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A synergistic reaction mechanism of a cycloalternan-forming enzyme and a D-glucosyltransferase for the production of cycloalternan in *Bacillus* sp. NRRL B-21195

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Abstract

Cycloalternan-forming enzyme (CAFE) was first described as the enzyme that produced cycloalternan from alternan. In this study, we found that a partially purified preparation of CAFE containing two proteins catalyzed the synthesis of cycloalternan from maltooligosaccharides, whereas the purified CAFE alone was unable to do so. In addition to the 117 kDa CAFE itself, the mixture also contained a 140 kDa protein. The latter was found to be a disproportionating enzyme (DE) that catalyzes transfer of a D-glucopyranosyl residue from the non-reducing end of one maltooligosaccharide to the non-reducing end of another, forming an isomaltosyl residue at the non-reducing end. CAFE then transfers the isomaltosyl residue to the non-reducing end of another isomaltosyl maltooligosaccharide, to form an α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-maltooligosaccharide, and subsequently catalyzes a cyclization to produce cycloalternan. Thus, DE and CAFE act synergistically to produce cycloalternan directly from maltodextrin or starch.

Keywords: Cycloalternan; Isomaltose agarose affinity chromatography; Disproportionating enzyme; Cycloalternan-forming enzyme; Alternanase

1. Introduction

Cycloalternan (**1**), the cyclic tetrasaccharide cyclo[→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(→3)-α-D-Glc*p*-(1→], has alternating α-(1→3) and α-(1→6) linkages.[1] It has a plate-like shape with a shallow depression in the center. The structure is not fully symmetrical because of an asymmetric hydrogen bond.[2] There are numerous reports on cyclic oligosaccharides, the best known being the cyclomaltodextrins (CDs).[3] The cyclomaltodextrins [CDs; cyclic oligosaccharides composed of 6, 7, or 8 D-glucosyl residues linked by α-(1→4) linkages] are used for stabilizing labile materials,[4] masking odors[5,6] and modifying viscosity[7,8] as they have hydrophobic cavities in the center.[9] Some other cyclic oligosaccharides, such as cycloisomaltodextran[10] [cyclic α-(1→6)-linked D-glucooligosaccharide] and cycloinulin[11] [cyclic β-(1→2)-linked D-fructooligosaccharide] are also enzymatically produced by bacteria. Among these cyclic oligosaccharides, only the CDs are produced industrially, mainly because they are made from starch, an abundant and inexpensive

Abbreviations: CAFE, cycloalternan-forming enzyme; DE, disproportionating enzyme; G_n, maltooligosaccharides; HPIC, high performance ion chromatography; IM(1→3)IMG_n, α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-maltooligosaccharides; IMG_n, α-isomaltosyl-(1→4)-maltooligosaccharides; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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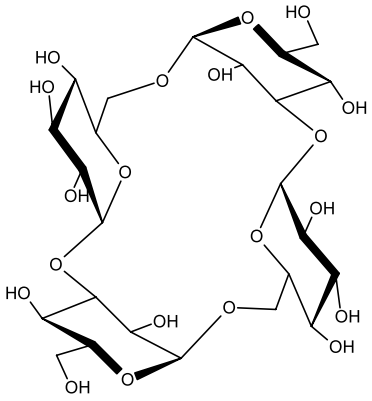
substrate.



Cycloalternan-forming enzyme (CAFE), also known as alternanase, endohydrolytically cleaves an alternating α-(1→3)-α-(1→6)-glucan,[1] alternan,[12] catalyzing a transglycosylation reaction to produce cycloalternan.[13] The CAFE also catalyzes the synthesis of cycloalternan from panose or α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc, apparently via pentasaccharide intermediates, with panose being converted at a much higher rate than the latter.[14] The rate of cycloalternan synthesis from panose is also much higher than that from alternan.[14] CAFE does not react with isomaltotriose and dextran, nor does it react with the trisaccharides α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc and 6-*O*-α-D-glucopyranosyltrehalose. When the tetrasaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc was used as the substrate,[14] it did not produce cycloalternan, but instead produced glucose and α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc, indicating that the hydroxyl group on C-3 of the D-glucopyranosyl group at the non-reducing end of the isomaltosyl group must be free for the transisomaltosylation and cyclization reactions to occur.

Based on the substrate specificities and inhibitory effect of isomaltose, Ahlgren and Côté[15] found that the enzyme could be partially purified from a concentrated cell-free culture fluid, using isomaltose immobilized to an agarose support. The yield was dramatically increased up to 66% by using this method, but the partially purified enzyme mixture contained two protein bands, as shown by SDS-PAGE analysis. It was suggested that the mixture included some other enzyme activity, possibly an α-(1→6)-D-glucosyltransferase. However, the properties and the role of this second enzyme component were not investigated in detail.

More recently, Nishimoto and coworkers[16] described two enzymes [α-(1→6)-D-glucosyltransferase and α-(1→3)-isomaltosyltransferase] involved in the production of cycloalternan from *Bacillus globisporus* C11. These two enzymes acted synergistically to produce cycloalternan from maltodextrins and starch. Using the combined reaction, Aga and coworkers[17] produced 544 g of cycloalternan hydrate as a crystalline powder from 3500 g of partially hydrolyzed starch. Their group also reported that the enzyme genes (*cts*Y and Z) form a gene cluster together with three other enzyme genes (*cts*U, V and W) (GenBank accession no. AB073929).[18]

In the present work, we describe both proteins [(CAFE, 117 kDa) and disproportionating enzyme (DE, 140 kDa)], including their purification and synergistic action to produce cycloalternan from starch and maltodextrins. Both enzymes should be classified into EC group 2.4.1.

2. Experimental

2.1. Materials

Maltooligosaccharides (G_2–G_7) were purchased from Kokusan Laboratory Chemicals (Tokyo, Japan) and Hayashibara Biochemical Laboratories (Okayama, Japan). Panose was purchased from Hayashibara Biochemical Laboratories. IMG_2 [α-isomaltosyl-(1→4)-maltose] used as a standard on high performance ion chromatography (HPIC) was produced by the action of isomaltodextranase from *Arthrobacter globiformis* T6.[19] All other chemicals were of reagent grade and used without further purification.

2.2. Bacterial growth and production of enzyme

Bacillus sp. NRRL B-21195 from National Center for Agricultural Utilization Research (Peoria, IL) was grown at 30 °C on a rotary shaker in a medium described by Ahlgren and Côté.[15] For production of larger amounts of CAFE, cultures were grown in several Erlenmeyer flasks, each containing 1 L of the standard tryptone-soytone medium with glucose. These were inoculated from starter cultures and grown with shaking at 30 °C for 3 days. Cells were removed by centrifugation (17000*g*, 30 min at 4 °C) and the cell-free supernatant was concentrated approximately 50-fold by ultrafiltration with a 5 kDa molecular-weight cut-off hollow-fiber cartridge (Macroza AHP13, Asahi Kasei, Tokyo, Japan). After ammonium sulfate precipitation (50%, w/v), the pelleted protein was dialyzed against 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0).

2.3. Purification of CAFE and DE

CAFE was partially purified by the method of Ahlgren and Côté.[15] Firstly, isomaltose immobilized on 4% agarose beads (Sigma Chemicals, St. Louis, MO) was rehydrated in water and washed with water several times to remove residual lactose. The swollen resin was equilibrated with 50 mM MOPS buffer (pH 7.0) and

transferred onto a column. The dialyzed crude enzyme was passed over the column, followed by washing with 5 column volumes of buffer. The enzyme that had bound to the resin was eluted with 50 mM MOPS buffer containing 0.5 M NaCl and the portion of column eluent showing enzyme activity was collected and used as the partially purified CAFE.

For further purification of the enzymes, the combined active fraction was applied to a DEAE Toyopearl 650S column (1 mL, Tosoh, Tokyo, Japan) equilibrated with 50 mM MOPS buffer (pH 7.0) and eluted with a 0–0.5 M gradient of NaCl (20 mL). The fractions from DEAE Toyopearl showing activity on panose were combined and applied to a MonoQ column (1 mL, Amersham Biosciences, Piscataway, NJ), which was then eluted with a 0–0.5 M gradient of NaCl in 50 mM MOPS buffer. Likewise, the fractions from DEAE Toyopearl showing activity on maltose were combined and purified by the same method. Protein concentration was determined by absorbance at 280 nm, using bovine serum albumin as the standard.

2.4. Enzyme activity

Mixtures were analyzed for their action on panose and maltose to identify both enzyme activities. CAFE activity was determined by measuring the amount of D-glucose liberated from 5 mM panose in 50 mM MOPS buffer (pH 7.0). Theoretically, two panose molecules will produce two D-glucose molecules and one cycloalternan molecule. In the case of DE, the enzyme reaction was performed with 10 mM maltose in 50 mM MOPS buffer (pH 7.0) and the activity was determined by calculating the amount of D-glucose liberated from maltose. Two maltose molecules will produce one glucose molecule and one panose molecule. The amount of D-glucose liberated from each substrate was measured by the glucose oxidase–peroxidase method,[20,21] using a Glucose CII test with mutarotase present (Wako Pure Chemical, Osaka). One unit (IU) of enzyme activity is defined as the amount of enzyme that liberates 1.0 μmol of D-glucose from maltose or panose per minute at 35 °C.

2.5. Preparation of α-isomaltosyl-(1→4)-maltooligosaccharides (IMG_n)

To produce IMG_n, the reaction solution contained 50 mg of each maltooligosaccharide and 2 μg of DE in 5 mL of 50 mM MOPS buffer (pH 7.0). After reaction at 30 °C for 160 h with G_2 or G_3, and 40 h with G_4–G_6, the reaction solutions were heated to 80 °C to inactivate the DE. Amberlite MB3 resin (Organo, Tokyo, Japan) was added to the inactivated reaction solution to remove the ionic compounds and the solutions were lyophilized. Each transfer product having d.p. $= n+1$, arising from maltodextrin G_n, was purified by using a size-exclusion chromatography column (Toyopearl HW40S, 2.5×90 cm, Tosoh) at room temperature with a 1 mL/min flow rate.

2.6. Reactions of CAFE, DE, and partially purified CAFE

To investigate the reaction patterns of DE and CAFE, reactions were monitored chromatographically. Reaction solutions contained 0.1 IU/mL of the partially purified CAFE or the purified DE and 10 mM

Table I
Summary of the purification of CAFE and DE

Step	Volume (mL)	Protein (mg)	Activity (IU)		Specific activity (IU/mg)		Yield (%)
			Panose [a]	Maltose [b]	Panose [a]	Maltose [b]	
Concentrated cell-free culture	50	290	26.1		0.09		100
$(NH_4)_2SO_4$ precipitate	10	30	20.6		0.75		79
Immobilized isomaltose	15	8.9	17.2		2.10 [c]		66
DEAE-Toyopearl							
CAFE	7	3.3	6.5		2.2		25
DE	7	4.3		1.6		0.4	nd
MonoQ							
CAFE	3	2.5	5.2		2.25[d]		20
DE	3	3		1.1		0.41[e]	nd

nd, not determined.
[a] Activity on panose.
[b] Activity on maltose.
[c] Partially purified CAFE.
[d] Purified CAFE.
[e] Purified DE.

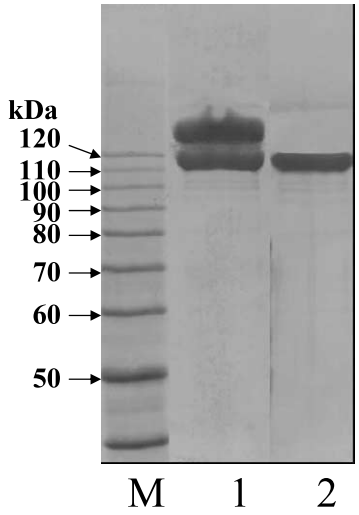


Fig. 1. SDS-PAGE of the partially purified CAFE and the purified CAFE: (1) CAFE partially purified by using isomaltose agarose affinity chromatography; (2) purified CAFE.

maltooligosaccharides, or 0.1 IU/mL of purified CAFE and 5 mM of the appropriate substrates (IMG_n). The reactions were carried out in 50 mM MOPS buffer (pH 7.0) at 35 °C. After stopping the reaction by heat treatment at 80 °C for 10 min, 2 μL samples were analyzed by thin-layer chromatography (TLC) or ion-exchange chromatography (see below).

2.7. Analytical methods

SDS-PAGE was performed by the method of Laemmli[22] using 9% acrylamide gels containing 0.1% SDS. The gel was stained with a 0.1% solution of Coomassie Brilliant Blue R-250 in 50% MeOH–10% AcOH. For the purpose of amino acid sequencing, the enzymes were separated on SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane by using the electro-blotting system (BioRad). N-terminal sequences of the blotted samples were determined using a protein sequencing system (Model G1001A; Hewlett-Packard Co., Corvallis, OR, USA).

TLC (Merck, Germany) was performed on 5 × 7.5 cm silica gel $60F_{254}$ plates, using the double-ascending method with a solvent system of 3:1 MeCN–water. To compare reducing and non-reducing sugars by TLC, two identical plates were prepared. All carbohydrates present were visualized on one with 5% H_2SO_4 in MeOH, and non-reducing sugars were visualized on the other with bicinchoninate reagents.[23]

The reaction products were also analyzed by HPIC (Dionex DX320, Dionex, Sunnyvale, CA) equipped with a Dionex Carbopac PA1 column (4 × 250 mm) as described by Percy and coworkers.[24] The column was eluted with a NaOAc gradient (0–20%).

3. Results

3.1. N-terminal sequences and substrate specificities of partially purified CAFE

The partially purified CAFE (Table I(a)) from isomaltose-agarose affinity chromatography of the concentrated cell-free culture fluid contained two main protein bands, one of 140 kDa and the other of 117 kDa (Fig. 1), indicating that both proteins bind to isomaltosyl groups. The N-terminal sequence of the 117 kDa protein starting from leucine, was LGGIWHDPYGDD-DLYTVQE, which is identical to that of the purified CAFE. The sequence of the 140 kDa protein which started with phenylalanine, was FAAALGNIINVD-TAPD, showing that the 140 kDa protein is a newly identified isomaltose-binding protein (Fig. 1). When compared with the N-terminal sequences of α-(1→3)-isomaltosyltransferase (IDGVYHAPYGIDDLYEIQS) and α-(1→6)-D-glucosyltransferase (YVSSLGN LISSSVTGD) from *B. globisporus* C11,[18] CAFE showed high similarity to the former (10/19 a.a.) and DE showed similarity (4/16 a.a.) to the latter. When the

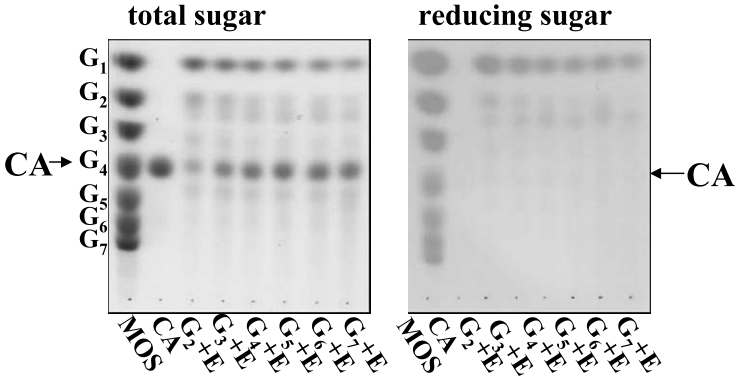


Fig. 2. Thin-layer chromatogram showing the action of partially purified CAFE on maltooligosaccharides (G_2–G_7). Total sugar was visualized with 5% sulfuric acid in methanol; reducing sugar was visualized with bicinchoninate reagents.

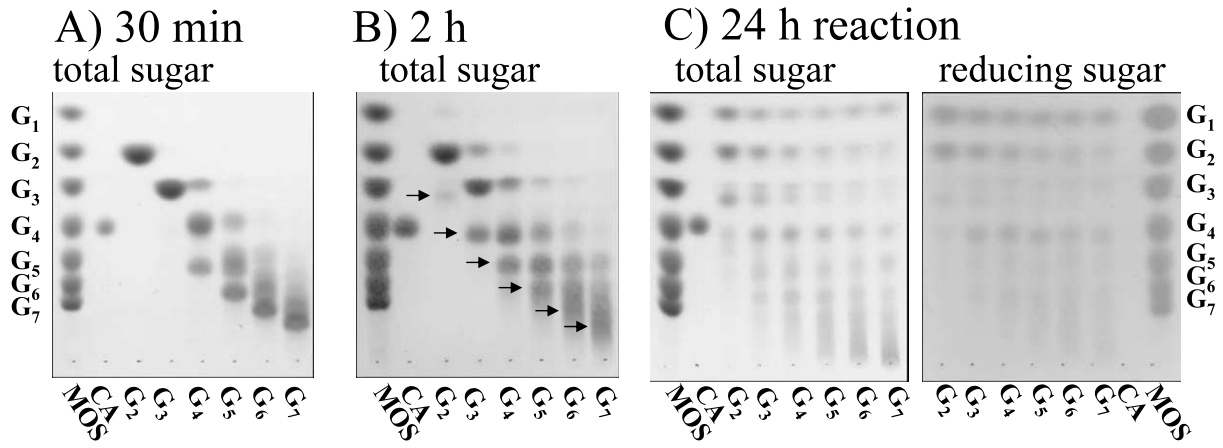


Fig. 3. Thin-layer chromatogram showing the action of the purified DE on maltooligosaccharides (G_2–G_7). Two microliters of the reaction products were spotted on the TLC plates after reaction for 30 min (A), 2 h (B), and 24 h (C). After double-ascending chromatography, the TLC plates were visualized with 5% sulfuric acid in methanol or with the bicinchoninate reagents. The products showing a single glucose higher d.p. than the starting substrates, indicated by arrows, were purified by size-exclusion chromatography and checked for reactivity with purified CAFE in Figs. 5 and 6. MOS, maltooligosaccharides; CA, cycloalternan.

partially purified CAFE was reacted with maltooligosaccharides, which did not react with purified CAFE, the substrates were completely consumed in the reaction within 24 h, leaving two main products with the same R_f values as cycloalternan and D-glucose, and several minor products migrating on TLC between maltose and maltotriose. Based on the TLC analysis (Fig. 2), the main reducing saccharide is presumed to be glucose and the other, non-reducing product, to be cycloalternan. This indicates that the partially purified CAFE produces some intermediates that can be used as substrates by the purified CAFE. Côté and Ahlgren[14] showed that the purified CAFE requires isomaltosyl residues to produce cycloalternan. Based on the substrate specificity of CAFE, it is strongly indicated that the intermediates produced by the partially purified CAFE must have an isomaltosyl group at the non-reducing end. It is further indicated that the 140 kDa protein contained in the partially purified CAFE fraction possesses α-(1→6)-D-glucosyltransferase activity (DE).

3.2. Reaction of 140 kDa protein (DE) with maltooligosaccharides

Reaction products of the DE (Table I[c]) from each maltooligosaccharide were determined by TLC after 0.5,

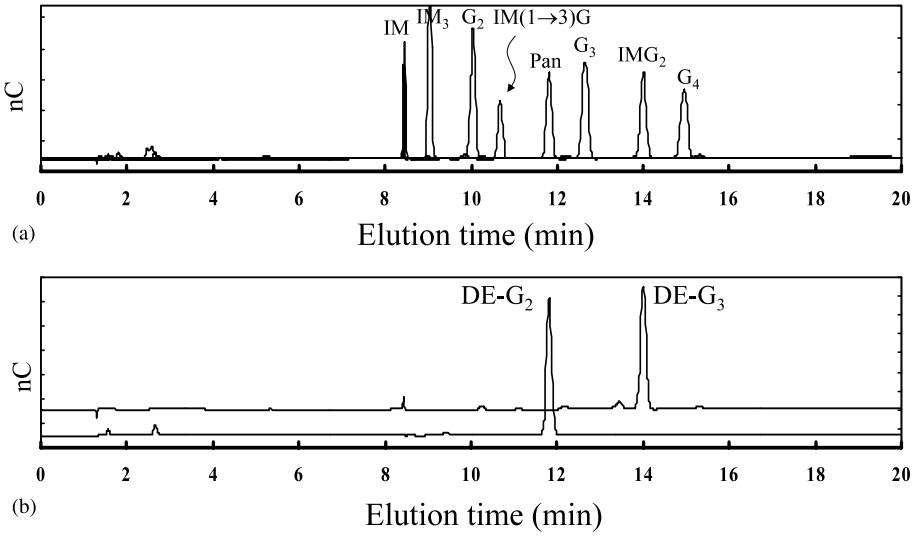


Fig. 4. HPIC of the purified DE products. A, Standards; B, Reaction products of DE reactions. IM, isomaltose; IM3, isomaltotriose; IM(1→3)G, α-isomaltosyl-(1→3)-D-glucose; Pan, panose; G_3, maltotriose; IMG_2, α-isomaltosyl-(1→4)-maltose; G_4, maltotetraose. DE-G_2 and DE-G_3, products of DE reaction showing a single D-glucose higher d.p. than the starting substrates G_2 and G_3, respectively.

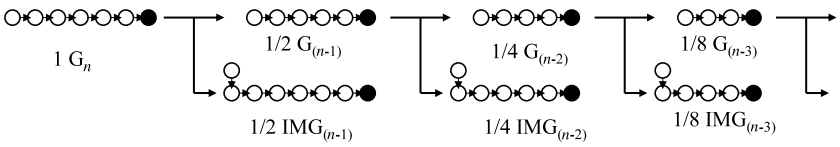


Fig. 5. Reaction scheme of DE on maltooligosaccharides: Open circle, D-glucopyranosyl residue; closed circle, D-glucose residue at reducing end; horizontal arrow, α-(1→4) linkage; vertical arrow, α-(1→6) linkage.

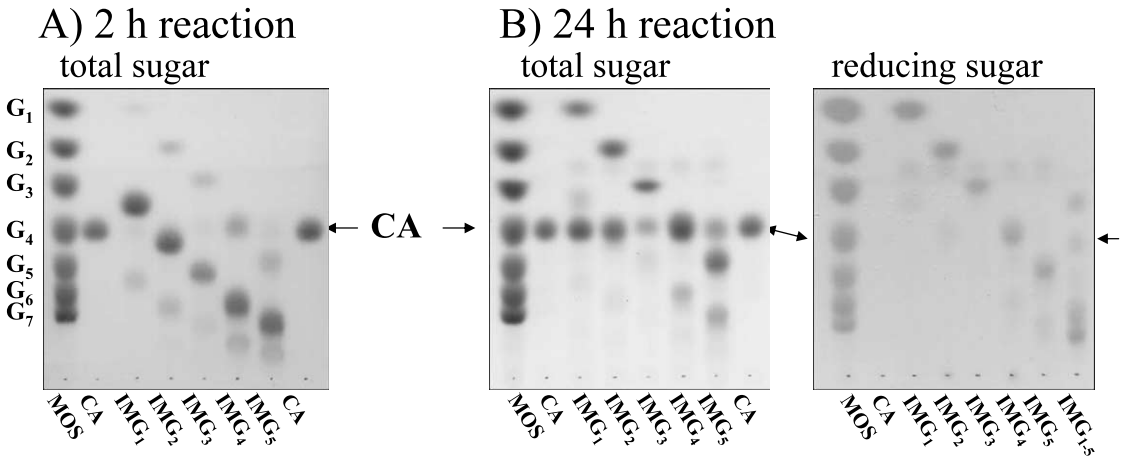


Fig. 6. Thin-layer chromatogram showing the action pattern of purifed CAFE on IMG_n. The reaction was stopped after 2 h (A) and 24 h (B) and 2 μL of the reaction products were spotted and analyzed on the TLC plates. Total sugar was visualized with 5% sulfuric acid in methanol; reducing sugar was visualized with bicinchoninate reagents. MOS, maltooligosaccharides; CA, cycloalternan.

2, and 24 h reaction times. In the earlier stage (30 min) of the reaction with maltooligosaccharides of d.p. ≥4, two main products appear, one with a single glucose added to the starting substrate and the other with one glucose unit less than the starting substrate. The same kinds of reaction products were also detected in the reaction with G_2 and G_3 after longer reaction times (24 h), showing that the reaction is more rapid with higher d.p. maltooligosaccharides (Fig. 3). When the reaction products with higher d.p. than those of the substrates used were purified and analyzed by HPIC, the reaction products from G_2 and G_3 showed the same retention times as panose (IMG_1) and IMG_2, respectively, showing that the transfer products must contain α-(1→6) linkages at their non-reducing ends (Fig. 4). This indicates that the DE is an enzyme that catalyzes the transfer of one D-glucosyl residue from the non-reducing end of G_n to the C-6 position of another identical substrate molecule to form $G_{(n-1)}$ and $IMG_{(n-1)}$. This transfer reaction proceeds until none of the maltooligosaccharides remain, ultimately producing a series of isomaltosyl maltooligosaccharides (Fig. 3(C) and Fig. 5).

3.3. Action pattern of the purified CAFE on IMG_n

Each IMG_n was checked for its ability to act as a substrate for purified CAFE (Fig. 6). The enzyme (Table I[b]) was capable of acting on all of these substrates, initially (2 h) producing two product series, one with the same R_f values as maltooligosaccharides and the other with somewhat lower R_f values than the added IMG_n (Fig. 6(A)). After 24 h, the latter compounds were consumed and cycloalternan and maltooligosaccharides were produced (Fig. 6(B)). From these results, it appears that the products from maltooligosaccharides are α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-maltooligosaccharides [IM(1→3)IMG_n] (Fig. 7).

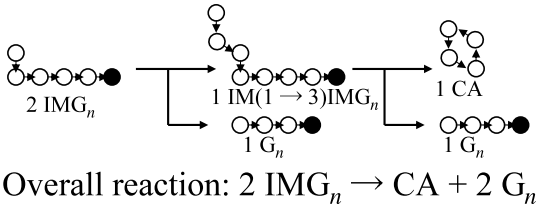


Fig. 7. Reaction scheme of purified CAFE on IMG_n. Open circles, D-glucopyranosyl residue; closed circles, D-glucose residue at reducing end; horizontal arrow, α-(1→4) linkage; vertical arrow, α-(1→6) linkage; diagonal arrow, α-(1→3) linkage. CA, cycloalternan; IMG_1, α-isomaltosyl-(1→4)-D-glucose (panose); IMG_2, α-isomaltosyl-(1→4)-maltose; IMG_3, α-isomaltosyl-(1→4)-maltotriose; IMG_4, α-isomaltosyl-(1→4)-maltotetraose; IMG_5, α-isomaltosyl-(1→4)-maltopentaose.

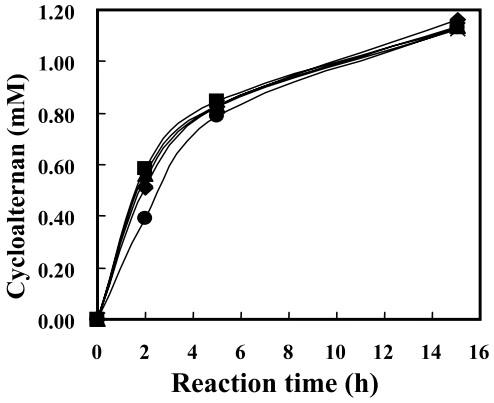


Fig. 8. Time course of the production of cycloalternan from IMG_n by the action of CAFE. The reaction solution containing 5 mM of each substrate (IMG_1–IMG_5) was diluted 50-fold and analyzed by HPIC (Dionex Carbopac PA1 column). Circle, IMG_1; square, IMG_2; triangle, IMG_3; ×, IMG_4; diamond, IMG_5.

3.4. Time course of the production of cycloalternan from IMG_n

Each IMG_n was reacted with CAFE, and the amount of cycloalternan produced from each substrate was determined using HPIC. Except in the case of IMG_1 (panose), which exhibited slightly lower reactivity, the production of cycloalternan from IMG_n by CAFE proceeded at identical rates, regardless of d.p. (Fig. 8).

4. Discussion

Côté and Biely[1] reported the direct production of cycloalternan from alternan by the action of CAFE (Fig. 9(A)). In the reaction of the purified CAFE with panose, Côté and Ahlgren[14] suggested that the initial transfer product was α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-D-glucose [IM(1→3)IMG_1]. In the present study we propose another pathway for the production of cycloalternan in conjunction with the DE (Fig. 9(B)). The DE is apparently an α-(1→6)-D-glucopyranosyltransferase, transferring a D-glucopyranosyl residue from the non-reducing end of one maltooligosaccharide to position 6 at the non-reducing end of another molecule of maltooligosaccharide to produce an isomaltosyl groups at the non-reducing end [$IMG_{(n-1)}$]. The overall reactions of DE are illustrated in Fig. 5. The reaction product of DE is then used as a substrate by CAFE. CAFE initially forms α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-maltooligosaccharide [IM(1→3)IMG_n] by an intermolecular α-(1→3)-transisomaltosylation. From these initial transfer products, CAFE catalyzes an intramolecular transglycosylation (cyclization) to produce cycloalternan. The overall reactions of CAFE are illustrated in Fig. 7. The maltooligosaccharide, which is released is reused as the substrate of DE until no further substrate is available (Fig. 9). Although DE prefers higher d.p. maltooligosaccharides for the

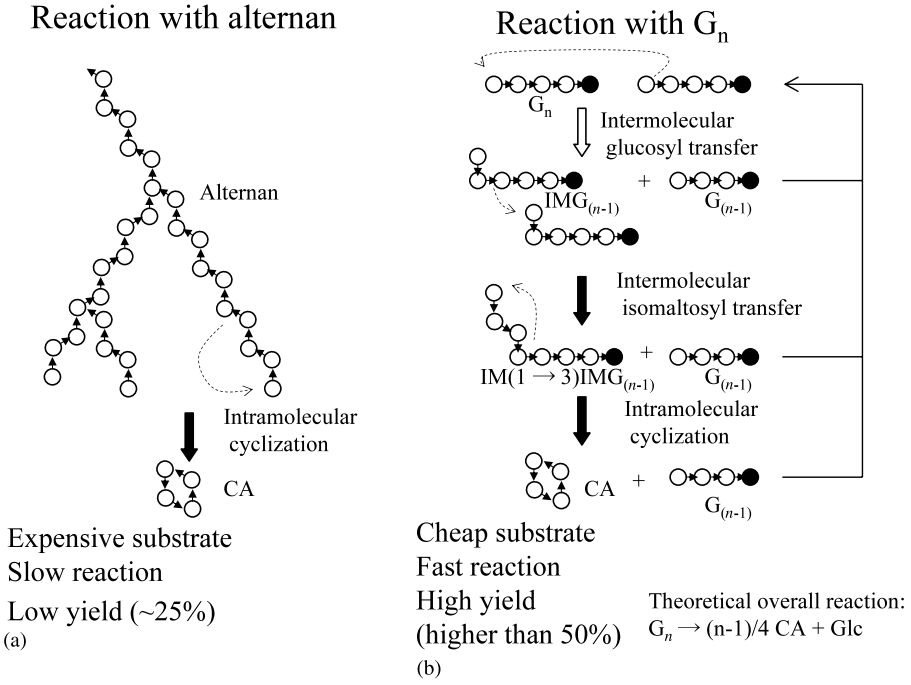


Fig. 9. Two pathways for the CAFE-catalyzed synthesis of cycloalternan. A, synthesis of cycloalternan from alternan by CAFE; B, synthesis of cycloalternan from maltodextrin or starch by synergistic action of CAFE and DE. CA, cycloalternan; closed broad arrow, catalyzed by CAFE; open broad arrow, catalyzed by DE; open circle, glucopyranosyl residue; closed circle, glucose residue at reducing end; horizontal arrow, α-(1→4) linkage; vertical arrow, α-(1→6) linkage; diagonal arrow, α-(1→3) linkage; broken line with arrow, direction of transglycosylation.

formation of IMG_n (Fig. 3(A)), CAFE is not highly dependent on the d.p. of G_n connected to the isomaltosyl residue (Fig. 6(A)). These results show that the synergistic pathway of DE and CAFE for production of cycloalternan is suitable for utilizing maltodextrins (Fig. 9).

The synergistic reaction system described here is presumably involved in the metabolism of starch by strain NRRL B-21195. Recently, Nishimoto and coworkers[16] reported a similar mechanism for the production of cycloalternan by *B. globisporus* C11.[16] The two-enzyme system for production of cycloalternan may be widespread among bacteria. A comparable synthetic mechanism involving two enzymes was reported for the synthesis of trehalose by *Arthrobacter* sp. Q36. In that reaction mechanism, maltooligosyltrehalose synthase (EC 5.4.99.15) first synthesizes a trehalose residue at the reducing end of maltooligosaccharides by transfer of a D-glucopyranosyl residue.[25,26] Maltooligosyltrehalose trehalohydrolase [4-α-D-[(1→4)-α-D-glucano]trehalose glucanohydrolase; EC 3.2.1.141] subsequently reacts with this substrate to produce trehalose and maltooligosaccharides.[27]

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