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Structure of the cyclic glucan produced from amylopectin by *Bacillus stearothermophilus* branching enzyme

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Abstract

The thermostable branching enzyme (BE, EC 2.4.1.18) from *Bacillus stearothermophilus* TRBE14 produces large cyclic glucans from waxy rice amylopectin similar to those obtained from amylose as described elsewhere [H. Takata, T. Takaha, S. Okada, M. Takagi, and T. Imanaka, *J. Bacteriol.*, 178 (1996) 1600–1606]. The structure of the product (P-1) from the late-stage reaction was analyzed in detail. The weight-average degree of polymerization ($\overline{dp}w$) of P-1 was 900. Its chain-length distribution was not significantly changed compared with that of amylopectin, although the amount of long chains (dp > 38) was slightly decreased. The cyclic component of P-1, which was isolated by the extensive action of glucoamylase, had $\overline{dp}w$ of 49. Three point five α-1,6 linkages were directly involved in the formation of the ring structure with several non-cyclic side chains linked to the ring. Based on these results, the action and new roles of BE are discussed. © 1996 Elsevier Science Ltd.

Keywords: Branching enzyme; Cyclization; Amylopectin; Glycogen

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1. Introduction

Starch, the major reserve polysaccharide of plants, consists of two components, amylose and amylopectin. Amylose is an essentially linear glucan, whereas amylopectin is highly branched. A cluster model (Fig. 1A, [1]) has been proposed for the molecular structure of amylopectin. The branching enzyme (BE, 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-glucano)-transferase, EC 2.4.1.18) synthesizes α-1,6-glucosidic linkages of amylopectin or glycogen [2,3]. The branching reaction [4–6] catalyzed by BE has been extensively studied and is considered to play an important role in determining the structure of amylopectin or glycogen [3]. We have cloned and sequenced the structural gene for the thermostable BE from *Bacillus stearothermophilus* TRBE14, and analyzed the active center and properties of the enzyme [7]. Recently, we reported that BE and D-enzyme (disproportionating enzyme, EC. 2.4.1.25) catalyze the cyclization of amylose [8,9].

In this paper, the action of BE on amylopectin was analyzed. The enzyme degraded amylopectin molecules to a large limited size by cyclization (Figs. 1 and 2). The detailed structure of the product from amylopectin at a late-stage of the reaction is described. The main product was cyclic glucans constructed from a cyclic part and non-cyclic side

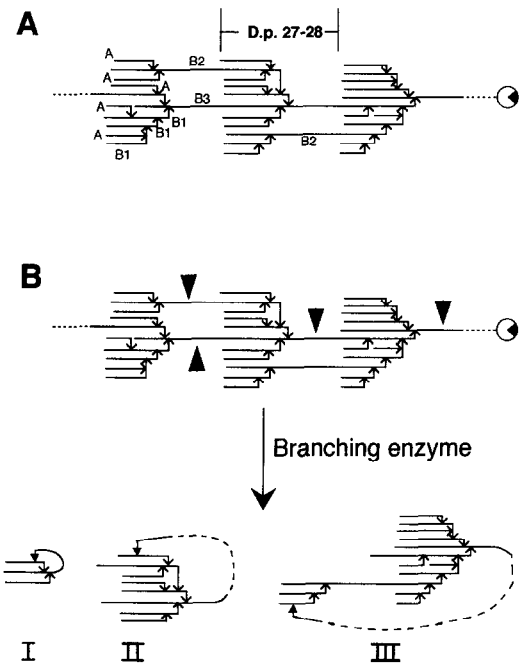

Fig. 1. Cluster structure of amylopectin, and action model of BE on amylopectin. (A) Cluster structure of amylopectin described by Hizukuri [1] are shown. (B) The action model of BE on amylopectin. Product I, II, and III are derived from a piece of cluster, one cluster unit, and two cluster units, respectively. ↓, α-1,6-glucosidic linkage; ↓, α-1,6-glucosidic linkage newly synthesized by BE; ◔, glucosyl residue with reducing end; ▼, attack point by BE.

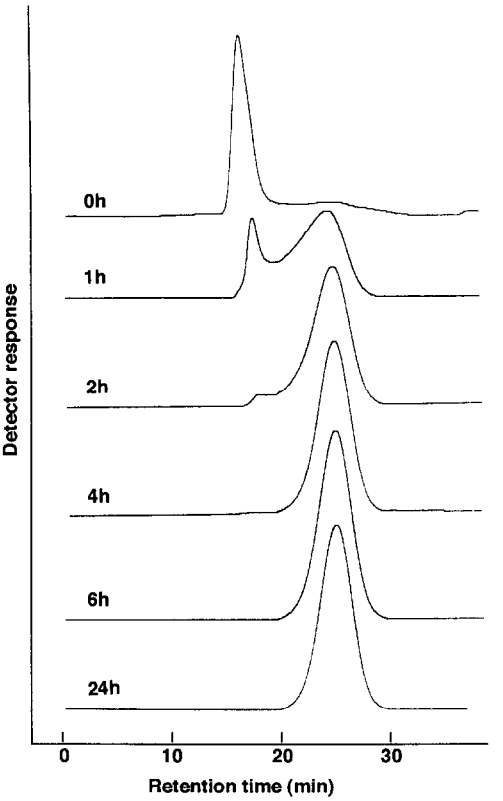

Fig. 2. Time course of action of BE on amylopectin. Amylopectin (0.4% (w/v)) was treated with 400 U/g substrate of BE at 50 °C. At intervals, the sample was removed and analyzed by gel-filtration chromatography (Superose 6 plus Superdex 30 columns) with a differential refractometer.

chains (Fig. 1B, I–III). The results demonstrated that the BE split the inner chains connecting the cluster units of amylopectin and transferred the cluster unit (multi-branched glucan) to another site on the same substrate molecule (intramolecular transfer). We believe that BE could also transfer the multi-branched glucan to other molecules (intermolecular transfer). Based on these findings, we discuss the new roles of BE on amylopectin or glycogen metabolism.

2. Experimental

Substrate and enzymes.—Amylopectin was prepared from waxy rice by the alkali method described by Yamamoto et al. [10]. Short chain amylose (average dp 17) was obtained from Hayashibara Biochemical Lab., Okayama. The BE from *Bacillus stearothermophilus* TRBE14 was purified to homogeneity from a cell extract of *Escherichia coli* MC1061Δglg carrying plasmid pTBE821 as described previously [7]. Glucoamylase of *Rhizopus* sp. was purchased from Toyobo, Osaka. Isoamylase was

obtained from Hayashibara. Bacterial α-amylase (saccharifying type) was donated from Nagase Biochemicals, Osaka. β-Amylase of sweet potato (Type I-B) was purchased from Sigma, St. Louis, MO, and purified by anion-exchange chromatography to remove α-glucosidase activity [11].

Assay of BE activity.—The BE activity was assayed by the method described previously [7] with slight modifications. The enzyme solution (50 μL) was mixed with the substrate solution (0.1% (w/v) amylose, 50 μL), and incubated at 50 °C for 30 min. The substrate solution was prepared by mixing 100 μL of 1% (w/v) type III amylose (Sigma) dissolved in dimethyl sulfoxide, 100 μL of M Tris–HCl (pH 7.5), and 800 μL of distilled water. Reactions were terminated by adding 1 mL of 0.4 mM HCl, and then 1 mL of iodine reagent was added to the solution. The iodine reagent was made daily by diluting 0.5 mL of a stock solution (0.26 g of I_2 and 2.6 g of KI in 10 mL of water) to 65 mL with distilled water. One unit of enzyme activity was defined as the amount of BE that decreases the absorbance at 660 nm by 1% per min.

Preparation of the cyclic material (P-1) from waxy rice amylopectin by the action of BE.—The reaction mixture (500 mL) consisted of 2 g of amylopectin in 20 mM sodium phosphate buffer (pH 7.5) and 800 U of BE. The mixture was incubated at 50 °C for 16 h, and heated at 100 °C for 15 min after eliminating oxygen by bubbling helium. The product was precipitated by adding 2 vol EtOH and then lyophilized. The yield of the product (P-1) was 98%.

Average degree of polymerization (dp) of P-1.—Weight-average dp ($\overline{dpw}$) is calculated from weight-average molecular weight ($\overline{M_w}$) by $\overline{M_w}/162$, and number-average dp ($\overline{dpn}$) is from number-average molecular weight (M_n) by $M_n/162$. The $\overline{dpw}$ was determined by a HPLC–LALLS–RI (high-performance liquid chromatography with a low-angle laser-light-scattering photometer and a differential refractometer) system which was similar to that described elsewhere [12,13]. The system is constructed with a HPLC pump (803D, Tosoh, Tokyo), a differential refractometer (RI-8011, Tosoh), a low-angle laser-light scattering photometer (LS-8000, Tosoh), and three tandem columns of Asahipak GS-520 (7.6 × 500 mm, Showa Denko, Tokyo), Asahipak GS-320 (7.6 × 500 mm, Showa Denko), and TSK-gel G2000PW (Tosoh). Elution was carried out at 40 °C with 0.1 M phosphate buffer (pH 6.1) containing 0.02% NaN_3 and 1.5% acetonitrile at a flow rate of 0.5 mL/min. Specimens (0.2 mL, 4 mg/mL) were injected into the HPLC after filtration through 0.22 μm membrane (Millex-GS, Millipore). Pullulan (MW 98,000, Hayashibara) was used as a standard. The $\overline{dpn}$ was determined from the ratio of total sugar to reducing sugar.

Isoamylolysis of amylopectin or P-1.—Isoamylolysis was performed at 45 °C for 3 h in 2.5 mL of 50 mM acetate buffer (pH 3.5) containing 10 mg of amylopectin or the P-1 and 1.2 U of isoamylase. One unit of the isoamylase activity was the amount of enzyme that releases 1 μmol of the reducing terminal from amylopectin per min at 40 °C. The isoamylolysis was complete because the reducing value due to the hydrolysis was constant in the incubation for 0.5–5.0 h.

High-performance anion exchange chromatography with a pulsed amperometric detector (HPAEC–PAD).—HPAEC was carried out with a Dionex DX-300 system (Dionex, CA) using a PAD (Model PAD-II). The column was a CarboPac PA-100 (4 mm × 250 mm) with a PA-100 guard column. Samples (25 μL) containing 0.2–0.5%

(w/v) glucan were injected and eluted at a flow rate of 1 mL/min with the following gradient: 0–2 min, 95% (v/v) eluent A and 5% (v/v) eluent B; 2–37 min, ratio of eluent B was increased up to 35% (v/v) with the curved gradient program 3; 37–45 min, ratio of eluent B was increased up to 85% (v/v) with the curved gradient program 7; 45–47 min, 15% (v/v) eluent A and 85% (v/v) eluent B. Eluent A was 150 mM NaOH, and eluent B was 150 mM NaOH containing M NaOAc. The curved gradient programs (3 and 7) used were prearranged and installed programs for the Dionex DX-300 system.

Glucoamylase (GA) treatment to obtain the GA-resistant glucan.—The reaction mixture (5 mL) consisting of 500 mg of the P-1 and 50 U of GA in 100 mM NaOAc acetate buffer (pH 5.5) was incubated at 40 °C for 16 h. The solution was heated at 100 °C for 3 min, and centrifuged to remove denatured GA. The 94% of the glucan was hydrolyzed to glucose with this GA treatment. Then, 10 vol EtOH was added to the supernatant, and the polysaccharide was collected by centrifugation (11,000 × g, 15 min). This cycle (GA treatment, inactivation of GA, and precipitation with EtOH) was repeated 15 times. Finally, 13 mg of GA-resistant glucan was derived. Since one more GA treatment of it scarcely released glucose ($< 0.4\%$ (w/w)), we concluded that the GA treatment was complete. On the other hand, no glucan (< 0.1 mg) was obtained from amylopectin (500 mg) after GA treatment. The GA-resistant glucan was eluted in 13 to 43 min by HPAEC (Fig. 5A). The elution time corresponds to the linear α-1,4 glucans with dp > 11.

Other procedures.—Non-reducing terminal residues were quantified after the rapid Smith-degradation reaction [14]. Glycerol produced from non-reducing ends of glucans was quantified using a glycerol assay kit (Boehringer Mannheim, Mannheim). Reducing terminal residues were quantified by the modified Park–Johnson method [15]. β-Amylolysis limit was determined as described by Takeda et al. [16]. The absorption spectrum of iodine–glucan complex was obtained from 1 mL of glucan solution (0.5 mg/mL) mixed with 3 mL of distilled water and 1 mL of iodine reagent (1% (w/v) KI, 0.1% (w/v) I_2). Unless otherwise specified, all chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

3. Results

The time course of the BE reaction on amylopectin was analyzed by gel-filtration chromatography (Fig. 2). The size of substrate was diminished during the early stage of the reaction without an increase in reducing power (< 1 μg/mL). After 4 h of reaction, the elution profile did not change significantly. These results suggested that amylopectin was degraded by a cyclization reaction (Fig. 1B). The product (P-1) from the late stage of the reaction was subjected to the structural analyses. Its elution profile resembled those of the 4–24 h samples shown in Fig. 2 (data not shown). P-1 eluted in a single peak by HPLC using Superose 6 plus Superdex 30 columns as shown in Fig. 2. However, it was separated to 3 fractions, EL, L, and S by HPLC–LALLS–RI (Fig. 3). Table 1 shows the $\overline{dp}w$ of fractions and their contents. A small amount of fraction EL having a large molecular weight may be a product liberated when the action of BE is

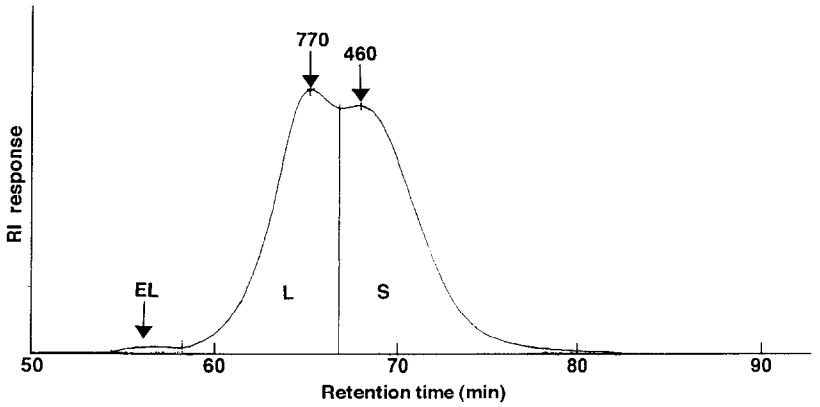

Fig. 3. Analysis of P-1 with HPLC–LALLS–RI. The elution profile monitored by RI is shown. Peak-pointing numbers are peak dp.

insufficient. The peak dp of fractions L and S were 770 and 460, respectively, which were 1 to 3 orders less than the estimated dp of amylopectin [17,18].

P-1 was completely debranched with isoamylase, and its chain-length distribution was compared with that of amylopectin by HPAEC (Fig. 4). In spite of the large decrease in the whole molecular size, the chain-length distribution was only slightly changed as follows. The chains with medium length (dp 22–35) of P-1 increased, and the long chains (dp > 38) decreased. In particular, the long chains (dp > 60) which eluted together as a peak at 42 min were not detected in P-1 (data not shown). These results indicated that the BE acted mainly on the long B chains of B2-, B3-, and B4-chains.

The structural parameters of P-1 and amylopectin are summarized in Table 2. P-1 showed almost no detectable reducing value. The $\overline{dp}n$ of P-1, assuming it to be a non-cyclic glucan, was 5900 but the $\overline{dp}w$ was 900. This discrepancy implies that P-1 is mainly cyclic glucan having no reducing ends.

If the enzyme acted on outer chains of amylopectin, the β-amylolysis limit would decrease. However, the β-amylolysis actually increased, although only slightly. This result also suggested that most of the BE action occurred on the inner chains of amylopectin (B2-, B3-, and B4-chains).

The cyclic component of P-1 was isolated by the extensive action of glucoamylase (GA). Fig. 5 shows the HPAEC profile of the GA-resistant glucan before and after

Table 1
Weight-average dp of fractions of P-1 and their contents

Fraction	$\overline{dp}w$	Content (%)
EL	17,000	0.79
L	1210	47.1
S	380	52.1
Whole	900	100

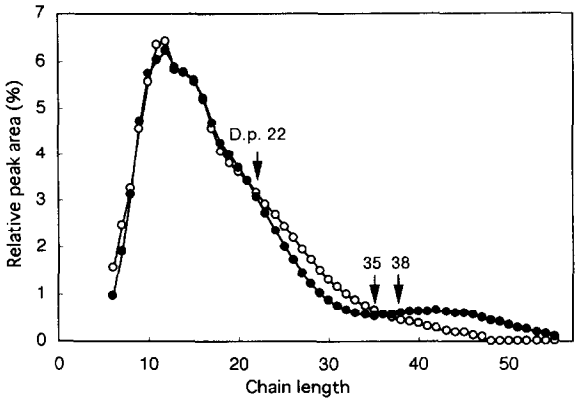

Fig. 4. Chain-length distributions of amylopectin and P-1. The unit chains of amylopectin and P-1 were separated by using HPAEC. The ratio of each peak area to the sum of the peak areas (dp 6–55) is shown. ●, amylopectin; ○, P-1.

treatments with various amylases. The $\overline{dpw}$ of the GA-resistant glucan was determined as 49 by HPLC–LALLS–RI. Since the GA can not hydrolyze the α-1,6 linkages of glucosyl–cyclodextrin [19,20], it was expected that the GA-resistant molecule from P-1 would have a cyclic structure with glucosyl stubs. We quantified the glucosyl stubs linked to the glucan, and α-1,4 and α-1,6 linkages constructing the ring structure as follows.

Isoamylase hydrolyzes α-1,6 linkages but is unable to release the glucosyl stubs [21]. The number of α-1,6 linkages constructing the ring structure was measured by quantifying reducing sugars after isoamylolysis of the GA-resistant glucan. The ratio of reducing sugar to total sugar was 7.1%. By the rapid Smith-degradation of GA-resistant glucan,

Table 2
Structural parameters of amylopectin and P-1

Parameters	Amylopectin	P-1
$\overline{Dpw}$	N.D. [a]	900
$\overline{Dpn}$ [b]	N.D.	5900
α-1,6 Linkage [c] (%)	5.2	6.3
$\overline{C.I.n}$ [d]	19	16
β-Amylolysis (%)	56	59
$\overline{E.c.l.}$ [e]	13	11
$\overline{I.c.l.}$ [f]	5	4
λ_{max} (nm)	520	489

[a] Not determined.
[b] Assuming non-cyclic structure.
[c] Molar ratio of α-1,6 linkage, [non-reducing end residues]/[total sugars as glucose]×100.
[d] Number-average chain length, $\overline{c.l.n}$ = [total sugars as glucose]/[non-reducing end residues].
[e] Exterior chain length, $\overline{e.c.l.} = \overline{c.l.n} \times$ β-amylolysis (%)/100 + 2.
[f] Interior chain length, $\overline{i.c.l.} = \overline{c.l.n} - \overline{e.c.l.} - 1$.

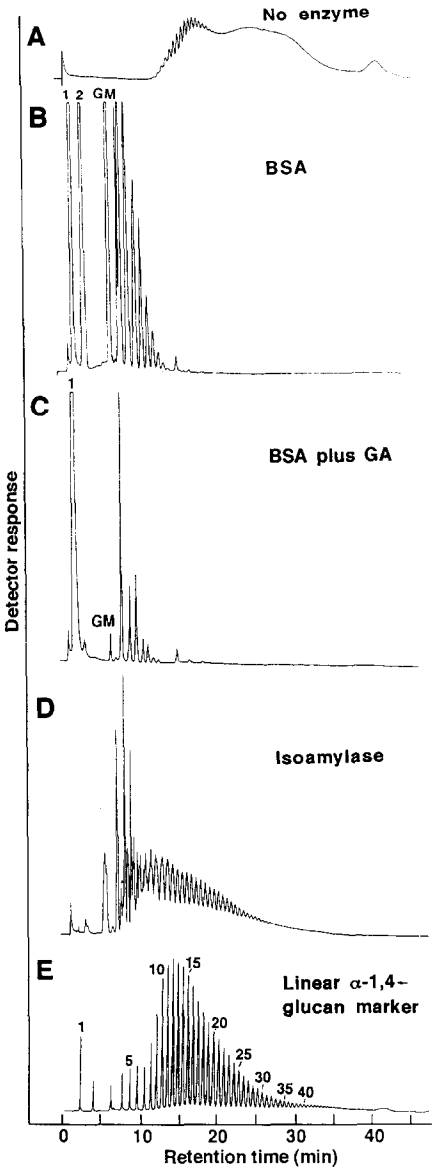

Fig. 5. Treatment of the GA-resistant glucan with various amylases. The GA-resistant glucan was digested with various amylases to analyze the structure. The elution profile of the GA-resistant glucan is shown in (A). The glucan was treated with 1.7 U/mL bacterial α-amylase (saccharifying type, BSA) (B), or 1.7 U/mL BSA plus 10 U/mL GA (C), or 1 200 U/mL isoamylase (D). The digests were analyzed by HPAEC. The peaks of glucose, maltose, and 6^3-*O*-α-glucosyl-maltotriose (Glcα1 → 6Glcα1 → 4Glc α1 → 4Glc) are indicated as 1, 2, and GM, respectively. The elution profile of linear α-1,4 glucan marker (mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose, and short chain amylose) is also indicated (E). The peak pointing number is dp.

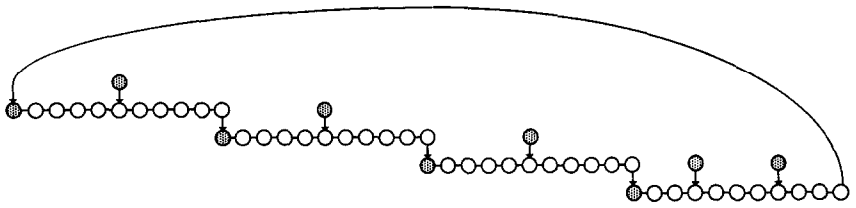

Fig. 6. Model structure of the GA-resistant, cyclic glucan. In this model, the cyclic structure consists of 44 glucosyl residues, and has 4 α-1,6-glucosidic linkages. Five glucosyl stubs bind to the cyclic structure. ↓, α-1,6-glucosidic linkage; -, α-1,4-glucosidic linkage; ○, glucosyl residue. The glucosyl residues releasing glycerol after rapid Smith-degradation are shaded.

glycerol should be released not only from non-reducing end residues (glucosyl stubs in this case) but also from glucosyl residues linked with both 1- and 6-position OH-groups (the residues at branch points in this case, see Fig. 6). It was found that the ratio of the sum of the stubs and α-1,6 linkages to the total sugar was 18%. The structural parameters of the GA-resistant glucan are summarized in Table 3, and the structure of GA-resistant molecule is illustrated in Fig. 6.

4. Discussion

The cluster unit of amylopectin has heavily branched and straight-chain regions (Fig. 1A). In the A type crystalline packing, chains have been suggested to form left-handed, parallel-stranded double helices (average diameter, 10.3 Å), and the helix is packed in a parallel manner, occupying 104 $Å^2$ of a cross section of the cluster [22,23]. If the cluster unit is cylindrical (length, 70 Å; diameter, 100 Å; and length of straight-chain region, 50 Å) [24] and the chains are packed uniformly, the number of glucosyl residues of a single cluster is calculated as about 2000. The results on structural analysis suggested that P-1 is a cyclic glucan although it included trace amounts of reducing impurities. The cyclic glucan may be derived mainly from a piece of the cluster unit, because its $\overline{dpw}$ was

Table 3
Structural parameters of the GA-resistant glucan

Parameters [a]	
$\overline{Dpw}$	49
Dp of the ring structure (A)	43.5
No. of α-1,6 linkage in the ring structure (B)	3.5
No. of α-1,4 linkage in the ring structure	40
No. of glucosyl stubs (C)	5.5
$\overline{C.I.n}$ [b] (D)	12.4
Span length [c]	4.8

[a] Assuming the $\overline{dpn}$ was similar to $\overline{dpw}$.
[b] $\overline{C.I.n} = A/B$.
[c] Average span length between glucosyl stubs = $D/(C/B + 1)$.

lower than the calculated dp of the cluster unit. However, some molecules may be derived from 2-cluster units (product III in Fig. 1B) because P-1 had chains corresponding to the B2-chain. P-1 was separated into two main fractions (S and L) with dp of 460 and 770, respectively, suggesting two possibilities. First, fraction L may be degraded very slowly to fraction S by BE. Second, two kinds of sub-structures corresponding to fractions S and L may comprise the cluster unit. Further investigations of the BE action and the structure of amylopectin are needed to clarify these points.

According to the mode of cyclization of BE (Fig. 1B), the cyclic portion of P-1 should include the heavily branched regions of amylopectin. Structural analyses of the GA-resistant glucan allowed us to illustrate the molecular structure as shown in Fig. 6. Most of the α-1,6 linkages probably originated from the heavily branched region of amylopectin, although at least one α-1,6 linkage was newly synthesized. The span length between branch linkages seems to be longer than that of the heavily branched region of amylopectin expected by Hizukuri [17]. This is probably due to the assumption that the $\overline{dp}w$ is the same to $\overline{dp}n$ but actually $\overline{dp}w$ is larger than $\overline{dp}n$.

The transfer reaction of BE on the linear α-1,4-glucan has been extensively studied [5,6,25]. BE catalyzes the transfer of linear α-1,4-glucan chain to produce a new α-1,6-branch point, but no data have been published on the transfer of branched glucan. The results presented in this paper demonstrated that BE catalyzed the transfer of multi-branched glucan to another site on the same molecule (intramolecular transfer) to form a cyclic structure. Also, intermolecular transfer of the multi-branched glucan can be catalyzed as in the case of the transfer of linear glucan.

The preliminary experiments suggested that BE from potato also catalyzes the cyclization of amylopectin. Boyer et al. analyzed the action of maize BE isozyme I by gel-filtration chromatography, and observed that the enzyme action retarded the retention time of amylopectin [26]. They attributed the retardation to a change of substrate shape caused by the enzyme, but we interpret it as due to the fragmentation by cyclization. It is conceivable that BEs from various sources catalyze the transfer of the multi-branched glucan.

From these observations, we propose new roles of BE on amylopectin or glycogen metabolism. First, BE could potentially change the structure of amylopectin by transferring their cluster units to another molecule (intermolecular transglycosylation). Second, BE may produce small cyclic glucans from amylopectin or glycogen through its intramolecular transglycosylation, and the glucans may be used by other starch/glycogen metabolic enzymes. Further investigation is needed to evaluate the contribution of the new reaction to the metabolism of amylopectin or glycogen.

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