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HYDROLYTIC ACTION ON VARIOUS MALTOSIDES BY AN ENZYME FROM *Bacillus coagulans*

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ABSTRACT

An enzyme ("cyclodextrinase") from *Bacillus coagulans* hydrolyzes maltotriose, maltotetraose, maltopentaose, maltohexaose, and cyclomaltohexa-, -hepta-, and -octa-ose by detaching one maltose unit. This enzyme recognizes the maltose residue at the non-reducing end of various maltosides and hydrolyzes (1→4), (1→2), (1→3), and (1→6)-α-D-glucosidic linkages and the glucosidic linkages between D-glucose and phenol, D-glucose and D-glucitol, and D-glucose and D-fructose, adjacent to the maltose molecule. The relative rates of hydrolysis for these maltosides are 1, 1/60, 1/10, 1/2.5, 5, 1/2, and 1/40, respectively.

INTRODUCTION

Cyclomalto-oligosaccharides (**1–3**), homogeneous, cyclic oligosaccharides of six or more (1→4)-linked α-D-glucopyranose residues[1], resist the action of such exo amylases as glucoamylase[2] and beta amylase[3], because of the absence of a non-reducing D-glucose end-group. Even such alpha amylases as those from *Bacillus subtilis* (saccharifying[4], liquefying[5]) and *Aspergillus oryzae*[6] hydrolyze **1–3** at rates much lower than that for starch.

Enzymes that can hydrolyze **1–3** faster than starch have been reported thus far from only three origins, namely, "cyclodextrinase"* from *B. macerans*[7], alpha amylase from *Pseudomonas sp.*[8], and glucoamylase from *Flavobacterium sp.*[9]. Among these enzymes, only that from *Pseudomonas sp.* was purified to homogeneity, by disc electrophoresis; the other two have not yet been so purified.

Recently, we isolated from soil a bacterium that produces an enzyme which hydrolyzes **1–3** faster than starch. The previous paper[10] reported the identification of the bacterium, and the purification and some properties of the enzyme. This enzyme hydrolyzed such linear malto-oligosaccharides (G_n) as maltotetraose (G_4), maltopentaose (G_5), and maltohexaose (G_6), and **1–3** faster than maltotriose (G_3) and short-chain amylose (d.p. 18), but did not hydrolyze maltose (G_2). This enzyme

*The nonsystematic name "cyclodextrinase" is used for convenience in relating to previous work, pending assignment of a systematic E.C. name.

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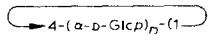
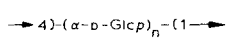
showed much lower activities for such polysaccharides as starch and amylose, and the rates of hydrolysis for these polysaccharides were <1% of those for **1–3**. It was found that this enzyme is a specific alpha amylase that hydrolyzes a variety of malto-oligosaccharides, including **1–3**, much faster than polysaccharides, and the its substrate specificity is different from those of other cyclomalto-oligosaccharide-hydrolyzing enzymes.

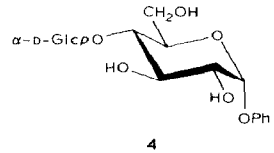
The present paper deals with the action of this enzyme on oligosaccharides, especially maltosides, containing various glucosidic linkages.



1 n = 6
2 n = 7
3 n = 8



Glc n = 1
G_2 n = 2
G_3 n = 3
G_4 n = 4
G_5 n = 5
G_6 n = 6



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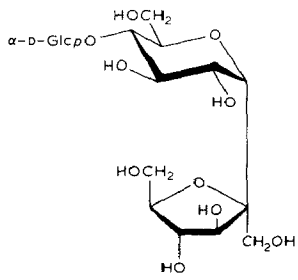

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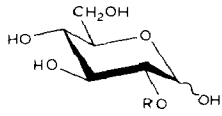
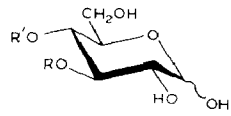


6 R = α-maltosyl



7 R = α-maltosyl, R′ = H
8 R = R′ = α-D-Glucopyranosyl

MATERIALS AND METHODS

Materials. — "Cyclodextrinase" from *B. coagulans* was purified to homogeneity as described in the previous paper[10]. Cyclomalto-hexa-(**1**), hepta-(**2**), and octa-ose (**3**), maltotriose (G_3), maltotetraose (G_4), panose, 4-*O*-α-isomaltosyl-maltose, maltotriitol, and 4^G-α-D-glucopyranosylsucrose (**5**) were supplied by Hayashibara Co., Ltd. Maltopentaose (G_5) was a gift from Dr. N. Saito of the Noda Institute for Scientific Research. Maltohexaose (G_6) was purchased from Seishin Pharmaceutical Co., Ltd. These oligosaccharides, except **1–3**, were purified by paper chromatography before use. Isopanose was a gift from Dr. Y. Sakano of Tokyo Noko University. Phenyl α-maltoside **4** was a gift from Dr. S. Ono, Emeritus Professor of the University of Osaka Prefecture. 2-*O*-α-Maltosyl-D-glucose **6** was prepared by the action of cyclomaltooligosaccharide glucanotransferase on

kojibiose and starch as described previously[11]. A 4:1 mixture of 3-*O*-α-maltosyl-D-glucose (**7**) and 3,4-di-*O*-α-D-glucosyl-D-glucose (**8**) was also prepared by the action of cyclomalto-oligosaccharide glucanotransferase on nigerose and starch[12].

Analytical methods. — Cyclodextrinase activity was assayed with **2** as the substrate by measuring the reducing sugar produced, as described in the previous paper[10]. The amount of reducing sugar was determined colorimetrically by the Somogyi–Nelson method[13,14]. D-Glucose was determined by using Glucostat, and phenol by the method of Lowry *et al.*[15].

Paper chromatography. — Paper chromatography was performed with Toyo No. 50 filter paper (20 × 20 cm) with 6:4:3 (v/v) 1-butanol–pyridine–water, with 4 ascents. Sugars on paper were detected with the silver nitrate reagent[16].

RESULTS

Reaction products from cyclomaltohexa-, hepta-, octa-ose, maltotetraose, maltopentaose, and maltohexaose. — "Cyclodextrinase" (1 U/mL, 20 μL) was incubated with 100 μL of 2% substrate at 40°. After 10, 30, and 180 min, 30-μL aliquots of the mixtures were examined by paper chromatography. After development, the sugars were detected with the silver nitrate reagent after treatment with glucoamylase according to the method of Kainuma and French[17]. As shown in Figs.

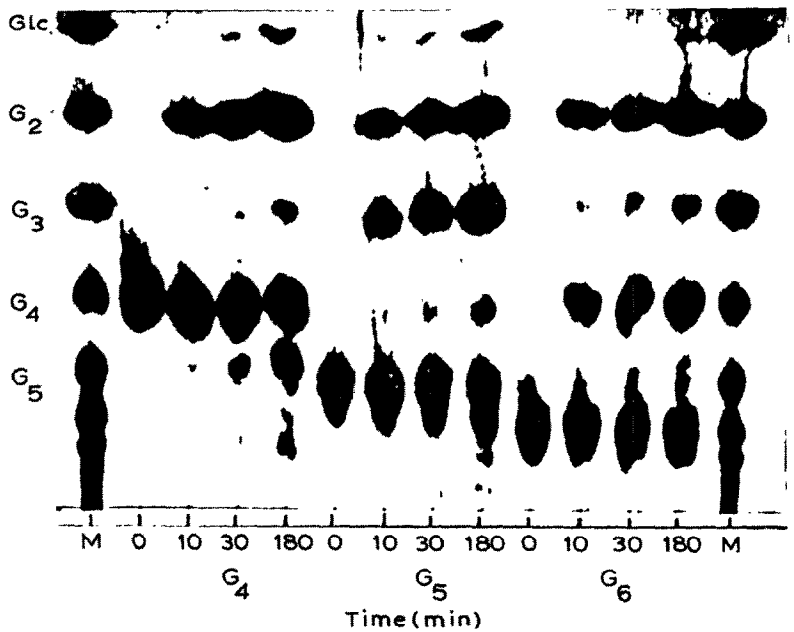


Fig. 1. Paper chromatogram of the hydrolyzates of maltotetraose, maltopentaose, and maltohexaose by "cyclodextrinase". M, standard oligosaccharides; Glc, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose.

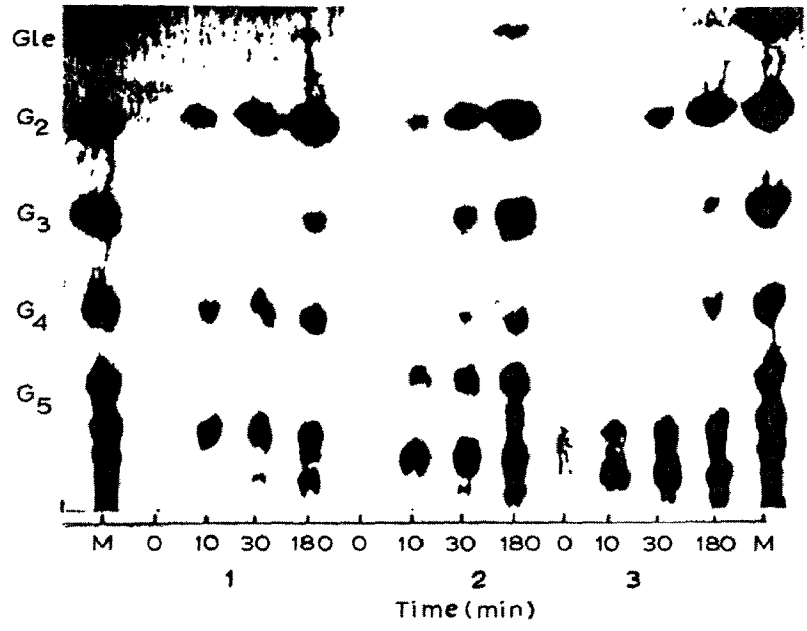


Fig. 2 Paper chromatogram of the hydrolyzates of **1**, **2**, and **3** by "cyclodextrinase". G_7, maltoheptaose; G_8, maltooctaose. Other abbreviations are as in Fig. 1.

1 and 2, G_4 was hydrolyzed mostly to G_2, whereas G_5 was hydrolyzed mainly to G_2 and G_3, and G_6 mainly to G_2 and G_4. Small amounts of other hydrolytic products (such as Glc and G_3 from G_4, Glc and G_4 from G_5, and Glc, G_5 and G_3 from G_6) were also observed. The hydrolytic products from **2** were mostly maltoheptaose (G_7) at the initial stage of reaction; subsequently, G_2 and G_5 were produced. This result indicates that the ring of **2** is initially opened, giving the linear G_7, and then G_7 is further degraded to G_2 and G_5. Other cyclomalto-oligosaccharides were hydrolyzed similarly. From G_4, G_5, and **1–3**, transfer products were also observed; these await characterization.

Action on maltotriose, panose, and 4-O-α-isomaltosylmaltose. — "Cyclodextrinase" (4.5 U/mL, 30 μL) was incubated with 70 μL of 2% substrate (G_3 and 4-*O*-α-isomaltosylmaltose) at 40°. After 15, 30, 60, and 180 min, 20-μL aliquots of the mixtures were examined by paper chromatography (Fig. 3). Maltotriose (G_3) was hydrolyzed to G_2 and Glc. 4-*O*-α-Isomaltosylmaltose was hydrolyzed to glucose and panose. (Panose was identified by its R_F value of the paper chromatogram). Even a "cyclodextrinase" solution having higher activity (27 U/mL) did not hydrolyze panose. These results indicate that this enzyme hydrolyzes the second glucosidic linkage toward the reducing end from the branching point in the branched oligosaccharides.

Action on maltotriitol, phenyl α-maltoside (**4**), *and* 4^G-O-α-D-*glucosylsucrose* (**5**). — "Cyclodextrinase" solution (40 μL, 20 U/mL in the case of maltotriitol and

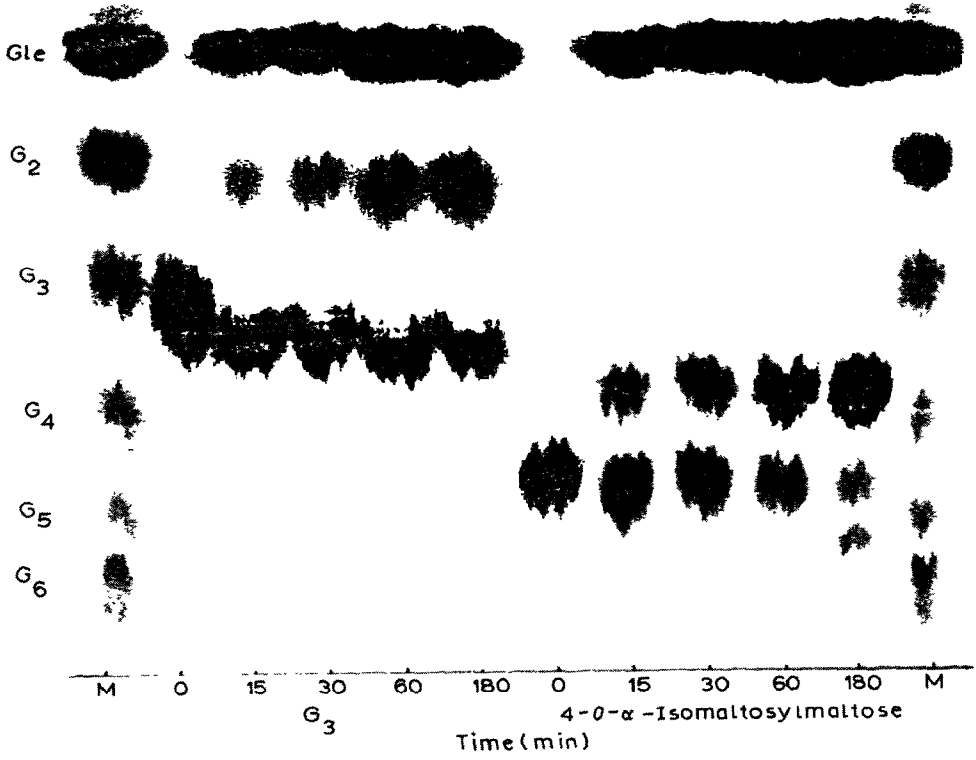


Fig. 3. Paper chromatogram of the hydrolyzates of maltotriitol and 4-*O*-α-isomaltosylmaltose by "cyclodextrinase". Abbreviations are as in Fig. 1.

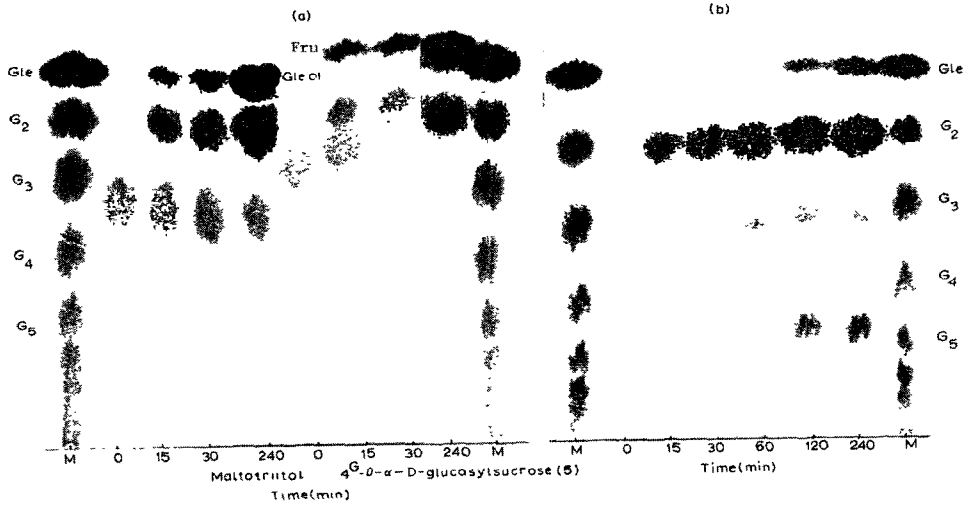


Fig. 4. (a),(b). Paper chromatogram of the hydrolyzates of maltotriitol and 4^{G}-*O*-α-D-Glucosylsucrose (**5**) (*a*) and phenyl α-maltoside (**4**) (*b*) by "cyclodextrinase" Glc-ol, glucitol; Fru, fructose. Other abbreviations are as in Fig. 1.

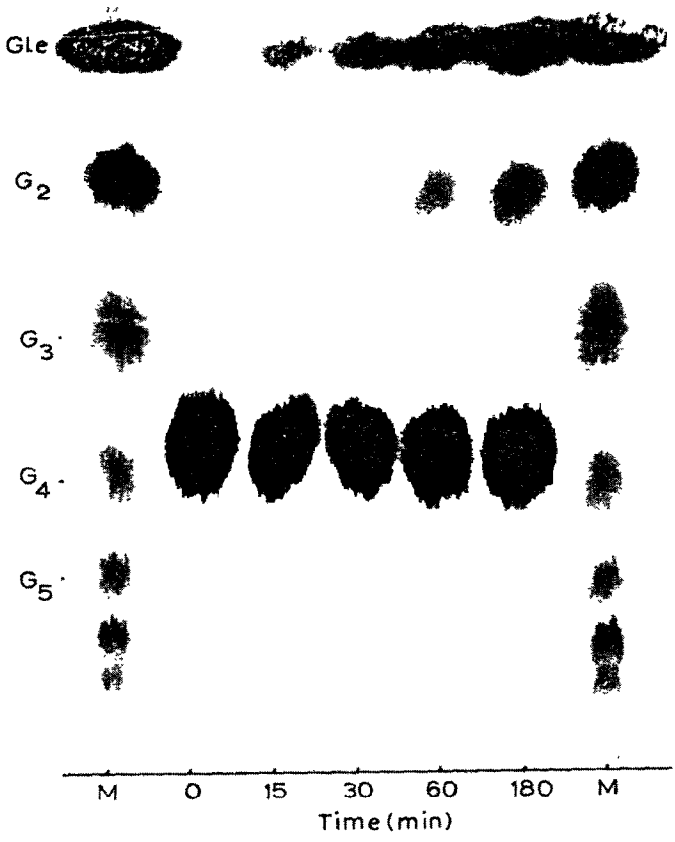


Fig. 5. Paper chromatogram of the hydrolyzates of isopanose by "cyclodextrinase". Abbreviations are as in Fig. 1

TABLE I

KINETIC PARAMETERS OF THE "CYCLODEXTRINASE" ON THE HYDROLYSIS OF VARIOUS OLIGOSACCHARIDES[a]

Substrate	K_m (mM)[b]	k_0 (min^{-1})[c]
Maltotriose	5.3	42
Phenyl α-maltoside (**4**)	5.5	220
Compounds **7** + **8** (4:1)	13	4.3
4^G-*O*-α-D-Glucosylsucrose (**5**)	17	1.0
2-*O*-α-Maltosylglucose (**6**)	21	0.67
Isopanose	28	17
Maltotriitol	110	20

[a]A mixture of 1.0 mL of substrate solution (in 50mM, pH 6.2 phosphate buffer) and 50 μL of "cyclodextrinase" was incubated at 40°. After 10 min, the reducing power (for **5** and maltotriitol), the amount of glucose (**7** + **8**, G_3, **6**, and isopanose) or the amount of phenol (for **4**) in the digests were assayed. The K_m values and the V_{max} values were calculated from Hofstee plots. [b]K_m and V_{max} values are the mean values of three runs. [c]Molecular activity (k_0) is expressed as V_{max}/e, e, the concentration of enzyme.

5, 1 U/mL for **4**) was incubated with 100 μL of 2% substrate solution at 40°. After 15, 30, and 240 min intervals, 40-μL aliquots of the mixtures were removed to determine D-glucose (Glucostat) and the other products by paper chromatography (Fig. 4a,b). No glucose was detected in the mixtures from maltotriitol and **5** and in the initial products from **4**. This result, in conjugation with the paper-chromatographic results, indicates that the hydrolytic products from **4** in the initial stage of reaction, maltotriitol, and **5** were G_2 and phenol, G_2 and glucitol, and G_2 and D-fructose, respectively. These results show that "cyclodextrinase" is able to hydrolyze not only the (1→4)-α-D-glucosidic linkage, but also glucosidic linkages between D-glucose and D-glucitol, D-glucose and D-fructose, and D-glucose and phenol, in maltotriitol, **5**, and **4**, respectively. This hydrolysis pattern was independent of the substrate concentration in the case of phenyl α-maltoside.

Action on isopanose, 2-O-α-maltosyl-D-glucose (**6**) *and compounds* **7** + **8**. — "Cyclodextrinase" solution (30 μL, 4.5 U/mL for isopanose, 27 U/mL for **6** and **7** + **8**) was incubated with 70 μL of 2% substrate solution at 40°. At intervals, 20-μL aliquots of the mixtures were examined by paper chromatography (Fig. 5). Glucose and G_2 were observed as the hydrolytic products from isopanose. G_2 and Glc were also observed as the hydrolytic products from **6** and from **7** + **8**. These results indicate that this enzyme is able to hydrolyze not only the (1→4)-α-D-glucosidic linkage but also (1→2), (1→3), and (1→6)-α-D-glucosidic linkages.

Kinetic parameters of the cyclodextrinase" for the hydrolysis of various maltosides. — The kinetic parameters, K_m and $k_0(V/e$; e, enzyme concentration) of the hydrolysis of (1→2), (1→3), and (1→6)-α-D-glucosidic linkages and the glucosidic linkages between D-glucose and D-fructose, D-glucose and D-glucitol, and D-glucose and phenol by "cyclodextrinase" were compared with those for the (1→4)-α-D-glucosidic linkage by using the trisaccharides or phenyl α-maltoside (**4**) as substrates (Table I). The K_m value of this enzyme for G_3 is about same as that for **4** and increase in the following order: **4**<**7** + **8**< **5** <2-*O*-α-maltosylglucose<isopanose<maltotriitol. The molecular activity (k_0) of the enzyme for **4** is about 5 times greater than that for G_3, and those for maltotriitol, isopanose, **7** + **8**, **5**, and 2-*O*-α-maltosylglucose are respectively about 2, 2.5, 10, 40, and 60 times smaller than that for G_3.

DISCUSSION

The "cyclodextrinase" from *B. coagulans* recognizes the α-maltosyl group in the non-reducing end of various maltosides and hydrolyzes not only (1→4)-α-D-glucosidic linkage, but also (1→2), (1→3), and (1→6)-α-D-glucosidic linkages and the linkages between D-glucose and phenol, D-glucose and D-glucitol, and D-glucose and D-fructose, adjacent to the maltose molecule. The enzyme also hydrolyzes G_3 labeled at the reducing end to produce labeled glucose (unpublished data). It hydrolyzes the glucosidic linkages between D-glucose and phenol of phenyl α-maltoside, between D-glucose and D-glucitol of maltotriitol, and between D-glucose

and D-fructose of 4^G-*O*-α-D-glucosylsucrose, at rates about 5, 1/2, and 1/40 times that of the (1→4)-α-D-glucosidic linkage of G_3. These broad substrate-specificities have, also been reported for bacterial saccharifying alpha amylase and *Asp. oryzae* alpha amylase. Bacterial saccharifying alpha amylase hydrolyzes **5**, to G_3 and D-fructose[18]. Bacterial saccharifying alpha amylase is also known to hydrolyze phenyl α-maltoside by two parallel process[19]; first, hydrolysis between phenol and D-glucose residues to produce phenol and maltose, and second, hydrolysis between two D-glucose residues to produce glucose and phenyl α-D-glucopyranoside. At lower substrate-concentration, the first process is predominant, whereas at higher substrate-concentration, the second preponderates. The alpha amylase of *Asp. oryzae* is reported to hydrolyze **4** to G_2 and phenol[20]. This "cyclodextrinase" hydrolyzes **4** to G_2 and phenol, regardless of its concentration, thus resembling the alpha amylase of *Asp. oryzae*.

The rates of hydrolysis for (1→6), (1→3), and (1→2)-α-D-glucosidic linkages of isopanose, **7** + **8**, and 2-*O*-α-maltosylglucose are respectively 1/2.5, 1/10, and 1/60 of that for the (1→4)-α-D-glucosidic linkage of G_3. Recently, such alpha amylases as bacterial saccharifying, *Asp. oryzae*, human salivary, *Streptococcus bovis*, and *Thermoactinomyces vulgaris* alpha amylase have been reported to be able to hydrolyze (1→6)-α-D-glucosidic linkages[21,22]. These alpha amylases are classified into two groups. Such alpha amylases as bacterial saccharifying, *Asp. oryzae*, human salivary, and *St. bovis* alpha amylase hydrolyze the (1→6)-α-D-glucosidic linkage adjacent to a maltotriose molecule at the side of the non-reducing end. On the other hand, the alpha amylase of *T. vulgaris* hydrolyzes the (1→6)-α-D-glucosidic linkage adjacent to the maltose molecule at the side of the non-reducing end. The "cyclodextrinase" here studied was found to belong to the latter group.

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