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An enzymatically produced novel cyclomaltopentaose cyclized from amylose by an α-(1→6)-linkage, *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}

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Abstract—A bacterial strain AM7, isolated from soil and identified as *Bacillus circulans*, produced two kinds of novel cyclic oligosaccharides. The cyclic oligosaccharides were produced from amylose using a culture supernatant of the strain as the enzyme preparation. The major product was a cyclomaltopentaose cyclized by an α-(1→6)-linkage, *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}. The other minor product was cyclomaltohexaose cyclized by an α-(1→6)-linkage, *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}. We propose the names isocyclomaltopentaose (ICG5) and isocyclomaltohexaose (ICG6) for these novel cyclic maltooligosaccharides having one α-(1→6)-linkage. ICG5 was digested by α-amylase derived from *Aspergillus oryzae*, cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus stearothermophilus*, and maltogenic α-amylase. On the other hand, ICG6 was digested by CGTase from *B. stearothermophilus* and *B. circulans*, and maltogenic α-amylase. This is the first report of enzymatically produced cyclomaltopentaose and cyclomaltohexaose, which have an α-(1→6)-linkage in their molecules.

Keywords: Cyclomaltopentaose; Cyclomaltohexaose; Cyclodextrin; α-(1→6)-Linkage; *Bacillus circulans*

1. Introduction

It is well known that bacterial enzymes produce cyclic glucans. Cyclomaltooligosaccharides (cyclodextrins, CDs, cyclic α-(1→4)-glucans), consisting of six glucose units or more, are produced from starch by (1→4)-α-D-glucan 4-α-(1→4)-α-D-glucanotransferase (cyclomaltodextrin glucanotransferase, EC 2.4.1.19).[1] Cyclodextran (cyclic α-(1→6)-glucan, seven to nine glucose units) is produced from dextran by an extracellular enzyme, called cycloisomaltooligosaccharide glucanotransferase, from *Bacillus circulans* T-3040.[2] All of these cyclic glucans have homogeneous linkages in their structure. Côté and co-workers first reported that a cyclic tetrasaccharide consisting of α-D-glucose, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}, was produced from a dextran-like polysaccharide, alternan, by its degrading enzyme.[3–5] This cyclic tetrasaccharide was called a cycloalternan (CA). Recently, we discovered two novel enzymes, 6-α-glucosyltransferase and 3-α-isomaltosyltransferase, in *Bacillus globisporus*, and succeeded in mass production of CA from starch by a joint reaction of both enzymes.[6–8] More recently, we found a new enzymatic system to synthesize a cyclic maltosyl-(1→6)-maltose (CMM), *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}, from starch.[9,10] We consequentially obtained the two kinds of cyclic tetrasaccharide by discovering CMM. To distinguish the two kinds of cyclic tetrasaccharide, in this paper we designate a cyclic nigerosyl-(1→6)-nigerose (CNN) for CA. CNN and CMM are smaller than the other cyclic glucans and have

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heterogeneous linkages in their structure. Although the characteristics of CNN or CMM are not yet clear in detail, potential applications of the saccharides for food, cosmetics, and medicines are anticipated because of their unique structure. Thus, cyclic oligosaccharides produced from starch by bacterial enzymes are of great interest from the functional and industrial points of view.

In the course of our search for soil bacteria producing novel nonreducing oligosaccharides from amylose, we obtained a bacterial strain AM7 that produced novel cyclic oligosaccharides. In this paper, we report the isolation and the structures of novel cyclic oligosaccharides that are enzymatically produced from amylose.

2. Results

2.1. Screening of bacterial strains producing nonreducing oligosaccharides

About 1000 bacterial strains from soil were tested for production of nonreducing oligosaccharides from amylose. After α-glucosidase digestion of the reaction mixtures, the samples were treated with alkali to degrade reducing saccharides and were analyzed by thin-layer chromatography (TLC). As shown in Figure 1, a strain AM7 produced an unknown oligosaccharide whose R_f value was 0.51 (Lane 1). This saccharide remained after α-glucosidase digestion (Lane 2) and alkaline treatment (Lane 3). The R_f value of this saccharide was different from those of other nonreducing oligosaccharides, such as trehalose, neotrehalose, cyclomaltooligosaccharides, CNN, and CMM. These results indicated that this saccharide should be a novel nonreducing oligosaccharide. The strain AM7 was isolated from a soil sample in Okayama, Japan. Morphological, cultural, and physiological characterizations classified the strain into *B. circulans*, according to *Bergey's Manual of Systematic Bacteriology*.[11]

2.2. High-performance liquid chromatography (HPLC) analysis of the reaction mixture containing amylose and the culture supernatant from *B. circulans* AM7

To examine the novel nonreducing oligosaccharide produced from amylose in detail, the reaction mixture containing amylose and the culture supernatant from *B. circulans* AM7 was analyzed by HPLC. After α-glucosidase digestion of the reaction mixture, two peaks other than glucose were mainly detected around retention times of 44 min (saccharide **1**) and 37 min (saccharide **2**) (Fig. 2). The analytical yields of saccharides **1**, **2**, and glucose were 23.9%, 3.7%, and 71.8%, respectively. In the LC–MS analysis, the adduct ions with sodium ion $[M+Na]^+$ at m/z 833 (saccharide **1**) and 995 (saccharide **2**) were monitored in the positive-ion electrospray ionization mode. These results show that saccharides **1** and **2** are novel cyclic oligosaccharides having a degree of polymerization of 5 and 6.

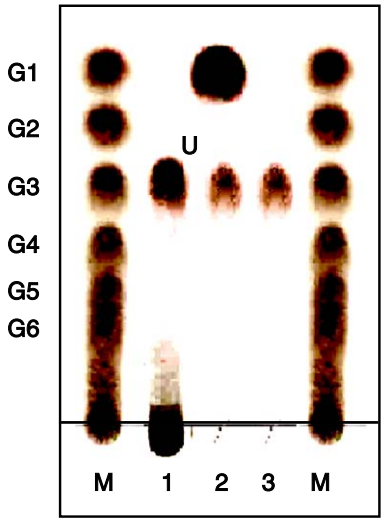


Figure 1. TLC analysis of the reaction products by *B. circulans* AM7. A reaction mixture containing 400 μL of 1.5% amylose EX-I in 100 mM acetate buffer (pH 6.0) and 200 μL of the culture broth from *B. circulans* AM7 was incubated at 40 °C for 72 h. Samples were spotted onto a TLC plate, developed twice, and then detected by spraying with 20% sulfuric acid in MeOH. Lane M, maltooligosaccharides standard solution (G1, glucose; G2, maltose, and so on); Lane 1, after the reaction; Lane 2, after α-glucosidase digestion; Lane 3, after alkaline treatment; U, unknown oligosaccharide spot.

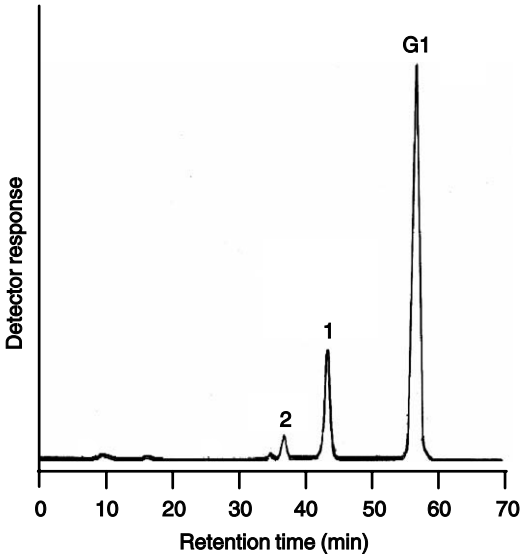


Figure 2. HPLC profile of the reaction mixture after α-glucosidase digestion. A reaction mixture containing 400 μL of 1.5% amylose EX-I in 100 mM acetate buffer (pH 6.0) and 200 μL of the culture broth from *B. circulans* AM7 was incubated at 40 °C for 72 h. After α-glucosidase digestion, the sample was analyzed by HPLC using an MCI GEL CK04SS column: (1) saccharide **1**; (2) saccharide **2**; (G1) glucose.

2.3. Preparation and isolation of saccharides 1 and 2

The culture supernatant of *B. circulans* AM7 was used as an enzyme preparation and reacted with 5.0 g of amylose. After α-glucosidase digestion of the reaction mixture, saccharides **1** and **2** reached 23.7% and 3.8% of the total sugar content. Saccharides **1** and **2** were isolated from this mixture using preparative reversed-phase column chromatography. Finally, we obtained the purified saccharides **1** (yield, 855 mg; purity, 97.6%) and **2** (106 mg, 100%).

2.4. Characterization of saccharide 1

The molecular weight of saccharide **1** was found to be 810 by measuring the $[M+Na]^+$ ion (m/z 833) by ESIMS. The molecular mass was determined to be $M = 162 \times n$ ($n = 5$), indicating that the pentasaccharide contained a cyclic structure. Methylation analysis gave 1 mol of 2,3,4-tri-*O*-methyl per-O-acetylated glucitol and 4 mol of 2,3,6-tri-*O*-methyl per-O-acetylated glucitol, as shown in Table 1. To confirm this structure, NMR spectroscopy measurements were performed. The ^{13}C NMR spectrum of saccharide **1** contained 27 signals for 30 carbon molecules (Fig. 3a). As listed in Table 2, all carbon resonances in the spectrum were assigned using ^1H–^1H COSY, ^1H–^{13}C COSY, distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple bond correlation (HMBC) measurements. Five signals of equal intensity in the C-1 anomeric region were detected, but no NMR peaks attributable to a reducing-end C-1 were detected. A large downfield shift (about 8–9 ppm) of the C-6 signal (residue V in Fig. 4a) was observed in the spectrum. In addition, ^1H–^{13}C COSY revealed the α-configurations of all glucose residues in saccharide **1** that were confirmed by the C-1 signals at 99.4 ppm {δ^1H, 4.87 ppm (d, $J_{1,2}$ 3.3 Hz)}, 103.4 ppm {δ^1H, 5.07 ppm (d, $J_{1,2}$ 3.5 Hz)}, 100.5 or 102.4 or 103.7 ppm {δ^1H, 4.98 or 4.99 or 5.00 ppm (d, $J_{1,2}$ 2.4 or 2.9 or 3.9 Hz)}. These results showed that the structure of saccharide **1** is *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→} (Fig. 4a). Thus, we determined the structure of saccharide **1**. Saccharide **1**, a novel cyclomaltopentaose, has one α-(1→6)-linkage in its molecule different from a CD. Saccharide **1** was then tentatively named isocyclomaltopentaose (ICG5).

Table 1. Methylation analysis of saccharides **1** and **2**

Methylation products	Ratio[a]	
	1	**2**
2,3,4-Tri-*O*-methyl 1,5,6-*O*-acetylglucitol	1.0	1.0
2,3,6-Tri-*O*-methyl 1,4,5-*O*-acetylglucitol	3.9	4.9

Data are normalized relative to the 2,3,4-tri-*O*-methyl derivative.
[a] Relative molar ratios of methylated alditol acetates.

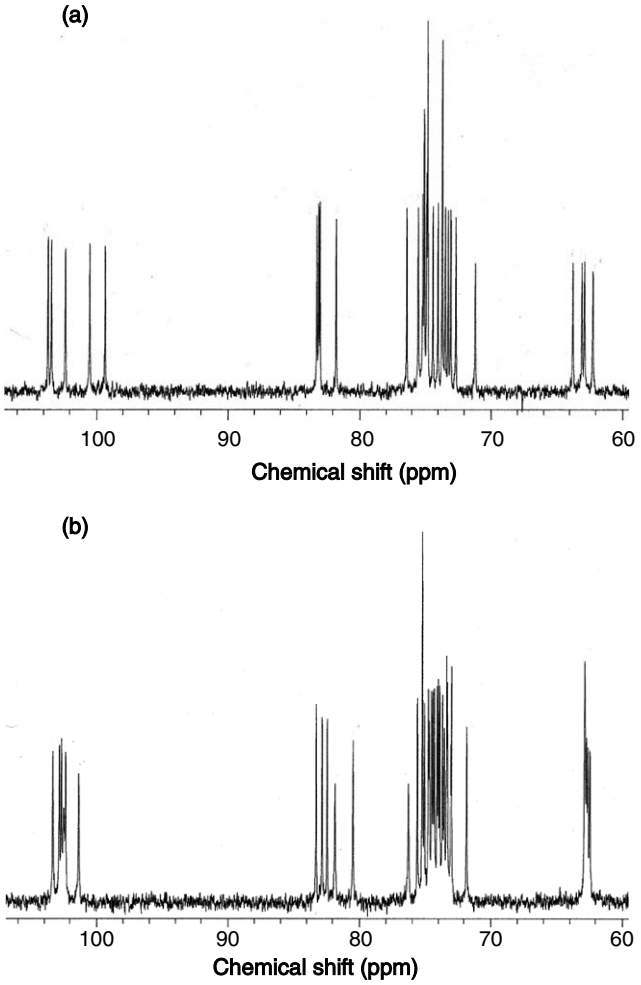


Figure 3. ^{13}C NMR spectra of saccharides **1** and **2**: (a) saccharide **1**; (b) saccharide **2**.

The R_f value of this saccharide was 0.51. The value for optical rotation of ICG5 was +71.9° at 20 °C. The reducing power of ICG5 was negligible; therefore, ICG5 caused no Maillard reaction with glycine or peptone.

2.5. Characterization of saccharide 2

The mass spectrum of saccharide **2** showed its molecular weight to be 972 by measuring the $[M+Na]^+$ ion (m/z 995). The molecular mass was determined to be $M = 162 \times n$ ($n = 6$), indicating that the hexasaccharide contained a cyclic structure. Methylation analysis gave 1 mol of 2,3,4-tri-*O*-methyl per-O-acetylated glucitol and 5 mol of 2,3,6-tri-*O*-methyl per-O-acetylated glucitol, as shown in Table 1. To confirm this structure, NMR spectroscopy measurements were performed. The ^{13}C NMR spectrum of saccharide **1** contained 34 signals for 36 carbon molecules (Fig. 3b). As listed in Table 2, all carbon resonances in the spectrum were assigned using ^1H–^1H COSY, ^1H–^{13}C COSY, DEPT,

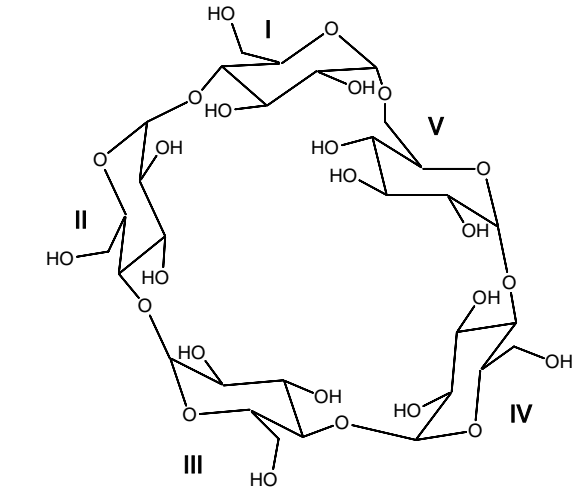
Table 2. ^{13}C NMR chemical shift data for saccharides **1** and **2**[a]

Residue[b]	Carbon atom	Saccharide **1**	Saccharide **2**
I	C-1	99.4	101.4
	C-2	72.7[e]	73.0[f]
	C-3	75.5	75.7
	C-4	81.8	81.9
	C-5	73.7	73.4
	C-6	62.3	62.5
II[c,d]	C-1	100.5	102.6
	C-2	74.8[e]	74.6[f]
	C-3	74.9	74.8
	C-4	83.0	82.5
	C-5	73.7	71.9
	C-6	62.9	62.6
III[c,d]	C-1	103.7	103.4
	C-2	73.5[e]	74.1[f]
	C-3	75.1	75.1
	C-4	83.3	83.3
	C-5	73.1	73.7
	C-6	63.1	62.8
IV[c,d]	C-1	102.4	102.7
	C-2	73.3[e]	73.6[f]
	C-3	75.1	75.3
	C-4	83.1	82.9
	C-5	74.4	74.2
	C-6	63.8	62.9
V[d]	C-1	103.4	102.9
	C-2	74.0[e]	73.4[f]
	C-3	75.2	75.3
	C-4	76.5	80.5
	C-5	74.8	74.0
	C-6	71.2	62.9
VI	C-1		102.4
	C-2		74.5[f]
	C-3		74.8
	C-4		76.3
	C-5		74.4
	C-6		73.1

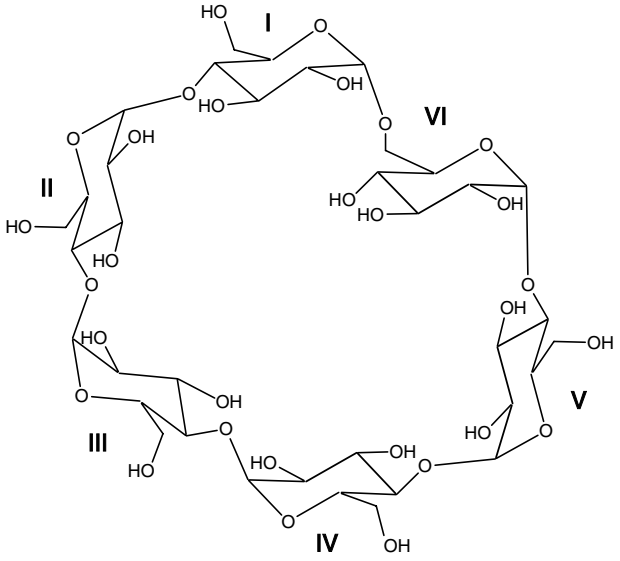
[a] NMR spectra data were recorded for solutions in D_2O at 27 °C. The chemical shifts were expressed in parts per million downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS), which was used as an internal standard.

[b] Roman numerals indicate the positions of the hexose residues in the saccharides **1** and **2** as shown in Figure 4.

[c,d,e,f] Possibly interchangeable.



(a) Saccharide **1**



(b) Saccharide **2**

Figure 4. Structure of saccharides **1** and **2**. The drawing is intended to show linkage information only, and no particular conformation is implied: (a) saccharide **1**; (b) saccharide **2**.

and HMBC measurements. Six signals in the C-1 anomeric region were detected, but no NMR peaks attributable to a reducing-end C-1 were detected. A large downfield shift (about 9–10 ppm) of the C-6 signal (residue VI in Fig. 4b) was observed in the spectrum. In addition, ^{1}H–^{13}C COSY revealed the α-configurations of all glucose residues in saccharide **2** that were confirmed by the C-1 signals at 101.4 ppm {$\delta^{1}H$, 4.81 ppm (d, $J_{1,2}$ 3.9 Hz)}, 102.4 ppm {$\delta^{1}H$, 5.15 ppm (d, $J_{1,2}$ 3.5 Hz)}, 102.6 or 102.7 or 102.9 or 103.4 ppm {$\delta^{1}H$, 4.91 or 4.92 or 4.93 or 4.95 ppm (d, $J_{1,2}$ 2.6 or 3.1 or 3.9 or 4.0 Hz)}. These results showed that the structure of saccharide **2** is *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→} (Fig. 4b). Saccharide **2**, a novel cyclomaltohexaose, has one α-(1→6)-linkage in its molecule different from α-CD. Saccharide **2** was then tentatively named an isocyclomaltohexaose (ICG6). The R_f value of this saccharide was 0.51, identical with that of ICG5.

2.6. Digestibility of ICG5 and ICG6 by various enzymes

We investigated the digestibility of ICG5 and ICG6 by various enzymes. ICG5 was digested by α-amylase derived from *Aspergillus oryzae*, cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus stearothermophilus*, and maltogenic α-amylase. On the other hand, ICG6

Table 3. Digestibility of ICG5 and ICG6 by various enzymes[a]

Enzyme	Origin	Digestibility	
		ICG5	ICG6
α-Glucosidase	*Aspergillus niger*	—	—
Glucoamylase	*Rhizopus* sp.	—	—
β-Amylase	Soybean	—	—
α-Amylase	*Bacillus subtilis*	—	—
	Aspergillus oryzae	+	—
G4-Forming α-amylase	*Pseudomonas stutzeri*	—	—
Maltogenic α-amylase	*Bacillus stearothermophilus*	+	+
Isoamylase	*Pseudomonas amylodelamosa*	—	—
Pullulanase	*Klebsiella pneumoniae*	—	—
Isomalto-dextranase	*Arthrobacter globiformis*	—	—
CGTase	*Bacillus stearothermophilus*	+	+
	Bacillus circulans	—	+

Reaction conditions: α-glucosidase (1000 U/g-DS, pH 5.0, 50 °C), glucoamylase (1000 U/g-DS, pH 5.0, 50 °C), β-amylase (1000 U/g-DS, pH 5.5, 50 °C), α-amylase from *B. subtilis* (1000 U/g-DS, pH 5.5, 50 °C), α-amylase from *A. oryzae* (1000 U/g-DS, pH 6.0, 40 °C), G4-forming α-amylase (100 U/g-DS, pH 6.0, 40 °C), maltogenic α-amylase (1000 U/g-DS, pH 5.0, 60 °C), isoamylase (10,000 U/g-DS, pH 4.0, 40 °C), pullulanase (1000 U/g-DS, pH 6.0, 30 °C), isomalto-dextranase (500 U/g-DS, pH 5.0, 50 °C), CGTase from *B. stearothermophilus* (1000 U/g-DS, pH 5.5, 50 °C), CGTase from *B. circulans* (100 U/g-DS, pH 6.0, 40 °C).
Other reaction conditions are described in Section 4.6.
[a] +: ICG5 or ICG6 spot disappears completely. —: No reaction.

was digested by CGTase from *B. stearothermophilus* and *B. circulans*, and maltogenic α-amylase. These results are summarized in Table 3.

3. Discussion

Recently, we have reported that a cyclic pentasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}, was produced from starch by two glycosyltransferases.[12] However, there was a problem in that the yield of this saccharide was very low. In this paper, we have found another cyclic pentasaccharide that has a high yield. Structural analyses revealed that the cyclic pentasaccharide is a novel cyclomaltopentaose cyclized by an α-(1→6)-linkage, *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}. Furthermore, we have also found a novel cyclomaltohexaose cyclized by an α-(1→6)-linkage, *cyclo*-{→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}, from the same reaction mixture. We propose the names isocyclomaltopentaose (ICG5) and isocyclomaltohexaose (ICG6) for these novel cyclic maltooligosaccharides having one α-(1→6)-linkage.

The R_f value of ICG5 was 0.51, identical with that of ICG6. The spot that had been detected as an unknown saccharide in the course of screening was thought to be a mixture of ICG5 and ICG6. The crystalline structures of ICG5 and ICG6 are not clarified yet; therefore, we examined the three-dimensional structure by computer-modeling studies using MOPAC and AM1.[13] ICG5 has a deep depression in the center of its molecule. The inner diameter of the depression is ~5 Å, and the depth is also ~5 Å. From this result, ICG5 should be able to hold a low-molecular-weight compound in its cavity. On the other hand, ICG6 has a hole that completely penetrates in the center of its molecule. When the molecular size of ICG6 was compared with that of α-CD,[14] both were considerably similar. ICG6 should be able to subsume various guest molecules.

Among the numerous enzymes tested, maltogenic α-amylase hydrolyzed both ICG5 and ICG6. Alpha-amylase derived from *Bacillus subtilis* did not hydrolyze either ICG5 or ICG6, but *Aspergillus oryzae* α-amylase hydrolyzed only ICG5. On the other hand, CGTase from *B. stearothermophilus* digested both ICG5 and ICG6, but *B. circulans* CGTase only digested ICG6. Interestingly, the digestibility of ICG5 and ICG6 was different according to the difference in the origin of the enzyme. The *B. stearothermophilus* enzyme produces equal amounts of α-CD and β-CD.[15] The *B. circulans* enzyme produces mainly β-CD.[15] The difference in such substrate specificity will be related to the digestibility. By using the difference in the digestibility of ICG5 and ICG6, we will be able to obtain only ICG5 or ICG6.

The characteristics of ICG5 and ICG6 are not yet clear in detail because these cyclic oligosaccharides have just been discovered as described in this paper. However, potential applications of the saccharides for food, cosmetics, and pharmaceuticals are anticipated due to their unique structures.

We have already purified one enzyme from *B. circulans* AM7 involved in the formation of both ICG5 and ICG6 from amylose. The characterization of the enzyme will be described in the near future.

4. Experimental

4.1. Saccharides

Partially hydrolyzed starch, Pinedex no. 4 (dextrose equivalent 19 ± 2), was purchased from Matsutani Chemical Industry (Hyogo, Japan). Amylose EX-I whose average degree of polymerization (DP) is 17 was prepared in our laboratory.

4.2. Enzymes

α-Glucosidase (EC 3.2.1.20) from *Aspergillus niger* was purchased from Amano Enzyme, Inc. (Aichi, Japan). Glucoamylase (EC 3.2.1.3) from *Rhizopus* sp. was

obtained from Seikagaku Corp. (Tokyo, Japan). α-Amylase (EC 3.2.1.1) from *B. subtilis* was purchased from Nagase ChemteX Corporation. (Hyogo, Japan). α-Amylase from *Aspergillus oryzae* was purchased from Shin Nihon Chemical Co. Ltd (Aichi, Japan). β-Amylase (EC 3.2.1.2) from soybeans was obtained from Showa Sangyo Co. Ltd (Tokyo, Japan). Maltogenic α-amylase (EC 3.2.1.133) from *B. stearothermophilus* was obtained from Novozymes Japan (Chiba, Japan). Isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), CGTase (EC 2.4.1.19) from *B. stearothermophilus* and *B. circulans*, and maltotetraose-forming α-amylase (EC 3.2.1.60) from *Pseudomonas stutzeri* were prepared in our laboratories. Isomalto-dextranase (EC 3.2.1.94) from *Arthrobacter globiformis* T6 was purified and assayed according to the method of Okada et al.[16] Glucoamylase, isoamylase, and pullulanase were of analytical grade. Other enzymes were of commercial grade. The activities of α-glucosidase, glucoamylase, α-amylase, β-amylase, and maltogenic α-amylase were assayed according to the methods recommended by their respective suppliers. The activities of isoamylase, pullulanase, CGTase, and maltotetraose-forming α-amylase were assayed as reported in previous publications.[17–20]

4.3. Screening of bacteria

Bacterial strains isolated from soil were incubated with reciprocal shaking at 27 °C for 5 days in a liquid medium containing 1.5% Pinedex no. 4, 0.5% polypeptone, 0.1% yeast extract, 0.1% K_2HPO_4, 0.06% $NaH_2PO_4 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% $CaCO_3$ (pH 6.8). Reaction mixtures containing 200 μL of the culture broth and 400 μL of 1.5% (w/v) amylose in 100 mM acetate buffer (pH 6.0) were incubated at 40 °C for 72 h and then boiled for 10 min to stop the reaction. α-Glucosidase (3.0 U) was added into the reaction mixture and incubated at 50 °C for 24 h. After alkaline treatment (pH 12) at 100 °C for 1 h, the nonreducing sugars in the reaction mixture were detected by TLC.

4.4. Preparation of condensed culture supernatant of strain AM7

B. circulans AM7 was incubated at 27 °C with reciprocal shaking for 5 days in the same medium described in Section 4.3. The bacterial cells were separated from the culture medium by centrifugation at 10,000*g* for 20 min. $(NH_4)_2SO_4$ was added, with stirring at 4 °C, to 300 mL of the culture supernatant to give 80% saturation. After the mixture was left overnight at 4 °C, it was centrifuged at 10,000*g* for 20 min. The precipitate was recovered, dissolved in 30 mL of 10 mM acetate buffer (pH 6.0), and dialyzed against the same buffer.

4.5. Isolation of saccharides 1 and 2

A reaction mixture (500 mL) containing 5.0 g of amylose, 25 mL of the condensed culture supernatant of strain AM7, and 0.5 mL of 1 M $CaCl_2$ in 10 mM acetate buffer (pH 6.0) was incubated at 40 °C for 72 h. After the reaction was stopped by boiling for 10 min, α-glucosidase (2500 U) was added to the reaction mixture and incubated at 50 °C for 24 h. The mixture was desalted by passing it through ion-exchange resins: 15 mL of Diaion SK1B (Mitsubishi Chemical Co., Tokyo, Japan), 30 mL of Diaion WA30 (Mitsubishi Chemical), and 15 mL of Amberlite IRA-411S (Organo Co., Tokyo, Japan). To purify saccharides **1** and **2** from the saccharide mixture, preparative ODS column chromatography on a YMC-pack ODS-AQ R-355-15-AQ column (50 mm i.d. × 500 mm: YMC Co., Ltd, Kyoto, Japan) was performed, eluting with 7.5% (v/v) MeOH as a solvent at a flow rate of 20 mL/min at 25 °C. The fractions containing saccharides **1** and **2** were separately collected and then evaporated. After 5 cycles of chromatography while injecting 5 mL (1.0 g-DS) of samples per cycle, two fractions, saccharides **1** and **2**, respectively, were obtained.

4.6. Digestibility of ICG5 and ICG6 by various enzymes

A reaction mixture (40 μL) containing 0.4 mg of ICG5 or ICG6 and 20 μL of each enzyme solution was incubated for 24 h. The other reaction conditions are summarized in Table 3. Digestibility of ICG5 and ICG6 was detected by TLC.

4.7. HPLC and TLC analyses

The amounts of neutral saccharides in samples were determined by HPLC. Samples were first treated by filtration using a filter kit, KC prep dura (0.45 μm, Katayama Chemical Co., Osaka, Japan) and by deionization using a microacilyzer G0 (Asahi Chemical Co., Tokyo, Japan). HPLC analysis was performed with an LC-10AD pump, an RID-10A refractive index monitor, and a C-R7A data processor (Shimadzu Corporation, Kyoto, Japan) equipped with an MCI GEL CK04SS column (10 mm i.d. × 200 mm × 2; Mitsubishi Chem. Co., Tokyo, Japan) at a flow rate of 0.4 mL/min, using water as a solvent at 80 °C. TLC was performed on a Kieselgel 60 plate (E. Merck, Darmstadt, Germany) developed twice with a solvent of 6:4:1 BuOH–pyridine–water. Sugar spots were detected by spraying with 20% sulfuric acid in MeOH, followed by heating the plates at 110 °C for 10 min.

4.8. Methylation analysis

A sample (2 mg) was methylated according to the method of Ciucanu and Kerek,[21] and the products were isolated by partitioning between $CHCl_3$ and water. The methylated sample was then hydrolyzed in acid, reduced with $NaBH_4$, and acetylated as described by Hakomori.[22] The resulting partially methylated alditol acetates were separated by gas chromatography (GC-14B; Shimadzu Co., Kyoto, Japan) on a silica gel capillary column (DB-5; 0.25 mm × 30 m; J&W Scientific Co., Folsom, CA, USA), with a temperature program of 130 °C for 2 min, followed by 5 °C/min to 250 °C.

4.9. LC–MS analysis

The molecular masses of the products were determined by LC–MS. The LC–MS was carried out using the same HPLC system as above, interfaced to an LCQ advantage ion-trap mass analyzer (Thermo Electron Corporation, Kanagawa, Japan) fitted with an electrospray-ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set at 5 kV, and the capillary temperature was 350 °C.

4.10. NMR measurements

NMR spectral data were recorded for 5% (w/v) solutions in D_2O at 27 °C with a JNM-AL300 spectrometer (^1H 300.4 MHz, ^{13}C 75.45 MHz: JEOL, Tokyo, Japan). The chemical shifts were expressed in parts per million downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS), which was used as an internal standard.

4.11. Computer modeling

The MOPAC2000 program was used for the semiempirical level of molecular orbital calculations.[13] Molecular structures were displayed using the Mercury graphic software. In the semiempirical calculation, the AM1 hamiltonian was used.[13]

4.12. Other analytical methods

Total sugar content was measured by the anthrone–sulfuric acid method, and the reducing sugar was measured by the Somogyi–Nelson method.[23,24] The reducing power was calculated as follows: (the amount of reducing sugar as glucose)/(the amount of total sugar as glucose) × 100%. Optical rotation was determined at 20 °C on a sample dissolved in distilled water (Horiba model SEPA-300; Horiba Ltd, Kyoto, Japan).

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