·HCl buffer (pH 8.0–9.0). In the pH range of 4.5 to 8.0, the pH had no effect on G_2-cG_6 formation (see Fig. 6).

Effect of temperature on formation of G_2-cG_6. — Acetate buffer (60mM, pH 5.0) was used in the following experiments. A mixture (0.2 mL) of 40mM α-G_2F

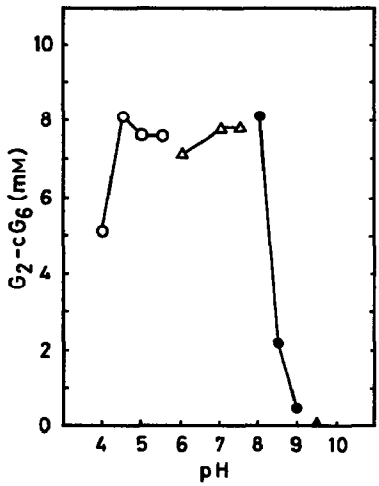

Fig. 6. Effect of pH on formation of G_2-cG_6. [Pullulanase (2.8 U/mL) was incubated with α-G_2F (40mM) and cG_6 (90mM) in various kinds of buffer for 1 h at 40°: ○, 60mM acetate buffer (pH 4.0–5.5); △, 60mM phosphate buffer (pH 6.0–7.5); ●, 60mM Tris·HCl buffer (pH 8.0–9.0); ▲, 30mM borate buffer (pH 9.5).]

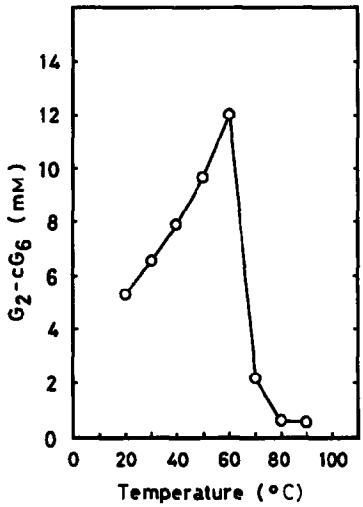

Fig. 7. Effect of temperature on formation of G_2-cG_6. [The solutions containing α-G_2F (40mM), cG_6 (90mM) and pullulanase (2.8 U/mL) were incubated for 1 h at the temperature indicated.]

and 90mM cG_6 was incubated with pullulanase (2.8 U/mL) for 1 h at various temperatures, and the amounts of G_2-cG_6 produced were determined by l.c. By the amounts of fluoride anion released, it was confirmed that α-G_2F is completely degraded in the reaction at 20, 30, 40, 50, and 60°. As shown in Fig. 7, the amounts of G_2-cG_6 produced increased with increase in the temperature, up to 60°, and then decreased because of inactivation of the pullulanase.

About 12mM G_2-cG_6 was produced when pullulanase (2.8 U/mL) was incubated with 40mM α-G_2F and 90mM cG_6 for 1 h at 60°, whereas, under conditions where α-maltose replaced α-G_2F, the concentration of G_2-cG_6 produced was 0.5mM. Thus, the yield of G_2-cG_6 by the transfer reaction with α-G_2F and cG_6 was twenty times that obtained by the condensation reaction with α-maltose and cG_6.

DISCUSSION

Pullulanase catalyzes the splitting of certain α-(1→6) linkages in pullulan, starch, glycogen, and suitably branched oligosaccharides[20]. Pullulanase also catalyzes the condensation reaction at high concentrations of substrate[21], to form G_2-cyclomalto-oligosaccharides from maltose and cyclomalto-oligosaccharides[8,9].

Recently, Mizokami and Hehre[22], using α-G_2F as the substrate, independently observed that *Aerobacter* pullulanase has transfer activity.

In this study, we showed that the crude pullulanase preparation from *Bacillus acidopullulyticus* catalyzes maltosyl transfer from α-G_2F to cyclomalto-oligosaccharides to produce 6-*O*-α-maltosylcyclomalto-oligosaccharides. The amount of G_2-cG_6 is not affected by the pH of the reaction (see Fig. 6), but increases with an increase of reaction temperature (see Fig. 7). This effect of temperature on the

production of G_2-cG_6 was found to be due to any contaminating enzymes, because similar results were obtained with purified enzyme (data not shown herein). The reason why the higher temperature favors the transfer ratio from α-G_2F to cG_6 has not yet been clarified.

Two methods have been reported to produce the branched cyclomalto-oligosaccharides. One uses condensation of maltose and cyclomalto-oligosaccharides by pullulanase[8,9], and the other uses the cyclizing reaction of cyclodextrin glucanotransferase[3,5–7]. Our method, using the transfer action of pullulanase, with α-G_2F as the donor molecule and a cyclomalto-oligosaccharide as the acceptor molecule, has some advantages, as follows. (*1*) High yield: the yield of G_2-cG_6 by our method is about twenty times that obtained by the condensation reaction. (*2*) High selectivity: in our method, the reaction products are maltose and branched cyclomalto-oligosaccharides. In the condensation reaction, such noncyclic branched oligosaccharides as *O*-α-maltosyl-(1→6)-maltose are also produced, rather than branched cyclomalto-oligosaccharides. Various kinds of products, such as D-glucose, cG_6, cG_7, cG_8, G_1-cG_6, G_1-cG_7, and G_1-cG_8, are formed in the cyclizing reaction. (*3*) Multi-branched cyclomalto-oligosaccharides: our method is able to synthesize such multi-branched compounds as $(G_2)_2$-cG_6, $(G_2)_2$-cG_7, and $(G_2)_3$-cG_7.

In further studies, we found that G_2-cG_6 was obtained by the action of *Aerobacter* pullulanase or *Pseudomonas* isoamylase in a yield about twice that given by *Bacillus* pullulanase.

Recently, we found that a slight proportion of $(G_2)_3$-cG_6 is obtained from a mixture of α-G_2F and cG_6. Branched cyclomalto-oligosaccharides [G_2-cG_8, $(G_2)_2$-cG_8, and $(G_2)_3$-cG_8] were also formed, using cG_8 as the acceptor (data not shown). G_2-cyclomalto-oligosaccharides had already been isolated by Sakano *et al.*[9], whereas such multi-branched compounds as $(G_2)_2$-cyclomalto- and $(G_2)_3$-cyclomalto-oligosaccharides have not yet been isolated. These multi-branched compounds will be useful in study of the mode of action of debranching enzymes, and of the active site of cyclodextrin glucanotransferase.

ACKNOWLEDGMENTS

The authors thank Dr. Y. Kusayama of Novo Industri Japan Co. for gift of *Bacillus* pullulanase. We also thank Dr. M. Nishimura of Tokuyana Soda Co. for helpful suggestions concerning the preparation and purification of α-maltosyl fluoride.

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