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High-performance anion-exchange chromatography with pulsed amperometric detection of linear and branched glucose oligosaccharides

Robert N. Ammeraal, Gregory A. Delgado, Francis L. Tenbarge, and Robert B. Friedman

Research Department, American Maize-Products Company, 1100 Indianapolis Boulevard, Hammond, IN 46320-1094 (U.S.A.)

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ABSTRACT

Variation of response according to degree of polymerization (d.p.) of linear malto-oligosaccharides was estimated for a high-performance anion-exchange chromatography system with pulsed amperometric detection (h.p.a.e.-p.a.d.). In the range of d.p. 1–20, response per μmol increased as the d.p. increased to about d.p. 14. Beyond this value, response was variable and showed no clear upward trend. Response per μg decreased with increasing d.p. The effect of α-(1→6) glycosidic linkages on retention times was also investigated with this system using linear pullulan oligosaccharides, as well as enzymatically generated maltosyl or glucosyl branched oligosaccharides from waxy maize starch. Retention times were reduced about 0.5 min for each α-(1→6) glycosidic linkage present. Isomers with single maltosyl branches could not be resolved from those with single glucosyl branches but were separated from α-amylase limit dextrins with multiple branching. Identity of the chromatographic peaks was confirmed (*i*) for the pullulan fragments by comparing to a commercial d.p. 36 standard; (*ii*) for d.p. 6–14 singly branched maltosyl oligosaccharides by fractionation, pullulanase hydrolysis, and analysis of the resultant products; and (*iii*) for the α-amylase limit dextrins by hydrolysis of a singly branched cyclodextrin, analysis of the products, and comparison to the known starch limit dextrin structures.

INTRODUCTION

The fine structure of amylopectin is usually defined in terms of chain lengths and their distribution. Techniques for elucidation of amylopectin structure were recently reviewed by Manners[1]. A powerful new technique for carbohydrate analysis has recently been described[2] whereby linear starch dextrins containing up to 70 dextrose units [degree of polymerization (d.p.) = 70] can be resolved using high-performance anion-exchange chromatography with pulsed amperometric detection (h.p.a.e.-p.a.d.). In seeking standards to calibrate the h.p.a.e.-p.a.d. column, a pullulanase digest of pullulan was examined since it primarily contained polysaccharide chains with d.p. values in even multiples of three glucose units[3]. The chromatographic behavior of these oligosaccharides, however, was so different from the α-(1→4)-linked linear isomers that they could not be used as standards.

Chromatographic systems have been described which can distinguish singly and doubly branched malto-oligosaccharides of limited size if the branches are limited to

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glucosyl or maltosyl residues[4]. Other h.p.l.c. methods for carbohydrate analysis have been reviewed by Hicks[5]. H.p.a.e.-p.a.d. is capable of resolving and identifying various charged carbohydrates[6,7]. The behavior of branched oligosaccharides from starch in this system has not yet been reported. It would be valuable if an h.p.a.e.-p.a.d. system could discriminate longer chains with branches from isomeric linear chains, since a much finer resolution can be obtained than in the gel-permeation chromatographic method used by Hizukuri and Maehara[8] to determine the frequency of A chains on B chains and variation with B chain size. At present the variation of p.a.d. detector response with chain length has not been satisfactorily defined, but we have a limited calibration that has been useful. Response calibration over an extended range of molecular weight appears necessary despite the lack of variation over the limited d.p. range described by Koizumi *et al.*[7]

The purpose of this work is to develop procedures to separate and identify branched polysaccharides by h.p.a.e.-p.a.d. chromatography. These methods will be used to define branching patterns in starch amylopectins, details of which will be reported elsewhere. This knowledge will contribute to the practical goal of relating starch structure to function especially for screening genetic varieties of corn for specific applications.

EXPERIMENTAL

Materials. — Waxy β-limit dextrin was a gift of the United States Department of Agriculture, Northern Regional Research Laboratory, Peoria, IL. Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa*, β-amylase (EC 3.2.1.2) Type 1-B crystalline from sweet potato, and pullulan were purchased from Sigma Chemical Co., St. Louis, MO. Pullulanase (EC 3.2.1.41) from *Aerobacter aerogenes* was obtained from Boehringer-Mannheim, Indianapolis, IN. *Aspergillus oryzae* α-amylase (EC 3.2.1.1), 3× crystallized and lyophilized, was obtained from Calbiochem-Behring Corp., La Jolla, CA. Enzymes were used without further purification. Enzyme activity units used were those defined by the suppliers. Polysaccharide standard MP5800 was prepared by Polymer Laboratories, Ltd. and purchased from Alltech Associates, Inc. The D-glucose standard was from Sigma Chemical Co., and oligosaccharide standards of d.p. 2–7 were from Nihon Shokuhin Kako Co., Ltd., Japan.

H.p.a.e.-p.a.d. chromatography. — Chromatography was performed on a Dionex Bio-LC system equipped with a gradient pump system, an Eluent Degas Module, and a Pulsed Amperometric Detector (p.a.d.). The p.a.d. contained a gold reference electrode. The column used was a Dionex CarboPac PA1 (4 × 250 mm) with a CarboPac PA Guard column (3 × 25 mm). Results were recorded on a Hewlett-Packard HP 3396A integrator. The sample injection loop volume was 25 μL. Pulse potentials and durations used for this work on the p.a.d. were as follows: $E_1 = 0.05$ V, $t_1 = 480$ ms, $E_2 = 0.60$ V, $t_2 = 120$ ms, $E_3 = -0.80$ V, $t_3 = 60$ ms. The response time of the detector was set for 1.0 s.

Eluent 1 was M sodium acetate. Eluent 2 was either 200mM or 375mM sodium hydroxide. Eluent 3 was water. Gradient programs were as follows: Elution program A

— (percent eluent 1), 5% at 0 min to 50% at 45 min; (percent eluent 2, 200mM NaOH), constant 50% from 0 to 45 min; elution program B — (percent eluent 1) 5% at 0 min, 15% at 5 min, 25% at 20 min, 35% at 45 min, and 45% at 65 min; (percent eluent 2, 375mM NaOH), constant 40% from 0 to 65 min. Flow rate was held constant at 1.0 mL/min throughout. Sodium hydroxide, 400mM, was added to the elution solvent post-column prior to detection at a rate of approximately 0.5 mL/min to stabilize the base line. All eluents were prepared with doubly deionized water, and were degassed by sparging with helium.

Calibration of the pulsed amperometric response. — Solutions of known concentration were prepared from purified standards of d.p. 1–7. Injections of 25 μL were made with a 50-μL syringe. The integrated area obtained was then divided by the μg or the μmol injected to determine the response. The μg response was on the order of 10^7 area counts/μg while the μmol response was on the order of 10^{10} area counts/μmol. For d.p. 12–21, fractions were collected from the h.p.a.e.-p.a.d. system after injecting a 2% solution of debranched amylopectin. The d.p. range of the sample before injection had been narrowed by ethanol precipitation and size-exclusion chromatography. These fractions (about 0.8 mL each) contained 2 to 3 major components. Total carbohydrate in the fractions was determined using an anthrone method[9] (the eluent salts did not interfere with the test). A 25-μL sample of the fraction was injected into the column, and the response was determined as before. The samples were low in concentration. Response was measured at 1000 nA sensitivity on the p.a.d.

Preparation of the pullulan digest. — The pullulan digest was prepared by dissolving 0.5 g of pullulan in 50 mL of water (30 min at 100°). One mL of 0.2M acetate buffer (pH 5.0) was added, followed by 10 μL of pullulanase suspension. This gave 40.6% maltotriose after 22 h at 30°. The reaction was stopped (5 min at 100°), treated with mixed-bed (Amberlite MB-3) ion-exchange resin, and filtered before h.p.a.e.-p.a.d. chromatography.

Isoamylase hydrolysis of waxy maize β-limit dextrin (Reaction 1). — To a 20-mL vial were added 1.25 mL of 0.2M acetate buffer (pH 3.5), 3 μL (52 U) of isoamylase enzyme, and 3.75 mL of a solution containing 50 mg of solubilized β-limit dextrin. The mixture was incubated for 20 h at 45° with the vial capped. The enzyme reaction was stopped by adding 1 mL of sodium phosphate buffer (pH 6.2) and heating the vial for 3 min in a boiling water bath. The solution (1.75 mL) was diluted to 10 mL with doubly deionized water. The solution was then treated with mixed-bed (Amberlite MB-3) ion-exchange resin and filtered before chromatography.

β-Amylase hydrolysis of the Reaction 1 product (Reaction 2). — To a 20-mL vial were added 2 mL (20 mg) of the Reaction 1 solution, 1 mL of 0.2M acetate buffer (pH 5.0), 7.0 mL of doubly deionized water, and 108 μL (3000 U) of the β-amylase enzyme. The vial was capped and incubated for 20 h at 35°. Finally, the reaction was stopped (3 min at 100°). The solution was treated with Amberlite MB-3 ion-exchange resin and filtered before chromatography.

Pullulanase hydrolysis of the product from Reaction 2 (Reaction 3). — To a 20-mL vial, 3.75 mL (15 mg) of the Reaction 2 hydrolysate, 1.25 mL of acetate buffer (pH 5.0),

and 0.1 mL (15 U) of pullulanase enzyme were added. The vial was capped and incubated for 20 h at 30°. The reaction was then stopped (3 min at 100°), and the solution was treated with Amberlite MB-3 ion-exchange resin and filtered before chromatography.

Pullulanase hydrolysis of the product from Reaction 1 (Reaction 4). — To a 20-mL vial, 2.0 mL (20 mg) of the Reaction 1 hydrolysis product, 1.25 mL of acetate buffer (pH 5.0), 1.75 mL of doubly deionized water, and 0.1 mL (15 U) of pullulanase were added. The vial was capped and incubated for 20 h at 30°. Then the enzyme was deactivated (3 min at 100°), and the solution was diluted with 5.0 mL of doubly deionized water. The digest was treated with Amberlite MB-3 ion-exchange resin and filtered before chromatographic analysis.

Fractionation and enzymatic analysis of Reaction 2 branched oligosaccharides. — A 2% solids solution of a Reaction 2 digest was prepared, and 25 μL was injected for h.p.a.e.-p.a.d. chromatography. Fractions were collected, beginning at the initial rise of each new peak. This was repeated until each collector tube contained 3–4 mL of solution. Water was added to bring the volume to 6 mL for each fraction, and 1.3 g of moist Amberlite MB-3 were added. Tubes were swirled until the color changed. The pH was then measured and adjusted if necessary to pH 5.0 with 0.5N NaOH. The solution was divided into two, 3-mL aliquots, and 2 μL of pullulanase (0.3 U) were added to one. This was incubated at least 0.5 h at ambient temperature. The other aliquot served as a control. The two solutions were filtered and chromatographed (elution program B) at 300 nA sensitivity. After pullulanase treatment, 1 μL of β-amylase was added (28 U), and the mixture was incubated at ambient temperature for about 1 h, filtered, and chromatographed.

Preparation of α-amylase limit dextrins from branched cyclodextrins. — Branched cyclodextrins were isolated from pilot- or commercial-scale digests of Lo-Dex 5® (American Maize-Products Co., Hammond, IN) with commercial grade cyclodextrin glycosyl transferase. Isolation and purification was accomplished using the procedures of Ammeraal *et al.*[10,11] ^{1}H-N.m.r. spectroscopy was performed on a Varian XL-400 spectrometer. The spectra were measured using the parameters of Gidley[12], with an inversion–recovery pulse sequence of (180°-*t*-135°). Anomeric proton chemical shifts occurred at (a) 5.00 p.p.m. [α-(1→6) linkage]; (b) 5.14 p.p.m. [cyclodextrin α-(1→4) linkage]; and (c) 5.40 p.p.m. [noncyclic α-(1→4) linkage]. From the areas of shifts a, b, and c (set d = b/7); (*i*) total d.p. = (a + b + c)/d, (*ii*) branch d.p. = (a + c)/d, and (*iii*) branches per molecule = a/d. Areas a and c were adjusted for impurities. Results from these calculations were consistent with estimates of total d.p. by h.p.l.c., steroid complexation stoichiometry, stoichiometry of pullulanase debranching, and h.p.a.e.-p.a.d. chromatography, as well as by gel-permeation chromatography, calibrated with the other estimates. (These results will be reported elsewhere.) The singly branched cyclodextrin sample was 98% branched cyclodextrin and had an average branch d.p. = 2 with a branch frequency of 1 per molecule. The multiply branched β-cyclodextrin was 94% branched and had an average branch d.p. of 18. The average branch frequency was about 3.4 per molecule by n.m.r. spectroscopy. Limit dextrins were generated in 10 mL

TABLE I

H.p.a.e.-p.a.d. detector response for linear malto-oligosaccharides[a]

Degree of polymerization, d.p. (glucose units)	*Response per μg (10^6 area counts)*	*Standard deviation (10^6 area counts)*	*Response per μmol (10^9 area counts)*
1[b]	92.9	3.0	16.7
2	51.9	1.9	17.8
3	44.2	1.9	22.3
4	43.6	1.5	29.0
5	35.9	1.8	29.8
6	34.8	2.4	34.5
7	31.0	4.7	35.7
11–12[c]	20.6	1.4	38.8
12–13	19.6	2.3	40.0
14–15	21.5	2.6	50.9
14–16	18.8	1.0	46.0
17–18	18.3	5.3	52.2
18–19	14.8	3.4	44.6
19–21	13.6	3.0	44.3
20–22	15.9	4.9	54.4

[a] Elution program A. [b] Based on prepared solution concentration. [c] Based on anthrone analysis of dilute chromatographic fractions.

of a 1.25% branched cyclodextrin solution in 0.02M phosphate buffer at pH 6.6 by addition of 300 U of *A. oryzae* α-amylase. Incubation was carried out for 6 h at 45°.

RESULTS AND DISCUSSION

Variation of p.a.d. response with d.p. — Table I lists the response data for d.p. 1–20. This was measured using elution program A and a sensitivity of 1000 nA. A steady increase in μmol response can be seen with increasing d.p. up to about d.p. 14. Beyond that point, the responses were variable with no clear upward trend. The present response calibrations have proven useful in making quantitative estimates from h.p.a.e.-p.a.d. data where results could be compared with independent estimates. These estimates involved branched cyclodextrins and will be reported elsewhere. Response values obtained while using elution program B were somewhat different from those obtained using program A. If the relative response changes under different conditions, then calibrations will need to be checked periodically or established for different analytical conditions.

H.p.a.e.-p.a.d. chromatography of pullulan oligosaccharides. — A digest of pullulan was prepared using pullulanase. This was analyzed on an Aminex HPX-42A column (Bio-Rad), which resolved oligosaccharides up to d.p. 11. A maltodextrin standard was used to calibrate the retention times. This column separated d.p. 3 to d.p. 15 from the pullulan digest with major peaks at d.p.s 3, 6, 9, 12, and 15. The corresponding peaks

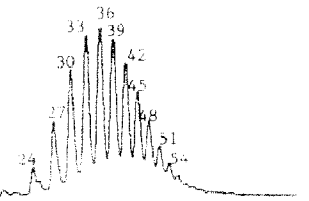

Fig. 1. H.p.a.e.-p.a.d. chromatogram of the commercial pullulan-derived standard, MP 5800. Peak d.p. is indicated above the peaks. Peak retention times are listed in Table II.

could then be identified by relative area on the h.p.a.e.-p.a.d. Assuming each successive peak represents a polysaccharide which is 3 d.p. larger than that of the previous peak, peaks up to d.p. 72 were well separated. Peak assignments for the linear starch oligosaccharides were made in an analogous manner using a starch isoamylase digest with peak d.p. assignment according to the method of Koizumi *et al.*[7]. To check the assignment of d.p. to peaks in this manner, a commercial standard for size-exclusion chromatography was subjected to h.p.a.e.-p.a.d. analysis (Fig. 1 and Table II).

The data for standard MP 5800 were provided by the supplier. The molecular weight was determined by sedimentation equilibrium in water[13]. The polydispersity was determined by gel-permeation chromatography according to Kawahara *et al.*[3]. The h.p.a.e.-p.a.d. chromatogram of MP 5800 is shown in Fig. 1. Peaks range in d.p. from 24 to 54. The relative detector response of these peaks is not known. The molecular weight

TABLE II

Average molecular weight of MP 5800 pullulan standard by h.p.a.e.-p.a.d. chromatography

D.p. *(glucose units)*	*Retention time (min)*[a]	*Model 1*[a] *(% of total response)*	*Model 2*[a] *(corrected*[b] *% of total response)*
21	25.5	1.7	2.24
24	26.1	6.6	8.13
27	26.8	11.2	12.92
30	27.5	14.5	15.72
33	28.0	15.6	15.95
36	28.6	14.6	14.14
39	29.2	12.3	11.30
42	29.6	9.5	8.31
45	30.1	6.7	5.58
48	30.5	4.4	3.51
51	31.0	1.9	1.45
54	31.4	1.0	0.73

[a] Integrated percent is mole % with equal response assumed for d.p. 21–54. [b] Integrated percent is corrected relative to the d.p. 20 response which was set at 48×10^9 area counts/μmol and assuming an increase in response of 1×10^9 area counts/d.p. from d.p. 20. [c] Correction factor = [(d.p. − 20) + 48]/48 for d.p. 21–54. [d] Elution program A.

TABLE III

Comparison of molecular weights for MP 5000

Method	M_n	M_w	*Polydispersity*
Sedimentation and g.p.c.	33.4	35.7	1.07
H.p.a.e.-p.a.d.			
Model 1	35.1	36.6	1.04
Model 2	34.3	35.8	1.04

of MP 5800 was calculated from the data of Table II on the basis of two models. In Model 1, no correction was made for differences in response related to d.p. In Model 2 the response per μmol of d.p. 20 was set at 48, and the response was increased by one for each incremental d.p. increase. This is about the rate of increase from d.p. 7 to d.p. 20 shown in Table I. In Table II, under Model 1, the percent of total response from the peaks of Fig. 1 is recorded. Under Model 2 these values are modified by a correction factor calculated as (peak d.p. + 28)/48.

M_n and M_w can be calculated for each model from the data of Table II. These results are shown in Table III and compared with the values assigned by the supplier to the standard. The response factors for the peaks in Fig. 1 are not known, but Models 1 and 2 should be reasonable limits for the variation of response. The values calculated from the two models are similar to each other and similar to the results obtained by the supplier of the standard. The Model 2 M_w is nearly identical to the supplier's M_w, and the Model 1 M_w is 0.9 d.p. higher. The polydispersity of Models 1 and 2 were the same and, since it is calculated from clearly defined peaks of known d.p., it is more reliable than one calculated from area slices on a gel-permeation chromatogram. Using the 1.04 polydispersity value, the M_n calculated from the M_w of the supplier becomes 34.3, which is within 1 d.p. of those calculated from Models 1 and 2. On the basis of the data of Table II, there is no reason to question the d.p. assignments of Fig. 1 or the conclusions reached from the data of Table IV. Further, it can also be seen that the calculation of M_n from h.p.a.e.-p.a.d. data is relatively insensitive to variations in the relative p.a.d. response of the various components involved.

The effect of the α-(1→6) glycosidic linkage on h.p.a.e.-p.a.d. retention times of pullulan oligosaccharides is shown in Table IV. Pullulan is a linear polymer of maltotriose, α-(1→6) linked. A pullulan oligosaccharide of d.p n thus has $(n - 3)/3$ such linkages per chain, and these linkages reduce retention time, in proportion to the number present, relative to the isomeric linear malto-oligosaccharides. The retention time reduction ranged from 0.86 to 0.39 min per α-(1→6) glycosidic linkage as the retention time increased. Elution program A was used, and the sensitivity was 1000 nA.

H.p.a.e.-p.a.d. chromatography of branched oligosaccharides. — Oligosaccharides with maltosyl branches were prepared according to the procedures of Hizukuri and Maehara[8]. A waxy maize β-limit dextrin with its maltosyl and maltotriosyl A-chain

TABLE IV

The effect of α-(1→6) linkages on h.p.a.e.-p.a.d. retention times in linear isomeric fragments of starch and pullulan

D.p. (glucose units)	*α-(1→6) Linkages per pullulan fragment*	*H.p.a.e.-p.a.d. retention times*			
		Pullulan (min)[a]	*Starch (min)*[b]	*Difference starch – pullulan (min)*	*Difference per α-(1→6) linkage (min)*
3	0	9.37	9.37	0.00	0.00
6	1	14.86	15.72	0.86	0.86
9	2	18.36	19.81	1.45	0.73
12	3	20.85	22.79	1.85	0.62
15	4	22.70	25.00	2.30	0.58
18	5	24.18	26.68	2.50	0.50
21	6	25.39	28.11	2.72	0.45
24	7	26.26	29.32	3.06	0.44
27	8	27.03	30.38	3.35	0.42
30	9	27.71	31.33	3.62	0.40
33	10	28.33	32.21	3.88	0.39

[a]Standard deviation was about 0.2 min, for elution program A. [b]Retention times adjusted by 0.17 min so that the d.p. 3 retention times are equal. These values are slightly different than those in Table III.

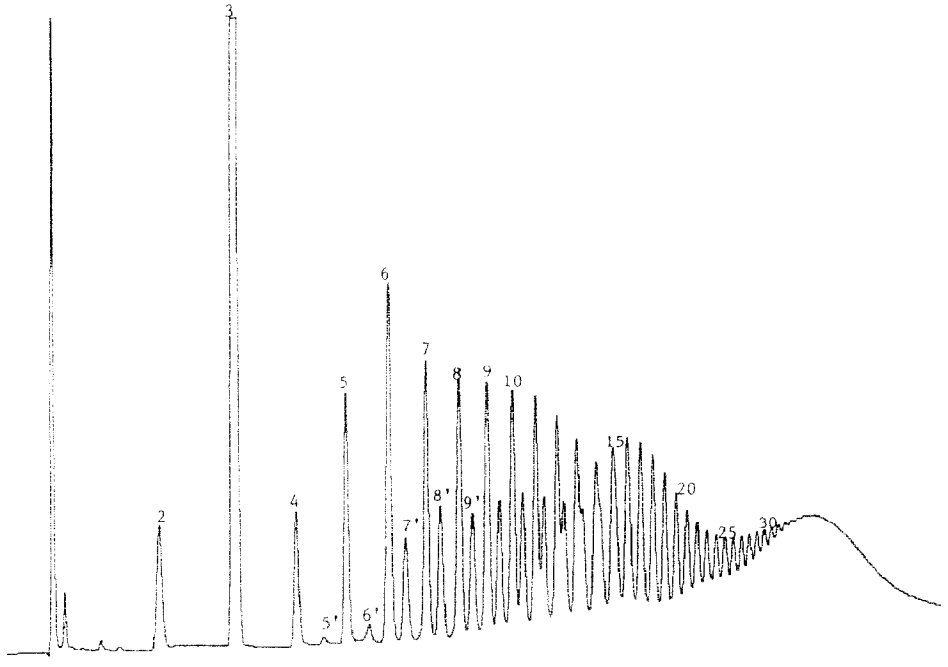

Fig. 2. H.p.a.e.-p.a.d. chromatogram of the Reaction 1 digest (an isoamylase digest of a waxy maize β-limit dextrin). The d.p. of individual peaks is indicated by normal integers for linear chains and by primed integers for branched chains. The branched chains have only maltosyl branches, with at least one near the non-reducing end of the chain. Typical retention times of the peaks are listed in Table V.

stubs was debranched with isoamylase (Reaction 1). The products were linear malto-oligosaccharides and chains with isoamylase-resistant maltosyl branches. The small branched chains separate from the linear chains in the h.p.a.e.-p.a.d. system (Fig. 2). After β-amylase treatment to remove the linear chains (Reaction 2), the branched chains, up to about 25 d.p., were separated (Fig. 3). The identity of peaks 5′ through 14′ was confirmed by pullulanase hydrolysis of isolated fractions as will be discussed later. When the Reaction 2 digest was treated with pullulanase, linear oligosaccharides were formed which could be separated to about d.p. 30 (Fig. 4). Some residual branched material, resistant to pullulanase, remained at d.p. 3–7. During Reaction 2 peaks 3′ and 4′ (not seen in Fig. 1) were generated, and peaks 5′ and 6′ increased substantially with β-amylase action (Fig. 2 and Table V). Peaks 3′ and 4′ were not susceptible to pullulanase, but peaks 5′ and 6′ were hydrolyzed (Fig. 3). The residual pullulanase-resistant peaks 4′ through 7′ seen in Fig. 4 are probably branched dextrins with a single glucosyl branch similar to those formed by α-amylases from porcine pancreas, *A. oryzae*, and human saliva[14]. Porcine pancreas α-amylase yields limit dextrins which have been characterized in the d.p. 4–10 range[15]. The formation of the pullulanase-susceptible d.p. 5′ and 6′ peaks suggests that they were derived from chains still susceptible to attack by β-amylase. Candidates for 5′ and 6′ are 6^2 maltosyl maltotriose and 6^2 maltosyl maltotetraose, respectively. Since these saccharides have branches adjacent to the reducing end, they were derived from places in the β-limit dextrin where an A–B branch was

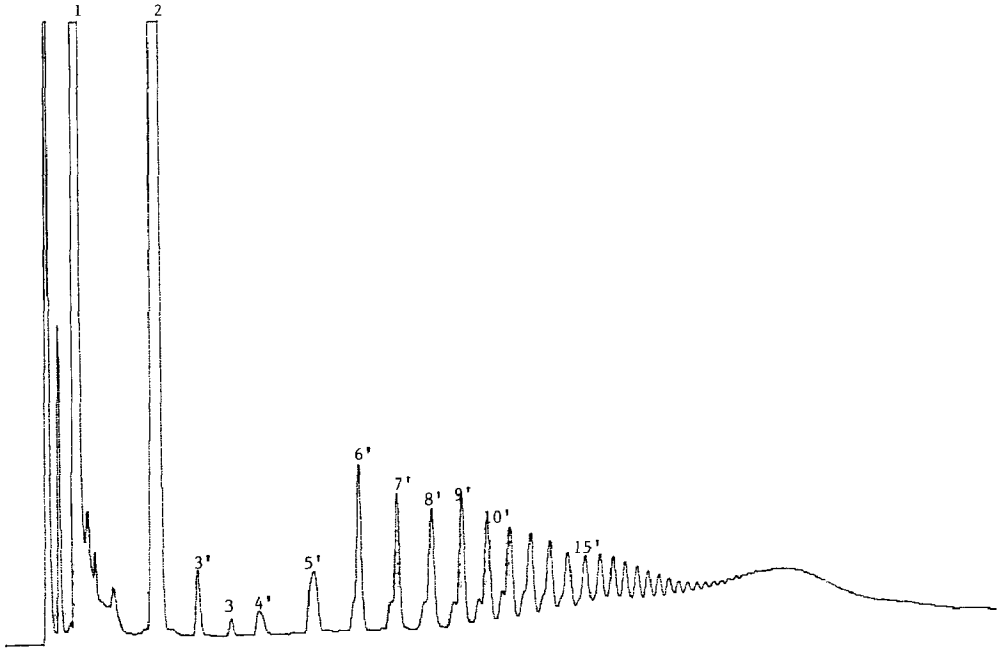

Fig. 3. H.p.a.e.-p.a.d. chromatogram of the Reaction 2 digest (a β-amylase digest of Reaction 1). Primed integers designate branched chains. Typical peak retention times are listed in Table V.

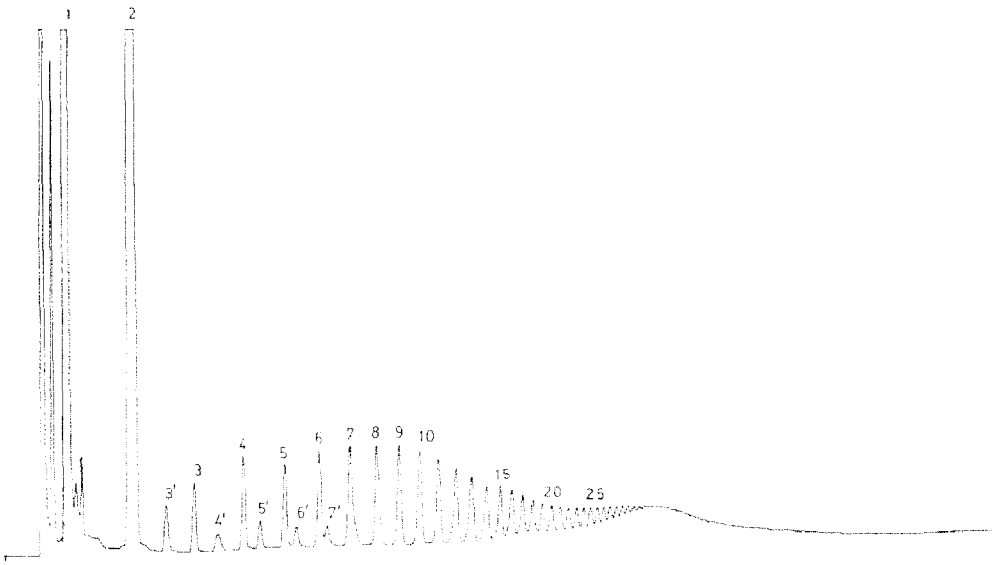

Fig. 4. H.p.a.e.-p.a.d. chromatogram of the Reaction 3 digest (Reaction 2 treated with pullulanase). Peak d.p. is indicated by integers above the peaks. Typical peak retention times are shown in Table V. Peaks representing branched chains have primed integers.

adjacent to a B–B branch. Where this occurs in starch the susceptibility to β-amylase at the non-reducing end may be reduced.

The effect of the α-(1→6) maltosyl branch on h.p.a.e.-p.a.d. retention times here (Table VI) was similar to that for the α-(1→6) linkage in pullulan chains (Table IV). Assuming the chains to be singly branched, this seems reasonable for d.p. 5–14. Based

TABLE V

Effect of β-amylase on branched chains from isoamylase digested waxy maize β-limit dextrin

Peak	*Retention time (min)*[a]	*Percent of total response*		
		Before β-amylase	*After β-amylase*	*Change after − before*
5′	13.2	0.1	1.5	+1.4
6′	15.0	0.3	2.6	+2.3
7′	16.6	1.6	2.1	+0.5
8′	18.2	2.0	2.2	+0.2
9′	19.4	1.7	2.1	+0.4
10′	20.6	1.8	1.7	−0.1
11′	21.5	1.9	1.5	−0.4
12′	22.4	1.8	1.6	−0.2
13′	23.2	1.6	1.4	−0.2

[a] Elution program A.

TABLE VI

H.p.a.e.-p.a.d. chromatography of starch-derived linear and branched isomers

D.p. (glucose units)	*Linear chains[a] (min)*	*D.p. (glucose units)*	*Isomeric branched chains[a] (min)*	*Difference linear – branched (min)*	*Difference per α-(1→6) linkage in pullulan (min)*
1	3.08[b]	—	—	—	—
2	6.36	—	—	—	—
3	9.54	—	—	—	—
4	12.11	—	—	—	—
5	14.18	5'[c]	13.20[b]	0.98	—
6	15.95	6'	15.10	0.85	0.86
7	17.56	7'	16.66	0.90	—
8	18.92	8'	18.14	0.78	—
9	20.08	9'	19.44	0.64	0.73
10	21.12	10'	20.54	0.58	—
11	22.08	11'	21.51	0.57	—
12	22.96	12'	22.39	0.57	0.62
13	23.77	13'	23.22	0.55	—
14	24.56	14'	23.98	0.58	—
15	25.26	15'	24.74	0.52	0.58
16	25.85	16'	25.37	0.48	—
17	26.40	17'	25.92	0.49	—
18	26.93	18'	26.44	0.49	0.50
19	27.42	19'	26.93	0.49	—
20	27.90	20'	27.41	0.49	—
21	28.34	21'	27.86	0.48	0.45
22	28.77	22'	28.29	0.48	—
23	29.17	23'	28.70	0.47	—

[a] Retention time. [b] Standard deviations were less than 0.08 min, for elution program A. [c] The prime indicates that the d.p. listed includes at least one maltosyl branch.

on the data in Table VII, the effect on retention time per branch ranges from 0.98 to 0.47 min as the retention time increases.

Peaks were characterized by h.p.a.e.-p.a.d. isolation and pullulanase treatment. The pullulan chromatography suggested that the maltosyl branched chain would travel about 0.5 min faster than its linear isomers. Initial d.p. assignments were made on this basis. To confirm this, a 2% solution of the Reaction 2 digest was chromatographed with elution program B, and fractions were collected. After dilution and pH adjustment, each fraction was chromatographed on h.p.a.e.-p.a.d. and showed two peaks. The d.p. assignments for these isolated peaks were made from the original digest retention times listed in Table VII. An aliquot was then treated with pullulanase and chromatographed. The retention times of the products were tabulated in Table VII against a reference isoamylase digest. That these peaks represented linear oligosaccharides was demonstrated by β-amylase digestion. They were identified by retention time against the reference and the d.p. values are listed in Table VII.

TABLE VII

The isolation and pullulanase digestion of branched oligosaccharides from the Reaction 2 digest[a]

		H.p.a.e.-p.a.d. retention times of fractions from the Reaction 2 digest[b]			*H.p.a.e.-p.a.d. retention times after pullulanase digestion of the fractions*	
Fraction	*D.p.*[c] *(glucose units)*	*Digest peaks (min)*	*Isolated fractions (min)*	*D.p.*[d] *(glucose units)*	*Reference isoamylase digest (min)*	*Isolated fractions (min)*
1	5′	11.08	10.92	3	8.63	8.87
	6′	12.49	12.43	4	10.24	10.30
2	6′	12.49	12.12	4	10.24	10.18
	7′	14.19	13.95	5	11.63	11.54
3	7′	14.19	13.83	5	11.63	11.50
	8′	15.92	15.68	6	13.30	13.08
4	8′	15.92	15.90	6	13.30	13.18
	9′	17.52	17.81	7	15.07	15.12
5	9′	17.52	17.42	7	15.07	14.77
	10′	18.97	19.26	8	16.72	16.78
6	10′	18.97	18.72	8	16.72	16.36
	11′	20.33	20.48	9	18.21	18.29
7	11′	20.33	20.03	9	18.21	17.79
	12′	21.62	21.74	10	19.63	19.60
8	12′	21.62	21.30	10	19.63	19.22
	13′	22.87	22.94	11	21.02	21.00
9	13′	22.87	22.50	11	21.02	20.57
	14′	24.06	24.08	12	22.33	22.28

[a] The Reaction 2 digest was the result of first isoamylase and then β-amylase digestion of waxy β-limit dextrin, which gives maltosyl branched oligosaccharides. [b] Each fraction contained two peaks. Tentatively assigned and confirmed from the products of pullulanase digestion. [c] Determined from retention times by comparison with standard linear oligosaccharides, linearity was established by β-amylase hydrolysis to d.p. 2 and 3.

The 6′ peak yielded d.p. 4 and maltose, which is consistent with a maltosyl maltotetraose. The 14′ peak yielded d.p. 12 and maltose, a result which is consistent with maltosyl maltododecaose. The other peaks follow the same pattern. If more than two peaks resulted from pullulanase, it would be evidence for multiple branching; however, such peak multiplicity was not observed. It is possible, but not likely, that in some fractions multiple peaks could be masked. Concentrations were too low in these fractions for good stoichiometric calculations, but relative peak areas did not suggest significant multiple branching. The conclusion was made that multiply branched material was not a substantial part of peaks 5′–14′. However, in Fig. 3, shoulders are evident on the left of the 7′–12′ peaks. Work to be discussed below suggests that these may represent components of multiply branched material.

Glucose oligosaccharides with the α-(1→6) glycosidic linkage can be separated and identified by h.p.a.e.-p.a.d. The number of such linkages determines the effect on retention time. Enzymatic characterization of isolated peaks can be carried out by a fast and simple procedure.

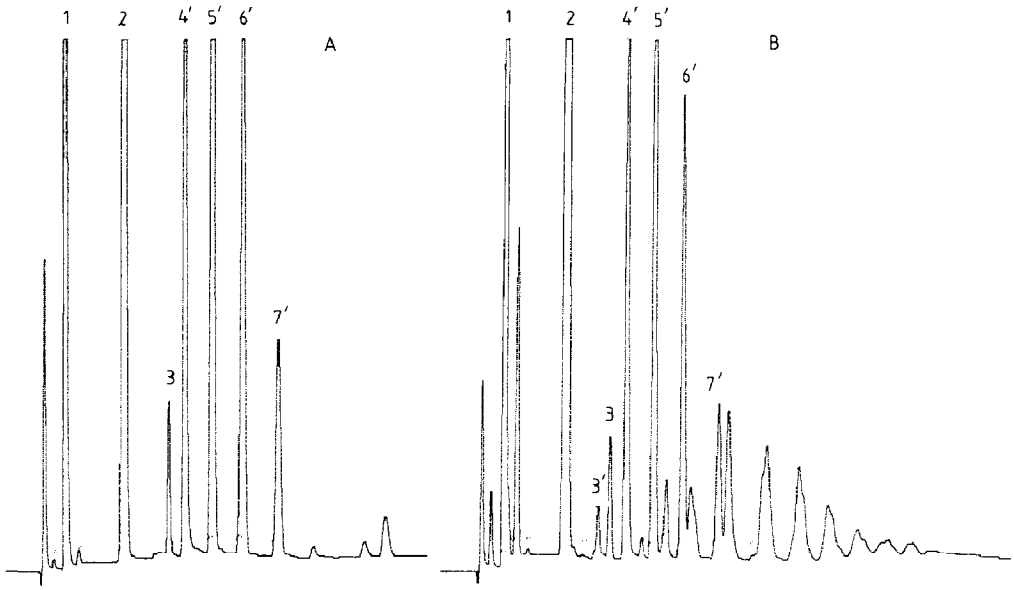

Fig. 5. H.p.a.e.-p.a.d. chromatograms of *A. oryzae* α-amylase digests of branched β-cyclodextrin; A, from singly branched cyclodextrin; B, from branched cyclodextrin with 3.4 branches. The peak d.p. is indicated by the integer above the peak. Singly primed peaks represent singly branched chains.

H.p.a.e.-p.a.d. chromatography of the A. aerogenes *α-amylase limit dextrins from branched cyclodextrins.* — Two branched β-cyclodextrin (cyclomaltoheptaose) preparations were used as substrates for the α-amylase. Characterized by n.m.r. spectroscopy[12], these had: (*i*) d.p. 9 with one branch per cyclodextrin residue, or (*ii*) d.p. 25 with 3.4 branches per cyclodextrin residue. The first can yield only singly branched limit dextrins. The second may yield multiply branched limit dextrins. After over 95% of the branched cyclodextrin was digested, they were analyzed by h.p.a.e.-p.a.d. with elution program B at 1000 nA sensitivity. The results are shown in Figs. 5A and 5B for the singly branched and multiply branched substrates, respectively. The substrates contained 2 and 6% acyclic impurity, respectively, as determined by h.p.l.c. The major products in Fig. 5A were peaks 4'–7'. The other peaks include unreacted branched cyclodextrin. The 4' peak is the smallest limit dextrin known from the *A. oryzae* α-amylase and is 6^3 glucosyl maltotriose. Structures of the porcine pancreas α-amylase (p.p.a.) limit dextrins from waxy starch have been determined[15] by Kainuma and French[16,17]. The *A. oryzae* and porcine pancreas α-amylases yield the same limit dextrins[18,19]. Porcine pancreas α-amylases yielded doubly branched d.p. 6 and 7 and triply branched d.p. 9 and 10[15]. Fig. 5B shows the product peaks from the larger branched cyclodextrin. There are the major 4' – 7' peaks seen in Fig. 5A. In addition, several other peaks are evident. These probably included doubly branched d.p. 6 and 7 and triply branched d.p. 9 and 10, all known α-limit dextrins from waxy maize starch. These peaks can be separated by elution program B from the 6' – 14' peaks of Reaction 2. However, peaks 4'–7' of Reaction 2 could not be separated from peaks 4'–7' of the α-amylase digest.

While more characterization work must be done on these substances, it is clear that oligosaccharide chains with two to three branches can be separated from their singly branched isomers. It was not demonstrated that doubly and triply branched isomers can be separated. This study does define how the α-(1→6) glycosidic linkage affected the h.p.a.e.-p.a.d. retention time. This, together with the simple method described for enzymatic characterization of isolated h.p.a.e. fractions, will assist in identification of various branched oligosaccharides. Coupled with response calibration, stoichiometric ratios of various limit dextrins can be determined to help characterize starch amylopectins. For example, multiply branched α-amylase limit dextrins form only when branching is closely spaced. The molar ratio of these to singly branched limit dextrins is thus a measure of branching density.

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