Carbohydrate Research, 240 (1993) 253–263
Elsevier Science Publishers B.V., Amsterdam

Branching of amylose by the branching isoenzymes of maize endosperm *

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(Received May 22nd, 1992; accepted in revised form August 31st, 1992)

ABSTRACT

A convenient, quantitative assay method of branching enzyme (BE) was devised with reduced amylose as the substrate. Using this assay, the properties of the purified branching isoenzymes from maize, BE I, IIa, and IIb, were studied. The method is based on determination of reducing power, by the modified Park–Johnson method, of the chains transferred by BE after they are released from the branched products with isoamylase. The optimum pH of the three enzymes is 7.5, and the optimum temperatures of BE I, IIa, and IIb are 33, 25, and 15–20°C, respectively. The specific activities are found to be the highest for BE I and the lowest for BE IIb, whereas in the conventional assay based on stimulation of unprimed phosphorylase activity, the specific activities are BE IIb > IIa > I. BE I has a lower K_m (2.0 μM of the nonreducing terminal) for the reduced amylose of average chain-length ($\overline{cl}$) 405 than BE IIa (10 μM) and IIb (11 μM), and the enzyme shows a higher K_m for reduced amyloses of smaller $\overline{cl}$. Gel-permeation chromatograms on Sephadex G-75SF of the chains transferred from the reduced amylose indicate that initially the three isoenzymes produce chains of various sizes (dp ~ 8 to > 200), and BE I preferentially transfers longer chains than BE IIa and IIb.

INTRODUCTION

Plant branching enzyme [(1 → 4)-α-D-glucan : (1 → 4)-α-D-glucan 6-glucosyltransferase, EC 2.4.1.18] is an enzyme involved in amylopectin biosynthesis. The enzyme of potato tuber was extensively purified, and the general properties and the mechanism of the chain transfer were investigated[1–5]. Multiple forms of the enzyme were found in spinach[6] and maize[7–9]. Maize endosperm contains three forms, BE I, IIa, and IIb, and they have been highly purified[8]. BE I was found to be distinct from BE IIa and IIb in immunological properties, amino acid composi-

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* Supported in part by USDA/DOE/NSF Plant Science Center Program #88-372721-3964 and by Penford Products Co., Cedar Rapids, IA, USA.

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tion, and peptide maps, but no difference was observed between BE IIa and IIb[10,11]. Other properties, such as the affinity for amylose and the branching of amylose and amylopectin were examined[7,8,11], but further information on the catalytic properties of the multiple forms was not provided. The conventional assay methods for the enzyme are based on the stimulation of phosphorylase[6,8,12] and change in iodine–glucan spectra[8,13]. These methods, however, are not quantitative. We have devised a convenient, quantitative assay method using reduced amylose and have examined the general properties of the three branching enzymes from maize endosperm. The characteristics of chain transfer from reduced amylose catalyzed by the different isoenzymes were studied by gel-permeation chromatography.

EXPERIMENTAL

Materials.—Potato amylose [Type I, number-average degree of polymerization ($\overline{dp}$) 640, determined by the colorimetric method[14]] was obtained from Sigma Chemical Co. Amylose having $\overline{dp}$ of 280 was prepared from alpha-amylase hydrolyzate of a mixture of potato starch and 1-butanol by the method previously described[15]. Short-chain amyloses [amyloses EX-I ($\overline{dp}$ 17) and EX-III ($\overline{dp}$ 82)] and crystalline *Pseudomonas* isoamylase (198 U/mL; one unit is defined[16] as one μmol of the reducing terminal released from amylopectin per min at 40°C) were obtained from Hayashibara Biochemical, Inc. (Okayama, Japan).

Maize branching isoenzymes, BE I, IIa, and IIb, were purified from kernels of W64A maize, which were harvested 22 days after pollination in 1988 and stored at −80°C, by the method previously described[8] with some modifications. The purification steps involved homogenization, ammonium sulfate precipitation, and DEAE-Sephadex chromatography where BE I, IIa, and IIb were separated. BE I was further purified by chromatography on ω-aminodecyl agarose (Sigma), Mono Q (Pharmacia) and Superose 12 (Pharmacia). Further purification of BE IIa and IIb was performed by chromatography on ω-aminooctyl agarose (Sigma) and Mono Q. The BE IIa preparation was chromatographed on Bio-Gel P-10 (Bio-Rad) to remove carbohydrate. Details of purification will be described elsewhere.

Preparation of reduced amylose.—A commercial amylose from potato was purified[17] to remove a possible contaminant of amylopectin, and then the purified specimen was reduced[18] with $NaBH_4$. The amylose (1.5 g) was dissolved in Me_2SO (15 mL) by heating, and water (135 mL) and butanol (15 mL) were added. After keeping at 0°C for 1 h, the resulting precipitate (butanol–amylose complex) was collected by centrifugation (8000*g*, 5 min, 4°C) and dissolved in hot water (~ 70°C, 150 mL) by heating. Insoluble materials were immediately removed by centrifugation at room temperature. To the supernatant (150 mL) was added butanol (15 mL), and the mixture was kept at 40°C for 1 h. The amylose precipitate, collected by centrifugation (8000*g*, 5 min, room temperature), was dissolved in 10% butanol (150 mL) by heating, and the amylose was again precipiated by keeping at 40°C for

1 h (this procedure was repeated three times). The yield of the purified amylose [$\overline{dp}$ 850, average chain length ($\overline{cl}$) 400] was 1.4 g. A portion of the amylose precipitate (1 g) was dissolved in water (final volume 100 mL) by heating, 0.2 M $NaBH_4$–0.01 M NaOH (10 mL) was added, and the solution was kept at 45°C for 1 h. An excess amount of $NaBH_4$ was removed by addition of 5.15 M acetic acid (10 mL), and the solution was then neutralized with 5 M NaOH. The reduced amylose was precipitated by addition of 0.1 vol of butanol and collected by centrifugation. The reduced amylose was dissolved in 10% butanol (100 mL) by heating and precipitated by cooling at 0°C for 1 h (repeated twice). The precipitate of the reduced amylose was twice washed with MeOH (80 mL), then with ether (80 mL), and the product was dried over silica gel. The yield of the reduced amylose ($\overline{cl}$ 405) was 0.8 g.

The potato amylose ($\overline{dp}$ 280) and amyloses EX-I and EX-III (0.4 g each) were dissolved in Me_2SO (4 mL) by heating, and water (36 mL) and 0.2 M $NaBH_4$–0.01 M NaOH (4 mL) were added. The solutions were kept at 40°C for 1 h. Further procedures in the case of the amylose of $\overline{dp}$ 280 were the same as above. The reduced amyloses EX-III and EX-I were precipitated by EtOH (final concn 66%) instead of butanol. The yields of the reduced amyloses from the potato amylose ($\overline{dp}$ 280) and amyloses EX-III and EX-I were 75, 85, and 21%, respectively.

The reduced amyloses were slightly branched as described later. But, they were used as substrate since the branch linkages are only partially hydrolyzed by isoamylase[13], although the linkages are alpha-(1 → 6). Even a partial hydrolysis requires a large amount of expensive isoamylase, and the reduced amyloses although branched, gave a low blank assay.

Assays of branching enzyme activity.—(1) Branch-linkage assay (Assay BL). The enzyme (diluted with water, 10 μL) was incubated at 30°C for 30 min with the substrate (90 μL). The substrate was prepared by dissolving the reduced amylose ($\overline{cl}$ 405, 7.5 mg dry weight) in M NaOH (150 μL), followed by addition of water (975 μL) and 0.5 M MOPS [3-(*N*-morpholino)propanesulfonate] buffer (pH 7.5, 75 μL), and adjusting to pH 7.5 with M HCl (~ 150 μL). After terminating the reaction by heating in a boiling water bath for 1 min, M acetate buffer, (pH 3.5, 10 μL) and isoamylase (5 μL of 2 U/mL) or water (5 μL) were added. The solution was then incubated at 45°C for 45 min. To the solution were added M NaOH (8 μL), water (277 μL), 0.1% potassium ferricyanide (200 μL), and 0.48% Na_2CO_3–0.92% $NaHCO_3$–0.065% KCN (200 μL), and the mixture heated in a vigorously boiling water bath for 15 min. After cooling for 2 min in tap water, 0.3% ferric ammonium sulfate in 50 mM H_2SO_4 (1 mL), was added in a hood, and the absorbance at 715 nm was read on a spectrophotometer after exactly 20 min. Standard (2 μg glucose) and blank solutions were treated simultaneously. One unit (U) of the enzyme activity was defined as one μmol of the branch linkage formed per min (equivalent to μmol of the reducing terminal per min).

(2) Phosphorylase-stimulation assay (Assay A). The stimulation by branching enzyme of α-D-glucan formation catalyzed by phosphorylase *a* was determined as previously described[8], but 4.4 U of phosphorylase *a* (Sigma) was used for each

assay. One unit (U) was defined as one μmol of glucose transferred from glucose-1-phosphate per min at 30°C.

Distribution of transferred chains from reduced amylose on Sephadex G75-SF.—The reduced amylose ($\overline{cl}$ 405, 1.2 mg) was incubated in 25 mM MOPS, pH 7.5, (200 μL) at 30°C with the branching enzyme (BE I, IIa, IIb, 1.5, 0.76, 0.75 mU by Assay BL, respectively). After 20 min to 6 h, the reaction was terminated by heating in a boiling water bath for 2 min, and M acetate buffer (pH 3.5, 20 μL) and isoamylase (0.6 μL of 198 U/mL) were added. After 2.5 h incubation[19] at 45°C, the solution was heated in a boiling water bath for 2 min, and water (20 μL) was added. An aliquot (200 μL) of the solution was applied to a column (1.2 × 25 cm) with Sephadex G-75SF and eluted at 37.5°C with 50 mM NaCl. The flow rate was 4 mL/h, and fractions of 1.26 or 1.31 mL were collected.

Analytical methods.—The reducing and nonreducing terminal glucosyl residues were determined by the modified Park–Johnson method[14] and the rapid Smith-degradation method[14], with modifications[20], respectively. Carbohydrate content was determined by the phenol–H_2SO_4 method[21]. The $\overline{dp}$ and $\overline{cl}$ values were (carbohydrate equivalent to glucose)/(reducing terminal) and (carbohydrate equivalent to glucose)/(nonreducing terminal), respectively. The blue value (absorbance at 680 nm of iodine–glucan complexes) and λ_{max} (the maximum wavelength of iodine–glucan complexes) were determined as previously described[22].

RESULTS AND DISCUSSION

Branching enzyme assay by determination of branch linkage (Assay BL).—Assay BL involves branching of the reduced amylose ($\overline{cl}$ 405) by branching enzyme, debranching of branched products by isoamylase, and then determination of the reducing terminal of the released chains. The reducing terminus was determined by the modified Park–Johnson method[13], which gives the same reducing power from glucose to amylose ($\overline{dp}$ 40) and shows a ~20-fold sensitivity compared with the Somogyi–Nelson method[23,24]. The reduced amylose produced <2% of the parent amylose as reducing power. The isoamylase amount used (10 mU) gave complete debranching under the conditions described above, since the debranched products were completely degraded into maltose with beta-amylase. Assay BL shows linearity of branch linkages introduced against the enzyme amount and incubation time up to 12 nmol and 90 min, respectively, under the conditions described above.

The conventional assay methods based on the phosphorylase stimulation[8] by production of nonreducing terminus by branching enzyme (Assay A), and the decrease in absorbance of the complex of iodine and amylopectin[13] or amylose[8] (Assay B) are useful, but not quantitative. Contaminating activity of amylases, if present, also decreases absorbance of iodine–glucan complexes and produces the nonreducing terminal which stimulates phosphorylase activity. However, Assay BL

determined the contaminating activity itself by assaying reducing power before isoamylolysis, whereas the Assays A and B could not. An alternative, quantitative assay method, reported by Krisman et al.[25], is based on determination of glucose released[16] by phosphorylase and transferase–amylo-(1 → 6)-glucosidase from glucan formed in Assay A. However, the method requires isolation of the glucan and the use of transferase–amylo-(1 → 6)-glucosidase, which is not commercially available. Thus, Assay BL is a convenient, quantitative assay method of branching enzyme activity.

MOPS buffer gave the lowest blank among buffers tested which were 25 mM citrate, bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], and triethanolamine. Phosphate (25 mM) could not be used because it precipitated Prussian blue color (ferric ferrocyanide). Dilution of branching enzyme with 50 mM Tris–acetate, pH 7.5, containing 10 mM EDTA and 2.5 mM 1,4-dithiothreitol (an assay buffer used in purification procedures) increased absorbance (~ 0.4).

Properties of branching isoenzymes from normal maize.—The branching isoenzymes of maize endosperm, BE I, IIa, and IIb, were purified to an almost homogeneous state (one main band with a few faint bands on gel electrophoreses with and without SDS). BE IIa and IIb were found to be contaminated by a small amount of beta-amylase, which was only detected by prolonged incubation (6 h) with relatively large amounts of the enzymes (0.38 mU per assay). Table I summarizes the specific activities measured by Assays BL and A. The specific activities measured by Assay BL were BE I > IIa > IIb, whereas those by Assay A and the activity ratios (Assay A)/(Assay BL) were BE I < IIa < IIb. To clarify the discrepancy between both assay methods, the $\overline{\text{cl}}$ of glucans formed in Assay A by using the same Assay A-units (0.04 U) was determined (Table I). All glucans had a similar $\overline{\text{cl}}$ of 250–330, indicating that a similar amount of branch linkages was introduced to the glucans by the respective branching isoenzymes. Therefore, the high specific activity of BE I measured by Assay BL appeared to be due to a higher affinity of BE I for amylose, while BE IIa and IIb may be favored when reacting with the growing glucan rather than amylose chain.

TABLE I

Activities (at 30°C) of the branching enzymes

Branching enzyme isoenzyme	BE I	BE IIa	BE IIb
Specific activity (U/mg protein)			
Branch linkage (a)	0.683	0.313	0.132
Phosphorylase stimulation (b)	213	484	1040
Ratio (a)/(b)	310	1550	7930
$\overline{\text{CI}}$ of glucan [a]	250	260	330

[a] Formed from glucose-1-phosphate by phosphorylase (4.4 U) and the branching enzyme [0.04 Assay A Units] at 30°C for 90 min.

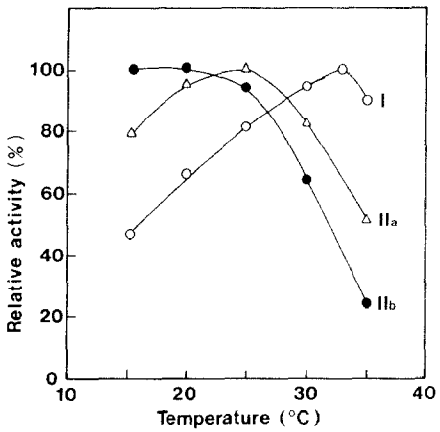

Fig. 1. Temperature dependence of the branching isoenzymes: ○, BE I; △, BE IIa; ●, BE IIb.

The branching isoenzymes showed the same pH optimum of 7.5. The value was in the range, as determined by Assay B, for maize endosperm[9] (pH 6–8.5) and spinach leaf[6] (6.5–8.0), and was a little higher than those for sweet corn[27] (6.8–7.4) and potato tuber[1] (6.6–7.2). The optimum temperatures were 33, 25, and 15–20°C for BE I, IIa, and IIb, respectively (Fig. 1), which is lower than that (37°C) required for sweet corn[27], and the value for BE IIa was similar to that of the branching enzyme of potato tuber[1] (25°C). The relatively low optimum temperatures were not due to heat denaturation of the enzymes, since the preincubation of BE IIa at 30°C for 1 h gave no decrease in the activity, and BE IIb proportionally increased branch linkages up to 90 min at 30°C. A higher temperature might weaken a stabilized conformation of amylose–chain complex[4] favored by the enzymes.

Table II summarizes the catalytic properties of the branching isoenzymes on reduced amyloses of $\overline{\text{cl}}$ 25–405. The reduced amyloses were composed of linear and slightly branched molecules, similar to amyloses isolated from various plants[28], since their beta-amylolysis limit was 72–90%, and their parent amyloses had

TABLE II

K_m and the relative activities of the three branching enzymes as measured with reduced amyloses of different properties

Reduced amylose			K_m (μM) [a]			Relative activity (50 μM) [a]		
$\overline{\text{CI}}$	$\overline{\text{Dp}}$ [b]	β-AL [c] (%)	BE I	BE IIa	BE IIb	BE I	BE IIa	BE IIb
405	850	72	2.0 (0.13) [d]	10 (0.66)	11 (0.72)	1	1	1
197	280	86	4.1 (0.13)	50 (1.6)	50 (1.6)	0.89	0.33	0.21
56	63	77	50 (0.45)	n.d.	n.d.	0.25	0.12	0.07
25	n.d.	90	n.d.	n.d.	n.d.	0.02	0.00	0.00

[a] Nonreducing terminal. [b] $\overline{\text{Dp}}$ of the parent amylose. [c] Beta-amylolysis limit. [d] In parenthesis, carbohydrate mg/mL; n.d., not determined.

1.1–2.1 chains on average. Therefore, the $\overline{cl}$ and molarity of the nonreducing terminal of the reduced amyloses were used in the calculation of the kinetic properties of the enzymes. The K_m values for the reduced amylose of $\overline{cl}$ 405 were 2.0, 10, and 11 μM for BE I, IIa, and IIb, respectively, and those for the reduced amylose with a smaller $\overline{cl}$ of 197 were higher (4.1, 50, and 50 μM, respectively). Only BE I gave a definite K_m of 50 μM for the reduced amylose with a much smaller $\overline{cl}$ of 56. The K_m values calculated on the basis of carbohydrate concentration (mg/mL) for the reduced amyloses showed a similar tendency to those by molarity except for BE I, which gave the same K_m of 0.13 mg/mL for the reduced amyloses of $\overline{cl}$ 405 and 197. This K_m value of BE I resembled that (0.16 mg/mL) previously determined[8] by Assay B using amylose of $\overline{dp}$ 280. Conversely, the K_m values (1.6 mg/mL) of BE IIa and IIb for the reduced amylose of $\overline{cl}$ 197 ($\overline{dp}$ probably 280) was higher than the published value (0.6 mg/mL) of BE II[8]. The rates of the three isoenzymes decreased with decrease in $\overline{cl}$ of reduced amyloses at 50 μM, and the rate decrease was BE I < IIa < IIb (Table II). The activity on the reduced amylose of $\overline{cl}$ 25 was almost undetectable, similar to the previous observation for potato tuber branching enzyme[2]. These results suggested that BE I had a higher affinity for amylose than the others, and all the isoenzymes favored a large MW amylose.

Cyclohexaamylose (5 mM) had no influence on the isoenzyme activity when assayed under the standard conditions.

Branching of amylose by the branching isoenzymes.—The incubation of the reduced amylose ($\overline{cl}$ 405) with the isoenzymes (BE I 1.25 U/g of the substrate, IIa and IIb 0.6 U/g, U was an Assay BL-unit) decreased the $\overline{cl}$ rapidly in 1 h and then gradually (Fig. 2). The $\overline{cl}$ obtained was 24 and 21 for BE I and IIa, respectively, in 6 h and 22 for BE IIb in 3 h. Prolonged incubation decreased $\overline{cl}$ gradually. The products obtained with BE IIa and IIb contained a small amount of maltose, which was identified by chromatography on Sephadex G-75SF, but no other oligosaccha-

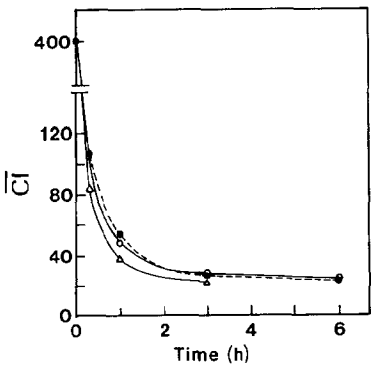

Fig. 2. Branching of reduced amylose by the branching enzymes as measured by the decrease in $\overline{cl}$: ○, BE I; ●, BE IIa; △, BE IIb. The reduced amylose ($\overline{cl}$ 405) was incubated at 30°C and pH 7.5 with BE I (1.25 U/g of the substrate), BE IIa and IIb (0.6 U/g).

TABLE III

Properties of branched products [a] of the branching enzymes from the reduced amylose

Product	Before isoamylolysis					After isoamylolysis		
	Blue value	λ_{max} (nm)	$\overline{CI}$	β-AL [b] (%)	Ecl [c]	Blue value	λ_{max} (nm)	β-AL (%)
BE I	0.078	534	24	58	16	0.298	562	100
BE IIa	0.231	568	21	48	12	0.445	580	98
BE IIb	0.351	574	22	48	13	0.563	586	98

[a] Products of incubation of the reduced amylose ($\overline{cl}$ 405) at pH 7.5 and 30°C with BE I (1.25 U/g of the substrate) for 6 h and BE IIa and IIb (0.6 U/g) for 6 h and 3 h, respectively. [b] Beta-amylolysis limit. [c] External chain length, calculated from the equation, $[(\overline{cl})\times(\beta\text{-AL}\%)/100]+2$.

rides having a reducing terminal were found. The maltose was due to beta-amylase contaminating activity. The amount of maltose in the BE IIa (6 h) and IIb (3 h) products was $< 5\%$ of the isoamylolyzates as reducing terminal and corresponded to ~ 1 glucosyl residue for their $\overline{cl}$, suggesting no great influence on the $\overline{cl}$ determined.

The products having a similar $\overline{cl}$ of 21–24, as described above, were characterized (Table III). The beta-amylolysis limits of the isoamylolyzates were 98–100%, indicating complete debranching of the products by isoamylase. BE I product and its isoamylolyzate showed lower blue value (absorbance at 680 nm of iodine–glucan complexes) and λ_{max} (the maximum wavelength of the complexes) than those of BE IIa and IIb, suggesting that the BE I product contained a smaller amount of long chains than the others (see below). The BE I product had a higher beta-amylolysis limit and a larger external $\overline{cl}$ (3-4 glucosyl residues) than those of BE IIa and IIb. These results suggested that BE I branched amylose in a different manner than BE IIa and IIb.

Fig. 3 shows the distributions of transferred chains on molar and weight bases on Sephadex G-75SF during incubation of the reduced amylose ($\overline{cl}$ 405) with the isoenzymes. The reduced amylose used as a substrate made it possible to differentiate transferred chains from the others (acceptor and donor segment) since the transferred chains showed reducing power after isoamylolysis. The reduced amylose was eluted at the void volume (0 time). At the early stage of the branching process (20 min), the isoenzymes showed a similar, wide distribution of transferred chains on a molar basis; that is, all the isoenzymes transferred chains with various sizes (dp ~ 8 to > 200). Later branching consisted of chain transfer from the transferred chains, and it was at this stage that the different behaviors between BE I and II became apparent, as can be seen in the chain distributions by both a molar and weight basis. BE I produced chains of various sizes, but having a narrow range compared with those at the early stage, with a slight peak of dp 70 and then shorter chains with the peaks of dp 31 and 14, accompanied by a rapid decrease of the number of the chains of dp ~ 70–100. A similar change of the distributions in molar and weight basis was observed when BE I acted on a smaller reduced

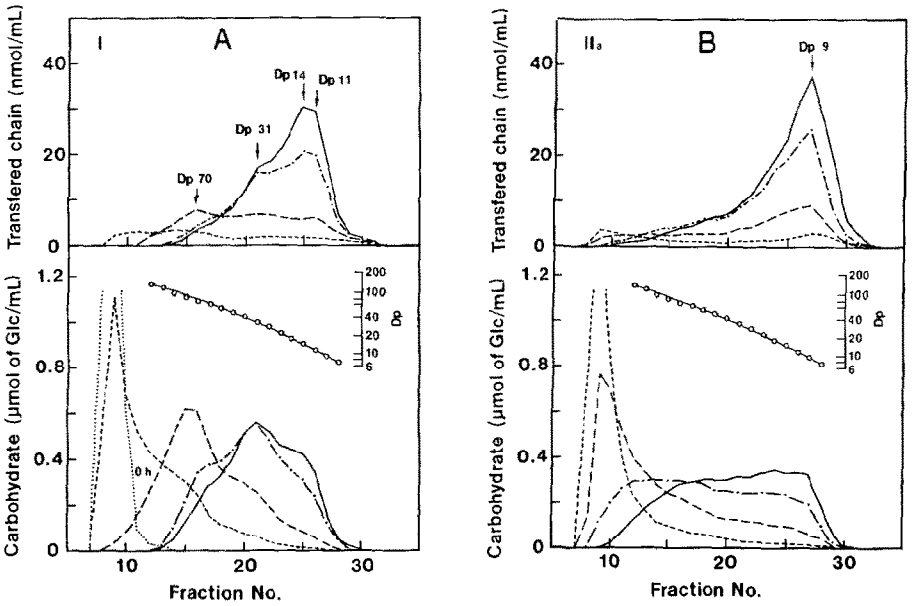

Fig. 3. Sephadex G-75SF chromatograms of the isoamylolyzates of the products by the branching enzymes at various times (- - - - - -, — — —, · – · – ·, and ——— 20 min, 1, 3, and 6 h, respectively). The fraction volumes were 1.26 mL for BE I and IIb and 1.31 mL for BE IIb. Top: number of chains. Bottom: amount of carbohydrate. The total amount of carbohydrate was estimated to be 5.5 mmol as glucose as calculated from the amount of transferred chains. A, BE I; B, BE IIa; C, BE IIb.

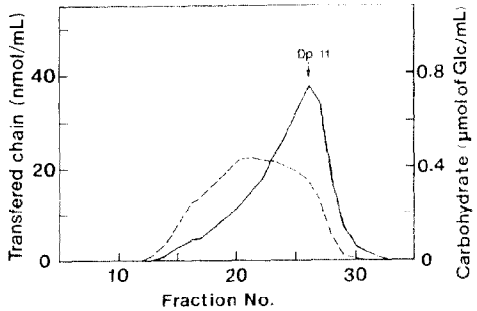

Fig. 4. Sephadex G-75SF chromatogram of the isoamylolyzate of the product of incubation (3 H) of reduced amylose with BE I (0.63 U/g) and IIa (0.3 U/g), indicating number of transferred chains (———) and amount of carbohydrate (- - - - -) in each fraction.

amylose ($\overline{cl}$ 197) (chromatograms not shown). On the other hand, BE IIa and IIb decreased gradually the number of long chains (dp > 100), and later a large increase in the number of short chains with a peak of dp ~ 9 was observed with a slow decrease in long chains (dp > 70). The simultaneous 3-h incubation with 0.63 U/g of BE I and 0.3 U/g of BE IIa (half the activity in the case of each isoenzyme) produced a product having a $\overline{cl}$ of 24. The product showed an intermediate chain distribution between those produced by the respective isoenzymes, that is, smaller and larger numbers of long and short chains, respectively (Fig. 4). Thus, BE I transferred chains in a different mode than BE IIa and IIb at the later stage. Namely, BE I preferentially transferred long chains, whereas BE IIa and IIb transferred short chains. The behavior of chain transfer of BE I resembled that of branching enzyme of potato tuber[2], judging from the change of distributions of transferred chains on a weight basis during incubation.

It seemed to be of interest to consider the relationship between the structure of maize starches and the branching isoenzymes. The branched molecule in maize amyloses comprised[15,16] a small number (5–6 on the average) of side chains with dp 6 to ~ 5000 (ref. 17). These side chains may also be introduced by the branching isoenzymes, since the enzymes transferred chains of various sizes. The amylopectin of amylomaizes (*amylose extender*) had[29,30] a larger $\overline{cl}$ of 31–32 than that ($\overline{cl}$ 20) of normal amylopectin[31]. The distribution of chains released from the amylomaize amylopectin by isoamylase showed higher and lower proportions of long- and short-chain fractions[30,32], respectively, than in case of normal amylopectin[31]. Their structural characteristics may be due to the low activity ratio (BE IIa + IIb)/(BE I) (0.81) (ref. 7) of the amylomaize, compared with normal maize (3.1) (ref. 8), since the amylomaize was lacking[7] in BE IIb, which transferred short chains.

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