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Te Poele, Evelien; Corwin, Sarah; Hamaker, Bruce R; Lamothe, Lisa; Vafeiadi, Christina; Dijkhuizen, Lubbert

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Article

Development of Slowly Digestible Starch Derived α-Glucans with 4,6-α-Glucanotransferase and Branching Sucrase Enzymes

E. M. te Poele, S. G. Corwin, B. R. Hamaker, L. M. Lamothe, C. Vafiadi, and L. Dijkhuizen*

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ABSTRACT: Previously, we have identified and characterized 4,6-α-glucanotransferase enzymes of the glycosyl hydrolase (GH) family 70 (GH70) that cleave (α1→4)-linkages in amylose and introduce (α1→6)-linkages in linear chains. The 4,6-α-glucanotransferase of *Lactobacillus reuteri* 121, for instance, converts amylose into an isomalto/malto-polysaccharide (IMMP) with 90% (α1→6)-linkages. Over the years, also, branching sucrase enzymes belonging to GH70 have been characterized. These enzymes use sucrose as a donor substrate to glucosylate dextran as an acceptor substrate, introducing single -(1→2,6)-α-D-Glc*p*-(1→6)- (*Leuconostoc citreum* enzyme) or -(1→3,6)-α-D-Glc*p*-(1→6)-branches (*Leuconostoc citreum*, *Leuconostoc fallax*, *Lactobacillus kunkeei* enzymes). In this work, we observed that the catalytic domain 2 of the *L. kunkeei* branching sucrase used not only dextran but also IMMP as the acceptor substrate, introducing -(1→3,6)-α-D-Glc*p*-(1→6)-branches. The products obtained have been structurally characterized in detail, revealing the addition of single (α1→3)-linked glucose units to IMMP (resulting in a comb-like structure). The *in vitro* digestibility of the various α-glucans was estimated with the glucose generation rate (GGR) assay that uses rat intestinal acetone powder to simulate the digestive enzymes in the upper intestine. Raw wheat starch is known to be a slowly digestible carbohydrate in mammals and was used as a benchmark control. Compared to raw wheat starch, IMMP and dextran showed reduced digestibility, with partially digestible and indigestible portions. Interestingly, the digestibility of the branching sucrase modified IMMP and dextran products considerably decreased with increasing percentages of (α1→3)-linkages present. The treatment of amylose with 4,6-α-glucanotransferase and branching sucrase/sucrose thus allowed for the synthesis of amylose/starch derived α-glucans with markedly reduced digestibility. These starch derived α-glucans may find applications in the food industry.

KEYWORDS: *branching sucrase, α-glucanotransferase, isomalto/malto-polysaccharide, digestibility, dextran*

INTRODUCTION

It is well-known that sugar reduction is a major challenge for the food industry. In many cases, maltodextrins, glucose syrups, and other starch derivatives are proposed and used as alternatives. Their consumption results in rapid and abrupt glucose delivery to the body and consequently causes a high glycemic response. Therefore, like sugars, these glycemic carbohydrates are viewed in an unfavorable light by consumers, the scientific community, and regulatory bodies.[1]

In recent years, we have characterized various novel starch modifying enzymes of glycosyl hydrolase family 70 (GH70) that cleave (α1→4)-linkages and introduce (α1→6)-linkages, resulting in the synthesis of α-glucans with various ratios of these linkage types, either in linear chains or with different degrees of branching. Such modified starches are likely to be digested slowly or to a lesser degree, releasing glucose less abruptly, turning them into more healthful carbohydrates compared to the original rapidly digestible starch ingredients.[1−5]

One example is the 4,6-α-glucanotransferase GtfB-ΔN-ΔV of *Lactobacillus reuteri* strain 121; it cleaves (α1→4)-linkages in amylose and introduces (α1→6)-linkages, resulting in the synthesis of linear isomalto/malto-polysaccharides (IMMP).[6−10] Another example is the 4,6-α-glucanotransferase GtfB of *Lactobacillus reuteri* NCC 2613 that modifies amylose/starch into a branched glucan with (α1→4)- and (α1→6)-linkages.[3] *Lactobacillus aviarius* subsp. *aviarius* DSM 20655 encodes both types of 4,6-α-glucanotransferase GtfB enzymes from adjacent genes.[11] The differences in product and substrate specificity between these GtfB enzymes are understood in molecular detail, involving a closed (*L. reuteri* 121 GtfB, acting on amylose, producing a linear α-glucan) or an open (*L. reuteri* NCC 2613 GtfB, acting on amylose, amylopectin, and starch, producing a branched α-glucan) active site cavity.[12,13] Differences in digestibility between these various α-glucan products with (α1→4)- and (α1→6)-linkages remain to be studied.

To digest the dietary available carbohydrates to the monosaccharides glucose, fructose, and galactose, the mammalian body employs the salivary and pancreatic α-amylases (EC 3.2.1.1.) and the small intestine mucosal two-enzyme complexes of maltase−glucoamylase (MGAM) (EC 3.2.1.20 and 3.2.1.3) and sucrose−isomaltase (SI) (EC 3.2.148 and 3.2.10). The α-amylases are classified in glycoside hydrolase (GH) family GH13 and the four catalytic subunits

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of MGAM and SI, in GH31.[14] The four enzyme subunits of these α-glucosidases have different roles in the conversion of glycemic carbohydrates to glucose (and of sucrose to glucose and fructose). α-Glucans with structures and linkages that are less easily hydrolyzed by these enzymes potentially are of interest as slowly digestible carbohydrates.[1] It is the synthesis and digestibility of such α-glucan structures that are the subject of this work.

The α-glucans with (α1→4)- and (α1→6)-linkages may still be digestible to some (considerable) extent by human oral, pancreatic, and intestinal tract enzymes.[1,8] Therefore, we also looked at the possible synthesis of α-glucans with (α1→3)-linkages. The characterized GH70 branching sucrase enzymes use sucrose as the donor substrate to glucosylate dextran as an acceptor substrate, introducing single -(1→2,6)-α-D-Glc*p*-(1→6)- (*Leuconostoc citreum* enzyme) or -(1→3,6)-α-D-Glc*p*-(1→6)-branches (*Leuconostoc citreum*, *Leuconostoc fallax*, *Lactobacillus kunkeei* enzymes) (Figure 1).[15−18] Interestingly, our results show that the GtfZ-CD2 catalytic domain of the *L. kunkeei* DSM 12361 branching sucrase uses not only dextran but also IMMP as an acceptor substrate, introducing -(1→3,6)-α-D-Glc*p*-(1→6)-branch points. A detailed structural analysis and the *in vitro* digestibility of such novel linear and branched α-glucans are reported in this paper.

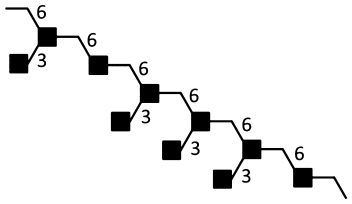

Figure 1. Incubation of dextran (70 kDa) (Meng et al.[16]) and IMMP (18.3 kDa) (this study) with GtfZ-CD2 and 200 mM sucrose resulting in the synthesis of comb-like structures consisting of single (α1→3)-branched glucose units on a linear (α1→6) glucose chain.

MATERIALS AND METHODS

Production of the Enzymes. The GtfZ-CD2 enzyme used in this study is the (α1→3)-branching sucrase (EC 2.4.1.362) catalytic domain CD2 (amino acids 121−2264) of the GtfZ protein of *Lactobacillus kunkeei* DSM 12361.[16] The 4,6-α-glucanotransferase enzyme GtfB-ΔN-ΔV (EC 2.4.1.B34) is a truncated variant (amino acids 761 to 1619) of GtfB of *L. reuteri* 121 lacking both the N-terminal variable domain and domain V.[12] Both GtfZ-CD2 and GtfB-ΔN-ΔV carrying a C-terminal His-tag were expressed from *E. coli* BL21 DE3 cells and purified by Ni^{2+} nitrilotriacetic acid (NTA) affinity chromatography as described by Meng et al.[16]

Standard Reaction Buffer and Conditions. All GtfZ-CD2 enzyme reactions were performed at 30 °C in 25 mM sodium acetate (pH 5.5) containing 1.5 mM $CaCl_2$. GtfB-ΔN-ΔV reactions were at 37 °C in 25 mM sodium acetate (pH 5.0) containing 1.0 mM $CaCl_2$.

Enzyme Activity Assays. Enzyme activity assays with 0.12 mg/mL GtfZ-CD2 were performed with 7−20 g/L dextran (M_w 10.2 kDa or M_w 70 kDa) (Sigma Aldrich, The Netherlands) or IMMP (see below), in both cases, with 200 mM sucrose. Samples of 25 μL were taken every min over a period of 7 min and immediately inactivated with 12.5 μL of 0.4 M NaOH; after enzyme inactivation, the samples were neutralized by adding 12.5 μL of 0.4 M HCl. The glucose and fructose concentrations in these samples were enzymatically determined by monitoring the reduction of NADP with the hexokinase and glucose-6-phosphate dehydrogenase/phosphoglucose isomerase assays (Roche Nederland BV, Woerden, The Netherlands).[19] The determination of the release of glucose and fructose from sucrose allowed one to calculate the total activity of the glucansucrase enzymes.[20] One unit (U) of enzyme is defined as the amount of enzyme required for producing 1 μmol of fructose per min in reaction buffer.

The total enzyme activity of GtfB-ΔN-ΔV was determined by the amylose-iodine staining method as described by Bai et al.[21] using 0.125% (w/v) amylose V from potato starch (M_w 170 kDa) (AVEBE, Foxhol, The Netherlands). The decrease in absorbance of the α-glucan−iodine complex resulting from transglycosylation and/or hydrolytic activity was monitored at 660 nm for 7 min at 40 °C. One unit of activity was defined as the amount of enzyme converting 1 mg of substrate per min.

Synthesis of Isomalto/Malto-Polysaccharides (IMMP) from Starch. A 4% amylose stock solution was prepared by solubilizing amylose V in 1 M NaOH. The stock was set to pH 5.0 with 1 M HCl and diluted to a concentration of 1% (w/v) in buffer. For the production of IMMP from amylose V, 40 μg/mL GtfB-ΔN-ΔV was incubated with 500 mL of 1% amylose V for 72 h at 37 °C. After incubation, the enzyme was heat-inactivated at 95 °C for 20 min. The incubation was dialyzed against 25 L of running tap water for 72 h in 3.5 kDa snake skin tubing (ThermoFisher), then dialyzed for 48 h in 25 L of demineralized water, and finally dialyzed for 24 h against 22 L of MilliQ water. The dialyzed IMMP was lyophilized to dryness.

Synthesis of α(1→3)-Branched IMMP and Dextran. The (α1→3)-branched polymers were synthesized by incubating 0.5 U/mL GtfZ-CD2 for 24 h with 20 g/L IMMP or dextran (M_w 70 kDa) with 200 mM sucrose. To obtain partially branched polymers, 20 g/L IMMP or dextran (M_w 10.2 kDa) were incubated with 0.5 U/mL GtfZ-CD2 for 24 h with different sucrose concentrations ranging from 0 to 200 mM. The concentrations of 10.2 and 70 kDa dextran and IMMP were expressed as the molar concentrations of the anhydroglucosyl units in the polymer. The 20 g/L polymer (dextran (M_w 70 kDa), dextran (M_w 10.2 kDa), and IMMP (M_w 18.3)) used in each case thus corresponds to a concentration of 123 mM anhydroglucosyl units. The incubations were heat-inactivated at 95 °C for 20 min and subsequently dialyzed as described above for IMMP synthesis.

High-pH Anion-Exchange Chromatography. High-performance anion-exchange chromatography (HPAEC) was performed on an ICS-3000 workstation (Dionex, Amsterdam, The Netherlands), equipped with an ICS-3000 ED pulsed amperometric detection system (PAD). Samples were diluted 1:100 in MilliQ water and filtered through a 0.2 μm cellulose filter prior to injection (25 μL injection volume). The oligosaccharides were separated on a CarboPac PA-1 column (Dionex; 250 × 4 mm) by using a linear gradient of 10−240 mM sodium acetate in 100 mM NaOH over 57 min at a flow rate of 1 mL/min. Commercial oligosaccharide standards were used to identify the peaks.

Methylation Analysis. Analysis of the glucosyl linkage composition of the (α1→3)-branched polymers of the 24 h incubations of 20 g/L (123 mM anhydroglucose) dextran (M_w 70 kDa) or IMMP with 200 mM sucrose was done as follows. Samples were permethylated using CH_3I and solid NaOH in $(CH_3)_2SO$, as described previously,[22] and then hydrolyzed with 2 M trifluoroacetic acid (2 h, 120 °C) to give the mixture of partially methylated monosaccharides. After evaporation to dryness, the mixture, dissolved in H_2O, was reduced with $NaBD_4$ (2 h, room temperature). Subsequently, the solution was neutralized with 4 M acetic acid, and boric acid was removed by repeated coevaporation with methanol. The obtained partially methylated alditol samples were acetylated with 1:1 acetic anhydride−pyridine (30 min, 120 °C). After evaporation to dryness, the mixtures of partially methylated alditol acetates (PMAA), dissolved in dichloromethane, were analyzed by GLC-EI-MS on an EC-1 column (30 m × 0.25 mm; Alltech), using a GCMS-QP2010 Plus instrument (Shimadzu Kratos Inc., Manchester, UK) and a temperature gradient (140−250 °C at 8 °C/min).[23]

NMR Spectroscopy. One-dimensional ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 600 MHz spectrometer (NMR Center, University of Groningen), using D_2O as solvent at a probe temperature of 300 K. Before analysis, 3 mg of

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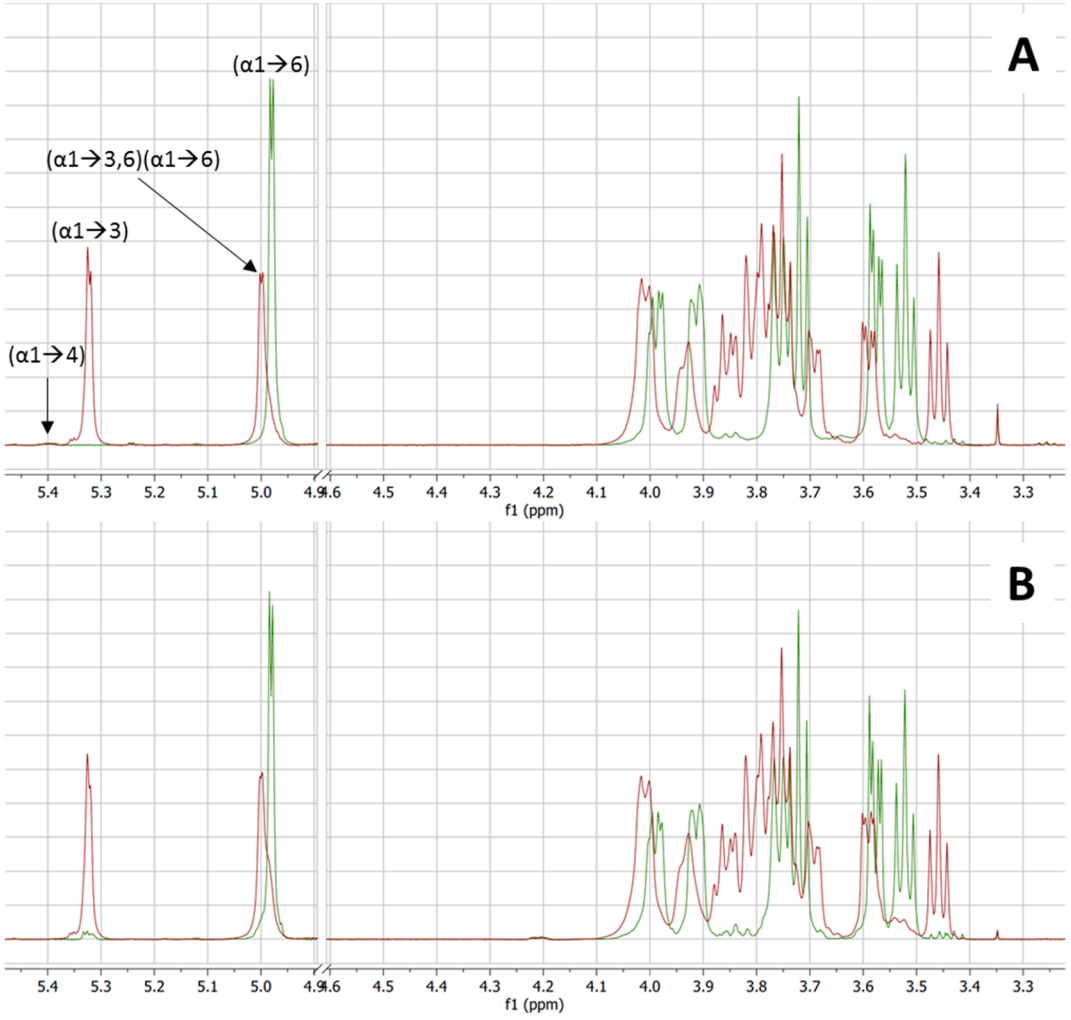

Figure 2. ^{1}H NMR spectra (D_2O, 300 K) of branched polymers formed by the incubation of GtfZ-CD2 with 20 g/L (123 mM anhydroglucose) (A) IMMP (18.3 kDa) and (B) dextran (70 kDa) with (red lines) or without (green lines) 200 mM sucrose. Chemical shifts are shown in parts per million (ppm) relative to the signal of internal acetone (δ 2.225).

freeze-dried polymer sample was exchanged twice in 500 μL of D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and finally dissolved in 650 μL of D_2O spiked with 0.005% acetone as an internal standard. The NMR data were processed using the MestReNova 12 program (Mestrelab Research SL, Santiago de Compostella, Spain). Chemical shifts (δ) were expressed in ppm by reference to internal acetone (δ_H 2.225 for ^{1}H). The ratio of different glycosidic linkages was determined by integration of the surface areas of the respective signal peaks in the ^{1}H NMR spectra.

High-Performance Size-Exclusion Chromatography. The molecular mass distribution of the products was determined by high-pressure size-exclusion chromatography (HPSEC) as described previously.[21,24] The HPSEC system (Agilent Technologies 1260 Infinity) was equipped with a multi angle laser light scattering detector (SLD 7000 PSS, Mainz, Germany), a viscometer (ETA-2010 PSS, Mainz), and a differential refractive index detector (G1362A 1260 RID Agilent Technologies). Separation was performed by using three PFG-SEC columns with porosities of 100, 300, and 4000 Å, coupled with a PFG guard column. DMSO-LiBr (0.05 M) was used as eluent at a flow rate of 0.5 mL/min. The system was calibrated and validated using a standard pullulan kit (PSS, Mainz, Germany) with M_w ranging from 342 to 708 000 Da. The specific RI increment (dn/dc) value for pullulan was determined to be 0.072 mL/g (PSS, Mainz). We assumed that the specific RI increment (dn/dc) values for the IMMP and dextran polysaccharides were the same as for pullulan. The molecular weight of the low molecular weight products ($<1 \times 10^5$ Da) was determined by the universal calibration method. WinGPC Unity software (PSS, Mainz) was used for data processing.

***In Vitro* Glucose Generation Rate (GGR) Assay Using Rat Intestinal Acetone Powder.** On the basis of the protocol of Shin et al.,[25] 100 mM sodium phosphate buffer at pH 6.0 with 0.005% w/v ampicillin salts[26] was freshly prepared for each experiment. Rat intestinal acetone powder (RIAP) (Sigma Aldrich, Burlington, MA, USA) was ground using a cell grinder (IKA A-10 Homogenizer, IKA Works, Inc., Wilmington, NC, USA), then pressed through a #20 standard (850 μm) sieve, and stored at −20 °C. Sufficient quantities of RIAP needed for all assays were ground and pooled together to ensure consistent enzyme activity. A suspension of 5.55% w/v RIAP was created and held at 4 °C for 60 min prior to beginning incubation of the samples. Substrate solutions (10% w/v) made with the above buffer were preincubated for 10 min at 37 °C, 600 rpm (Eppendorf ThermoMixer C, Eppendorf, Hauppauge, NY, USA). Quantities of 1% substrate solutions sufficient to dilute RIAP to 5% w/v and substrate to 0.1% w/v were added. RIAP−substrate suspensions were incubated at 37 °C; aliquots were taken and inactivated at 100 °C and 600 rpm for 5 min at 15, 30, and 45 min and 1, 2, 3, and 6 h. Inactivated aliquots were centrifuged (Microfuge 20R Centrifuge, Beckman Coulter, Indianapolis, IN, USA) at 9160*g* for 10 min and held at 4 °C until the time of analysis. Pure 0.1% α-glucans in water were used for the 0 min time point. The amount of released glucose in digested and inactivated supernatant was diluted 10 times and then

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analyzed using the glucose oxidase/peroxidase (GOPOD) method and a microplate spectrophotometer (SpectraMax 190 Absorbance Microplate Reader, Molecular Devices, LLC, San Jose, CA, USA),[27] using glucose to prepare the standard curve. Digestion assays were performed in triplicate at each time point. Raw wheat starch was used as a positive slowly digestible carbohydrate benchmark control[28] and was digested fully in 6 h with RIAP containing residual α-amylase and α-glucosidases. Percent digestibility was determined on the basis of the full hydrolysis of raw wheat starch to glucose (100%) at 360 min.

RESULTS AND DISCUSSION

IMMP Synthesis and Structural Characterization. To synthesize IMMP, amylose V (M_w 170 kDa) was incubated for 72 h with the GtfB-ΔN-ΔV enzyme. After inactivation of the GtfB-ΔN-ΔV enzyme, the reaction products were purified from glucose, oligosaccharides, and salts by dialysis. Structural analysis of the GtfB-ΔN-ΔV reaction products with 1D ^{1}H NMR revealed that the amylose V was almost completely converted into IMMP, showing an NMR profile nearly identical to that of dextran (M_w 70 kDa) (Figure 2). The (α1→4) signal (δ 5.41), corresponding to linear (α1→4)-linked glucose units, had disappeared and a peak corresponding to linear (α1→6)-linked glucose units had appeared at δ 4.98. Methylation analysis showed that the IMMP product consisted of terminal [Glc*p*(1→], 4-substituted [→4)-Glc*p*(1→], and 6-substituted [→6)Glc*p*(1→] glucosyl units in a molar ratio of 3.8%, 6.8%, and 89.4%, respectively (Table 1).

Table 1. Methylation Analysis (%) of the Carbohydrate Moieties in IMMP (18.3 kDa), Dextran (70 kDa), and their Branched Derivatives[a]

PMAA	R_t	IMMP GtfZ-CD2	IMMP-	dextran GtfZ-CD2	dextran-
Glc(1→	1.00	50.8	3.8	39.4	3.9
→3)Glc(1→	1.16			1.5	
→4)Glc(1→	1.19		6.8		
→6)Glc(1→	1.23	5.5	89.4	12.9	89
→3,6)Glc(1→	1.38	43.7		46.2	6.2
→4,6)Glc(1→	1.40				0.9

[a]These branched polymers were produced during a 24 h incubation of the GtfZ-CD2 enzyme with 20 g/L (123 mM anhydroglucose) IMMP or dextran (70 kDa) and 200 mM sucrose. PMAA = partially methylated alditol acetates. R_t = relative retention time to Glc(1→.

The molar ratio of terminal [Glc*p*(1→] and 6-substituted [→6)Glc*p*(1→] glucosyl units in 70 kDa dextran was nearly identical to that of IMMP, i.e., 3.9% and 89%, respectively, but consisted of 6.2% 3,6-substituted [→3,6)Glc*p*(1→] glucosyl units instead of 4-substituted [→4)Glc*p*(1→] glucose units (Table 1). HPSEC analysis of the IMMP and 10.2 kDa dextran showed monodisperse peaks with M_w of 18.30 and 9.75 kDa, respectively (Table 2).

Branching Sucrase Activity of GtfZ-CD2 on IMMP. GtfZ-CD2 of *L. kunkeei*[16] and the related branching sucrase enzymes of *L. citreum* and *L. fallax*[17,18] use sucrose as a glucose donor to decorate dextran molecules, adding single (α1→3)-branched glucose units on the linear (α1→6) glucose chain (Figure 1). Here, we show that GtfZ-CD2 also had (α1→3)-branching activity on the IMMP product (with ~90% (α1→6)). The effects of (α1→3)-branching on the digestibility of the dextran and IMMP derived products subsequently were analyzed.

Table 2. Molecular Weights (M_w) of a Range of Purified Branched Polymers Derived from IMMP (18.3 kDa) and Dextran (10.2 kDa) as Determined by HPSEC Analysis (Also See Figure 5)[a]

[sucrose] (mM)	ratio [suc]/[IMMP or dextran]	M_w (kDa) IMMP products	M_w (kDa) dextran products	M_w increase (%) IMMP	M_w increase (%) dextran
200	1.63	25.50	13.10	39.34	34.36
150	1.22	23.00	12.20	25.68	25.13
100	0.81	23.20	12.30	26.78	26.15
75	0.61	21.30	11.90	16.39	22.05
50	0.41	21.00	11.40	14.75	16.92
25	0.20	19.00	10.40	3.83	6.67
12.5	0.10	18.70	10.10	2.19	3.59
6.25	0.05	18.50	9.90	1.09	1.54
0	0.00	18.30	9.75	0.00	0.00

[a]The branched polymers were produced during a 24 h incubation of the GtfZ-CD2 enzyme with 20 g/L (123 mM anhydroglucose) IMMP or dextran (M_w 10.2 kDa) at increasing molar ratios of [sucrose]/[IMMP or dextran anhydroglucose]. Sucrose was provided at 0−200 mM.

To determine whether GtfZ-CD2 had (α1→3)-branching activity on IMMP, enzyme activity assays were performed with IMMP, with and without 200 mM sucrose. Assays with 70 kDa dextran with and without 200 mM sucrose served as controls. GtfZ-CD2 had clear transglucosylating activity on both dextran and IMMP; a somewhat lower initial rate was observed with IMMP, i.e., 10.4 and 14.8 U/mg protein for IMMP (M_w 18.3 kDa) and dextran (M_w 70 kDa), respectively (Figure 3). In the absence of sucrose as a glucosyl donor, no activity was observed with dextran or IMMP alone. With sucrose alone, GtfZ-CD2 showed minor transglucosylase activity and mainly catalyzed sucrose hydrolysis and synthesis of leucrose, as reported before (Figure 3).[16]

Structural Analysis of the GtfZ-CD2 Reaction Products with Dextran and IMMP. Using dextran (M_w 70 kDa) as acceptor substrate, Meng et al.[16] showed that, at a molar ratio of [sucrose]/[dextran] of 0.65 and higher, the dextran glucosylation products obtained maximally had 41% (α1→3)-linkages. In this work, we used both IMMP (M_w 18.3 kDa) and

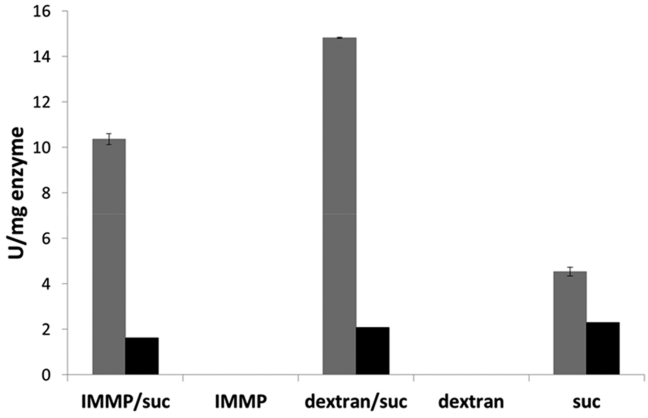

Figure 3. Enzyme activities of GtfZ-CD2 on 7 g/L IMMP (18.3 kDa) or dextran (70 kDa) with and without 200 mM sucrose. Transglucosylation activity (gray bars); sucrose hydrolysis (black bars). Hydrolysis standard deviations were very small and therefore not apparent in this figure.

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70 kDa dextran (M_w 70 kDa) (as positive control) as substrates for GtfZ-CD2, incubated with 200 mM sucrose for 24 h (molar ratio of [sucrose]/[dextran] of 4.63). Structural analysis of the reaction products with 1D ^{1}H NMR showed that GtfZ-CD2 indeed also decorated IMMP with (α1→3)-branched glucosyl units. The NMR profiles of the products from the IMMP and dextran incubations with GtfZ-CD2 were almost identical (Figure 2), suggesting that the branched polymers produced from IMMP and from dextran were very similar. Treatment with the GtfZ-CD2 enzyme in the presence of sucrose resulted in the disappearance of the (α1→6) signal (δ 4.98), corresponding to linear (α1→6)-linked glucose units, in both the IMMP and dextran products. Instead, a structural unit -(1→3,6)-α-D-Glc*p*-(1→6)- at δ 5.00, corresponding to an (α1→3) branch point on linear (α1→6)-linked glucose units, appeared in the profiles of both polymer products. The high intensity of H-4 signals (δ 3.40−3.45) stemming from terminal glucose units in the IMMP product indicated a high percentage of branching linkages, as seen previously for the dextran product (Figure 1).[16] Furthermore, a peak appeared at δ 5.32 that is typical for (α1→3)-linkages. Integration of the surface areas of the (α1→3)-linkage signal at δ 5.32 and the -(1→3,6)-α-D-Glc*p*-(1→6)-linkage signal at δ 5.00 revealed that their ratios were close to one, 0.85 and 0.72 for IMMP and dextran, respectively (Figure 2). This indicates that the (α1→3) signal stems from branched (α1→3)-linkages and not from consecutive (α1→3)-linkages and that most of the (α1→6)-linked glucose units were decorated with an (α1→3)-linked glucose (cp. Figure 1). The absence of the [→6)-α-D-Glc*p*-(1→3)-] epitope at δ 4.20[29] suggests that the (α1→3) glucosyl units were not elongated with (α1→6)-linked glucose units. Methylation analysis showed that the branched IMMP and 70 kDa dextran polymers consisted of 44% and 46% (α1→3)-linkages (Table 1), respectively, representing comb-like structures consisting of single (α1→3)-branched glucose units on a linear (α1→6) glucose chain (Figure 1). The branched IMMP polymer had terminal [Glc*p*(1→], 6-substituted [→6)Glc*p*(1→], and 3,6-substituted [→3,6)-Glc*p*(1→] glucosyl units in a molar ratio of 50.8%, 5.5%, and 43.7%, respectively. No detectable levels of 3-substituted [→3)Glc*p*(1→] glucosyl units were observed, confirming the absence of linear (α1→3) stretches (Table 1, Figure 1).

Synthesis of Partially Branched IMMP and Dextran with GtfZ-CD2. The degree of (α1→3)-branching may well influence the digestibility of the branched IMMP and dextran polymers. To test this, GtfZ-CD2 incubations were performed with fixed IMMP or dextran concentrations and a varying sucrose concentration to modulate the degree of (α1→3)-branching in the polymer products. For comparison, 10.2 kDa dextran was used in this experiment, since its molecular weight is more similar to that of IMMP (18.3 kDa) than 70 kDa dextran. The incubation of GtfZ-CD2 with 20 g/L (123 mM anhydroglucose) 10.2 or 70 kDa dextran, and 200 mM sucrose (molar ratio of [sucrose]/[dextran anhydroglucose] of 1.63) resulted in 57% and 46% of (α1→3)-linkages in the end products, respectively.

NMR analysis of purified reaction products showed that GtfZ-CD2 was also able to partially branch IMMP and dextran (Figures 4 and S1). As observed with 70 kDa dextran, branching of the 10.2 kDa dextran leveled off at [sucrose]/[dextran] molar ratios above 0.65, whereas with IMMP the decoration with (α1→3)-linkages was less at lower [sucrose]/[IMMP] molar ratios and had not reached a maximum at a ratio of 1.63. HPSEC analysis of the polymers indeed showed that the M_w of the branched dextran (10.2 kDa) products increased less above a ratio of 0.61, while the M_w of the branched IMMP (18.3 kDa) products displayed a linear increase from 0 to 1.63 (Table 2, Figure 5). This suggests a lower branching efficiency of GtfZ-CD2 with IMMP compared to dextran, which is also reflected by its lower initial transglucosylase activity with IMMP. On the basis of linkage type distributions, the (branched) IMMP and dextran (products) are rather similar, but there may be other structural differences that affect the branching efficiency of GtfZ-CD2, e.g., the presence of 6.8% 4-substituted [→4)Glc*p*(1→] linkages in IMMP.

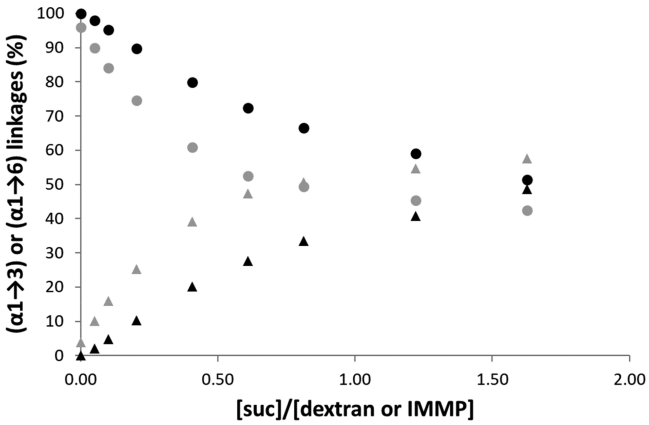

Figure 4. Percentages of (α1→3)-linkages (triangles) and (α1→6)-linkages (circles) in a range of purified branched polymers, derived from IMMP (18.3 kDa) (black) and dextran (10.2 kDa) (gray), based on the integrated peak areas of their 1D ^{1}H NMR profiles. The polymers were produced during a 24 h incubation of the GtfZ-CD2 enzyme with 20 g/L (123 mM anhydroglucose) IMMP or dextran (M_w 10.2 kDa) at increasing molar ratio of [sucrose]/[IMMP or dextran anhydroglucose]. Sucrose was provided at 0−200 mM. For ratios, also see Table 2.

***In Vitro* Glucose Generation Rate (GGR) Assay Using Rat Intestinal Acetone Powder (RIAP).** The digestibility of the branched polymers was tested using the *in vitro* RIAP assay. This assay simulates the digestive power of the human gastrointestinal tract by using rat intestinal maltase−glucoamylase and sucrase−isomaltase enzymes that digest (α1→6)- and (α1→3)-linkages[30] and residual α-amylase. Digestibility was measured by the release of free glucose from enzymatic hydrolysis of the polymers over time. Polymers that are slowly digestible are less easily hydrolyzed by these enzymes, thus resulting in a decreased glucose release over time. Raw wheat starch was found to be a good representative of a slowly digestible carbohydrate[31,32] and was used here as a benchmark.

In vitro digestion analysis showed that, compared to the slowly digestible raw wheat starch benchmark, the unmodified dextran and IMMP α-glucans [0 mM sucrose] had a reduced digestibility (Figure 6). The introduction of (α1→3)-linked branching decreased their digestibility even further, displaying an incremental effect with a greater degree of branching correlating to lower digestibility. Highly branched polymers were essentially not digested. Of interest, IMMP [0 mM sucrose] reached 44% digestion by 6 h; dextran [0 mM sucrose] reached 53% digestion by 6 h. Note that [200 mM

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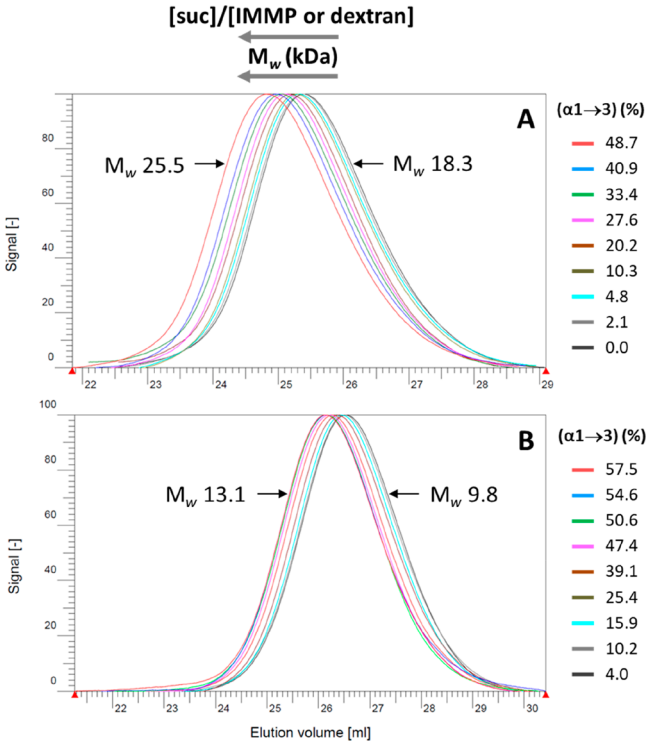

Figure 5. HPSEC chromatograms of a range of purified branched polymers derived from IMMP (18.3 kDa) (A) and dextran (10.2 kDa) (B). The percentages of (α1→3)-linkages in the branched polymers are based on the integrated peak areas of their 1D ^{1}H NMR profiles (see % color coding on the right). The polymers were produced during a 24 h incubation of the GtfZ-CD2 enzyme with 20 g/L (123 mM anhydroglucose) IMMP or dextran (M_w 10.2 kDa) at increasing molar ratios of [sucrose]/[IMMP or dextran anhydroglucose]. Sucrose was provided at 0−200 mM. For ratios, also see Table 2.

sucrose] and [150 mM sucrose] dextran and [200 mM sucrose] and [0 mM sucrose] IMMP were very hygroscopic and viscous when hydrated and required additional heat and shear application to create homogeneous mixtures in solution. These solutions were incubated at 37 °C at 1000 rpm for 30 min prior to the digestion assay.

A 6 h timeline was used for the *in vitro* studies in view of the observation that RDS and SDS controls were 100% digested at 6 h of *in vitro* digestion.[25,30] The alignment of digestion times between an *in vitro* assay and human digestion is difficult for a number of reasons, including unknown α-glucosidase expression and activity levels along the course of the small intestine and unknown concentrations of pancreatic α-amylase relative to types of food ingested and location in the small intestine. Thus, it is not possible to directly compare 1 h of *in vivo* digestion to 1 h of *in vitro* digestion; hence, digestions of carbohydrates were considered over 6 h in this *in vitro* model (Figure 6).

In conclusion, treatment of amylose with 4,6-α-glucanotransferase and branching sucrase/sucrose allowed the synthesis of starch derived α-glucans with markedly reduced digestibility. These starch derived α-glucans may find application in the food industry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c01465.

^{1}H NMR spectra of polymers formed by the incubation of GtfZ-CD2 and IMMP or dextran with different [sucrose]/[IMMP or dextran] ratios (PDF)

■ AUTHOR INFORMATION

Corresponding Author

L. Dijkhuizen − *Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, 9747AG Groningen, The Netherlands; CarbExplore Research BV, 9747AN Groningen, The Netherlands;* orcid.org/0000-0003-2312-7162; Email: L.Dijkhuizen@rug.nl

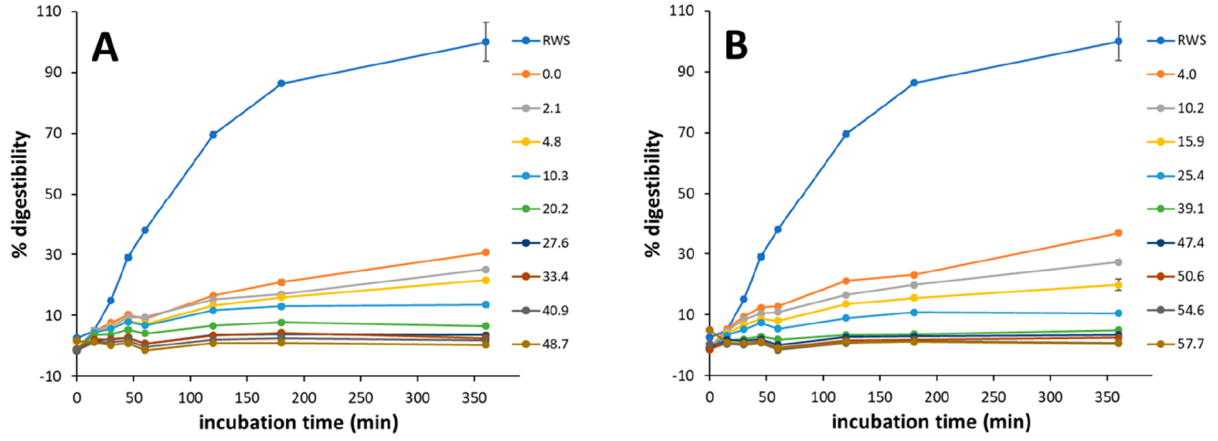

Figure 6. Digestibility of raw wheat starch (RWS) and a range of purified branched polymers derived from IMMP (A) and dextran (B) in an *in vitro* digestion assay (6 h) with rat intestinal acetone powder enzymes. Digestibility was measured by the release of free glucose from enzymatic hydrolysis of the polymers over time (in triplicate). Standard deviations were very small and therefore in most cases not apparent in this figure. The percentages of (α1→3)-linkages in the branched polymers are based on the integrated peak areas of their 1D ^{1}H NMR profiles (see % color coding on the right). The polymers were produced during a 24 h incubation of the GtfZ-CD2 enzyme with 20 g/L (123 mM anhydroglucose) IMMP or dextran (M_w 10.2 kDa) at increasing molar ratios of [sucrose]/[IMMP or dextran anhydroglucose]. Sucrose was provided at 0−200 mM. For ratios, also see Table 2.

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Authors

E. M. te Poele − *Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, 9747AG Groningen, The Netherlands; CarbExplore Research BV, 9747AN Groningen, The Netherlands;* orcid.org/0000-0002-8193-9516

S. G. Corwin − *Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, Indiana 47907, United States;* orcid.org/0000-0003-1834-2642

B. R. Hamaker − *Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, Indiana 47907, United States;* orcid.org/0000-0001-6591-942X

L. M. Lamothe − *Nestlé Research, 1000 Lausanne, Switzerland;* orcid.org/0000-0002-5270-1875

C. Vafiadi − *Nestlé Research, 1000 Lausanne, Switzerland;* orcid.org/0000-0002-4511-7582

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c01465

Notes

The authors declare the following competing financial interest(s): L.M.L. and C.V. are employed by the Nestle Research Center. B.R.H. and L.D. have received grant/research support from Nestle Research Center, at least partly used to employ E.M.t.P. and S.G.C. Authors have jointly filed multiple patent applications around this topic of research and potential products.

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