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Note

The branched malto-oligosaccharides resulting from the action of *Bacillus macerans* cycloamylose glucanotransferase on 6-*O*-α-D-glucopyranosyl-cyclomaltohexaose plus D-glucose*

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Bacillus macerans cycloamylose glucanotransferase (BME) catalyzes three reactions[1,2]. These are (*1*) cyclization [by which cycloamyloses are formed from (1→4)-α-D-glucans, (*2*) coupling (by which the rings of cycloamyloses are opened and transferred to such cosubstrates as D-glucose, maltose, and sucrose, to afford various oligosaccharides[3]), and (*3*) disproportionation (which is transfer between linear oligosaccharides[4,5]). Furthermore, BME has hydrolyzing activity[6]. Therefore, study of the action mechanism of the enzyme has, because of its complexity, been very difficult.

According to our study, on addition of surfactants, BME can cyclize branched dextrins to branched cycloamyloses. It is difficult for this reaction to proceed on the substrates under the usual reaction conditions[7], because, usually, the branching points interfere with cyclization. The branching points may also interfere with coupling. In fact, the reaction rate of coupling of 6-*O*-α-D-glucopyranosyl-cyclohexaamylose (G-α) with D-glucose is less than one hundredth of the coupling action of cyclohexaamylose with D-glucose; but, by using intense reaction conditions, various oligosaccharides are formed, by coupling and redistribution, from G-α plus D-glucose.

In this report, we have concentrated on determining the structures of these oligosaccharides, in order to acquire more information about the action of BME.

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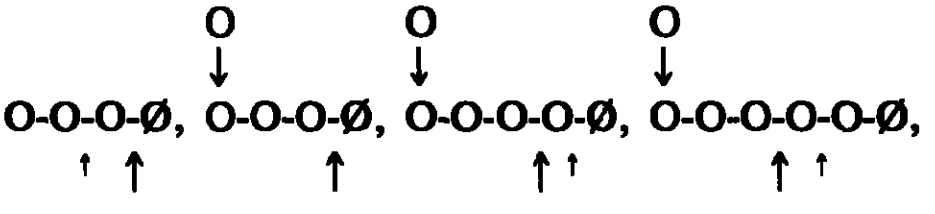
EXPERIMENTAL

Materials. — G-α was prepared from a branched dextrin by the action of BME, and crystallized[7]. D-Glucose (dextrose, anhydrous granular, Mallinckrodt, Inc.) was used after purification by passage of a solution through a charcoal column. Radioactive D-glucose was D-[U-^{14}C]glucose (aqueous solution containing 3% of ethanol, 50 μCi/250 μL, 333 mCi/mmol; Batch 108, the Radiochemical Centre, Amersham, England; and 1 mCi in 1 mL of 50% ethanol, 240 mCi/mmol, Lot No. CR-2867, Schwarz–Mann). BME was prepared, purified, and crystallized by following the method previously reported[6]. The enzyme activity (THU) was determined by the method of Tilden and Hudson[8].

Two hundred μL of the original enzyme suspension of porcine pancreatic alpha-amylase (PPA, 50 mg/5 mL; No. 1227409, Boehringer–Mannheim GmbH, made in West Germany) was dialyzed in 500 mL of 0.1M phosphate buffer, pH 6.57, overnight at 4°, and the dialyzate was used as PPA solution (concentration of the enzyme protein was 1 mg/200 μL). The actions of PPA on various substrates are* as follows: (arrows indicate the linkages split)



Beta-amylase (from *Ipomoea batatas*, 5 mg/mL; No. 1367106) was purchased from Boehringer–Mannheim. The action of the enzyme on (1→4)-α-D-glucans proceeds from the nonreducing ends of the substrates to form maltose, but it does not skip the branching points in such branched substrates as amylopectin.

Pullulanase (from *Aerobacter aerogenes*, crystalline, 10 mg = 400 U/mL, 2.7M ammonium sulfate solution; Code No. EN 200) was kindly donated by Hayashibara Co., Ltd., Okayama, Japan. Pullulanase acts on branched substrates that have a maltosyl, or longer than maltosyl, branch, and does not act on D-glucosyl-branched substrates absolutely.

Original suspensions of beta-amylase and pullulanase were dialyzed in the same way as for PPA, and the concentration of enzyme protein in each enzyme solution used in this study was half that of the original enzyme suspension.

Isopullulanase (1.35 IU in 1 mL of 0.1M acetate buffer, pH 3.50) was kindly donated by Dr. Y. Sakano and Dr. T. Kobayashi, Tokyo Noko University. The actions of the enzyme on D-glucosyl-branched substrates are as follows[9,10]:

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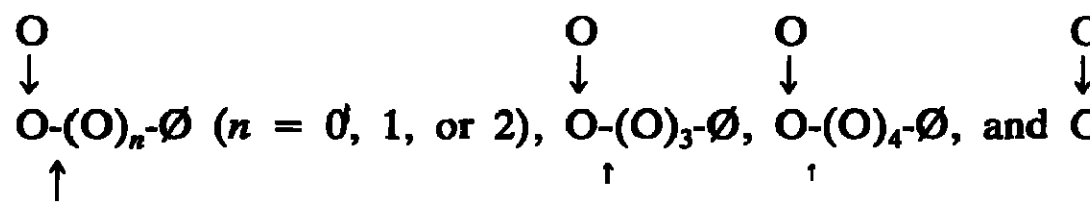
*The symbols G_1, G_2, G_3,$\cdots G_7$ are abbreviations for D-glucose, maltose, maltotriose,$\cdots$maltoheptaose; O, D-glucose unit; Ø, reducing-end D-glucose residue; -, (1→4)-α-D-glucosidic bond; ↓, (1→6)-α-D-glucosidic bond.



Glucoamylase was Cryst., 25 U/mg, Lot. GATC6710, purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

Standard solutions of radioactive, linear malto-oligosaccharides were prepared as follows: Fifty μL (10 μCi) of radioactive D-glucose, 10 mg of cyclo-maltohexaose, and 14 THU of BME were mixed, to make 1 mL of reaction mixture, and reacted for 3 h at 40°. The enzyme was inactivated by keeping it in a boiling-water bath for 15 min. The solution was then used as the standard.

Time course of BME reaction. — Fortly μL of 10% G-α (4 mg) solution, 20 μL of radioactive D-glucose (4 μCi) solution, and 330 μL of water were mixed, 10 μL of BME (1.12 THU) solution was added, and the mixture was allowed to react at pH 6.0 at 40°. Fifty μL of the mixture was periodically taken out and spotted on paper for chromatography (see Fig. 1).

Preparation of reducing-end, ^{14}C-labelled B_5–B_8 fractions. — The sugars were prepared under the following reaction conditions; 400 μL (40 mg) of G-α, 80 μL (80 μCi) of radioactive D-glcose, 1440 μL of water, and 80 μL of BME (44.8 THU) were mixed, reacted for 3 h at 40°, and the enzyme inactivated in a boiling-water

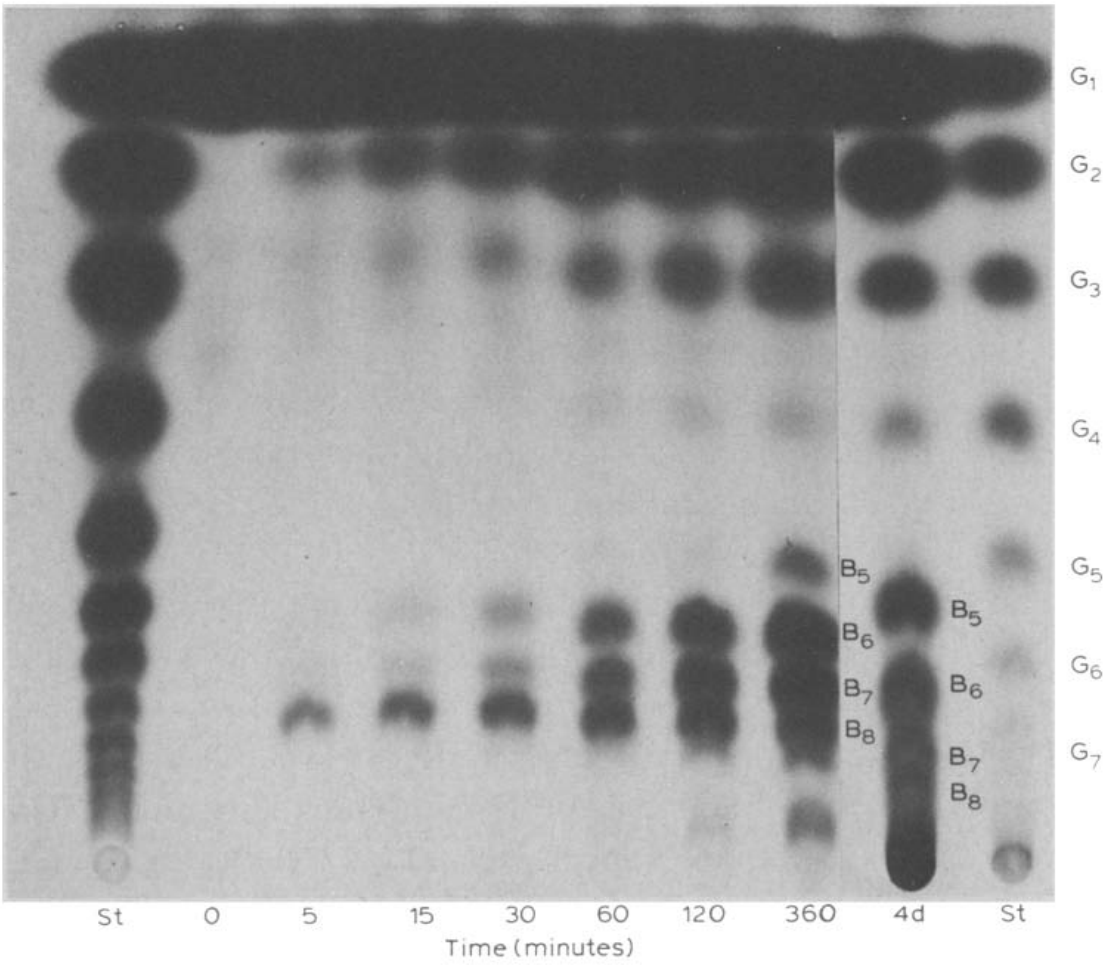


Fig. 1. Time course of BME reaction (autoradiogram). Key: St, spotted 20 μL of radioactive malto-oligosaccharides in standard solution; 4d, reacted for 4 days.

bath for 30 min. Fifty μL (10 IU) of pullulanase solution was added to the mixture, to remove longer chained branches than D-glucose which might be formed by the action of BME, and allowed to react for 4 h at 40°. The mixture (1 mL) was streaked on chromatography paper (Whatman filter paper No. 3, qualitative, 20 cm wide), and the paper was irrigated, and then exposed to make an autoradiogram. Two sheets were used for the preparation. Then, bands of fraction B_5–B_8 were cut off, and eluted with water. Each eluate was evaporated to dryness overnight at 65°, the residue dissolved in 500 μL of water, and the solution streaked on paper 10 cm wide, to purify it; then, autoradiograms were made. In the same way as just described, 500 μL of purified fractions B_5, B_7, and B_8, and 1 mL of B_6 solution were obtained. The relative yields were 100 of fraction B_5, 406 of fraction B_6, 98 of fraction B_7, and 88 of fraction B_8 (compared with the radioactive count of fraction B_5 as 100).

Paper chromatography for autoradiogram. — The paper size was 20 cm long × 25 cm wide (Whatman filter paper No. 3, qualitative). The paper spotted with labelled sample was irrigated 3 times in the solvent system 3:2:2 1-butanol–pyridine–water at 65° (ascending), and the irrigated paper was attached firmly to a sheet of X-ray film (X-Omat R film, XR-5, Kodak, size 35 × 43 cm), and exposed for 3 days.

Determination of the structures of the components of fractions B_5–B_8. — To each sugar solution (50 μL) was added 5 μL (25 IU) of PPA solution; reacted for 4 h at 40°, and the total of each reaction mixture spotted. In the same way, 25 μL (50 IU) of beta-amylase solution was added to 50 μL of each sugar solution. Twenty μL (0.027 IU) of isopullulanase solution and 20 μL of 0.1M acetate buffer (pH 3.50) were added to 50 μL of each sugar solution. Five μL (1 IU) of pullulanase solution was added to 50 μL of each sugar solution, and allowed to react for 4 h at 40°. Autoradiograms were made as in Figs. 2a and 2b. Fraction B_6 solution (200 μL) and 20 μL of glucoamylase solution (0.05 U in 20 μL of 0.1M acetate buffer, pH 5.20) were mixed, and allowed to react at 40°. A 25 μL aliquot of the reaction mixture was periodically taken out and spotted on paper to make the paper chromatogram and the autoradiogram (see Fig. 3).

RESULTS

As in Fig. 1, with regard to branched oligosaccharides formed at the initial stage of the action of BME on the mixture of G-α and D-glucose (5 min), fraction B_8 was the main radioactive oligosaccharide, and after reaction for 4 days, radioactive fraction B_5, fraction B_6, fraction B_7, fraction B_8, and higher radioactive oligosaccharides were also formed. At exhaustive reaction (more than 6 days of reaction), non-radioactive B_4 fraction, which was the smallest branched oligosaccharide, was formed.

Fraction B_5 was found to consist of a single component. It was characterized as 6^4-*O*-α-D-glucosylmaltotetraose by the action of isopullulanase (IPul), which

Fig. 2. Determination of the structure of components in fraction B_5–B_8 by use of various enzymes (autoradiogram). Key: St, spotted 20 μL of radioactive malto-oligosaccharides in standard solution; B_5–B_8, fractions B_5–B_8; Pul, treated with pullulanase; Ipul, treated with isopullulanase; βAm, treated with beta-amylase; and PPA, treated with porcine pancreatic alpha-amylase.

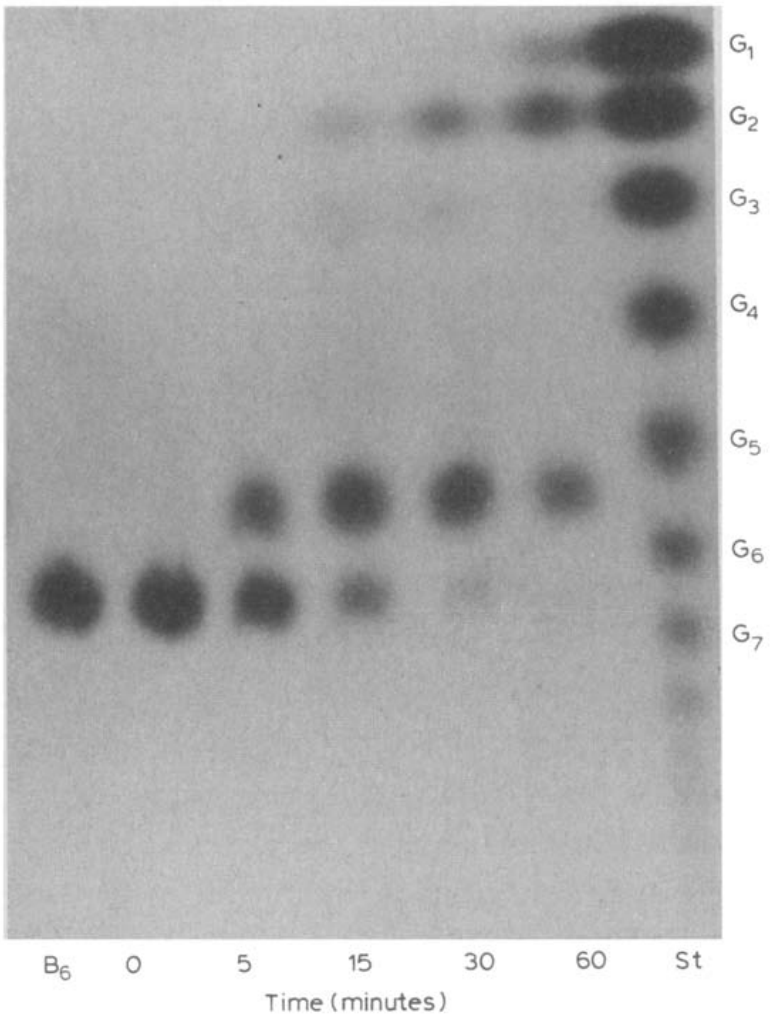


Fig. 3. Determination of the structure of components in fraction B_6 by use of glucoamylase (autoradiogram). Key: St, spotted 20 μL of radioactive malto-oligosaccharide solution; B_6, spotted 25 μL of fraction B_6 solution.

gave isomaltose and linear radioactive G_3 (see Fig. 2a); this was confirmed by use of PPA, which degrades the linkage between the reducing residue and the next D-glucosyl unit of 6^4-*O*-α-D-glucosylmaltotetraose[11].

Fraction B_6 was found to consist of two components, which were mainly 6^4-*O*-α-D-glucosylmaltopentaose mixed with a small proportion of 6^5-*O*-α-D-glucosylmaltopentaose. D-Glucose was the main product from the action of PPA on fraction B_6. This suggests that the structure of the main component in fraction B_6 is 6^4-*O*-α-D-glucosylmaltopentaose, but PPA can also degrade G_2 to G_1, although the action is very slow. If IPul can completely degrade 6^5-*O*-α-D-glucosylmaltopentaose to isomaltose and G_4, it should be possible to determine successfully the amount of the saccharide in fraction B_6. However, the action of the enzyme on B_{6+n} ($n = 0$, $1 \cdot \cdot$) is quite slow[10]. Therefore, by use of IPul, the formation of G_4 merely shows the existence of 6^5-*O*-α-D-glucosylmaltopentaose. As in Fig. 3, the branched oligosaccharide formed from fraction B_6 by the action of glucoamylase was only 6^4-*O*-α-D-glucosylmaltotetraose, with subsequent formation of G_1, G_2, and G_3. This result confirmed that the structure of main component in fraction B_6 is 6^4-*O*-α-D-glucosylmaltopentaose.

Fraction B_7 was found to consist of three components, which were mainly 6^5-*O*-α-D-glucosylmaltohexaose mixed with small proportions of 6^4- and 6^6-*O*-α-D-glucosylmaltohexaose.

By the action of beta-amylase, 6^4-*O*-α-D-glucosylmaltotetraose was formed from fraction B_7. This shows that this fraction contains 6^4-*O*-α-D-glucosylmaltohexaose. Also, by the action of IPul, a trace of G_5 was formed (see Fig. 2b); consequently, the fraction contains 6^6-*O*-α-D-glucosylmaltohexaose. To confirm the structure of the main product, a sample that was free from 6^4-*O*-α-D-glucosylmaltohexaose was prepared as follows: 250 μL of fraction B_7 solution and 50 μL of beta amylase solution were mixed, allowed to react for 24 h at 40°, and then streaked on a 5-cm wide paper. An autoradiogram was made, the band of non-reacted fraction B_7 cut off, and finally, 300 μL of the sample solution was obtained.

Branched oligosaccharide formed from the non-reacted fraction B_7 by the action of glucoamylase at the initial stage of the reaction was only B_6, followed by gradual formation of G_3, G_2, and G_1. This result shows that the main structure of the beta-amylase-nonreactive fraction B_7 is 6^5-*O*-α-D-glucosylmaltohexaose. In addition, the respective percentages of 6^4-*O*-β-D-glucosylmaltotetraose and 6^5-*O*-α-D-glucosylmaltohexaose on the line of βAm in Fig. 2b were 11 and 84, compared with the total radioactive count taken as 100.

Fraction B_8 was also determined in the same way as for the determination of B_7 (see Fig. 2b). From fraction B_8, a considerable amount of 6^4-*O*-α-D-glucosyl maltopentaose was formed by the action of beta-amylase (on the line of βAm in Fig. 2b).

The respective percentages of 6^4-*O*-α-D-glucosylmaltopentaose and 6^6-*O*-α-D-glucosylmaltoheptaose were 29 and 68, compared with the total count taken as 100. Thus, fraction B_8 contains 6^6-*O*-α-D-glucosylmaltoheptaose as the major component, and 6^5- and 6^4-*O*-α-D-glucosylmaltoheptaose as minor components.

Furthermore, by the exhaustive action of BME, 6^4-*O*-α-D-glucosylmaltotetraose was degraded to 6^3-*O*-α-D-glucosylmaltotriose and D-glucose, and 6^3-*O*-α-D-glucosylmaltotriose was completely nonreactive to the enzyme. Consequently, 6^3-*O*-α-D-glucosylmaltotriose is the limit dextrin for BME, and therefore, the smallest, radioactive, branched oligosaccharide was 6^4-*O*-α-D-glucosylmaltotetraose and the non-radioactive one was 6^3-*O*-α-D-glucosylmaltotriose.

DISCUSSION

The coupling reaction of BME had been studied by French's group[1,2], and they found that the oligosaccharides formed from a mixture of cyclomaltohexaose and ^{14}C-labelled D-glucose by the action of BME had radioactivity on the reducing end.

Therefore, it was reasonable to expect that reducing-end-labelled D-glucosyl-branched oligosaccharides would be obtained from the coupling reaction of BME on G-α with ^{14}C-labelled D-glucose.

The structures of the oligosaccharides comprising fractions B_5–B_8 are summarized in Fig. 4. Fraction B_8, formed after reaction for 30 min (see Fig. 1), was collected, and its structure was studied by use of glucoamylase. As a result, it

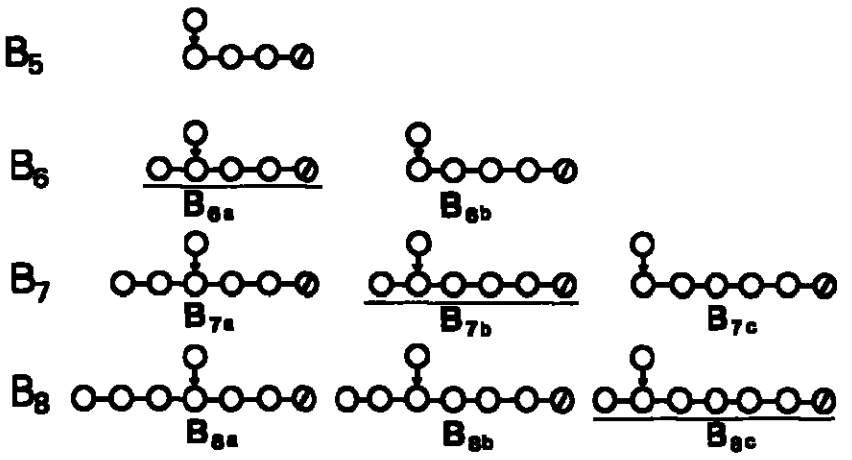


Fig. 4. The structures of the branched oligosaccharides obtained from G-α and radioactive D-glucose by the action of BME. Underlined are main structures, and all of the reducing ends have radioactivity.

was found that 6^4-*O*-α-D-glucosylmaltotetraose, 6^5-*O*-α-D-glucosylmaltopentaose, and 6^6-*O*-α-D-glucosylmaltohexaose are formed. This implies that fraction B_8, formed at the initial stage of the coupling reaction, contains $B_{8a,b,c}$, as shown in Fig. 4. Accordingly, B_{8a} may be easily hydrolyzed by the action of BME from the non-reducing end, to afford B_{7a}, B_{6a}, and B_5. In the same way, B_{8b} may be converted into B_{7b} and B_{6b}, and B_{8c} into B_{7c}, even though, by the disproportionation, various kinds of oligosaccharides may be formed.

As shown in Fig. 1, from the initial stage of the reaction G_2 and G_3 were formed. This implies that BME can also hydrolyze radioactive fraction B_8 from the reducing end.

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