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Penta-, hexa-, and heptasaccharide acceptor products of alternansucrase☆

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Abstract—In the presence of suitable acceptor molecules, dextransucrase makes a homologous series of oligosaccharides in which the isomers differ by a single glucosyl unit, whereas alternansucrase synthesizes one trisaccharide, two tetrasaccharides, etc. For the example of maltose as the acceptor, if one considers only the linear, unbranched possibilities for alternansucrase, the hypothetical number of potential products increases exponentially as a function of the degree of polymerization (DP). Experimental evidence indicates that far fewer products are actually formed. We show that only certain isomers of DP >4 are formed from maltose in measurable amounts, and that these oligosaccharides belong to the oligoalternan series rather than the oligodextran series. When the oligosaccharide acceptor products from maltose were separated by size-exclusion chromatography and HPLC, only one pentasaccharide was isolated. Its structure was α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc. Two hexasaccharides were formed in approximately equal quantities: α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc and α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc. Just one heptasaccharide was isolated from the reaction mixture, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc. We conclude that the enzyme is incapable of forming two consecutive α-(1→3) linkages, and does not form products with more than two consecutive α-(1→6) linkages. The distribution of products may be kinetically determined.

Keywords: Oligosaccharides; Alternansucrase; Prebiotic; Low-glycemic sweetener; Maltose; Sucrose; Glucansucrase

1. Introduction

Dextransucrase (EC 2.4.1.5) and alternansucrase (EC 2.4.1.140) are bacterial enzymes that transfer glucosyl units from sucrose into long-chain polymers of glucose.[1] In the presence of suitable acceptor molecules, glucosyl transfer can also occur to the acceptor, forming glucose oligosaccharides of varying molecular sizes (degree of polymerization, DP).[2] Commercial dextran, produced by *Leuconostoc mesenteroides* NRRL B-512F, contains approximately 95% α-(1→6) linkages, whereas alternan contains alternating α-(1→3) and α-(1→6) linkages.[3] A major difference between commercial dextransucrase and alternansucrase is that dextransucrase acceptor products consist of an homologous series of oligosaccharides in which the isomers differ by a single glucosyl unit, whereas alternansucrase synthesizes one DP 3 product, two DP 4 products, etc.[2] For the example of maltose as the acceptor, if one considers only the linear, unbranched possibilities for alternansucrase, the hypothetical number of potential products increases exponentially as a function of DP. Experimental evidence indicates that far fewer products are actually formed. It was shown previously that the enzyme does not synthesize an α-(1→3) linkage by acceptor reactions unless an α-(1→6) linkage is present, and that the enzyme is

☆ Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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incapable of forming two consecutive α-(1→3) linkages.[2] Currently, alternansucrase acceptor products are under consideration as additives for food and feed uses.[4–6] It would be very useful to know the structures of the higher DP acceptor products in order to understand the structure–function relationships of these novel ingredients.

2. Experimental

2.1. Carbohydrates

Sucrose and maltose were reagent-grade from Sigma–Aldrich Corp. (St. Louis, MO). Panose was prepared using *L. mesenteroides* NRRL B-512F dextransucrase.[7]

2.2. Enzymes

Alternansucrase was prepared from *L. mesenteroides* NRRL B-21297 as previously described.[4] *Arthrobacter globiformis* NRRL B-4425 isomaltodextranase was prepared according to the method described by Okada et al.[8] Glucoamylase from *Rhizopus* sp. was purchased from Sigma–Aldrich and was dialyzed against buffer before use. Unless otherwise stated, enzyme reactions were carried out in 20 mM pH 5.4 NaOAc buffer containing 0.01% NaN_3.

2.3. Preparation of oligosaccharides

Acceptor reactions were carried out as previously described.[4] After the sucrose was all consumed, as ascertained by TLC, the reaction mixtures were chromatographed over a 5×150 cm column of Bio-Gel P-2 (fine mesh) with water as the eluent. Oligosaccharides in the eluate, separated by size according to DP, were monitored by TLC. Appropriate fractions were combined and freeze-dried. For separation of isomeric oligosaccharides of the same size, preparative HPLC was carried out using a Waters 625 system with a refractive index detector. A Phenomenex Synergi 10-μm Hydro-RP 80 column (21.2×250 mm) was eluted at room temperature with 1% (vol) MeOH in water. Samples represented by detector peaks were collected manually and freeze-dried. It should be pointed out that RP (reversed-phase) columns separate α and β anomers of reducing sugars, so each compound was represented by a pair of peaks.

2.4. Analytical methods

Carbohydrate content was determined by the phenol–sulfuric acid method.[9] Thin-layer chromatography was performed on Whatman K5 silica gel plates as previously described.[2,4] Developed TLC plates were scanned using a desktop scanner in the reflectance mode, and densitometry was carried out on the images using Un-Scan-It Gel version 6.1 (Silk Scientific, Orem, UT). Molecular weights were measured using a Bruker Daltonics Omniflex MALDI-TOF mass spectrometer. Aqueous solutions of oligosaccharides were mixed with an equal volume of saturated 2,5-dihydroxybenzoic acid solution in acetonitrile, allowed to dry on the probe, and subjected to MALDI-TOF mass spectrometry. Methylation analysis was carried out using a modified Hakomori procedure[10] in which hydrolysis was accomplished using 2 M CF_3CO_2H. The permethylated derivatives were analyzed by capillary GC–MS as the peracetylated aldononitriles.[11]

2.5. NMR spectroscopy

Individual samples of oligosaccharides (~15 mg) were dissolved in D_2O (0.6 mL) plus acetone (5 μL). All NMR experiments were performed on a Bruker Avance 500 spectrometer equipped with a 5-mm broadband inverse probe with *z*-gradient. Experiments on DP 5 and DP 6-B were performed at 27 °C and those on DP 6-A and DP 7 were performed at 50 °C. The proton spectral width was 1600 Hz for all experiments except for the 1D ^1H spectra where it was set at 3000 Hz for the inclusion of the peak of acetone. The carbon spectral width was 1500 and 3000 Hz for excitation of C-1 and C-2 to C-6 carbons, respectively. Typically, $2 \times 256 \times 2048$ data points were recorded for the gradient-enhanced band-selective HSQC and HSQC–TOCSY experiments and 128×2048 data points were recorded for the gradient-enhanced band-selective HMBC experiment. In the band-selective HSQC and HSQC–TOCSY experiments, quadrature detection in the t_1 dimension was carried out by the Echo–Antiecho method. The band-selective HMBC spectrum was recorded in magnitude mode. Isotropic mixing in the band-selective HSQC–TOCSY experiments was achieved by the DIPSI2 pulse sequence[12] with effective field strength of 6.9 kHz. The duration of isotropic mixing was 20, 50, 75, 100, and 180 ms for the band-selective HSQC–TOCSY experiments covering C-1 region and 10 ms for that covering the C-2 to C-6 region. Band-selective excitation and inversion were accomplished by eBURP and iBURP pulse[13] with durations of 1.5 and 1.3 ms, respectively. Carbon decoupling was done by the GARP pulse sequence.[14]

The data were processed with X-Win NMR software. Typically, an exponential window function (lb = 1.00 Hz) was applied in the t_2 dimension and a shifted squared sine-bell window function was applied in the t_1 dimension. Zero-filling was applied in both dimensions. Chemical shifts were referenced to acetone at 2.05 and 29.92 ppm for ^1H and ^{13}C NMR spectra, respectively.

3. Results[†]

Preliminary experiments indicated that 0.5 M sucrose and 0.1 M maltose were optimal for producing oligosaccharides in the DP 5–7 range. Variations in substrate concentrations within this range did not qualitatively alter the distribution of products, but did affect the relative distribution according to DP. Quantitation of products was carried out after gel-filtration by weighing the freeze-dried fractions of each individual sample according to DP. Results appear in Figure 1.

TLC and HPLC analysis of each individual fraction from the gel-filtration column demonstrated the presence of a single pentasaccharide, two hexasaccharides in roughly equal proportions, and a single heptasaccharide. These were further purified by preparative HPLC (Fig. 2). HPLC-purified products were subjected to NMR spectroscopic analysis, methylation analysis, and enzymatic digestion.

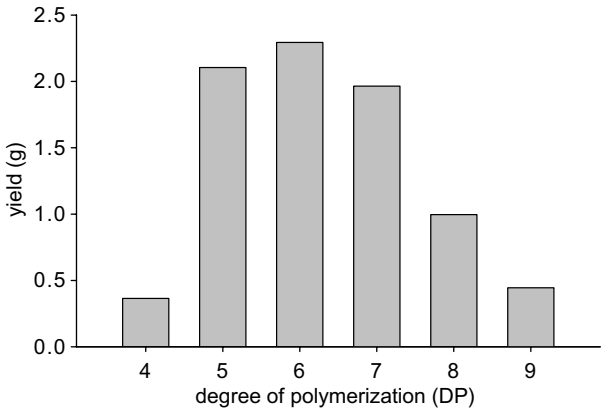

Figure 1. Yield of maltose acceptor products according to size (DP). Initial sucrose concentration was 0.5 M, and initial maltose concentration was 0.1 M. Reaction volume was 100 mL.

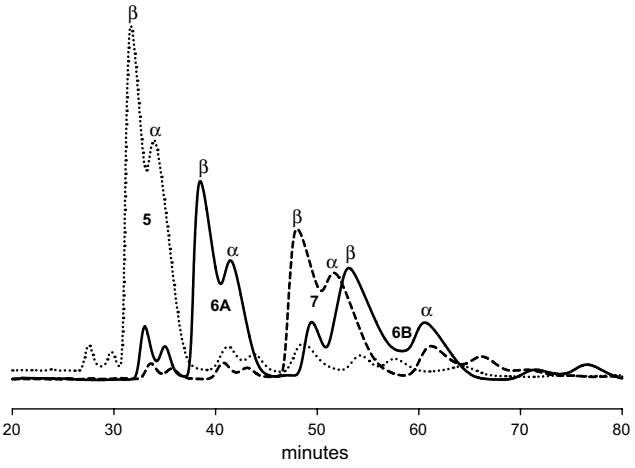

Figure 2. Preparative HPLC of alternansucrase oligosaccharide acceptor products from a Bio-Gel P-2 column chromatographic separation. Note that a C_{18} column separates α and β anomers. The dotted line represents the pentasaccharide fraction, a solid line the hexasaccharide fraction, and a dashed line the heptasaccharide fraction. Main contaminants of the hexasaccharide were shown to be pentasaccharide 5 and heptasaccharide 7 by TLC and MALDI-TOF MS. (Experimental details described in text.)

3.1. Pentasaccharide

Only a single pentasaccharide was isolated in significant quantities (Fig. 2). It contained a nonreducing end glucose, a single 4-substituted glucose residue, a single 3-substituted residue, and two 6-substituted residues, according to the methylation analysis. Comparison with the dextransucrase acceptor product series showed a markedly different chromatographic mobility on HPLC and TLC relative to the dextransucrase DP 5 product. (For a TLC densitogram, see Supplementary data.) Degradation by isomaltodextranase yielded isomaltose as the major product, with somewhat less glucose. Assignment of NMR peaks as described below for the DP 7 product appears in Table 1. Taken together, these results show the structure of the pentasaccharide to be α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc.

3.2. Hexasaccharides

A mixture of two hexasaccharide products was isolated by gel-filtration chromatography. The two major components were separated by preparative HPLC from each other and from contaminating heptasaccharide and pentasaccharide (Fig. 2), and referred to as 6A and 6B. Saccharide 6A migrated ahead of 6B on TLC. (For a TLC densitogram, see Supplementary data.) Neither exhibited the same mobility as the DP 6 dextransucrase product, which is α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc. Methylation analysis of saccharide 6A indicated the presence of a single nonreducing end unit, a single 4-substituted unit, two 3-substituted glucose residues, and two 6-substituted residues. Isomaltodextranase digestion yielded isomaltose, glucose, and a trisaccharide comigrating with an authentic sample of α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc.[15] NMR data (Table 1), along with the chemical evidence, demonstrate that 6A is α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc.

Methylation analysis of 6B indicated the presence of a single non-reducing end unit, a single 4-substituted unit,

[†] *Note added in proof:* After this paper was accepted for publication, it came to our attention that some of the oligosaccharides described herein had previously been mentioned in the Ph.D. thesis of Marc Raemaekers (Raemaekers, M. Ph.D. Thesis, University of Ghent, 1995). His suggested structures Ac3B, Ac3C, Ac4A and Ac4B correspond respectively to the pentasaccharide, two hexasaccharides, and the heptasaccharide isolated and characterized in this paper. We thank Dr. ir. Erick Vandamme for sending us a copy of the thesis.

Table 1. NMR chemical shift assignments for penta-, hexa-, and heptasaccharide acceptor products

	Glc^{I_α}	Glc^{I_β}	Glc^{II}	Glc^{III}	Glc^{IV}	Glc^{V}	Glc^{VI}	Glc^{VII}
Pentasaccharide: α-D-Glc*p*^{V}-(1→6)-α-D-Glc*p*^{IV}-(1→3)-α-D-Glc*p*^{III}-(1→6)-α-D-Glc*p*^{II}-(1→4)-D-Glc*p*^{I}								
H-1	5.05	4.47	5.23	4.79	5.16	4.49		
H-2	3.40	3.10	3.44	3.49	3.41	3.38		
H-3	3.80	3.60	3.51	3.68	3.56	3.56		
H-4	3.48	3.48	3.34	3.49	3.39	3.25		
H-5	3.78	3.43	3.76	3.56	4.02	3.53		
H-6	3.69	3.76	3.82	3.67	3.85	3.67		
H-6′	3.69	3.63	3.56	3.59	3.50	3.59		
C-1	91.57	95.44	99.48	97.75	99.17	97.54		
C-2	71.04	73.64	71.36	69.72	71.28	72.78		
C-3	72.78	75.81	72.78	80.24/80.12	72.88	71.16		
C-4	77.09	76.93	69.03	69.53	68.95	69.22		
C-5	69.53	74.19	70.93	72.78	70.07	71.47		
C-6	60.43	59.92	65.40	60.33	64.86	60.14		
	Glc^{I_α}	Glc^{I_β}	Glc^{II}	Glc^{III}	Glc^{IV}	Glc^{V}	Glc^{VI}	
Hexasaccharide A: α-D-Glc*p*^{VI}-(1→3)-α-D-Glc*p*^{V}-(1→6)-α-D-Glc*p*^{IV}-(1→3)-α-D-Glc*p*^{III}-(1→6)-α-D-Glc*p*^{II}-(1→4)-D-Glc*p*^{I}								
H-1	5.06	4.48	5.20	4.81	5.14	4.80	5.17	
H-2	3.40	3.11	3.44	3.51	3.42	3.49	3.40	
H-3	3.81	3.60	3.52	3.68	3.57	3.71	3.58	
H-4	3.46	3.46	3.32	3.49	3.41	3.48	3.28	
H-5	3.78	3.43	3.77	3.58	4.00	3.54	3.82	
H-6	3.72	3.77	3.80	3.68	3.87	3.68	3.66	
H-6′	3.66	3.63	3.59	3.58	3.52	3.58	3.61	
C-1	91.61	95.52	99.58	97.88	99.32	97.88	98.87	
C-2	71.03	73.73	71.22	69.81	71.39	69.91	71.47	
C-3	72.82	75.81	72.82	80.90/81.03	73.00	79.77	72.72	
C-4	77.54	77.38	69.21	69.48	69.06	69.72	69.21	
C-5	69.72	74.32	71.03	71.22	70.30	71.39	71.47	
C-6	60.52	60.61	65.78	60.11	65.14	60.11	60.20	
Hexasaccharide B: α-D-Glc*p*^{VI}-(1→6)-α-D-Glc*p*^{V}-(1→6)-α-D-Glc*p*^{IV}-(1→3)-α-D-Glc*p*^{III}-(1→6)-α-D-Glc*p*^{II}-(1→4)-D-Glc*p*^{I}								
H-1	5.05	4.48	5.23	4.79	5.17	4.80	4.79	
H-2	3.40	3.11	3.44	3.49	3.41	3.41	3.38	
H-3	3.80	3.60	3.52	3.69/3.68	3.56	3.56	3.55	
H-4	3.48	3.48	3.34	3.50	3.39	3.34	3.26	
H-5	3.76	3.44	3.78	3.56	4.03	3.71	3.54	
H-6	3.69	3.76	3.82	3.68	3.88	3.80	3.68	
H-6′	3.69	3.63	3.58	3.60	3.51	3.58	3.59	
C-1	91.58	95.46	99.50	97.78	99.15	97.53	97.43	
C-2	70.97	73.64	71.31	69.73	71.17	71.17	71.15	
C-3	72.83	75.82	72.77	80.26/80.11	73.06	73.06	72.74	
C-4	77.11	76.93	69.05	69.55	68.96	69.43	69.18	
C-5	70.87	74.21	69.60	71.16	69.98	69.90	71.49	
C-6	60.36	60.45	65.42	59.92	64.92	65.20	60.16	
	Glc^{I_α}	Glc^{I_β}	Glc^{II}	Glc^{III}	Glc^{IV}	Glc^{V}	Glc^{VI}	Glc^{VII}
Heptasaccharide: α-D-Glc*p*^{VII}-(1→6)-α-D-Glc*p*^{VI}-(1→3)-α-D-Glc*p*^{V}-(1→6)-α-D-Glc*p*^{IV}-(1→3)-α-D-Glc*p*^{III}-(1→6)-α-D-Glc*p*^{II}-(1→4)-D-Glc*p*^{I}								
H-1	5.05	4.48	5.20	4.81	5.15	4.81	5.15	4.79
H-2	3.41	3.11	3.44	3.51	3.44	3.49	3.42	3.39
H-3	3.80	3.59	3.52	3.68	3.57	3.70	3.57	3.57
H-4	3.46	3.47	3.32	3.49	3.42	3.48	3.38	3.25
H-5	3.78	3.44	3.76	3.58	4.01	3.54	4.01	3.53
H-6	3.72	3.77	3.80	3.68	3.87	3.68	3.83	3.68
H-6′	3.66	3.63	3.59	3.60	3.52	3.60	3.53	3.59
C-1	91.60	95.50	99.57	97.85	99.31	97.85	99.09	97.60
C-2	71.01	73.71	71.35	69.79	71.35	69.88	71.35	71.26
C-3	72.81	75.78	72.81	80.97/81.09	72.94	80.37	72.94	72.87
C-4	77.56	77.37	69.28	69.44	69.06	69.63	69.15	69.37
C-5	69.73	74.31	71.01	71.18	70.29	71.26	70.13	71.51
C-6	60.49	60.58	65.80	60.08	65.11	60.08	65.20	60.30

one 3-substituted glucose residue, and three 6-substituted residues. Isomaltodextranase digestion gave the same products as 6A. Glucoamylase hydrolyzed 6B slowly to give glucose plus the pentasaccharide

described above. Taken along with the NMR evidence, it is concluded that 6B is α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc.

3.3. Heptasaccharide

A single major heptasaccharide was isolated by gel-filtration chromatography and purified by HPLC. Small amounts of other saccharides were present prior to HPLC (Fig. 2), and were shown by MALDI-TOF MS to be mainly DP 6 and DP 8, but the possibility of minor traces of other DP 7 products cannot be rigorously excluded at this point. Methylation analysis of the heptasaccharide showed the presence of a single, nonreducing, terminal, glucose residue, a single 4-substituted residue, two 3-substituted glucose residues, and three 6-substituted residues.

The 1D ^{1}H NMR spectrum of DP 7 showed eight resonances in the anomeric region, which was consistent with the structure of a heptasaccharide. The eight resonances were labeled as I–VII with I consisting of I_{α} and I_{β} indicating that the resonance I was attributable to the sugar residue at the reducing end. The 1D ^{1}H spectrum revealed substantial overlapping even in the anomeric region. The signals labeled as IV and VI, and III, V, and VII could not be assigned based on conventional 2D COSY and TOCSY experiments. To carry out the complete assignment, a combination of 2D band-selective heteronuclei correlation experiments that allowed one to take the advantage of the relatively large ^{13}C chemical shift dispersion was carried out.

The assignment of the sugar ring skeleton protons of DP 7 was achieved by the use of band-selective HSQC–TOCSY spectra recorded with different mixing times ranging from 20 to 180 ms. The short mixing time (20 ms) only allowed the magnetization to be transferred from H-1 protons to H-2 protons in the band-selective HSQC–TOCSY spectra centered at C-1 carbons. As the mixing time was gradually increased, the magnetization was transferred further away from the H-1 protons, which permitted the assignment of the sugar ring protons in a stepwise manner. Based on this strategy, complete sugar ring proton assignment was achieved for residues II, IV, VI, I_{β}, and VII, and the assignment of H-1 to H-3 protons for residues I_{α}, III, and V. The remaining sugar ring protons were assigned in conjunction with the ^{13}C assignments.

The C-1 carbons were assigned using the band-selective HSQC spectra covering the C-1 region. The high degree of degeneracy in ^{1}H chemical shifts in the H-2 to H-6 region made the assignment of ^{13}C chemical shifts based on a conventional HSQC experiment impossible. To carry out the complete ^{13}C assignment, a 2D band-selective HSQC–TOCSY spectrum covering the C-2 to C-6 region was used in conjunction with a 2D band-selective HSQC spectrum. The mixing time in the HSQC–TOCSY experiment was 10 ms, which allowed the magnetization to be transferred from the parent protons only to their nearest neighbor protons. In general, each H-*n* proton was correlated to C-*n* carbon and H-($n-1$) and H-($n+1$) protons. For each sugar residue, the H-1 proton was correlated to the respective H-2 whose interaction with H-1 resulted from the magnetization transfer occurring during the TOCSY step of the pulse sequence. The H-2 was correlated to C-2 where the magnetization was transferred during the HSQC step of the pulse sequence. The H-2 proton was also correlated to H-3, its other nearest neighbor. Following this algorithm, we assigned all the ^{13}C resonances plus those of the ^{1}H resonances that were not assigned previously using band-selective HSQC–TOCSY spectra, and confirmed the assignment of the ^{1}H resonances assigned previously.

To assign the configuration of each anomeric center, the values of the $^{1}J_{CH}$ coupling constants for the anomeric resonances obtained from the band-selective HMBC spectrum were used. Typically, the $^{1}J_{CH}$ values of about 160 and 170 Hz are associated with β- and α-pyranosyl linkages, respectively.[16] In DP 7, all the glucosyl residues except for I_{β} showed the value of ~171 Hz and were assigned α configuration. The residue I_{β} showed the value of 162 Hz and was assigned the β configuration.

The band-selective HMBC spectrum of DP 7 revealed long-range C-1 → H-x' correlations for the seven residues. The observation of cross-peaks between C-1 of Glc^{II} and H-4 of Glc^{I}, C-1 of Glc^{IV} and H-3 of Glc^{III}, C-1 of Glc^{VI} and H-3 of Glc^{V}, and C-1 of Glc^{VII} and H-6 of Glc^{VI} indicated that Glc^{II} was linked to Glc^{I} through a (1→4) linkage, Glc^{IV} and Glc^{VI} were linked to Glc^{III}, and Glc^{V} through (1→3) linkages, respectively, and Glc^{VII} and Glc^{VI} were linked through a (1→6) linkage. The band-selective HMBC did not provide unambiguous assignment for the remaining two linkages due to the almost identical ^{13}C chemical shift of C-1 carbons in Glc^{III} and Glc^{V}. A priori, Glc^{III} could be linked to Glc^{II} or Glc^{IV} through a (1→6) linkage. If Glc^{III} were linked to Glc^{II}, Glc^{V} had to be linked to Glc^{IV}, and vice versa. However, Glc^{VII} was the nonreducing end residue, which left the combination of Glc^{III} (1→6) Glc^{II} and Glc^{V} (1→6) Glc^{IV} as the only possibility. Thus, the primary structure of DP 7 was determined to be α-D-Glcp^{VII}-(1→6)-α-D-Glcp^{VI}-(1→3)-α-D-Glcp^{V}-(1→6)-α-D-Glcp^{IV}-(1→3)-α-D-Glcp^{III}-(1→6)-α-D-Glcp^{II}-(1→4)-D-Glc^{I}.

4. Discussion

Previously, it was shown that alternansucrase produced only one trisaccharide acceptor product from maltose,

namely, panose.[2] Panose was subsequently capable of serving as an acceptor for further acceptor reactions, resulting in the formation of two tetrasaccharide products, α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc (major) and α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc (minor). From these results, it was concluded that the structure α-D-Glc*p*-(1→6)-α-D-Glc was necessary for the formation of a (1→3) linkage, and that two (1→3) linkages could not be formed sequentially.[2] However, it was unknown at that point whether or not multiple sequences of (1→6) linkages could be formed via acceptor reactions, as the presence of α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc suggested that at least two adjacent (1→6) linkages could be formed. Based on our 1982 results,[2] Castillo et al.[17] hypothesized that alternansucrase should synthesize three pentasaccharide acceptor products from maltose, namely α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc, and α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc. If one were to extrapolate the possible number of acceptor products based solely on this presumption, then there ought to be five possible structures for DP 6 products, eight for DP 7, etc. The number of possible structures increases exponentially with DP in a Fibonacci series. If the possibility of branching at higher DP is considered, the number becomes even greater. It became evident, based on our chromatographic analyses, that the actual number of products was far fewer. Comparison of acceptor reaction mixtures from alternansucrase with those from dextransucrase suggested that the two made quite a different series of products,[18] but the structures of the alternansucrase products larger than DP 4 were unknown prior to this work. It is now apparent that alternansucrase is more specific in its action than previous evidence might suggest.

The data presented here show that alternansucrase forms α-(1→3) linkages only when the acceptor is α-(1→6)-linked, thereby prohibiting the formation of sequences of α-(1→3) linkages. Furthermore, the enzyme appears not to make products containing more than two sequential α-(1→6) linkages, thereby prohibiting the formation of dextran-like linkage sequences. This is summarized in Figure 3. The foregoing strictly applies only to acceptor reactions in which the product is smaller than DP 8. However, it may explain the presence of an alternating linkage structure for alternan. It should be noted that alternan contains a higher proportion of α-(1→6) linkages than α-(1→3) linkages.[3,19,20] The ability to form two adjacent α-(1→6) linkages would explain this structural feature of the polysaccharide as well.

The finding that the alternanuscrase acceptor products isolated from reaction with maltose do not contain more than two consecutive α-(1→6) linkages is interesting, especially in light of our previous results showing isomaltotriose and α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc to be good acceptors for alternansucrase.[4] This can only be explained by assuming that the distribution of acceptor products is kinetically controlled. The fact that nigerose is a better acceptor than isomaltose suggests that structures terminated by an α-(1→3) linkage more rapidly undergo acceptor reactions, yielding new α-(1→6) linkages. This is also supported by our observation that more α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc is formed relative to the amount of α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc.[2] This difference in rates will eventually result in a buildup of products with alternating linkages, since consecutive α-(1→3) linkages are not formed.[2] Kinetic control of the relative rates of formation of each type of linkage may also explain why alternan from different strains of alternan-producing *L. mesenteroides* differ slightly in the relative proportions of each linkage type.[19] To better understand this phenomenon, future experiments will necessitate the isolation of each acceptor product in sufficient quantities to study its ability to undergo additional acceptor reactions.

These experiments do not address the formation of branch linkages by alternansucrase. The polysaccharide alternan contains approximately 11% branching through 3,6-disubstituted glucose residues.[19] We have observed no branching in maltose acceptor products up to DP 7. One reason we did not describe the structure of DP 8 and higher acceptor products is due to the difficulties we have encountered in separating very similar

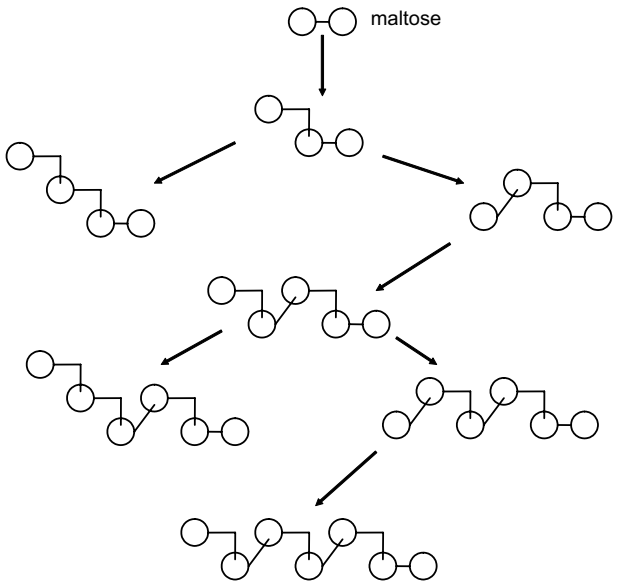

Figure 3. Schematic representation of the alternansucrase-catalyzed formation of acceptor products from maltose, showing the kinetically favored products. Circles represent α-D-glucopyranosyl units. Linkages are represented according to the convention proposed by Robyt:[23] (⌐) α-(1→6) linkage; (/) α-(1→3) linkage; (—) α-(1→4) linkage.

glucose oligosaccharides of this size. However, preliminary analysis of the mixture suggests the presence of two or more structures in the DP 8 product. One of these seems to be the homologue of the DP 7 product in which an additional glucose is added to the nonreducing end via an α-(1→3) linkage. The presence of 3,6-disubstituted glucose and an excess of nonreducing terminal residues in the mixture indicate that some branched product occurs at or above DP 8. This makes sense in light of the fact that the polysaccharide contains approximately one branch unit in nine glucose residues. Branch formation by alternansucrase has not been investigated, but branching in some complex dextrans can arise via acceptor reactions, and varies according to enzyme source.[21,22]

The data presented here show that alternansucrase acceptor products from maltose do not contain dextran-type linkage sequences. Instead, the product series is comprised of an alternan-type linkage sequence, with some pairs of consecutive α-(1→6) linkages in the even-numbered members of the series. The distribution and sequence of linkages is apparently kinetically controlled. Branch formation was not detected below DP 8.

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Supplementary data

Supplementary material (TLC densitograms of products of DP 5 and DP 6) is provided in the Supplementary material section that is available online at doi:10.1016/j.carres.2006.04.044.

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