Carbohydrate Research, 53 (1977) 263–267

Note

Synthesis of oligosaccharides of the isomaltose series labelled with ^{14}C at the reducing end

GWEN J. WALKER

Institute of Dental Research, 2 Chalmers Street, Sydney, N. S. W. 2010 (Australia)

(Received June 21st, 1976, accepted for publication, June 29th, 1976)

End-labelled oligosaccharides are suitable substrates for studying the action pattern of carbohydrases. Analysis of the labelled products allows a determination of the frequency distribution of hydrolysis of each glycosidic linkage, and the data contribute to the information required for mapping the specificity site of the enzyme. The number of subsites and the position of the catalytic site have been determined for a number of alpha-amylases[1,2] from the chain-length dependence of bond-cleavage frequencies.

A series of isomaltose oligosaccharides labelled at the non-reducing terminal[3] has recently been used to study the action pattern of a bacterial dextranase[3] (Calbiochem) and a fungal dextranase[4] (*Penicillium lilacinum*). Both enzymes were able to hydrolyse linkages close to the reducing end of the oligosaccharides, and if secondary attack, or multiple attack towards the non-reducing end had occurred, the values for bond-cleavage frequencies would have been in error. Clearly, the use of two series of oligosaccharides, labelled at opposite ends, would test the accuracy of the frequency distribution of hydrolysis at each linkage, and the results would also prove or eliminate the possibility of multiple attack.

The preparation of a second series of isomaltose oligosaccharides, labelled at the reducing end, was made possible because of the ability of an exo-dextranase[5] of *Arthrobacter globiformis* T6 to transfer isomaltosyl residues[6] from dextran to D-glucose-^{14}C to give end-labelled trisaccharides. Isomaltose saccharides of higher d.p. were then synthesized by the transfer of D-glucosyl residues from sucrose to the non-reducing end of isomaltotriose-^{14}C with a D-glucosyltransferase[3] of *Streptococcus mutans*.

Dextran (Sigma, mol. wt. 2×10^6, 30 mg) and D-glucose-^{14}C (35 mg, 0.3 mCi) were incubated with exo-dextranase (1.2 i.u.) in 50mM sodium acetate buffer (pH 4.5, 1 ml) at 35°. Preliminary tests with unlabelled D-glucose showed that the production of trisaccharide, as observed by paper chromatography (p.c.), increased up to 1 h, and then remained constant for up to 4 h, due to the synthesis being balanced by hydrolysis to isomaltose and D-glucose. The reaction was therefore ended after 2 h by boiling, the mixture was deionised with Amberlite MB3 resin (carbonate form),

and the labelled trisaccharide fraction was separated from D-glucose-^{14}C, isomaltose (IM_2) dextran, and traces of IM_4 and IM_5 by chromatography on Whatman No. 3MM paper in nitromethane–ethanol–water[7] (41:36:23). Examination of a radioautogram revealed one labelled product in the position corresponding to trisaccharide. Hydrolysis of labelled trisaccharide had therefore given unlabelled isomaltose, indicating that the ^{14}C-label was at the reducing D-glucose residue.

Because the specificity of *A. globiformis* exo-dextranase for the hydrolysis of (1→6)-α-D-glucosidic linkages is not absolute (panose[5] is hydrolysed to isomaltose and D-glucose), the possibility that the enzyme had transferred isomaltosyl groups to positions other than C-6 on the acceptor (D-glucose-^{14}C), giving several trisaccharides, had to be considered. The trisaccharide fraction was eluted with water (1 ml) from the appropriate area of the chromatogram. A portion (60 μg, 25 μl) was incubated with a specific exo-dextranase, *Streptococcus mitis* dextranglucosidase[8] (0.05 i.u.), in 15mM sodium citrate buffer (pH 6) for 18 h at 35°. A determination of D-glucose, with D-glucose oxidase reagent[9], showed that the conversion of trisaccharide into D-glucose was apparently complete, but paper chromatography of the products in ethyl acetate–pyridine–water (10:4:3), followed by radioautography, revealed 3 small spots in the region between nigerose and maltose, in addition to a large D-glucose spot. Two areas of the paper were sectioned and placed into vials, and their radioactivity determined by liquid scintillation spectrometry. The area corresponding to D-glucose contained 97% of the total counts, while the remaining 3% was associated with the disaccharides. This proved that the trisaccharide fraction was a mixture of isomaltosyl-glucoses, isomaltotriose being the major component. Isomaltotriose was separated from the other trisaccharides by p.c. in ethyl acetate–pyridine–water (10:4:3), followed by elution with water. The position of ^{14}C-labelling in the isolated isomaltotriose was determined by sodium borohydride reduction and acid hydrolysis as previously described[3]. Separation of the sugars by p.c.[10] gave glucitol as the only labelled product, proving that isomaltotriose (IM_3) was labelled exclusively at the reducing end.

End-labelled oligosaccharides with higher d.p. were synthesized by incubating the reducing-end-labelled IM_3 (2 mg) with unlabelled sucrose (8 mg) and *S. mutans* D-glucosyltransferase[3] (0.02 i.u.) in 0.5 ml of solution at 35° and pH 6 for 18 h. The same enzyme preparation had been used for synthesis of isomaltose saccharides labelled at the non-reducing end. In the previous work[3], the weight of acceptor was in excess of the donor (sucrose-^{14}C), so that the d.p. of the major product was one unit higher than the acceptor. In the present experiment, where the donor (sucrose) was in excess, it was possible to synthesize a complete series from IM_4 to IM_9 in one incubation. Analysis of a portion of the solution by p.c. showed that isomaltotriose-^{14}C was almost completely consumed, giving a trace of IM_4, and IM_5–IM_9 as the main products. Small spots corresponding to branched oligosaccharides were also seen at positions between those of the higher isomaltose saccharides on the radioautogram. The labelled products were separated by p.c.[7] for 5 days to allow IM_5 to reach the bottom of the paper, and to give a complete separation of

linear from branched products. The yields and radioactivity of the eluted isomaltose saccharides are shown in Table I. A sample (50 μg) of IM_5–IM_8 was incubated with dextranglucosidase, as described above for the trisaccharide fraction. A test with D-glucose oxidase reagent showed that each substrate was completely hydrolysed to D-glucose, and a radioautogram prepared from a paper chromatogram revealed that D-glucose was the only labelled product. No labelled oligosaccharides were seen, proving that anomalous linkages, which arrest dextranglucosidase action[11], were absent.

TABLE I

SPECIFIC ACTIVITIES OF END-LABELLED ISOMALTOSE SACCHARIDES

D.p.	*Weight (mg)*	*Specific activity (counts/sec/mg)*
3	2.4	85,980
5	0.51	68,880
6	0.76	54,710
7	0.60	47,180
8	0.38	37,530

The first series of end-labelled isomaltose saccharides was prepared by the transfer of D-glucosyl-^{14}C residues from sucrose-^{14}C to the non-reducing end of each isomaltosaccharide acceptor. Robyt *et al.*[12] commented that, under the conditions of excess acceptor, an insertion mechanism could explain the pattern of labelling. Since the ratio (4) of sucrose:acceptor chosen for the present work was higher than that (0.25) used for the preparation of the first series, it was necessary to establish the position of the label in the new series, in case D-glucosyl residues from unlabelled sucrose had been inserted at the reducing end. The test was made by reacting IM_5 and IM_7 (0.125 μmol of each) with *A. globiformis* exo-dextranase (0.5 i.u.) in 25mM acetate buffer (pH 4.2, 0.35 ml). This enzyme, being an isomaltohydrolase, hydrolyses oligosaccharides having an odd d.p. to give not only isomaltose, but also one D-glucose residue from the reducing end of the chain. After incubation for 18 h, when a sample taken for the determination of D-glucose with D-glucose oxidase reagent showed that the hydrolyses were complete, the digests were boiled, and deionized, and the products were separated by p.c.[7]. A radioautogram revealed that D-glucose was the only labelled product and, when portions of the papers corresponding to D-glucose and isomaltose were counted, >99.7% of the radioactivity was found to be associated with D-glucose.

Action of Streptococcus mutans *dextranase on isomaltopentaoses labelled at opposite ends.* — One oligosaccharide from the new series was further tested by comparing the pattern of its hydrolysis by dextranase with the pattern obtained from the corresponding isomaltose oligosaccharide labelled at the non-reducing end. Since the main aim was to confirm the linkage and position of the label at the reducing end of the new series, the endo-dextranase of *S. mutans*[13] was chosen because of

its ability to release D-glucose from the reducing end of oligosaccharides. IM_5 was selected as substrate, because the linkage at the reducing end (linkage 1) is preferentially cleaved, whereas the proportion of the hydrolysis occurring at linkage 1 decreases[14] markedly with increasing d.p. The two oligosaccharides (0.2mM) were incubated at 35° with *S. mutans* dextranase (0.09 i.u.) in 4mM sodium citrate buffer (pH 6) in a final volume of 0.5 ml. When the extent of reaction was ~ 5%, the enzyme was inactivated, and the products were separated by paper chromatography[7]. From a radioautogram, the radioactivity of sections of the paper that corresponded to labelled products was measured. The bond-cleavage frequencies were identical for the two substrates (Fig. 1).

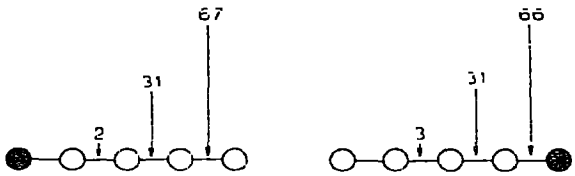

Fig. 1. Distribution of linkage hydrolysis of end-labelled isomaltopentaose by *Streptococcus mutans* dextranase. ○, D-glucose residue; ●, D-glucose-^{14}C residue; ———, (1→6)-α-D-glucosidic linkage. Arrows indicate the point and extent of hydrolysis (%).

These results prove conclusively that the new series of isomaltose saccharides is labelled only at the reducing end, and that the linkage at the reducing end is α-(1→6). The two series were synthesized with a D-glucosyltransferase preparation that transfers to the non-reducing end of isomaltosaccharide acceptors. Recent evidence has indicated that some polysaccharide molecules may grow from the reducing chain-end[11,15]. It is not intended to convey that the results presented here on the direction of *oligosaccharide* synthesis are contrary to those of Robyt *et al.*[11], who reported that a different enzyme system, insolubilized dextransucrase of *Leuconostoc mesenteroides* NRRL B-512(F), acted *via* an insertion-type mechanism when synthesizing *polysaccharide* from sucrose alone.

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.

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