Carbohydrate Research, 222 (1991) 239–244
Elsevier Science Publishers B.V., Amsterdam

Branched saccharides formed by the action of His-modified cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* M 5 al on starch

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(Received February 20th, 1991; accepted for publication April 23rd, 1991)

ABSTRACT

Digestion of potato starch with His-modified α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* M 5 al yielded branched tetra- to nona-saccharides, as revealed by debranching with pullulanase. Maltose and maltotriose stubs preponderated together with small proportions of D-glucose stubs. The branched saccharides accounted for ~1.2% of the starch.

INTRODUCTION

In the context of the mechanism of the reversible (1→4)-α-D-glucopyranosyl-transfer reactions, catalysed by the cyclodextrin glycosyltransferases [(1→4)-α-D-glucan:(1→4)-α-D-glucopyranosyltransferase (cyclising), EC 2.4.1.19; CGTase], the influence of (1→6)-α-D-glucosidic branches is of interest. Like the alpha-amylases, which are structurally related to the CGTases[1,2], the CGTases can by-pass branch points to some extent. Thus, in addition to the well-known cyclisation (coupling) and disproportionation reactions, the enzymes catalyse rapid endo-cleavage of the longer B-chains of amylopectins[3,4] and long amylose chains[5]. Moreover, small amounts of branched cyclodextrins (cyclomalto-oligosaccharides, CDs) are formed[6,7]. From the results of beta-amylolysis and debranching of the non-cyclic compounds produced by the action of the α-CGTase from *Klebsiella pneumoniae* M 5 al (*K. pneumoniae*) on starch, the smallest branched saccharides were assumed[4], but not proved, to be hexasaccharides.

Ethoxyformylation with diethyl pyrocarbonate of the His-residues of the CGTases[8] (→H*CGTases) resulted in a delayed formation of CDs and a marked increase in non-cyclic products. This modification also affected the disproportionation reactions so that larger amounts of branched saccharides were formed from starch. The detection and identification of such saccharides are now reported.

EXPERIMENTAL

Enzymes. — Purified α-CGTase from *K. pneumoniae* was the enzyme used in previous studies[8]. Beta-amylase [(1→4)-α-D-glucan maltohydrolase, EC 3.2.1.2] from

0008-6215/91/$ 03.50

Ipomoea batatas (500 U/mg), glucoamylase [(1→4)-α-D-glucan glucohydrolase, EC 3.2.1.3] from *Aspergillus niger* (14 U/mg), and pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) from *Aerobacter aerogenes* (30 U/mg) were obtained from Boehringer.

Substrate. — Potato starch was obtained from the Consortium für Elektrochemische Industrie, Munich.

Analytical methods. — Modification of the CGTase (→H*CGTase) with diethyl pyrocarbonate and determination of the cyclising activity were performed as described[2,8]. Total carbohydrate was determined with the anthrone reagent[9] and reducing end groups with the Nelson reagent[10]. Beta-amylolysis, debranching with pullulanase, digestion with glucoamylase, and quantitative h.p.l.c. were performed as described[3,11].

Digestion of starch. — Gelled potato starch (10%) in 10mM sodium phosphate buffer (pH 6.8) was incubated (30°) separately with CGTase and H*CGTase (cyclising activities of 2.8 and 0.8 μkat/g of starch, respectively) for 24 h. The CDs were removed as their insoluble complexes with bromobenzene and cyclohexane, and the non-cyclic products were fractionated by successive precipitation with methanol to 55% (fraction I), ethanol to 60% (fraction II), and acetone to 70% (fraction III). After each precipitation step, the organic solvent was evaporated. The final supernatant solution was concentrated to half volume to give fraction IV. An aqueous 8% solution of fraction III was fractionated further by precipitation of the larger compounds with ethanol to 60% at −18° to give fraction IIIa, and precipitation of the smaller saccharides with acetone to 75% to give fraction IIIb. Fractions obtained with H*CGTase are designated with H*.

RESULTS AND DISCUSSION

Digestion of starch with H*CGTase gave markedly less CDs than did CGTase, but large proportions of non-cyclic products (Table I). Due to an altered binding mode[8], the high-molecular-weight substrates (fraction I) decreased at a higher rate, and smaller non-cyclic products (fraction III) were the main products.

H.p.l.c. of fraction IIIbH* revealed a mixture (Fig. 1) of malto-oligosaccharides (G_n) that were formed by disproportionation reactions, together with minor peaks (indicated by arrows) due to other saccharides. The degree of beta-amylolysis of fraction IIIbH* was 69%, and digestion with pullulanase increased the proportion of glucose equivalents to 160 mg/g. As shown by h.p.l.c., G_2–G_7 were the main products of the debranching, which indicated the presence of small branched saccharides. Since ~90% of the branched compounds were resistant to beta-amylase, fraction IIIbH* was subjected to beta-amylolysis and the increased proportion of branched saccharides was precipitated by the addition of acetone (to 60%) to give fraction IIIbH*β. Of the larger compounds, ~60% were recovered with the precipitate and their total proportion comprised ~1.2% of the starch. Fraction IIIbH*β contained G_2, G_3, αCD (eluted with the retention time of G_4), and a series of saccharides, the retention times of which were different from those of G_n (Fig. 2A, peaks A–H). On debranching fraction IIIbH*β, C,

TABLE I

Products obtained from potato starch[a] by digestion with CGTase and H*CGTase[b]

Fraction	*CGTase (% of total carbohydrate)*	*H*CGTase*
CDs	43.8	17.0
I	28.6 (140)[c]	10.5 (96)
II	14.6 (45) [540 nm][d]	22.7 (34) [500 nm]
III	7.2 (21.4)	31.4 (15.8)
IIIa	4.3 (28.2)	11.2 (21.2)
IIIb	2.9 (11.4)	20.3 (7.9)
IV	5.8 (7.1)	18.4 (3.4)

[a] Gelled 10% potato starch in 10mM sodium phosphate buffer (pH 6.8) was digested for 24 h at 30°; 1.35 and 7.3 g/L of glucose equivalents, respectively, were found in the digests with CGTase and H*CGTase. [b] Cyclising activities of 2.8 (CGTase) and 0.8 μkat (H*CGTase) per g of starch were employed. [c] D.p. in brackets. [d] λ_{max} of the iodine complexes; aqueous 0.3% solutions were mixed with 100 μL of 13mM I_2 in 40mM KI. For fractionation of the products, see Experimental.

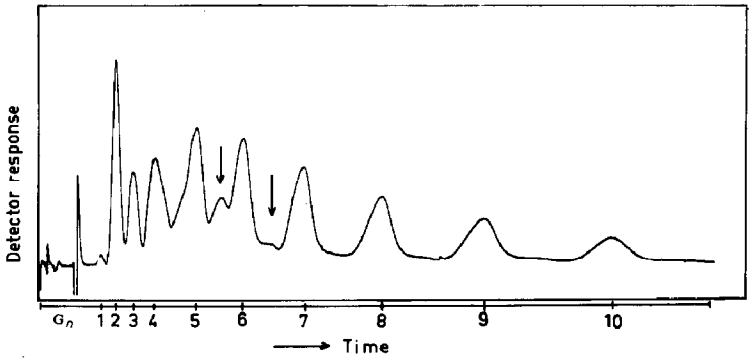

Fig. 1. H.p.l.c. of fraction IIIbH*; 50 μL of an aqueous 10% solution was injected. For fractionation of the starch digests, see Experimental. H.p.l.c. as described[8,12]. G_n denotes glucose and malto-oligosaccharides with *n* glucose residues.

TABLE II

Tentative structures of the branched saccharides[a]

H.p.l.c. peak	*Structure*[b]
B	6^2-*O*-α-G_2-G_3 (6^3-*O*-α-D-G_1-G_4)
C	$6^{2(3)}$-*O*-α-G_2-G_4 (6^2-*O*-α-G_3-G_3)
D	6^n-*O*-α-G_1-G_5
E	6^3-*O*-α-G_2-G_5 (6^3-*O*-α-G_3-G_4)
G (H)	6^3-*O*-α-G_2-G_6 (6^3-*O*-α-G_3-G_5)

[a] Derived after debranching by digestion with beta-amylase or glucoamylase. [b] Alternative structures in brackets.

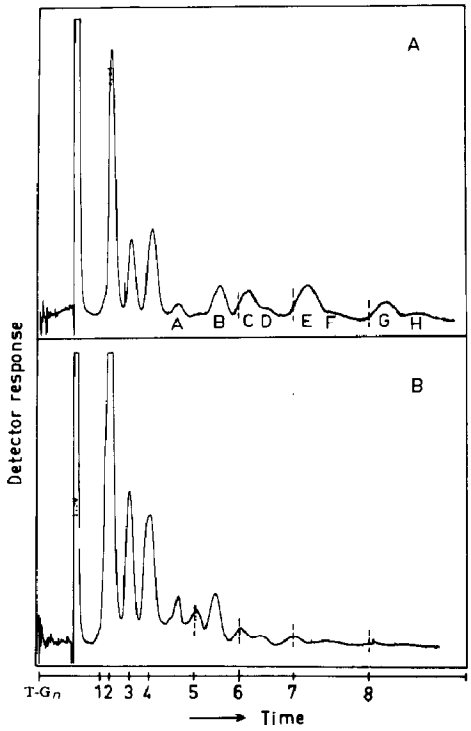

Fig. 2. H.p.l.c. of fraction IIIbH*β: A before and B after debranching with pullulanase; 50 μL of an aqueous 5% solution was injected. Debranching[3] and h.p.l.c.[8,12] as described. *T*-G_n denotes the retention times of glucose and malto-oligosaccharides with *n* glucose residues.

E, G, and H vanished, the proportions of G_2–G_4, A, and B increased, and G_5–G_7 were formed (Fig. 2B). Thus, C, E, G, and H represented branched hexa- to octa-saccharides, respectively, which are substrates for pullulanase[12] (Table II). The small proportion of G_7 found in the pullulanase digest pointed to the presence of some nonasaccharide, the peak of which was within the noise of the detection system. Because of the low concentration, the compound in the shoulder F could not be identified. The nature of A and B (the proportions of which increased on debranching) and of D was not clear.

Digestion of debranched fraction IIIbH*β with beta-amylase and glucoamylase (Figs. 3A and 3B) hydrolysed G_4–G_7, and glucoamylase also hydrolysed B and D. Thus, B was a branched pentasaccharide, and D might be G_6 carrying a glucose stub and thus be a poor substrate for pullulanase[12] (Table II). Since B was still branched, that part produced by the action of pullulanase must have been derived from a multiply branched saccharide. The enzymes did not attack A, which, therefore, could be a tetrasaccharide containing (1→3)-α-D-glucosidic bonds, since some transfer to HO-3 of the acceptors during the disproportionation reactions was ascertained[13,14]. It could not be decided solely from the retention time whether peak K of the glucoamylase digest contained nigerose. Peak I of the beta-amylase digest had the retention time of panose, and could be isopanose or 6^1-*O*-α-D-glucosylmaltose, derived from a saccharide that carried a glucose stub.

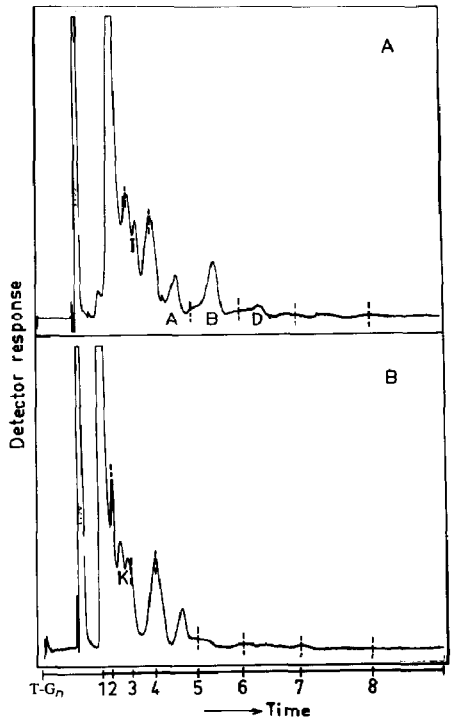

Fig. 3. H.p.l.c. of the products obtained by digestion of debranched fraction IIIBH*β with A beta-amylase and B glucoamylase. Conditions of beta-amylolysis[3], digestion with glucoamylase[3], and h.p.l.c.[8,12] as described. T-G$_n$ denotes the retention times of glucose and malto-oligosaccharides with n glucose residues.

The results showed that H*CGTase formed branched saccharides from starch, *i.e.*, the enzyme could attack B-chains in close proximity to their branch points. However, relative to the proportion of (1→6)-α-linked branches in the substrate, only a low percentage was recovered with the branched saccharides, which came from the less branched regions, whereas the highly branched clusters, apparently resistant to CGTase, were found in fractions I and II[3,4]. From the distances of the branch points from the reducing ends of the saccharides, it is probable that some of the branched saccharides are products of secondary disproportionation, *i.e.*, the branches did not strongly affect this reaction. Branched CDs could not be detected. The increase in G$_4$/αCD in fraction IIIbH*β caused by debranching (Fig. 2B) decreased to the original level in the beta-amylase and glucoamylase digests, and indicated that the compound released by pullulanase was G$_4$.

Besides glucose and small G$_n$, fraction IVH* contained small proportions of A and B. Larger branched saccharides or compounds other than those found in IIIbH* could not be detected. Fraction IIIbβ from the digests with CGTase contained only traces of branched saccharides, the concentrations of which were within the noise of the detection system.

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