Arthrobacter globiformis M6における環状マルトシルマルトースの合成および分解に関与する酵素

誌名	Journal of applied glycoscience
ISSN	13447882
著者名	森,哲也 西本,友之 向井,和久 渡邊,光 大倉,隆則 茶圓,博人 福田,恵温
発行元	日本応用糖質科学会
巻/号	56巻2号
掲載ページ	p. 127-136
発行年月	2009年4月

J. Appl. Glycosci., **56**, 127–136 (2009)

Proceedings of the Symposium on Amylases and Related Enzymes, 2008

Enzymes Involved in the Biosynthesis and Degradation of Cyclic Maltosyl-maltose in *Arthrobacter globiformis* M6

(Received December 22, 2008; Accepted January 6, 2009)

Tetsuya Mori,[1,*] Tomoyuki Nishimoto,[1] Kazuhisa Mukai,[1] Hikaru Watanabe,[1] Takanori Okura,[1] Hiroto Chaen[1] and Shigeharu Fukuda[1]

[1]*Glycoscience Institute, Research Center, Hayashibara Biochemical Laboratories, Inc. (675-1, Fujisaki, Okayama 702-8006, Japan)*

Abstract: A bacterial strain M6, isolated from soil and identified as *Arthrobacter globiformis*, produced a novel nonreducing oligosaccharide from starch. This oligosaccharide had a cyclic structure consisting of four glucose residues joined by alternate α-1,4 and α-1,6 linkages. The cyclic tetrasaccharide, *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→}, was designated cyclic maltosyl-maltose (CMM). CMM was not hydrolyzed by various amylases, such as α-amylase, β-amylase, glucoamylase, isoamylase, pullulanase, maltogenic α-amylase and α-glucosidase, but hydrolyzed by isomalto-dextranase to give rise to isomaltose. A glycosyltransferase involved in the synthesis of CMM from starch was purified to homogeneity from the culture supernatant of *A. globiformis* M6. The enzyme acted on maltooligosaccharides that have degrees of polymerization more than 3, amylose, and soluble starch to produce CMM but failed to act on cyclomaltodextrins, pullulan and dextran. The CMM-forming enzyme catalyzed both intermolecular and intramolecular α-1,6-maltosyl transfer reaction and found to be a novel maltosyltransferase (6MT). To reveal the degradation pathway of CMM, we identified two enzymes, CMM hydrolase (CMMase) and α-glucosidase, as the responsible enzymes from the cell-free extract of the strain. CMMase hydrolyzed CMM to maltose via maltosyl-maltose as intermediates; however, it did not hydrolyze CMM to glucose, suggesting that it is a novel hydrolase that hydrolyzes the α-1,6-linkage of CMM. α-Glucosidase degraded maltosyl-maltose to glucose *via* panose and maltose as intermediates; however, it did not degrade CMM. Furthermore, when CMMase and α-glucosidase existed simultaneously in the reaction mixture containing CMM, glucose was detected as the final product. It was found that CMM was degraded to glucose by synergistic action of CMMase and α-glucosidase. The genes for 6MT, CMMase and α-glucosidase were cloned from the genomic library of *A. globiformis* M6. The four conserved regions common in the α-amylase family enzymes were also found in 6MT, CMMase and α-glucosidase, indicating that these enzymes should be assigned to this family. In the cloning experiments, three other open reading frames (ORFs) were found. These ORFs were expected to encode proteins concerned with incorporation of CMM *via* cell membrane. The genes for CMMase and α-glucosidase and three ORFs were located downstream of the gene for 6MT, and expected to form gene cluster. The results of gene analysis suggested that *A. globiformis* M6 has a unique starch utilization pathway *via* CMM.

Key words: cyclic maltosyl-maltose, *Arthrobacter globiformis*, maltosyltransferase, CMM hydrolase, α-glucosidase

Enzymatic syntheses of various nonreducing oligosaccharides from starch have been reported by many researchers. Nonreducing oligosaccharides are generally divided into two groups: linear and cyclic oligosaccharides. Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a typical linear nonreducing oligosaccharide occurring in bacteria, yeasts, fungi, plants and invertebrates. Mass production of trehalose from starch has been developed using two bacterial enzymes, maltooligosyltrehalose synthase (EC 5.4.99.15) and maltooligosyltrehalose trehalohydrolase (EC 3.2.1.141),[1-3] and now this saccharide is used in the food, cosmetics and the pharmaceutical industries. Cyclomaltohexaose (α-cyclodextrin), one of the most well-known cyclic oligosaccharides, is produced from linear α-1,4-glucans by the intramolecular α-1,4-transglycosylation reaction of a cyclomaltodextrin glucanotransferase (EC 2.4.1.19).[4] The cyclic oligosaccharide has a hydrophobic cavity in the center of the structure. Guest molecules of suitable size can enter the cavity, and the formation of the inclusion complex is used for stabilizing labile materials,[5] masking odors[6] and modifying viscosity.[7] Côté and coworkers 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.[8-10] The cyclic oligosaccharide was designated cycloalternan (CA). Recently, we discovered two novel enzymes, 6-α-glucosyltransferase and 3-α-isomaltosyltransferase, in *Bacillus globisporus*, and succeeded in the mass production of this saccharide from starch by the joint reaction of the two enzymes.[11-13] Although the characteristics of CA has not yet been totally clarified, potential applications of the saccharide in food, cosmetics and medicines are anticipated because of its unique structure. Hence nonreducing oligosaccharides pro-

* Corresponding author (Tel. +81-86-276-3142, Fax. +81-86-276-8670, E-mail: Tmori@hayashibara.co.jp).

duced from starch by bacterial enzymes are of great interest from the functional and industrial points of view.

During the course of our screening for microorganisms that produce nonreducing oligosaccharides from starch, we obtained the bacterial strain *Arthrobacter globiformis* M6 from soil, which produces a novel tetrasaccharide designated cyclic maltosyl-(1→6)-maltose (CMM). In this review, we first describe the isolation and structure of CMM. Next, we report the purification and characterization of CMM-forming enzyme, CMM hydrolase and α-glucosidase. Finally, we describe the gene analysis of these enzymes and four open reading frames (ORFs).

Screening of bacterial strains producing nonreducing oligosaccharides.

About 1500 bacterial strain from soil were tested for production of nonreducing oligosaccharides from soluble starch. After glucoamylase digestion of the reaction mixtures, samples were treated with alkali to degrade reducing saccharides, and analyzed by thin-layer chromatography (TLC). As shown in Fig. 1, a strain M6 produced two kinds of oligosaccharides (saccharides 1 and 2), whose *Rf* values were 0.32 and 0.15, respectively, in addition to glucose and maltose (lane 1). Saccharide 1 remained after alkaline treatment (lane 3) and showed a different *Rf* value from those of other nonreducing oligosaccharides, such as trehalose, cyclomaltodextrins and CA. These results indicated that saccharide 1 produced by the strain M6 might be a novel nonreducing oligosaccharide. Morphological, cultural and physiological characterizations classified the strain into *Arthrobacter globiformis*, according to 'Bergey's Manual of Systematic Bacteriology'.[14]

Structure of saccharide 1.

APCI-MS of saccharide 1 showedan $[M+Na]^+$ ion peak with an m/z ratio of 671, for an apparent mass of 648. The value was consistent with that of maltotetraose anhydride, indicating that saccharide 1 consisted of four glucose residues linked circularly, as in CA. Methylation analysis gave equimolar ruantities of 2,3,4-tri-*O*-methyl 1,5,6-*O*-acetyl glucitol and 2,3,6-tri-*O*-methyl 1,4,5-*O*-acetyl glucitol (Table 1). Saccharide 1 was not hydrolyzed by α-amylase, β-amylase, glucoamylase, isoamylase, pullulanase, maltogenic α-amylase and α-glucosidase. However, treatment with isomalto-dextranase resulted in its complete conversion to isomaltose. From these results, we determined the structure of saccharide 1 is *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→} (Fig. 2). We designated this saccharide as cyclic maltosyl-maltose (CMM).

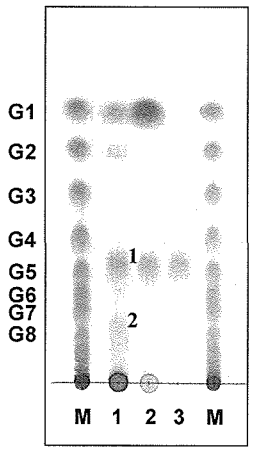

Fig. 1. TLC analysis of the reaction products by *A. globiformis* M6.

A reaction mixture containing 50 μL of 4% soluble starch in 50 mM acetate buffer (pH 6.0) and 50 μL of the culture broth from *A. globiformis* M6 was incubated at 40°C for 24 h. Samples were spotted onto a TLC plate, developed twice, and then detected by 20% sulfuric acid. Lane M, maltooligosaccharides standard solution (G1, glucose; G2, maltose and so on); lane 1, after the reaction; lane 2, after glucoamylase digestion; lane 3, after alkaline treatment.

Table 1. Methylation analysis of saccharide 1.

Methylation products	Ratio
2,3,4-Tri-*O*-methyl 1,5,6-*O*-acetyl glucitol	1.00
2,3,6-Tri-*O*-methyl 1,4,5-*O*-acetyl glucitol	0.97

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

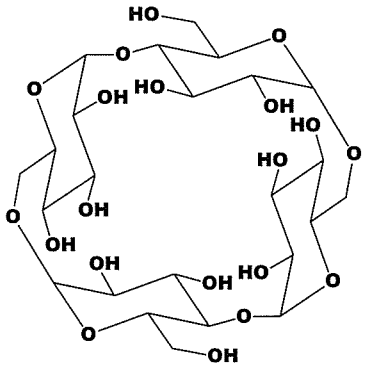

Fig. 2. Structure of saccharide 1.

The drawing is intended to show linkage information only, and no particular conformation is implied.

Purification of the CMM-forming enzyme.

The enzyme producing CMM from starch was purified from the culture supernatant of *A. globiformis* M6 by successive column chromatography on DEAE-TOYOPEARL 650S and Phenyl-TOYOPEARL 650M, following the ammonium sulfate precipitation. Native PAGE of the purified enzyme showed a single protein band. The enzyme was purified 79-fold in a yield of 40%.

Physical and enzymatic properties of the CMM-forming enzyme.

The molecular mass of the enzyme was estimated to be 71.7 kDa by SDS-PAGE. The p*I* of the enzyme was found to be 3.6 by gel IEF. The N-terminal sequence up to 30th residue was determined as follows: DPTTSPGPLAEGDVIYQVLVDRFEDGDPTN. The enzyme was the most active at pH 6.0 and was stable in a pH range of 5.0 to 9.0 when kept at 4°C for 24 h. The optimum temperature for the enzyme was 50°C. The enzyme was stable up to 30°C when heated at various temperatures for 60 min. The addition of 1 mM Ca^{2+} enhanced the thermal stability of the enzyme up to 45°C. At a concentration of 1 mM, Cu^{2+}, Hg^{2+}, Al^{3+}, Fe^{3+}, Pb^{2+} and EDTA inhibited the enzyme activity at a loss of 99, 98, 88, 68, 64 and 75%, respectively.

Substrate specificity and action on maltooligosaccharides.

As shown in Table 2, CMM was produced from maltooligosaccharides with DP3 or greater, amylose, soluble starch and glycogen. Maltotriose was a poor substrate compared to the other maltooligosaccharides. Amylose produced the highest yield (44%) of CMM. The enzyme did not act on cyclodextrins, pullulan and dextran. For a better understanding of the action of the CMM-forming enzyme on the substrates, the reaction products from maltotetraose were analyzed by HPLC with an ODS AQ-303 column, and the change with time of the products were monitored. The products from maltotetraose by the action of the enzyme were maltose, CMM, maltosyl-maltose and maltohexaose, together with unknown saccharides A and B (Fig. 3 (A)). As shown in Fig. 4, the amount of maltohexaose, saccharide A, and saccharide B increased during the initial stage of the reaction but decreased in late stage. The amount of saccharide A after a 4-h reaction was about three- to fourfold as much as those of maltohexaose and saccharide B. Therefore, we postulated that saccharide A was the main intermediate for CMM. Treatment of the reaction mixture with pullulanase resulted in hydrolysis of maltosyl-maltose, saccharide A, and saccharide B, with concomitant production of maltose (Fig. 3 (B)). Based on these results, it was strongly suggested that the CMM-forming enzyme mainly catalyzed the α-1,6-maltosyl transfer reaction and could be called 6-α-maltosyltransferase (6MT).

Identification of saccharide A.

The mass spectrum of saccharide A showed an $[M+Na]^+$ ion peak with an m/z ratio of 1013, indicating that the saccharide has a molecular mass of 990 and consist of six glucose residues. Methylation analysis yielded 1 mol of 2,3,4,6-tetra-*O*-metyl glucitol, 1 mol of 2,3,4-tri-*O*-metyl glucitol and 4 mol of 2,3,6-tri-*O*-metyl glucitol. Pullulanase and isomalto-dextranase hydrolyzed the saccharide into maltose and maltotetraose, and into isopanose

Table 2. Effect of substrate on CMM formation.

Substrate	CMM yield (%)
Maltose	ND
Maltotriose	2.4
Maltotetraose	28.6
Maltopentaose	24.7
Maltohexaose	41.6
Maltoheptaose	36.6
Amylose	44.0
Soluble starch	31.4
Glycogen	29.5
Cyclodextrins	ND
Pullulan	ND
Dextran	ND

Reaction mixtures (2 mL) containing 1% of each substrate, 25 mM acetate buffer (pH 6.0), 1 mM $CaCl_2$ and 0.02 U of enzyme were incubated at 40°C for 48 h. After glucoamylase treatment of the reaction mixture, the yield of CMM was measured by HPLC with a Shodex SUGAR KS-801 column. ND, not detected.

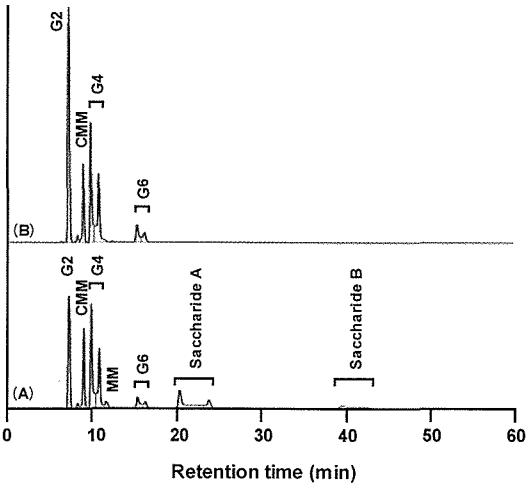

Fig. 3. HPLC profiles of the reaction of CMM-forming enzyme on maltotetraose.

The reaction mixture (2 mL) was the same as described in Table 2 using maltotetraose as a substrate. After incubation at 40°C for 4 h, the reaction was stopped by boiling for 10 min. Two units of pullulanase in 25 mM acetate buffer (pH 6.0) were then added to the reaction mixture and incubated at 40°C for 20 h. Samples before and after the pullulanase treatment were analyzed by HPLC using an ODS AQ-303 column. (A) before the pullulanase treatment, (B) after the pullulanase treatment. G2, maltose; G4, maltotetraose; G6, maltohexaose; CMM, cyclic maltosylmaltose; MM, maltosylmaltose.

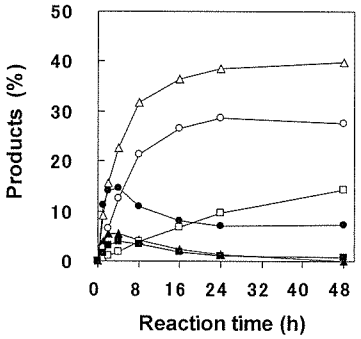

Fig. 4. Time course of the reaction products from maltotetraose.

Reaction mixture (10 mL) containing 1% maltotetraose, 25 mM acetate buffer (pH 6.0), 1 mM $CaCl_2$, and 0.1 U of enzyme was incubated at 40°C. Samples were collected at intervals, boiled for 10 min to stop the reaction, and analyzed by HPLC with an ODS AQ-303 column. △, maltose; ○, CMM; □, maltosylmaltose; ▲, maltohexaose; ●, saccharide A; ■, saccharide B.

(6-*O*-α-maltosyl-glucose) and maltotriose, respectively. Based on these results, saccharide A was concluded to be α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p* (6^4-*O*-α-maltosyl-maltotetraose).

The purified 6MT from *A. globiformis* M6 acted on 6^4-*O*-α-maltosyl-maltotetraose to produce nearly equimolar amounts of CMM and maltose. The yield of CMM from 6^4-*O*-α-maltosyl-maltotetraose reached about 40% of the total sugar. Thus, it was demonstrated that CMM was synthesized by 6MT from maltotetraose *via* 6^4-*O*-α-maltosyl-maltotetraose as the main intermediate.

The mechanism for the synthesis of CMM.

The mechanism for the synthesis of CMM from maltooligosaccharides was proposed as shown in Fig. 5. First, 6MT breaks down the α-1,4-glucosidic bond between the

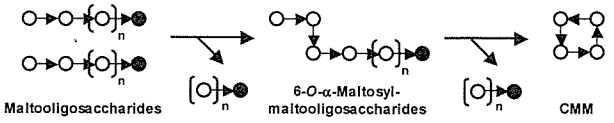

Fig. 5. Scheme for the formation of CMM from maltooligosaccharides by the action of 6MT.

Open circle, glucopyranosyl residue; closed circle, glucose residue at reducing end; horizontal arrow, α-1,4-linkage; vertical arrow, α-1,6-linkage. n=1, 2, 3···.

second and third residues from the non-reducing end of the maltooligosaccharide. The maltosyl part bound to the enzyme is then transferred to the 6-OH of the non-reducing end glucose of another molecule to produce 6-*O*-α-maltosyl-maltooligosaccharide. Second, 6MT cuts off the α-1,4-linkage between the fourth and fifth residues from the non-reducing end of the intermediate, and cyclizes it through the intramolecular α-1,6-transglycosylation to finally produce CMM. Thus, 6MT was found to be a novel glycosyltransferase catalyzing both the intermolecular and the intramolecular α-1,6-maltosyl transfer reactions. At the same time, maltooligosaccharides, with a DP decreased by two, were formed by the 6MT reactions. If the maltooligosaccharides have a DP3 or more, they could be substrates for the enzyme again. The formation of maltohexaose from maltotetraose indicates that 6MT also has a weak α-1,4-maltosyl transferring activity (Fig. 3 (A)).

Purification of CMM-degrading enzymes from cell-free extract of A. globiformis M6.

The cell-free extract of *A. globiformis* M6 was incubated with CMM in 20 mM Tris-HCl buffer (pH 7.0) and the products were analyzed by thin-layer chromatography (TLC). As shown in Fig. 6, most of the CMM was degraded to glucose. This result suggests that CMM-degrading activity exists in the cell-free extract of the strain. Therefore, we tried to purify the CMM-degrading

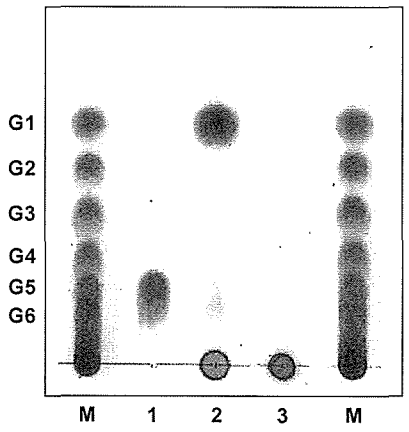

Fig. 6. CMM-degrading activity of the cell-free extract of *A. globiformis* M6.

The cell-free extract (50 μL) was added to the substrate solution (100 μL) containing 1.0% CMM and 20 mM Tris-HCl buffer (pH 7.0), and incubated at 35°C for 16 h. Samples were spotted on a TLC plate, developed, and then detected by spraying 20% sulfuric acid. Lane M, maltooligosaccharides standard solution (G1, glucose; G2, maltose and so on); lane 1, CMM standard; lane 2, after the reaction in 20 mM Tris-HCl buffer (pH 7.0); lane 3, cell-free extract of *A. globiformis* M6 without CMM.

enzyme from the cell-free extract. As the first step of purification, the proteins in the cell free extract were separated by a DEAE-TOYOPEARL 650S column. All the fractions were examined for their reactivity to maltooligosaccharides, soluble starch, or CMM. Fractions 35 and 36 exhibited degrading activity towards maltooligosaccharides, and especially towards maltose. These fractions also degraded *p*NPαG and liberated glucose. Therefore, we concluded that these fractions contained α-glucosidase. Conversely, CMMase activity was noted in fractions 72 and 73. These fractions produced maltosyl-maltose from CMM; however, they did not produce glucose. We concluded that these fractions contained CMMase. The other fractions did not act on the saccharides mentioned above. Therefore, we tried to purify α-glucosidase and CMMase independently from the indicated fractions through Butyl-TOYOPEARL 650M and DEAE-5PW column chromatographies successively. Native PAGE analysis of each purified enzyme showed a single protein band. The CMMase enzyme was purified 199-fold with a yield of 27.8% and a specific activity of 20.7 U/mg protein, whereas the α-glucosidase enzyme was purified 82.2-fold with a yield of 11.4% and a specific activity of 8.3 U/mg protein.

Physical and enzymatic properties of CMMase.

The molecular mass of CMMase was estimated as 48.6 kDa by SDS-PAGE and 136 kDa by gel filtration column chromatography. The N-terminal sequence up to the 20th residues was MTAPDWLADAVFYQIFPERF. The effects of pH and temperature on enzyme activity and stability were determined as follows: The enzyme had an optimal pH of 6.5 and was stable in the pH range of 5.5 to 8.0 when kept at 4°C for 24 h. The optimum temperature for the enzyme activity was 30°C. The enzyme was stable up to 25°C when heated at various temperatures for 60 min at pH 6.5. The enzyme activity was inhibited strongly by Cu^{2+}, Hg^{2+} and Pb^{2+} (residual activity, 0–17%). Mn^{2+} and Zn^{2+} moderately inhibited its activity (64–67%). Conversely, Al^{3+}, Fe^{2+} and Fe^{3+} slightly enhanced its activity (124–129%). Other metal ions and EDTA had almost no effect. As shown in Table 3, CMMase degraded CMM most efficiently (hydrolysis ratio, 99.4%) and produced maltosyl-maltose or maltose. Isopanose, maltosyl-maltose and pullulan were also good substrates for the enzyme (28.7–76.2%). Isopanose was degraded to glucose and maltose. Maltosyl-maltose and pullulan were degraded to maltose and maltotriose, respectively. These results indicate that CMMase hydrolyzes the α-1,6-linkage of the substrates. In our previous study, the reactivity of various enzymes for CMM was examined.[15] Among the enzymes, only isomalto-dextranase from *A. globiformis* T6[16,17] could hydrolyze CMM and produce isomaltose. Isomalto-dextranase is the enzyme that hydrolyzes the α-1,4-linkage of CMM. CMMase is obviously different from isomalto-dextranase as unlike isomalto-dextranase, CMMase hydrolyzed the α-1,6-linkage of CMM and produced maltosyl-maltose or maltose. As far as we know, CMMase is the only enzyme that can hydrolyze the α-1,6-linkage of CMM.

Table 3. Reactivity of the enzymes against various substrates.

Substrate	CMMase		α-Glucosidase	
	Hydrolysis ratio (%)	Products (molar ratio)	Hydrolysis ratio (%)	Products (molar ratio)
Methyl-α-glucoside	0	ND	0	ND
Methyl-β-glucoside	0	ND	0	ND
*p*NPαG	0	ND	8.6	G1
*p*NPβG	0	ND	0	ND
Maltose (G2)	0	ND	99.7	G1
Maltotriose (G3)	0	ND	42.1	G1
Maltotetraose (G4)	4.2	G2	14.4	G1 (1.00), G3 (0.27)
Maltopentaose (G5)	1.8	G2 (1.00), G3 (0.99)	12.6	G1 (1.00), G3 (0.09), G4 (0.78)
Maltohexaose (G6)	0	ND	3.4	G1 (1.00), G3 (0.03), G4 (0.14), G5 (0.82)
Maltoheptaose (G7)	0	ND	2.1	G1 (1.00), G3 (< 0.01), G4 (0.02), G5 (0.16), G6 (0.94)
Trehalose	0	ND	0	ND
Neotrehalose	0	ND	3.6	G1
Kojibiose	0	ND	4.2	G1
Nigerose	0	ND	7.9	G1
Isomaltose (IG2)	0	ND	0	ND
Isomaltotriose	0	ND	0	ND
Cellobiose	0	ND	0	ND
Sucrose	0	ND	42.1	G1 (1.00), Fructose (1.03)
Maltitol	0	ND	5.4	G1 (1.00), Sorbitol (0.97)
Panose	0	ND	98.3	G1
Isopanose	28.7	G1 (1.00), G2 (0.98)	24.6	G1 (1.00), IG2 (0.96)
CMM	99.4	G2 (1.00), MM (0.22)	0	ND
Maltosyl-maltose (MM)	76.2	G2	99.4	G1 (1.00), Panose (0.07)
Isocyclomaltopentaose	3.2	G5	0	ND
Cyclodexrtins	0	ND	0	ND
Amylose	0	ND	0	ND
Soluble starch	0	ND	0	ND
Pullulan	34.6	G3	0	ND
Dextran	0	ND	0	ND

One percent of each substrate solution in 25 mM acetate buffer (pH 6.0) was incubated with 0.1 U/mL of CMMase at 20°C for 24 h. One percent of each substrate solution in 25 mM Tris-HCl buffer (pH 7.5) was incubated with 0.1 U/mL of α-glucosidase at 35°C for 24 h. Hydrolysis ratio of substrate and molar ratio of products were determined by HPLC. ND, not detected.

Table 4. Relative activity of α-glucosidase against various substrates.

Substrate	Relative activity (%)
Maltose	100
Isomaltose	ND
Maltotriose	11.4
Panose	61.1
Maltotetraose	6.5
Maltosyl-maltose	232

Five micromoles of each substrate in 25 mM Tris-HCl buffer (pH 7.5) were incubated with α-glucosidase (0.1 U/mL) at 40°C for 20 min. The liberated glucose was measured by the glucose oxidase-peroxidase method. Data were normalized relative to maltose. ND, not detected.

Physical and enzymatic properties of α-glucosidase.

The molecular mass of α-glucosidase was determined as 60.1 kDa by SDS-PAGE and 69.5 kDa by gel filtration column chromatography. The N-terminal sequence up to the 12th residue was SHTIERPSRLDT. The effects of pH and temperature on the activity and stability of the enzyme were determined as follows: α-glucosidase had an optimal pH of 7.0 and was stable in the pH range of 7.0 to 9.5 when kept at 4°C for 24 h. The optimum temperature for the enzyme was 35°C. The enzyme was stable up to 35°C when heated at various temperatures for 60 min at pH 7.5. The enzyme activity was strongly inhibited by Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (residual activity, 1.4–15%). Al^{3+}, Fe^{2+} and Fe^{3+} moderately inhibited its activity (49–57%). Other metal ions and EDTA had almost no effect on the enzyme activity. As shown in Table 3, maltose, panose and maltosyl-maltose are the preferable substrates for α-glucosidase (hydrolysis ratio, 98.3–99.7%). This enzyme did not degrade CMM at all. Although this enzyme did not degrade isomaltotriose, which consist of 3 glucose residues joined only by the α-1,6-linkage, it degraded panose to glucose efficiently. This result indicates that this enzyme can hydrolyze the α-1,6-linkage at the non-reducing end, which is next to the α-1,4-linkage. Relative activity of α-glucosidase against maltose, panose, and maltosyl-maltose was greater than that against isomaltose, maltotriose and maltotetraose (Table 4). It seemed that this enzyme is specialized in hydrolyzing maltosyl-maltose (a lineared molecule of CMM) and its partial structures (panose and maltose).

Analysis of degradation process of CMM by the enzymes.

First, we examined changes in sugar content during degradation of CMM by CMMase. Maltosyl-maltose transiently increased up to 74.0% in 4 h, and then decreased. Maltose increased during the experiment time and in-

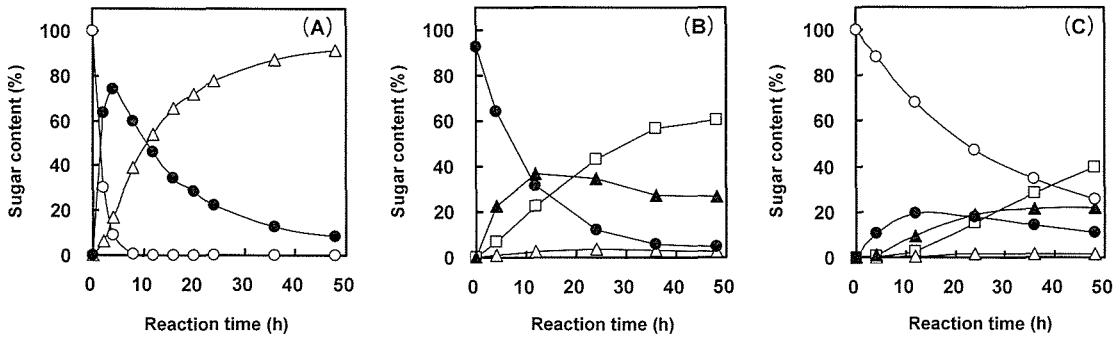

Fig. 7. Effect of enzymes on CMM or maltosyl-maltose degradation.

(A) Effect of CMMase on CMM degradation. One percent CMM in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ was incubated with CMMase (1.0 U/mL) at 20°C for 48 h. (B) Effect of α-glucosidase on maltosyl-maltose degradation. One percent maltosyl-maltose in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ was incubated with α-glucosidase (0.1 U/mL) at 20°C for 48 h. (C) Effect of CMMase and α-glucosidase on CMM degradation. One percent CMM in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ was incubated with CMMase (0.1 U/mL) and α-glucosidase (0.1 U/mL) at 20°C for 48 h. Each enzyme reaction was stopped at the indicated points. The sugar composition was analyzed by HPLC. ○, CMM; ●, maltosyl-maltose; △, maltose; ▲, panose; □, glucose.

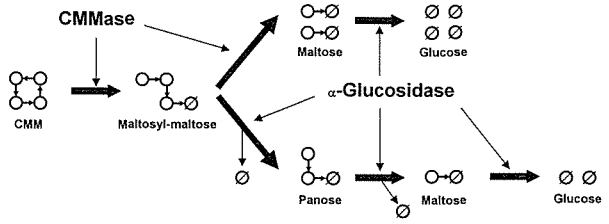

Fig. 8. Degradation pathway of CMM by the action of CMMase and α-glucosidase from *A. globiformis* M6.

○, glucopyranosyl residue; ∅, glucose or glucose residue at reducing end; horizontal arrow, α-1,4-linkage; vertical arrow, α-1,6-linkage.

creased to 91.6% at 48 h. Glucose was not detected during the experiment time (Fig. 7 (A)). Next, we examined changes in sugar content during degradation of maltosyl-maltose by α-glucosidase to gain a better understanding of the reaction profile of the enzyme because the enzyme did not degrade CMM but degraded maltosyl-maltose to glucose (Table 3). Maltosyl-maltose was degraded rapidly, while panose, glucose, and a small amount of maltose were generated (Fig. 7 (B)). This result indicates that maltosyl-maltose was degraded to glucose by the action of α-glucosidase via panose and maltose as intermediates. Based on the results of CMM and maltosyl-maltose degradation described above, we supposed that CMM is degraded to glucose by the synergistic action of these 2 enzymes. Therefore, we examined the synergistic action of CMMase and α-glucosidase against CMM. The sugar content of the reaction mixture containing CMM, CMMase, and α-glucosidase was analyzed simultaneously (Fig. 7 (C)). CMM, maltosyl-maltose, panose, glucose, and a small amount of maltose were detected in the reaction mixture. This result indicates that CMM is degraded to glucose by synergistic action of CMMase and α-glucosidase.

On the basis of the results shown in Figs. 7 (A) and (B), we hypothesized that 2 degradation pathways of CMM exist in *A. globiformis* M6. In one pathway, CMM is degraded to glucose *via* maltosyl-maltose and maltose as intermediates. In the other pathway, CMM is degraded to glucose *via* maltosyl-maltose, panose and maltose as the intermediates, although as shown in Fig. 7 (C), the amount of maltose was low during the degradation of CMM by the synergistic action of CMMase and α-glucosidase. From this result, we supposed that maltosyl-maltose was degraded more rapidly to panose by α-glucosidase than to maltose by CMMase. Therefore, we hypothesized that the latter pathway is the main pathway of CMM degradation. Based on this idea, we proposed a degradation pathway of CMM by the synergistic action of CMMase and α-

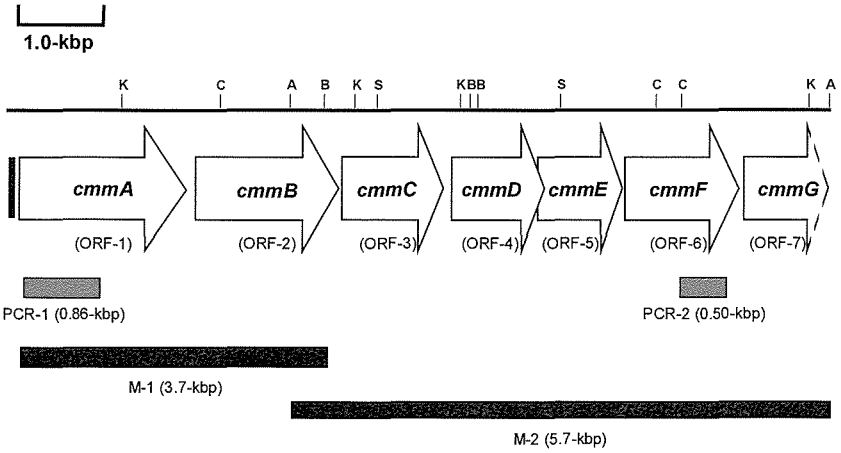

Fig. 9. Structure and restriction map of the gene cluster containing genes for synthesis, degradation and transport of CMM.

Arrows show the localization of each gene and the orientation of the coding sequences. The vertical solid line indicates a possible promoter sequences. K, *Kpn*I; C, *Cla*I; A, *Apa*I; B, *Bam*HI; S, *Sca*I.

glucosidase (Fig. 8). First, CMMase degrades CMM to maltosyl-maltose. Next, α-glucosidase degrades maltosyl-maltose to glucose as a final product via panose and maltose as intermediates. Thus, CMM is degraded to glucose by the synergistic action of CMMase and α-glucosidase.

Gene cloning.

The amino-acid sequence analysis found seven internal sequences for 6MT. During several trials of PCR using primers designed on the basis of the internal amino-acid sequences, we succeeded in amplifying a 861-bp fragment of 6MT gene (designated *cmmA*) from the genomic DNA of *A. globiformis* M6. The amino-acid sequence encoded by this DNA fragment contained four internal sequences of 6MT. Hence we concluded that the DNA fragment was part of *cmmA*. To obtain the full-length *cmmA*, we screened a genomic DNA library of *A. globiformis* M6 by colony hybridization using a 861-bp fragment (PCR-1 in Fig. 9) of *cmmA* as a probe. As a result, one positive clone, M-1, was obtained. The M-1 fragment was a 3750-bp DNA that encoded the full-length *cmmA* and an incomplete open reading frame (ORF), designated *cmmB*. This DNA fragment did not contain CMMase gene, because nucleotide sequences encoding internal amino-acid sequences of CMMase was not found in the DNA fragment. To obtain the full-length *cmmB* and CMMase gene, we re-screened a genomic DNA library using a 495-bp fragment (PCR-2 in Fig. 9) of CMMase gene as a probe. As a result, one positive clone, M-2, was obtained. The M-2 fragment contained a 5675-bp DNA encoding a C-terminal region of *cmmB*, the full-length CMMase gene (designated *cmmF*), three ORFs (designated ORF-3, ORF-4, ORF-5, respectively), and one incomplete ORF (designated ORF-7) at the 3′-region of M-2. Thus two DNA fragments, M-1 and M-2, laid to overlap each other, were cloned from a genomic DNA library of *A. globiformis* M 6, and an 8778-bp sequence containing *cmmA, cmmB* and *cmmF* was analyzed. The nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB190187.

The 6MT gene.

The 6MT gene (*cmmA*) encoded a protein with 623 amino-acid residues calculated a molecular mass of

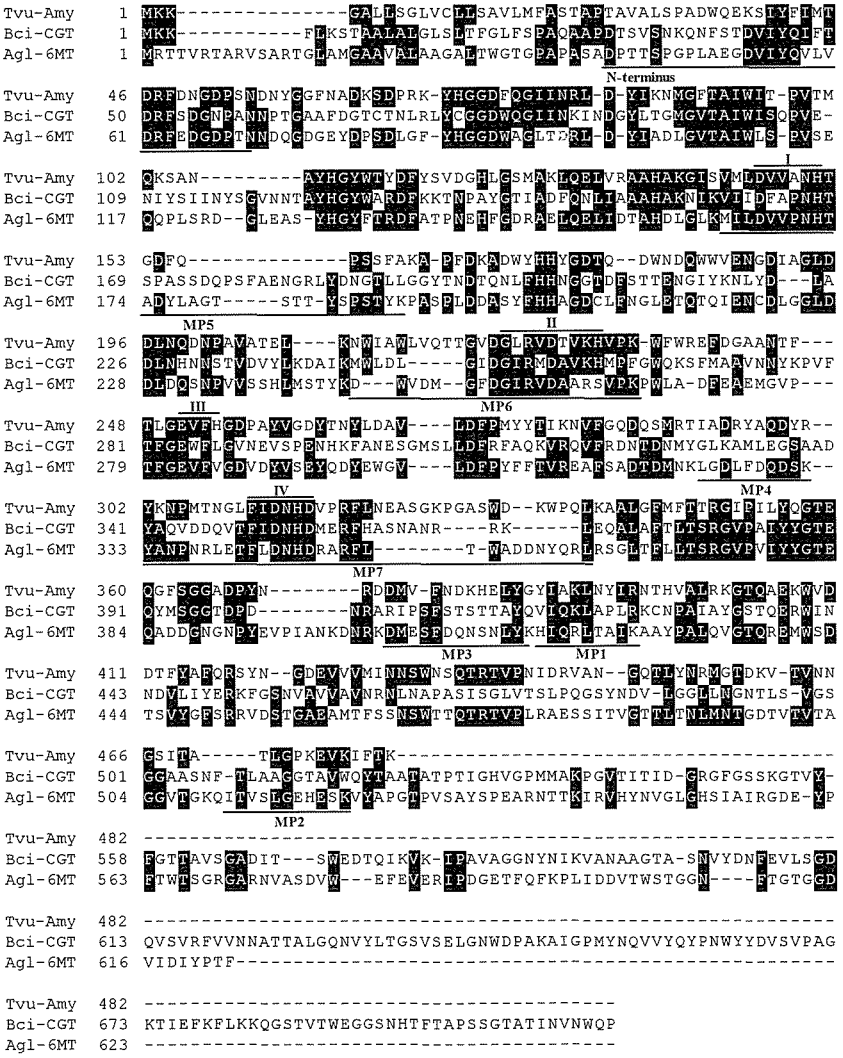

Fig. 10. Alignment of the amino acid sequences among 6MT from *A. globiformis* M6 and α-amylase family enzymes.

The identical amino acid residues in each column are outlined in black boxes. The N-terminal and internal amino acid sequences of the mature 6MT are underlined. The four conserved regions (I, II, III and IV) are lined above the amino acid sequences. Agl-6MT, 6-α-maltosyltransferase from *A. globiformis* M6 (this study); Bci-CGT, cyclomaltodextrin glucanotransferase from *Bacillus circulans* strain 251 (P43379 in Swiss-plot); and Tvu-Amy, α-amylase from *Thermoactinomyces vulgaris* 94-2A (Q60051).

68,460 Da. Upstream of the coding region, the putative −35 and −10 promoter sequences with a distance of 17-bp between them were observed. No possible terminator sequence was found downstream from the stop codon. The deduced amino acid sequence contained the seven internal peptide fragments (MP1-7 in Fig. 10) of 6MT protein. The N-terminal sequence of the mature 6MT started from Asp-41 of the deduced amino acid sequence, indicating that the preceding 40 residues might be a signal sequence for secretion. The molecular mass of the gene product without the putative signal sequence was calculated to be 64,637 Da in agreement with that (71.7 kDa) of the purified 6MT estimated by SDS-PAGE.

Homology searches performed with BLASTP program revealed that 6MT showed similarities to glycoside hydrolase family 13 (GH 13) or α-amylase family enzymes;[18] 38 and 31% identities to α-amylase (EC 3.2.1.1) from *Thermoactinomyces vulgaris*[19] and cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *Bacillus circulans*,[20] respectively. The four conserved regions that are common in the family enzymes were also found in 6MT (Fig. 10).

The α-glucosidase gene.

The α-glucosidase gene (*cmmB*) was expected to encode a protein with 567 amino-acid residues (calculated molecular mass, 63,014 Da). The deduced amino-acid sequence contained all of the N-terminal and internal sequences of α-glucosidase.

Homology searches showed that the deduced amino-acid sequences showed 51% identities to the α-glucosidase from *Thermomonospora curvata*. The four conserved regions that are common in GH 13 family enzymes were also found in the α-glucosidase from *A. globiformis* M6, indicating that this enzyme should be assigned to this family.

The CMMase gene.

The CMMase gene (*cmmF*) encoded a protein with 450 amino-acid residues. The molecular mass of the gene product was calculated to be 49,344-Da, in agreement with the 48.6 kDa of the purified enzyme (SDS-PAGE). The deduced amino-acid sequence contained all of the N-terminal and internal sequences of CMMase.

Homology searches performed with the BLASTP program revealed that the deduced amino-acid sequences of CMMase (amino-acid positions 1 to 450) showed similarities to the glycoside hydrolase family 13 (GH 13) or α-amylase family enzymes,[18] and 46, 46 and 45% identities to cyclodextrinase from *Geobacillus kaustophilus*, maltogenic α-amylase from *Bacillus* sp. and neopullulanase from *Bacillus stearothermophilus*[21−23] respectively. The four conserved regions that are common in this family of enzymes were also found in CMMase, indicating that this enzyme should be assigned to this family. CMMase has characteristic amino-acid sequence within conserved region II, namely Pro203-Tyr204-Phe205 (PYF). Proline, tyrosine and phenylalanine have an aromatic or a cyclic aliphatic side chain in its structure, and are hydrophobic. Therefore, we expected that the structure in the vicinity of the substrate binding region of CMMase was different from cyclodextrinase, maltogenic α-amylase, neopullulanase, and Pro203-Tyr204-Phe205 limited the direction of the substrate. Because the direction of the substrate was limited, the substrate which efficiently bound to the substrate binding region of CMMase was expected to be limited to CMM. Crystal and mutational analyses of CMMase should provide insight into the relationship between the structure and substrate specificity.

Other ORFs.

ORF-3 was located just downstream of *cmmB* through 53 bp flanking region. The structural gene expected to encode a protein with 421 amino-acid residues (calculated molecular mass, 44,547 Da).

Homology searches revealed that the deduced amino acid sequences of ORF-3 showed 27% identities to the sugar-binding protein of the ABC-type sugar transport systems from *Streptomyces avermitilis*.[24]

ORF-4 lies in the rear of the ORF-3 through a short flanking region of 10 bp. This ORF was expected to encode a protein with 320 amino-acid residues (calculated molecular mass, 34,651 Da).

Homology searches revealed that the deduced amino acid sequences of ORF-4 showed 44% identities to the permease of the ABC-type sugar transport systems from *Deinococcus geothermalis.*

ORF-5 encodes a protein of 284 amino-acid residues (calculated molecular mass of 31,764 Da). This ORF lies in the rear of the ORF-4 overlapping with the 37-bp region. The deduced amino-acid sequences showed 49% identities to the permease of the ABC-type sugar transport systems from *Bacillus clausii*. Described above, ORF-4 was similar to the permease from *D. geothermalis*. The identities suggest that the ORF-3 products may be a CMM-binding protein and the products encoded by ORF-4 and ORF-5 form a subunit structure and function as permease. Accordingly, the three ORF (ORF-3, ORF-4 and ORF-5) were designated *cmmC, cmmD* and *cmmE*, respectively.

ORF-7 is an incomplete ORF lacking the 3′-region that lies rear of *cmmF* through a short flanking region of 17 bp. The amino-acid sequence (194 residues) deduced from the ORF shows similarity to the N-terminal region of a transcriptional regulator from *Gluconobacter oxydans* (35% identity).[25] This ORF was named *cmmG.*

Promoter and transcriptional terminator were not found between *cmmA* and *cmmG*. Hence we considered that *cmmB, cmmC, cmmD, cmmE, cmmF* and *cmmG* form a gene cluster together with *cmmA*, namely *cmmABCDEFG*. In the case of cyclodextrins (CDs), the *cgtBACDE* cluster was found out in the genome of *Thermococcus* sp. B1001; *cgtB* for the intracellular CD-degration enzyme, *cgtA* for the exrtacellular CD-synthesis enzyme (CGTase), *cgtC* for the CD-binding protein, *cgtDE* for the membrane transporter proteins.[26] It has been reported that *Klebsiella oxytoca* M5a1 has a CD-synthesis/uptake/degradation system similarly to that of *Thermococcus.*[27] This system is considered to be advantageous for these microorganism assimilating starch as a carbon source, because CDs synthesized from starch by the action of CGTase show various degrees of registance and inhibition

to amylolytic enzymes produced by competitive microorganisms. Additionally, the competitors having a usual transport system for linear maltooligosaccharides are unable to take CDs into cells. We suppose that CMM has the advantage similarly to CDs. The host strain, *A. globiformis M6*, grow well in a medium with CMM as the sole carbon source. Although the role of CMM in the host strain is not clear, it is likely to it as a carbon source after the starch is converted into CMM, which is not easily used by other microorganisms.

REFERENCES

1) A. Tabuchi, T. Mandai, T. Shibuya, S. Fukuda, T. Sugimoto and M. Kurimoto: Formation of trehalose from starch by novel enzymes. *J. Appl. Glycosci.*, **42**, 401–406 (1995).
2) K. Mukai, A. Tabuchi, T. Nakada, T. Shibuya, H. Chaen, S. Fukuda, M. Kurimoto and Y. Tsujisaka: Production of trehalose from starch by thermostable enzymes from *Sulfolobus acidcaldarius*. *Starch/Stärke*, **49**, 26–30 (1997).
3) H. Chaen: Production of trehalose by a novel enzymatic method and its applications. *J. Appl. Glycosci.*, **44**, 115–120 (1997).
4) D. French: The Schardinger dextrins. *Adv. Carbohydr. Chem.*, **12**, 189–260 (1957).
5) T. Loftsson and M.E. Brewster: Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J. Pharm. Sci.*, **85**, 1017–1025 (1996).
6) I. Goubet, C. Dahout, E. Semon, E. Guichard, J.L. Le Quere and A. Voilley: Competitive binding of aroma compounds by beta-cyclodextrin. *J. Agric. Food Chem.*, **49**, 5916–5922 (2001).
7) C.D. Tran and S.H. De Paoli Lacerda: Determination of binding constants of cyclodextrins in room-temperature ionic liquids by near-infrared spectrometry. *Anal. Chem.*, **74**, 5337–5341 (2002).
8) G. L. Côté and P. Biely: Enzymically produced cyclic α-1,3-linked and α-1,6-linked oligosaccharides of D-glucose. *Eur. J. Biochem.*, **226**, 641–648 (1994).
9) A. Misaki, M. Torii, T. Sawai and I.J. Goldstein: Structure of the dextran of *Leuconostoc mesenteroides* B-1355. *Carbohydr. Res.*, **84**, 273–285 (1980).
10) P. Biely, G.L. Côté and A. Burgess-Cassler: Purification and properties of alternanase, a novel endo-α-1,3-α-1,6-D-glucanase. *Eur. J. Biochem.*, **226**, 633–639 (1994).
11) T. Nishimoto, H. Aga, K. Mukai, T. Hashimoto, H. Watanabe, M. Kubota, S. Fukuda, M. Kurimoto and Y. Tsujisaka: Purification and characterization of glucosyltransferase and glucanotransferase involved in the production of cyclic tetrasaccharide in *Bacillus globisporus* C11. *Biosci. Biotechnol. Biochem.*, **66**, 1806–1818 (2002).
12) H. Aga, T. Higashiyama, H. Watanabe, T. Sonoda, T. Nishimoto, M. Kubota, S. Fukuda, M. Kurimoto and Y. Tsujisaka: Production of cyclic tetrasaccharide from starch using a novel enzyme system from *Bacillus globisporus* C11. *J. Biosci. Bioeng.*, **94**, 336–342 (2002).
13) H. Aga, T. Nishimoto, M. Kuniyoshi, K. Maruta, H. Yamashita, T. Higashiyama, T. Nakada, M. Kubota, S. Fukuda, M. Kurimoto and Y. Tsujisaka: 6-α-Glucosyltransferase and 3-α-isomaltosyltransferase from *Bacillus globisporus* N75. *J. Biosci. Bioeng.*, **95**, 215–224 (2003).
14) P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, Williams & Wilkins, Baltimore (1984).
15) K. Mukai, H. Watanabe, K. Oku, T. Nishimoto, M. Kubota, H. Chaen, S. Fukuda and M. Kurimoto: An enzymatically produced novel cyclic tetrasaccharide, *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→} (cyclic maltosyl-(1→6)-maltose), from starch. *Carbohydr. Res.*, **340**, 1469–1474 (2005).
16) G. Okada, T. Takayanagi and T. Sawai: Improved purification and further purification of an isomalto-dextranase from *Arthrobacter globiformis* T6. *Agric. Biol. Chem.*, **52**, 495–501 (1988).
17) M. Torii, K. Sakakibara, A. Misaki and T. Sawai: Degradation of alpha-linked D-gluco-oligosaccharides and dextrans by an isomalto-dextranase preparation from *Arthrobacter globiformis* T6. *Biochem. Biophys. Res. Commun.*, **70**, 459–464 (1976).
18) B. Henrissat and G. Davies: Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.*, **7**, 637–644 (1997).
19) B. Hofemeister, S. Koenig, V. Hoang, J. Engel, G. Mayer, G. Hansen and J. Hofemeister: The gene *amyE* (TV1) codes for a nonglucogenic α-amylase from *Thermoactinomyces vulgaris* 94–2A in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **60**, 3381–3389 (1994).
20) C. L. Lawson, R. van Montfort, B. Strokopytov, H.J. Rozeboom, K.H. Kalk, G.E. de Vries, D. Penninga, L. Dijkhuizen and B.W. Dijkstra: Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. *J. Mol. Biol.*, **236**, 590–600 (1994).
21) H. Takami, Y. Takaki, G.J. Chee, S. Nishi, S. Shimamura, H. Suzuki, S. Matsui and I. Uchiyama: Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acid Res.*, **32**, 6292–6303 (2004).
22) B. Liu, Y. Wang and X. Zhang: Characterization of a recombinant maltogenic amylase from deep sea thermophilic *Bacillus* sp. WPD616. *Enzyme Microb. Technol.*, **39**, 805–810 (2006).
23) T. Kurik and T. Imanaka: Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*. *J. Gen. Microbiol.*, **135**, 1521–1528 (1989).
24) H. Ikeda, J. Ishikawa, A. Hanamoto, M. Shinose, K. Kikuchi, T. Shiba, Y. Sakaki, M. Hattori and S. Omura: Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.*, **21**, 526–531 (2003).
25) C. Prust, M. Hoffmeister, H. Liesegang, A. Wieser, W.F. Fricke, A. Ehrenreich, G. Gottschalk and U. Deppenmeier: Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nat. Biotechnol.*, **23**, 195–200 (2005).
26) Y. Hashimoto, T. Yamamoto, S. Fujiwara, M. Takagi and T. Imanaka: Extracellular synthesis, specific recognition, and intracellular degradation of cyclomaltodextrins of the hyperthermophilic archaeon *Thermococcus* sp. strain B1001. *J. Bacteriol.*, **183**, 5050–5057 (2001).
27) G. Fiedler, M. Pajatsch and A. Bock: Genetics of a novel starch utilisation pathway present in *Klebsiella oxytoca*. *J. Mol. Biol.*, **256**, 279–291 (1996).

Arthrobacter globiformis M6における環状マルトシルマルトースの合成および分解に関与する酵素

森　哲也[1]，西本友之[1]，向井和久[1]，渡邊　光[1]
大倉隆則[1]，茶圓博人[1]，福田恵温[1]
[1]株式会社林原生物化学研究所研究センター糖質研究部門
(702-8006 岡山市藤崎 675-1)

澱粉から酵素的に生成する非還元性糖質を広く検索したところ，土壌由来細菌 *Arthrobacter globiformis* M6株の培養上清中に未知非還元性糖質を見出した．この未知糖質の構造を決定したところ，グルコース4分子がα-1,4結合とα-1,6結合を交互に繰り返して環状化した新規環状四糖，*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→} であった．我々は，この糖質を環状マルトシルマルトース (CMM) と命名した．*A. globiformis* M6株の培養上清からCMM生成酵素を精製し，その諸性質を調べたところ，本酵素は，重合度3以上のマルトオリゴ糖や澱粉に作用してCMMを生成した．CMM生成機構を調べたところ，CMM生成酵素はマルトース単位でα-1,6分子間転移反応，およびα-1,6分子内転移反応を触媒することでCMMを生成することがわかった．このことから，本酵素は新規転移酵素であり，我々はこの酵素を6-α-マルトシルトランスフェラーゼ (6MT) と命名した．CMMの分解に関与する二つの酵素，CMM hydrolase (CMMase) およびα-グルコシダーゼを *A. globiformis* M6株の菌体破砕液から精製した．CMMaseはCMMに最もよく作用しマルトシルマルトース (MM) もしくはマルトースを生成するが，グルコースは生成しなかった．α-グルコシダーゼはCMMには作用しないが，MMやパノース，マルトースによく作用し，グルコースを生成した．CMMはCMMaseとα-グルコシダーゼの共同作用により最終的にグルコースにまで分解されることがわかった．M6株のゲノムDNAライブラリーから，6MT，CMMase，およびα-グルコシダーゼ遺伝子をクローニングした．これら遺伝子の塩基配列を解析したところ，6MTは623アミノ酸残基から，CMMaseは450アミノ酸残基から，α-グルコシダーゼは567アミノ酸残基から成ることがわかった．これらの酵素はいずれもそのアミノ酸配列中に，グリコシドヒドロラーゼファミリー13に属するα-アミラーゼファミリーに保存されている4カ所の共通領域が存在し，これらの酵素はα-アミラーゼファミリーに属することが示唆された．α-グルコシダーゼ遺伝子とCMMase遺伝子の間に，糖結合タンパク質をコードすると考えられる遺伝子が一つ，透過酵素をコードすると考えられる遺伝子が二つ見出された．これら遺伝子解析から，*A. globiformis* M6株はCMMを介した澱粉の資化経路を有している可能性が示唆された．

＊＊＊＊＊

〔質問〕　江崎グリコ　栗木

1）6MTによるアミロペクチンの非還元末端へのマルトース残基のα-1,6転移は，分子内か分子間転移か？その反応系のドナー分子は何か？

2）6MTによる6^2-*O*-α-maltosyl-maltose (マルトシルマルトース) を生成する反応は自然に感じるが，それに続いてさらにα-1,6転移によりCMMが生成する反応は，グルコシド結合がエネルギー的に安定になると考えにくいと思うが，どうか？

〔答〕

1）6MTをマルトテトラオースに作用させると，反応初期にはマルトシル-(1→6)-マルトテトラオースとマルトースが生成することから，主反応は分子間転移と考えています．しかしながら，分子内転移の可能性も否定できません．

2）CMMのグルコシド結合の安定性については検討を行っておりません．この点に関しましては今後の検討課題にしたいと思います．

〔質問〕　北畑

CMM合成酵素は分子内転移以外に分子間転移作用を触媒し，マルトテトラオースを基質とした場合，基質濃度を10%以上に増すと極端にCMMの合成を阻害するとのことですが，澱粉を基質にした場合も同じでしょうか．

〔答〕

澱粉を基質にした場合も，マルトテトラオースを基質にした場合と同様に，基質濃度が高くなるにしたがってCMMの生成率は低下します．

〔質問〕　北大院・農　木村

1）生成したCMMが長時間の反応で減少していた理由を知りたい．

2）この減少は，CMMがドナー基質となり，他のアクセプター分子 (例えばマルトテトラオース) に転移させるような反応によるものなのか？

3）水分子への転移作用は？

〔答〕

1）6MTは，分子間および分子内α-1,6転移反応を触媒しますが，弱いながらα-1,6結合を加水分解いたします．CMMの生成反応を長時間行いますと，生成したCMMが6MTにより加水分解されマルトシルマルトースを生成します．その結果，生成したCMMが長時間の反応で減少いたします．

2）CGTaseで知られているようなカップリング反応を触媒するかについては調べておりません．他のアクセプター分子への転移反応を触媒する可能性は否定できません．

3）非常にわずかながら水分子への転移も起こりうると考えています．