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HYDROLYSIS AND SYNTHESIS OF BRANCHED CYCLOMALTOHEXAOSES WITH *Pseudomonas* ISOAMYLASE

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ABSTRACT

The action of *Pseudomonas* isoamylase on branched cyclomaltohexaoses (α-cyclodextrins, cG_6s) with side chains of various lengths and the reverse condensation reaction between malto-oligosaccharides (G_2–G_7) and cG_6 have been studied. The rates of reaction for the liberation and the attachment of maltotriose were maximal in both the hydrolysis and the condensation reactions, and the activity decreased with increasing length of the side chain. The values of V_{max} (U/mg) for the hydrolytic reactions for G_2-, G_3-, G_4-, and G_5-cG_6 were 2.6, 690, 320, and 290, respectively, and the values of K_m (mM) were 72, 204, 92, and 47, respectively. The structures of the new branched cG_6s (G_4-cG_6 and G_5-cG_6), obtained through the condensation reaction, were identified by means of enzymic analyses, and ^{13}C-n.m.r. and f.a.b.-mass spectra. The haemolytic activities of these branched cG_6s are reported.

INTRODUCTION

Isoamylase (EC 3.2.1.68), which hydrolyses the (1→6)-α-D-glucosidic linkages in amylopectin and glycogen, is an essential enzyme for analysis of the molecular structures of these polysaccharides and related oligosaccharides, and plays an important role in the industrial production of glucose and maltose from starch. *Pseudomonas* isoamylase[1] is commercially available, and its structure and action have been investigated in detail[2]. However, the relationship between the activities and the structures of substrates has not been elucidated fully because suitable substrates are not readily available. This difficulty was overcome by

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following the reverse condensation action[3,4] instead of the forward hydrolytic action. The main purpose of this study was to elucidate the action of the enzyme in relation to the length of the side chains of the substrate in both the condensation and hydrolytic reactions. The condensation reactions were carried out between cyclomaltohexaose (cG_6) and a series of malto-oligosaccharides [maltose (G_2)–maltoheptaose (G_7)], and the hydrolytic reactions were performed on the products, *i.e.*, branched cG_6s with G_2–G_5 branches.

Branched cyclomalto-oligosaccharides (cyclodextrins, cG_ns) with glucose or malto-oligosaccharides attached at positions 6 of their glucosyl residue(s) are produced in minor quantities by the action of cyclodextrin glucanotransferase [(1→4)-α-D-glucan 4-D-glucosyltransferase (cyclising), EC 2.4.1.19] on starch[5–7]. Branched cG_ns have some superior properties compared to those of non-branched cG_ns, *i.e.*, they are highly soluble in water and form water-soluble complexes with hydrophobic materials[8–11]. The preparation of maltosyl-(G_2-) and maltotriosyl-(G_3-)cG_ns by means of *Pseudomonas* isoamylase[3,4] has been reported. Branched cG_ns were also synthesised by means of the reverse[12–14] or transfer[15,16] actions of pullulanase and isoamylase. Some properties of branched cG_6s are now described.

EXPERIMENTAL

Materials. — Purified *Pseudomonas* isoamylase, a series of malto-oligosaccharides (G_2–G_7), and cG_6 were the products of Hayashibara Biochemical Laboratories Inc. (Okayama). Beta-amylase[17] and glucoamylase GIII[18] were purified from sweet potato and a crude commercial preparation of *Rhizopus delemar*, respectively.

Analyses. — The products of reaction were quantified by h.p.l.c. on TSKgel NH_2-60 with acetonitrile–water (65:35) as the eluant, at 0.8 mL/min, with monitoring with a differential refractometer (RI-8000, Tosoh). Reducing power and total carbohydrate (as glucose) were assayed by the colorimetric method of Nelson[19] and Somogyi[20], and an anthrone–sulfuric acid method[21], respectively. The solubility and haemolytic activity of branched cG_6s were determined as described[10].

^{13}C-N.m.r. spectra (50.10 MHz) were recorded at 50° for 2–3% solutions in D_2O, using a JEOL JNM-FX200 spectrometer. The 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 detailed conditions are given elsewhere[3].

F.a.b.-mass spectra in the negative mode were obtained with a JEOL JMS-HX 110 mass spectrometer, using glycerol as the matrix[4].

Preparation of branched cG_6. — G_3-, G_4-, and G_5-cG_6 were synthesised by condensation of the respective malto-oligosaccharides and cG_6 with isoamylase. The pH and temperature for the reaction were based on those for the preparation[4] of G_3-cG_7, but the concentrations of the substrates were not optimised. The reaction mixtures, each containing 275mM cG_6, 605mM oligosaccharide, and 8.2 U/mL of isoamylase, in 50mM acetate buffer (pH 4.2) were incubated for 48 h at 58°. The

reaction was terminated by boiling, and the products were isolated by gel-permeation chromatography on Bio-Gel P-2 and Toyopearl HW40 and then purified by h.p.l.c. as described[3,4,14]. G-cG_6 was produced from G_3-cG_6 by hydrolysis with glucoamylase (0.1 U/μmol of G_3-cG_6) in 10mM acetate buffer (pH 4.5) for 24 h at 45°, and was isolated using a column (5.5 × 46 cm) of Bio-Gel P-2 and elution with distilled water. G_2-cG_6 was prepared by the condensation of maltose and cG_6 with *Klebsiella aerogenes* pullulanase under conditions similar to those used for the preparation[14] of G_2-cG_8. These products were purified by h.p.l.c. on ODS-Hypersil-5 (Shandon) with aqueous ~8% methanol as the solvent, each giving a single peak.

RESULTS AND DISCUSSION

Action of isoamylase. — *Pseudomonas* isoamylase releases maltotriose in preference to maltose from the beta-limit dextrin of amylopectin[22], and causes[3] the much faster condensation of maltotriose than of maltose to cG_n. The activities of the enzyme toward malto-oligosaccharides up to G_7 have now been examined (Table I). Experiments 1 and 2 were carried out with constant concentrations of the substrate (mol and weight, respectively). Experiment 2 avoided the possible decrease in the activity in the reactions of large substrates due to insufficient water[4]. In each experiment, maltose and maltotriose were the least and most effective substrates, respectively. Maltotetraose was a little less effective than maltotriose as substrate; for the higher malto-oligosaccharides, the enzyme showed less than half the activity for maltotetraose. The time courses of the formation of G_2- to G_5-cG_6 (Fig. 1) showed that G_2-cG_6 was produced only in a small quantity even on prolonged reaction, and thus the isoamylase was unsuitable for the production of G_2-cG_6 as reported[3].

The relationships between the rates of hydrolysis and the lengths of the side chain of the substrates are shown in Table II. The K_m for G_2-cG_6 was about one-third but the V_{max} only 0.4% of those for G_3-cG_6. The V_{max} for G_3-cG_6 was considerably higher than that for G_2-cG_6, but the affinity for this substrate was lower

TABLE I

CONDENSATION ACTIVITIES OF *Pseudomonas* ISOAMYLASE BETWEEN cG_6 AND MALTO-OLIGOSACCHARIDES

Malto-oligosaccharide	G_2	G_3	G_4	G_5	G_6	G_7
1[a] Activity (μmol/min/mg)	0.097	2.57	1.96	0.97	n.d.[c]	n.d.
(relative)	1.0	26.5	20.2	10.0		
2[b] Activity (μmol/min/mg)	0.15	2.80	2.44	0.97	0.91	0.86
(relative)	1.0	18.7	16.2	6.5	6.0	5.7

[a]Each reaction mixture (1 mL) contained 275mM cG_6, 605mM malto-oligosaccharide, and 8.5 U of isoamylase in 50mM acetate buffer (pH 4.2) and was incubated at 58°. [b]Each reaction mixture contained 550 mg/mL of oligosaccharide and cG_6 in the molar ratio 2:1, and the other conditions were as for Experiment 1. [c]Not determined.

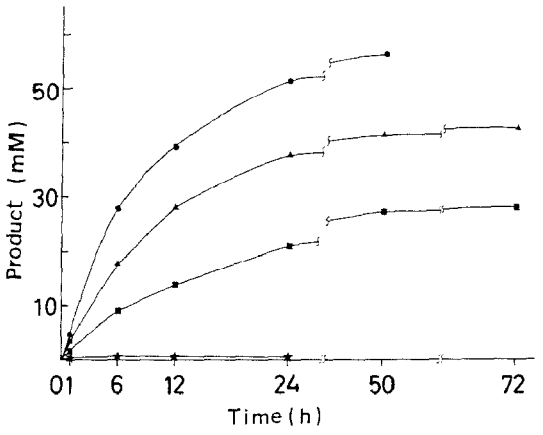

Fig. 1. Time courses of the formation of branched cG_6s. Each reaction mixture was at pH 4.2 and contained 275mM cG_6, 605mM G_2–G_5, and 8.2 U of isoamylase/mL, and was incubated at 58°: ★, G_2-cG_6; ●, G_3-cG_6; ▲, G_4-cG_6; ■, G_5-cG_6.

than that for G_2-cG_6 which was hydrolysed least. The values of K_m and V_{max} decreased with increasing chain length in the range of G_3–G_5. This finding implies that the enzyme has at least 5 binding sites for the side chains of the substrates, and the affinity is higher with the longer side-chains. It is reasonable, because of its marked debranching activity toward amylopectin and glycogen, that the enzyme shows similar activities toward the G_4 and G_5 side-chains. The activity appears to be modified by the structure of the main chain as reported[3], and G_2-cG_7 and G_2-cG_8 may be better substrates than G_2-cG_6. Therefore, it should not be concluded that the G_2-stubs in the beta-limit dextrins of glycogen and amylopectin are not hydrolysed significantly by the enzyme. The relationships between the activities and the structures of the main chains remain to be determined.

Structures of the branched-cG_6. — The branched cG_6s, including the new compounds G_4-cG_6 and G_5-cG_6, were characterised by means of enzymic analyses, and ^{13}C-n.m.r. and f.a.b.-mass spectra, G_3-, G_4-, and G_5-cG_6 in dilute solutions were hydrolysed into equimolar amounts of the respective malto-oligosaccharides

TABLE II

KINETIC PARAMETERS OF HYDROLYTIC ACTIVITIES FOR BRANCHED cG_6S

Substrate	K_m *(mM)*	V_{max} *(U/mg)*	V_{max}/K_m
G_2-cG_6	72	2.6	0.04
G_3-cG_6	204	690	3.4
G_4-cG_6	92	320	3.5
G_5-cG_6	47	290	6.2

and cG_6 by isoamylase. G_2–G_5-cG_6 gave 1–4 mol/mol glucose with glucoamylase (*Rhizopus delemar* GIII[18]) and an equimolar amount of G-cG_6, respectively. G_4- and G_5-cG_6 were converted into maltose and G_2- and G_3-cG_6, respectively, with sweet-potato beta-amylase. G_2- and G_3-cG_6 were resistant to this enzyme. These findings accord with the action of the enzyme on branched oligosaccharides[23].

The ^{13}C-n.m.r. spectra of G- to G_5-cG_6 were assigned as shown in Table III by comparison with those of branched cG_7S[3,4] and cG_8S[14]. The chemical shifts and intensities of the signals for C-1, C-4, and C-6 were consistent with the structures. The $J_{C\text{-}1,H\text{-}1}$ values for the side chains of G- to cG_5-cG_6 involved in the linkages between the ring residues were ~170 Hz, indicating[24] the linkages between the malto-oligosaccharides and cG_6 to be α.

The f.a.b-mass spectra (negative mode) of G_4-cG_6 and G_5-cG_6 indicated the

TABLE III

CHEMICAL SHIFTS AND RELATIVE INTENSITIES OF THE SIGNALS FOR C-1, C-4, AND C-6 IN THE ^{13}C-N.M.R. SPECTRA OF SOLUTIONS OF THE BRANCHED cG_6S IN D_2O

cG₆	*C-1*[a]			*C-4*[a]			*C-6*[a]	
	Rs and R	*Sr*	*So and S*	*Rs and R*	*So*	*Sr and S*	*R, S, So, and Sr*	*Rs*
G-	102.17	99.74		82.08	70.52		61.33	67.40
				82.30			61.48	
				82.40				
	6:1[b]			6:1			6:1	
G_2-	102.17	99.43	100.85	82.06	70.38	78.75	61.34	68.13
				83.18		78.76	61.36	68.17
				82.48			61.37	
							61.50	
	6:1:1			6:1:1			7:1	
G_3-	102.17	99.39	100.77	82.07	70.37	78.23	61.38	68.08
			100.84	82.15		79.24	61.45	
				82.48			61.53	
	6:1:2			6:1:2			8:1	
G_4-	102.19	99.42	100.58	82.13	70.38	78.23	61.46	68.22
			100.82	82.53		79.29	61.56	
	6:1:3			6:1:3			9:1	
G_5-	102.19	99.43	100.53	82.12	70.38	78.07	61.47	68.23
			100.63	82.54		78.15		
			100.71			78.39		
			100.83			79.18		
	6:1:4			6:1:4			10:1	

[a]Key: Rs, ring Glc linked to a side chain; R, ring Glc other than Rs; Sr, side-chain Glc linked to a ring Glc; So, side-chain Glc with HO-4 unsubstituted (identical to Sr in G-cG_6); S, side-chain Glc other than Sr and So. [b]Relative intensity.

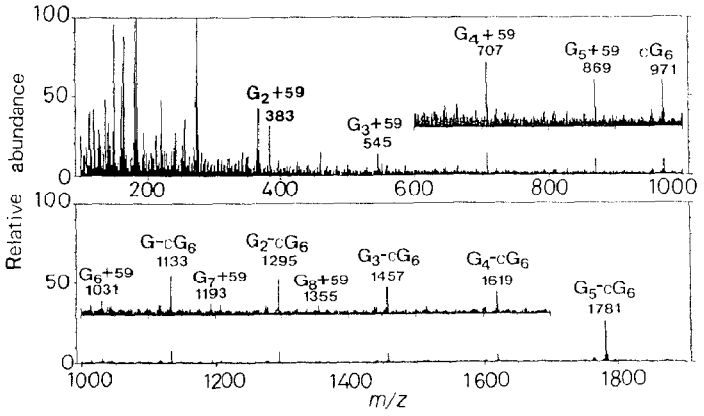

Fig. 2. F.a.b.-mass spectrum of G_5-cG_6 (mol. wt. 1782) in the negative mode.

molecular weights. The f.a.b.-mass spectrum of G_5-cG_6 (Fig. 2) shows a series of fragment ions corresponding to the shorter side-chains of similar abundance, suggesting a single and linear side-chain[4,14]. Another series of fragment ions, with masses of *n*.glucosyl + 59, is due to the cleavage of the cG_6 ring and a glucosyl residue[3,4]. A similar f.a.b.-mass spectrum was obtained for G_4-cG_6.

Properties. — The solubilities of cG_n in water were enhanced greatly by the attachment of glucose or malto-oligosaccharides[10]. cG_6 is moderately soluble in water at 25°, but its solubility is enhanced[10] up to ~5-fold on attachment of G_3. Fig. 3 shows the solubilities of the series of branched cG_6s at 25°, 45°, and 55°. The

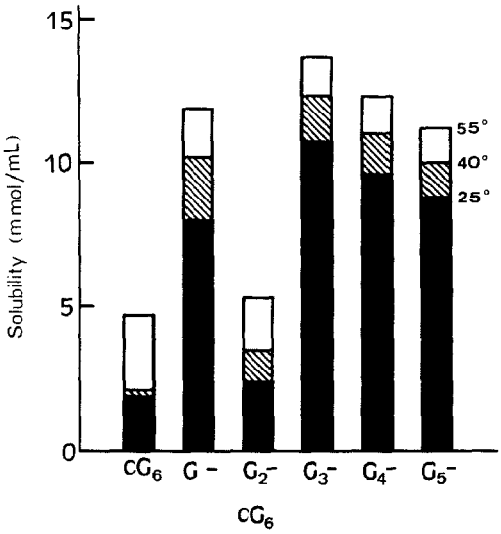

Fig. 3. Solubilities of cG_6s in water at 25°, 40°, and 55°.

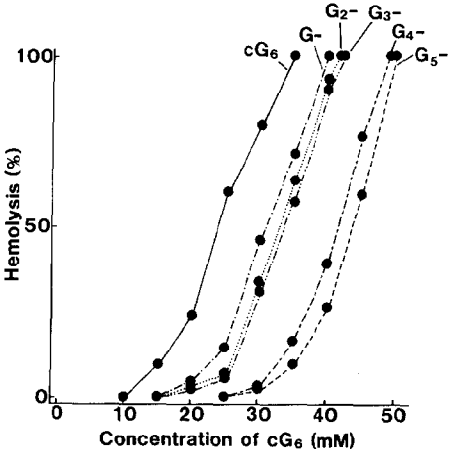

Fig. 4. Haemolytic activities of cG_6s.

solubility of G_2-cG_6, which was the least soluble among the branched cG_6s, was enhanced ~2.2-fold by elevation of the temperature from 25° to 55°, and those of other, highly soluble, branched CDs by 1.3–1.5-fold. The low solubility of G_2-cG_6 suggests that it may have a rigid, less hydrophilic structure, due to the formation of some intermolecular and/or intramolecular hydrogen bonds. The $[\alpha]_D^{22}$ values (water) were as follows: cG_6, +150°; G-cG_6, +157°; G_2-cG_6, +165°; G_3-cG_6, +167°; G_4-cG_6, +170°; and G_5-cG_6, +172°. The haemolytic activity toward human red cells of cG_6, which is intermediate among cG_6, cG_7, and cG_8, was considerably reduced on attachment of the long side-chains (Fig. 4).

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REFERENCES

1 T. HARADA, K. YOKOBAYASHI, AND A. MISAKI, *Appl. Microbiol.*, 10 (1968) 1939–1944.
2 T. HARADA, *Biotechnol. Genetic Eng. Rev.*, 1 (1984) 39–63.
3 J. ABE, N. MIZOWAKI, S. HIZUKURI, K. KOIZUMI, AND T. UTAMURA, *Carbohydr. Res.*, 154 (1986) 81–92.
4 J. ABE, S. HIZUKURI, K. KOIZUMI, Y. KUBOTA, AND T. UTAMURA, *Carbohydr. Res.*, 176 (1988) 87–95.
5 J. ABE, Y. TAKEDA, S. HIZUKURI, K. YAMASHITA, AND N. IDE, *Carbohydr. Res.*, 131 (1984) 175–179.

6 S. KOBAYASHI, K. KAINUMA, AND S. SUZUKI, *Nippon Nogei Kagaku Kaishi,* 51 (1977) 691–698.
7 K. KOIZUMI, T. UTAMURA, M. SATO, AND Y. YAGI, *Carbohydr. Res.,* 153 (1986) 55–67.
8 K. KOIZUMI, Y. KUBOTA, Y. OKADA, T. UTAMURA, S. HIZUKURI, AND J. ABE, *J. Chromatogr.,* 437 (1988) 47–57.
9 K. KOIZUMI, Y. OKADA, Y. KUBOTA, AND T. UTAMURA, *Chem. Pharm. Bull.,* 35 (1987) 3413–3418.
10 Y. OKADA, Y. KUBOTA, K. KOIZUMI, S. HIZUKURI, T. OHFUJI, AND K. OGATA, *Chem. Pharm. Bull.,* 36 (1988) 2176–2185.
11 S. HIZUKURI AND K. KOIZUMI, in M. FUJIMAKI, K. IWAI, Y. KAWAMURA, H. IMURA, S. MATSUSHITA, T. YANO, K. YAMAUCHI, H. CHIBA, AND S. ARAI (Eds.), *Reports of Systematic Analysis and Development of Food Functionalities,* Gakkai Shuppan Center, 1988, pp. 416–421 (in Japanese).
12 M. ABDULLAH AND D. FRENCH, *Arch. Biochem. Biophys.,* 137 (1970) 483–493.
13 Y. SAKANO, M. SANO, AND T. KOBAYASHI, *Agric. Biol. Chem.,* 49 (1985) 3391–3398.
14 S. HIZUKURI, S. KAWANO, J. ABE, K. KOIZUMI, AND T. TANIMOTO, *Biotech. Appl. Biochem.,* in press.
15 S. KITAHATA, Y. YOSHIMURA, AND S. OKADA, *Carbohydr. Res.,* 159 (1987) 303–313.
16 Y. YOSHIMURA, S. KITAHATA, AND S. OKADA, *Carbohydr. Res.,* 168 (1987) 285–294.
17 Y. TAKEDA AND S. HIZUKURI, *Biochim. Biophys. Acta,* 85 (1969) 469–471.
18 J. ABE, H. NAGANO, AND S. HIZUKURI, *J. Appl. Biochem.,* 7 (1985) 235–247.
19 N. NELSON, *J. Biol. Chem.,* 153 (1944) 375–380.
20 M. SOMOGYI, *J. Biol. Chem.,* 195 (1952) 19–23.
21 L. H. KOEHLER, *Anal. Chem.,* 24 (1952) 1576–1579.
22 K. YOKOBAYASHI, A. MISAKI, AND T. HARADA, *Biochim. Biophys. Acta,* 212 (1970) 458–469.
23 R. SUMMER AND D. FRENCH, *J. Biol. Chem.,* 222 (1956) 469–477.
24 K. BOCK AND C. PEDERSEN, *J. Chem. Soc., Perkin Trans. 2,* (1974) 293–297.