Carbohydrate Research, 82 (1980) 404–410

Note

Branching-enzyme activity of an α-D-glucosyltransferase of *Streptococcus mutans**

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(Received July 24th, 1979; accepted for publication in revised form, October 7th, 1979)

The ability to transfer a D-glucosyl group from sucrose to glucose di- and oligosaccharide acceptors[1–3] is a general property of dextransucrases. The reaction between *Streptococcus mutans* α-D-glucosyltransferase, [^{14}C]sucrose, and isomaltose saccharides (IM_n) having values of chain-length (n) from 2–6 D-glucose residues has been used to prepare isomaltose oligosaccharides[4] labelled at terminal D-glucosyl groups (Eq. *1*). The products were reported to contain (1→6)-α-D-glucosidic linkages exclusively.

$$[^{14}C]\text{Sucrose} + IM_n \rightarrow \text{D-}[^{14}C]\text{Glucosyl-}IM_n + \text{D-}[^{14}C]\text{Fructose} \qquad (1)$$

When the acceptor (IM_n) was added in 4-fold excess over [^{14}C]sucrose, polysaccharide synthesis was eliminated, and 80% of the transferred D-glucosyl groups appeared in the major product, IM_{n+1}, which contained one labelled D-glucosyl group at the nonreducing terminal. A minor product, IM_{n+2}, in which two glucose residues at the nonreducing end were labelled, accounted for the remainder of the D-glucosyl groups transferred.

This note describes the new products observed in a similar system, when isomaltose saccharides containing more than 6 glucose residues were added as alternative acceptors. The mixtures, containing [^{14}C]sucrose (1.47 mg, 60.3 μCi), isomaltose saccharide (18 μmol), and *S. mutans* OMZ176 α-D-glucosyltransferase (20 munit), were buffered to pH 6.5 with sodium citrate buffer (20mM), and incubated for 14 h at 35°. The enzyme was then inactivated by boiling, and the solutions were deionized with Amberlite mixed-bed resin MB-3 (carbonate form). The products were separated from the unlabelled substrate by descending chromatography (p.c.) on Whatman No. 3MM paper in 41:36:23 nitromethane–ethanol–water. The positions of ^{14}C-labelled oligosaccharides were revealed by preparing radioautograms using X-ray film. *New*, minor products from acceptors IM_7, IM_8, and IM_9 were located on the chromatograms inbetween the acceptor and the main product IM_{n+1}, and also in a

*Dedicated to Professor Stephen J. Angyal on the occasion of his retirement.

TABLE I

OLIGOSACCHARIDE PRODUCTS OF GLUCOSYLTRANSFERASE ACTION ON [^{14}C]SUCROSE AND ISOMALTOSE SACCHARIDE ACCEPTORS (IM_n)

Acceptor	*10^6 Counts per min*					
	Major products (n + 1)			*Minor products (n + 2)*		
	D.p.	*IMS*[a]	*BS*[b]	*D.p.*	*IMS*[a]	*BS*[b]
IM_4	5	36.69	0.409	6	5.26	
IM_5	6	29.39	0.443	7	4.91	0.241
IM_6	7	26.90	0.637	8	4.91	0.483
IM_7	8	27.31	2.34	9	4.44	0.812
IM_8	9	22.96	3.62	10	3.49	1.63

[a]Isomaltose saccharide; the subscript indicates the number of D-glucose residues. [b]Branched saccharide.

position between IM_{n+1} and IM_{n+2}. As the new products probably contained a secondary linkage that was most likely a branch linkage, the two series were designated B_{n+1} and B_{n+2}, respectively. The radioautograms clearly indicated that only trace amounts of $B_{n+1,n+2}$ were produced from IM_4, IM_5, and IM_6, and that thereafter, the proportion of branched product increased greatly. Labelled oligosaccharide-products from IM_4 to IM_8 were sufficiently well separated by p.c. for the papers to be sectioned and eluted with water. The radioactivity of the oligosaccharides, measured by liquid scintillation spectrometry, is shown in Table I. The average value for the specific radioactivity of the linear products IM_{n+1} was 11.07×10^6, and that of the IM_{n+2} series, 22.56×10^6 counts per min per μmol. The distribution of products between IM_{n+1} and IM_{n+2} was 23:2 for all values of n. Both series of linear products were completely hydrolyzed to D-glucose by the (1→6)-α-D-glucan glucohydrolase of *Streptococcus mitis*[5]. Further evidence that the IM_{n+1} series contained (1→6)-α-D-glucosidic linkages only was provided by hydrolysis with *S. mutans* K1-R endodextranase[6]. No branched oligosaccharides were found among the products[7].

Identification of branched-oligosaccharide products B_{n+1}. — The low counts (Table I) for the "branched" products from IM_4, IM_5, and IM_6 acceptors explained why these were not seen on radioautograms in the earlier work, in which the specific radioactivity of the [^{14}C]sucrose substrate for the preparations was 8 times lower than that used in the present work. The position of the posited branch or secondary linkages in the B_{n+1} series was investigated with three exoglucanases of known specificity. First, portions (~5%) of the products obtained from IM_5–IM_8, that is B_6–B_9, were incubated for 48 h at 35° with 33 munit of *S. mitis* (1→6)-α-D-glucan glucohydrolase[5], an enzyme that does not hydrolyze or by-pass secondary linkages. The products of the hydrolysis were then separated by p.c. as already described. Radioautograms revealed that, whereas the IM_{n+1} series had given D-[^{14}C]glucose as the only labelled product, each member of the B_{+n1} series yielded one spot in the

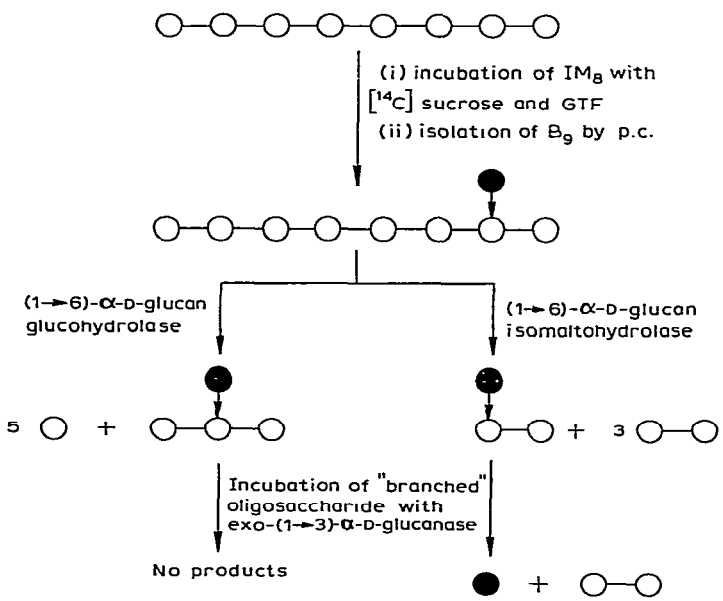
region corresponding to B_4, and there was no trace of a labelled product corresponding to glucose. The (1→6)-D-glucan glucohydrolase of *S. mitis* has no action on α-(1→6) linkages attached to D-glucose residues that are also substituted at C-2, C-3, or C-4, but α-D-(1→6) linkages adjacent to secondary linkages are hydrolyzed, provided a branch point is not involved[8]. An original branch-linkage in each B_{n+1} would therefore remain as a branch linkage in the limit oligosaccharide B_4, whereas a secondary linkage in the main chain of B_{n+1} would be exposed at the nonreducing terminal of B_4. An investigation into the position of the secondary linkage in B_4 was therefore undertaken.

The secondary linkages in the $B_{n+1,n+2}$ series were expected to be (1→3) because the *S. mutans* OMZ176 glucosyltransferase system reacts with sucrose to give glucans that contain only (1→6)- and (1→3)-α-D-glucosidic linkages. The structure of B_4 was explored with a (1→3)-α-D-exoglucanase[9] that could distinguish between secondary linkages at nonreducing terminals and those that constituted branch linkages. Authentic 3^3-α-D-glucosylisomaltotriose (25 μg) and a similar weight of the unknown B_4 (obtained from B_8 as already described), were each incubated for 20 h at 35° with (1→3)-α-D-exoglucanase of *Cladosporium resinae* (4 munit). The enzyme completely converted 3^3-α-D-glucosylisomaltotriose into D-glucose and isomaltotriose, but the limit oligosaccharide B_4 was totally resistant to hydrolysis. The (1→3)-α-D-exoglucanase of *C. resinae* has no action on (1→3)-α-D-glucosidic branch-linkages, and its lack of activity towards B_4 indicated that the tetrasaccharide was most likely 3^2-α-D-glucosylisomaltotriose, a structure that was consistent with its resistance to both exo-(1→6)- and exo-(1→3)-glucanases.

Two oligosaccharides from the B_{n+1} series were also incubated with another (1→6)-α-D-exoglucanase, namely the (1→6)-α-D-glucan isomaltohydrolase of *Arthrobacter globiformis*[10]. This enzyme releases isomaltose from nonreducing terminals, and can hydrolyze secondary linkages and by-pass branch linkages if, thereby, the products are either isomaltose or a linear glucosylisomaltose having a secondary linkage at the nonreducing end[11]. The R_F value of the labelled product (B_4) released from B_8 by isomaltohydrolase was identical to that produced by *S. mitis* glucohydrolase. This B_4 was resistant to hydrolysis by the (1→3)-α-D-exoglucanase of *C. resinae*, confirming the absence of a (1→3) linkage at the nonreducing terminal. As 3^1- and 3^3-α-D-glucosylisomaltotriose would not survive as limit oligosaccharides of *A. globiformis* isomaltohydrolase, the only structure for B_4 compatible with the enzymic analyses was 3^2-α-D-glucosylisomaltotriose. Thus, the two (1→6)-exoglucanases reacted with B_8 to give the same limit-oligosaccharide, despite the ability of the isomaltohydrolase to hydrolyze secondary linkages in the main chain.

Incubation of *A. globiformis* isomaltohydrolase with B_9 (Scheme 1) gave a single labelled product, located by p.c. in a position that corresponded to a trisaccharide (B_3) containing one primary and one secondary glucosidic linkage. This result was in accord with the ability of the enzyme to hydrolyze the (1→6) linkage that is adjacent to a substituted D-glucose residue, thus giving a limit oligosaccharide that contained one less D-glucose residue than that obtained with *S. mitis* gluco-

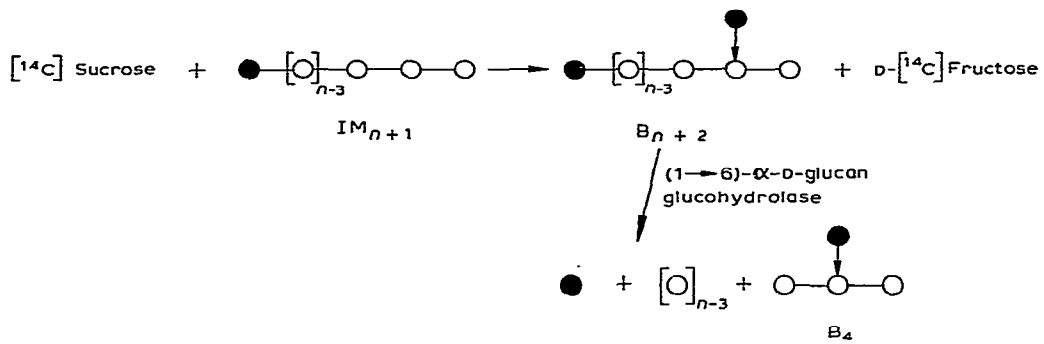
hydrolase, from substrates having an even number of D-glucose residues external to the branch point. B_3 was incubated with the (1→3)-α-D-glucanase of *C. resinae* under the same conditions as in the earlier experiments with the limit tetrasaccharides. In contrast to the total resistance of each preparation of B_4, the trisaccharide was completely hydrolyzed to D-glucose and isomaltose. The only labelled product observed on a radioautogram was glucose. Regions of the paper chromatogram corresponding to glucose and isomaltose were excised and counted, and 99.9% of the total radioactivity was found associated with glucose (Scheme 1). This result indicated that the secondary linkage was at the nonreducing terminal of B_3. We can therefore postulate that the limit oligosaccharides B_3 and B_4 are 3^2-α-D-glucosylisomaltose and 3^2-α-D-glucosylisomaltotriose, respectively. These assignations imply that the D-glucosyltransferase preparation from *S. mutans* converted isomaltose saccharides into branched saccharides by the introduction of a D-glucosyl side-chain to the D-glucose residue penultimate to the reducing terminal. Some results that provided the logical basis for this conclusion are illustrated in Scheme 1 below.



Scheme 1. Symbolic representation of the enzymic synthesis and degradation of a branched oligosaccharide from IM_8. ○, D-glucose residue or group; ●, D-[^{14}C]glucosyl group; ——, primary α-D-glucosidic linkage; ↓, secondary α-D-glucosidic linkage; GTF, α-D-glucosyltransferase.

Identification of branched-oligosaccharide products B_{n+2}. — The minor products B_{n+2}, obtained from IM_5–IM_8, were incubated with (1→6)-α-D-glucan glucohydrolase of *S. mitis* under the conditions already described for the B_{n+1} series. Radioautography of the products separated by p.c. showed that both D-glucose and B_4 were labelled. This result indicated that the structure near the reducing terminal was likely to be similar to that deduced for the B_{n+1} series, whereas the structure at the non-

reducing terminal was analogous to that deduced for the IM_{n+1} series. A doubly-branched chain, or a 2-unit nigerosyl chain were both excluded, for those would yield limit branched-oligosaccharides having d.p. > 4. Moreover, the glucohydrolase would not release D-[^{14}C]glucose from the side chains of 3^2-α-nigerosylisomaltosaccharides. The most probable reaction for the synthesis of B_{n+2} saccharides was considered to be the addition of a D-glucosyl side-chain to IM_{n+1} (Eq. *2*), by the same mechanism as the conversion of IM_n to B_{n+1}. The radioactivity of B_{n+2} would thus be the sum of a chain-lengthening and a chain-branching activity of the enzyme system.



Determination of chain-branching activity of S. mutans D-*glucosyltransferase.* — Although the separation and counting of the linear (IMS) and branched (BS) products of the reaction between [^{14}C]sucrose and isomaltose saccharide acceptors could be used to determine the relative chain-lengthening and chain-branching activities of the enzyme, the technique was time-consuming, and was not well adapted for systematic analysis. Furthermore, with acceptors of greater chain-length ($n > 8$), complete separation of the products was even more difficult to achieve. A simpler and more rapid determination of branching activity was clearly required, and a different approach, utilizing the difference in susceptibility of each product to hydrolysis by (1→6)-α-D-glucan glucohydrolase, was therefore assessed. D-[^{14}C]Glucosyl groups that were transferred to the nonreducing terminal of the acceptor by means of a (1→6)-α-D-glucosidic linkage were released by the glucohydrolase, and their radioactivity was thus a measure of chain-lengthening activity. By contrast, D-[^{14}C]-glucose residues attached with a branch linkage to the main chain appeared as glucohydrolase limit-oligosaccharides, and their radioactivity was a measure of chain-branching activity. The products of glucosyltransferase action could therefore be incubated with the (1→6)-α-D-glucan glucohydrolase of *S. mitis* without the need for prior separation of the IMS and BS series, and it was only necessary to separate D-[^{14}C]glucose from the branched limit-oligosaccharide, [^{14}C]B_4 (Eq. *3*).

$$[^{14}C]IM_{n+1,n+2} + [^{14}C]B_{n+1,n+2} \rightarrow D\text{-}[^{14}C]\text{Glucose} + [^{14}C]B_4 \qquad (3)$$

The counts associated with the latter, as compared to the counts in the total products (D-glucose + B_4), then provided a value for the relative branching activity.

Duplicate incubation-mixtures were prepared with IM_8 acceptor, and the products were applied to paper. One chromatogram was developed for a few h to remove D-[^{14}C]fructose from the labelled oligosaccharides. Then the oligosaccharides were eluted all together, and incubated with (1→6)-α-D-glucan glucohydrolase of *S. mitis*. The products, D-glucose and B_4, were separated by p.c. for 18 h. The second chromatogram was developed for 12 days, and each oligosaccharide was eluted and counted separately. The two methods gave results in close agreement. Thus, the relative branching activity, as determined by the more-rapid method, was expressed by:

$$\frac{\text{Counts corresponding to } B_4}{\text{Total counts}} \times 100 = 14.3\%,$$

whereas the original method, in which each product was counted separately, was expressed by:

$$\frac{\text{Counts corresponding to } B_{n+1} + 0.5\,(B_{n+2})}{\text{Total counts } (IM_{n+1} + IM_{n+2} + B_{n+1} + B_{n+2})} \times 100 = 14.0\%.$$

The more-rapid method was therefore suitable for testing the effect of various conditions on the relative branching-enzyme activity of *S. mutans* D-glucosyltransferase.

McCabe and Smith[12] found that a *S. mutans* K1-R D-glucosyltransferase system modified primer dextrans so that they became both more efficient as acceptors and more highly branched. This result indicated that one of the transferases recognized internal D-glucose residues, and catalyzed the formation of branch points. In this context, it is noteworthy that IM_8 was reported[13] to be the smallest effective primer for glucan synthesis by *S. mutans* 6715 D-glucosyltransferase, in good agreement with our conclusion that isomaltose oligosaccharides must contain 7 or 8 glucose residues to elicit the branching activity of *S. mutans* OMZ176 D-glucosyltransferase. However, we do not consider that these reactions, which occurred only when sucrose was present, constitute the only means of introducing α-D-(1→3) linkages into *S. mutans* glucans. Other types of branching enzymes may be produced, and transfer of D-glucosyl groups to position 3 of a D-glucose residue of a co-substrate could also occur by disproportionation[14].

ACKNOWLEDGMENTS

The author thanks Professor Teruo Sawai for providing a generous sample of *Arthrobacter globiformis* dextranase. This investigation was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

1 H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenka, C. A. Hoffman, E. S. Sharpe, and R. W. Jackson, *J. Biol. Chem.*, 200 (1953) 793–801.

2 F. YAMAUCHI AND Y. OHWADA, *Agric. Biol. Chem.*, 33 (1969) 1295–1300.
3 G. J. WALKER, *J. Dent. Res.*, 51 (1972) 409–414.
4 G. J. WALKER, *Carbohydr. Res.*, 30 (1973) 1–10.
5 G. J. WALKER AND A. PULKOWNIK, *Carbohydr. Res.*, 29 (1973) 1–14.
6 A. PULKOWNIK AND G. J. WALKER, *Carbohydr. Res.*, 54 (1977) 237–251.
7 A. PULKOWNIK, J. A. THOMA, AND G. J. WALKER, *Carbohydr. Res.*, 61 (1978) 493–497.
8 G. J. WALKER AND A. PULKOWNIK, *Carbohydr. Res.*, 36 (1974) 53–66.
9 G. J. WALKER AND M. D. HARE, *Carbohydr. Res.*, 77 (1980) 289–292.
10 T. SAWAI, K. TORIYAMA, AND K. YANO, *J. Biochem. (Tokyo)*, 75 (1974) 105–112.
11 M. TORII, K. SAKAKIBARA, A. MISAKI, AND T. SAWAI, *Biochem. Biophys. Res. Commun.*, 70 (1976) 459–464.
12 M. M. MCCABE AND E. E. SMITH, *Carbohydr. Res.*, 65 (1978) 223–239.
13 G. R. GERMAINE, A. M. CHLUDZINSKI, AND C. F. SCHACHTELE, *J. Bacteriol.*, 120 (1974) 287–294.
14 J. H. PAZUR, T. BUDOVICH, AND C. L. TIPTON, *J. Am. Chem. Soc.*, 79 (1957) 625–628.