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Starch with a Slow Digestion Property Produced by Altering Its Chain Length, Branch Density, and Crystalline Structure

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The hypothesis of increasing the branch density of starch to reduce its digestion rate through partial shortening of amylopectin exterior chains and the length of amylose was investigated. Starch products prepared using β-amylase, β-amylase and transglucosidase, maltogenic α-amylase, and maltogenic α-amylase and transglucosidase showed significant reduction of rapidly digested starch by 14.5%, 29.0%, 19.8%, and 31.0% with a concomitant increase of slowly digested starch by 9.0%, 19.7%, 5.7%, and 11.0%, respectively. The resistant starch content increased from 5.1% to 13.5% in treated starches. The total contents of the prebiotics isomaltose, isomaltotriose, and panose (Isomaltooligosaccharides) were 2.3% and 5.5%, respectively, for β-amylase/transglucosidase- and maltogenic α-amylase/transglucosidase-treated starches. The molecular weight distribution of enzyme-treated starches and their debranched chain length distributions, analyzed using high-performance size-exclusion chromatography with multiangle laser light scattering and refractive index detection (HPSEC-MALLS-RI) and HPSEC-RI, showed distinctly different patterns among starches with different enzyme treatments. A larger proportion of low molecular weight fractions appeared in starches treated additionally with transglucosidase. All enzyme-treated starches showed a mixture of B- and V-type X-ray diffraction patterns, and ^{1}H NMR spectra showed a significant increase of α-1,6 linkages. Both the increase of the starch branch density and the crystalline structure in the treated starches likely contribute to their slow digestion property.

KEYWORDS: Starch; slow digestion property; branch density; prebiotic function; β-amylase; maltogenic α-amylase; transglucosidase; ^{1}H NMR

INTRODUCTION

Starch is the main glycemic carbohydrate in starchy foods. According to the rate and extent of starch digestion in vitro, starch has been classified into three major fractions (*1*): (1) rapidly digestible starch, the portion of starch digested within the first 20 min of incubation, (2) slowly digestible starch, the portion of starch digested from 20 to 120 min, and (3) resistant starch, the remaining portion that cannot be further digested. A highly significant positive correlation between the glycemic index (GI) and rapidly digestible starch was reported (*2*). GI is defined as the incremental area under the glucose response curve after a standard amount of carbohydrate from a test food is consumed relative to that of a control food (glucose or white bread) (*3*). The human physiological consequences of GI have been related to diabetes, prediabetes, cardiovascular disease, and obesity (*4*). High GI meals promoted fat deposition in mice, resulting in almost twice the body fat of those consuming low GI meals (*5*). In obese teenagers, the rapid absorption of glucose after consumption of high GI meals induced a sequence of hormonal and metabolic changes that were related to excessive food intake (*6*). Low GI meals decreased nonfasting plasma glucose, plasma triacylglycerols, and adipocyte volume in rats (*7*) and prolonged satiety in obese adolescents (*8*). Starch with a slow digestion property would provide for extended glucose (energy) release along with a low glycemic response and, thus, may have commercial application as a healthy ingredient of processed foods. There are no commercial slowly digestible starch-based products available in the current food market to our knowledge.

It is known that starch is composed of two distinct types of macromolecules: amylose and amylopectin with molecular weights of 10^4 to 10^6 and 10^7 to 10^8, respectively. Amylose, a polymer of α-D-glucopyranosyl units mainly combined by α-1,4 linkages, is defined as essentially a linear molecular chain. Amylopectin is a very large branched polymer consisting of linear α-1,4-linked D-glucopyranosyl chains connected by α-1,6 branch linkages, the latter comprising 4.0−5.5% of the total linkages (*9*). The chains of amylopectin are described as A, B, and C chains (*10*). The A chains are linked only through their reducing termini to C_6 of a glucose unit of other chains. The B chains are linked in the same way, but the B chain has at least

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one other chain (either an A or a B chain) attached at C_6 of one of its glucose units. The C chain carries other chains as branches and contains the sole reducing terminal residue. The average chain lengths of amylopectin range between 19 and 31, depending on the botanical source (*11*). While amylose is generally defined as an unbranched molecule, pure amylose isolated from various starch sources contains certain amounts of branch linkages. It was found that the molar ratio of branched to unbranched molecules of amylose in maize was 0.44−0.56 (*12*). The branched molecules of maize amylose have short side chains with DP 18 and long side chains with DP 100−5000 which peak at DP 200 (*13*).

A strategy was used to *partially shorten the length* of exterior branch chains of amylopectin, as well as increase the branch density of normal maize starch, through enzyme treatments to reduce its overall digestion rate. It is known that, as amyloglucosidase approaches the branch linkage, its rate decreases (*14*−*16*). Thus, partial shortening of branch chains would slow the hydrolysis rate. β-Amylase and maltogenic α-amylase hydrolyze α-1,4-D-glucosidic linkages of starch and its derivatives to remove maltose residues and reduce the chain length. Transglucosidase catalyzes hydrolytic and transfer reactions to form new α-1,6 linkages. Thus, our research modified the normal maize starch structure by using either β-amylase or maltogenic α-amylase, with or without combination of transglucosidase, to produce starches with shorter and more branch chains (A, B_1, B_2, and longer chains). Thereby, the α-1,6 linkages in amylopectin were concentrated, as well as chain distributions altered, to change the digestion properties.

MATERIALS AND METHODS

Materials. Normal maize starch was a gift from Tate and Lyle, Inc. (Decatur, IL). β-Amylase from barley (Optimalt BBA) and transglucosidase L-500 from *Aspergillus* were gifts from Genencor International, Inc. (Rochester, NY). Maltogenic α-amylase (Novamyl, 10000 BG) from *Bacillus subtilis* was from Novozymes North America, Inc. (Franklinton, NC). Amyloglucosidase from *Aspergillus niger* (A7095), pancreatin from porcine pancreas (A7545), α-amylase from porcine pancreas (A3176), glycogen from rabbit liver (G8876), pullulan (A4516), glucose (G7528), maltose (M9171), isomaltose (I7253), isomaltotriose (I0381), and panose (P2407) were purchased from Sigma Chemical Co. (St. Louis, MO). Isoamylase from *Pseudomonas* sp. and a glucose assay kit were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Pullulan standards were purchased from Polymer Laboratories Inc. (Amherst, MA).

Preparation of Starch Products with a Slow Digestion Property. Preliminary studies showed the potential of using β-amylase and maltogenic α-amylase to affect the starch digestion property, as well as transglucosidase to change the branching structure. A simple experimental design used four enzyme treatments to prepare starches for testing of digestion properties and structural characteristics: β-amylase, β-amylase and transglucosidase, maltogenic α-amylase, and maltogenic α-amylase and transglucosidase. Normal maize starch (80 g, dry weight) was mixed with water to an 8% suspension by weight. The suspension was heated in a 2 L beaker at 80 °C for 10 min with stirring. Then the sample was autoclaved at 121 °C for 20 min and placed in a water bath at 55 °C. Sodium acetate buffer (360 mL, 0.5 mol/L, pH 5.0) was added and the mixture stirred for 10 min. Water was added to bring the total volume of the suspension to 1800 mL. The suspension was incubated with β-amylase or maltogenic α-amylase, with and without transglucosidase. The enzyme amounts used were 0.64% (dry weight of starch), 7.0 U/g (dry weight of starch), and 0.6% (dry weight of starch) for β-amylase, maltogenic α-amylase, and transglucosidase, respectively. In the case of maltogenic α-amylase, calcium chloride (1.78 mmol/L) was added to maintain the activity of the enzyme. Incubation with the enzyme was for 5 h. Then 1 volume of absolute ethanol was added followed by centrifugation at 3000 rpm for 10 min. The collected precipitate was dispersed in 800 mL of deionized water and heated with stirring in a boiling water bath for 30 min. The suspension was then stirred at ambient temperature overnight followed by spray-drying using a laboratory-scale unit with a flow rate of 3 mL/min and inlet and outlet temperatures of 120 and 75 °C, respectively (Pulvis GB21, Yamato Scientific Co., Tokyo, Japan). Four products were prepared: β-amylase-treated maize starch (BA-starch); β-amylase- and transglucosidase-treated maize starch (BATG-starch); maltogenic α-amylase-treated maize starch (MA-starch), and maltogenic α-amylase- and transglucosidase-treated maize starch (MATG-starch). The products were stored in sealed bottles for further analysis. Starch modification experiments were performed at least twice.

In Vitro Digestion with Porcine Pancreatic α-Amylase. The digestion property was analyzed using the method of Zhang and Hamaker (*17*) with modification. Starch (50 mg) with 5 mL of sodium glycerophosphate−HCl buffer (1 mmol/L, pH 6.9) containing 25 mmol/L NaCl and 5 mmol/L $CaCl_2$ was cooked in a boiling water bath for 20 min. The solution was equilibrated at 37 °C for 5 min, and 2 U of porcine pancreatic α-amylase was added. Enzyme digestion was carried out at 37 °C, and 0.3 mL aliquots of hydrolyzed solution were withdrawn at different time intervals. The aliquots were immediately put in a boiling water bath for 5 min to deactivate the enzyme. The equivalent reducing sugar value of maltose was determined using the Somogyi−Nelson method (*18*). Each sample was analyzed in duplicate.

In Vitro Digestion with Pancreatin and Amyloglucosidase. The digestion property was also analyzed by a modification of the Englyst assay (*1*), using decreased concentrations of substrate and enzyme to differentiate better how starch structural changes affect enzyme digestion. Enzyme solution containing pancreatin and amyloglucosidase was prepared immediately before use. Starch (50 mg) with 10 mL of sodium acetate buffer (0.1 mol/L, pH 5.2) was cooked in a boiling water bath for 20 min. The suspension was equilibrated at 37 °C for 5 min, and 0.2 mL of the specified enzyme solution and 0.44 mL of calcium chloride (0.1 mol/L) were added. Enzyme digestion was carried out at 37 °C, and 0.5 mL aliquots of hydrolyzed solution were withdrawn at different time intervals. Aliquots were immediately put in a boiling water bath for 5 min to deactivate the enzymes. The glucose content of the hydrolyzates was determined using the Megazyme glucose assay kit (GOPOD method). Each sample was analyzed in duplicate.

Isomaltooligosaccharide (IMO) Analysis. The contents of isomaltooligosaccharides (isomaltose, isomaltotriose, and panose) in prepared samples, dissolved in deionized water, were analyzed using a high-performance anion-exchange chromatograph equipped with a pulsed amperometric detector (Dionex, Sunnyvale, CA). A PA-100 anion-exchange analytical column (Dionex, Sunnyvale, CA) equipped with a guard column was used for sample separation. The profile of the separation gradient composed of eluent A (100 mmol/L sodium hydroxide) and eluent B (100 mmol/L sodium hydroxide and 300 mmol/L sodium nitrate) was as follows: 0−5 min, 99% A and 1% B; 5−30 min, linear gradient to 8% B. Each sample was analyzed in duplicate.

Molecular Weight Distribution by High-Performance Size-Exclusion Chromatography with Multiangle Laser Light Scattering and Refractive Index Detection (HPSEC-MALLS-RI). Starch samples (20 mg) were solubilized in 4 mL of deionized water and heated in a boiling water bath for 20 min. The solutions were then filtered through a 5 μm nylon filter (Daegger, Vernon Hills, IL) before injection into the HPSEC-MALLS-RI system consisting of a pump (model LC-10ATvp, Shimadzu, Columbia, MD), an injector valve with a 200 μL loop (Rheodyne, Cotati, CA), an intermediate-pressure size-exclusion column (1.6 × 50 cm, Amersham Biosciences, Piscataway, NJ) containing Sephacryl S-500 HR gel filtration media (exclusion range $M_w = 4 \times 10^4$ to 2×10^7 (Amersham Biosciences), a MALLS detector (Dawn DSP-F, Wyatt Technology, Santa Barbara, CA) fitted with an argon laser (488 nm), and an Optilab 903 refractive index detector (Wyatt Technology). The samples (200 μL) were injected into the system and run at a flow rate of 1.3 mL/min. The mobile phase used was deionized water with 0.02% sodium azide that had been passed through a 0.2 μm filter and degassed under vacuum. The molecular

weights were calculated using the ASTRA 4.9 software program (Wyatt Technology) and pullulan standards. Each sample was analyzed in duplicate.

Chain Length Distribution of Debranched Samples using High-Performance Size-Exclusion Chromatography with Refractive Index Detection (HPSEC-RI). Debranched samples were prepared using the previously reported method (*19*) with slight modification. The samples (20 mg) were wetted with 0.04 mL of deionized water followed by addition of 0.36 mL of dimethyl sulfoxide (DMSO). The samples were heated in a boiling water bath for 10 min. Sodium acetate buffer (3.6 mL, pH 3.5, 0.1 mol/L) was added to the sample suspensions. The suspensions were mixed well and kept in a water bath at 37 °C for 5 min. Isoamylase (0.5 U) was added to each dispersion, and the mixtures were incubated at 37 °C with shaking for 24 h. The solution was then heated in a boiling water bath for 10 min to deactivate the enzyme. The debranched sample solution (0.5 mL) was concentrated in a vacuum evaporator (SpeedVac SC100H, Savant) to remove water. Concentrated debranched sample solutions were diluted with 0.5 mL of 90% DMSO followed by centrifugation at 10000 rpm for 1 min.

The HPSEC-RI system was equipped with a Waters 1515 isocratic pump, a Waters 2414 refractive index detector (Waters, Milford, MA), two Zorbax PSM 60-S columns (Quantum Analytics Inc., Foster City, CA), and a 20 μL sample loop. The column and detector temperatures were set at 35 and 40 °C, respectively. The samples were injected into the system and run at a flow rate of 0.5 mL/min using DMSO as the mobile phase. Pullulan molecular weight standards were used for column calibration. Each sample was analyzed in duplicate.

X-ray Diffraction Patterns. X-ray diffraction patterns were obtained using a Kristalloflex diffractometer (Siemens, Germany) with a rate of 0.05 deg/s from $2\theta = 4°$ to $2\theta = 40°$. Cu Kα ($\lambda = 1.5406$ Å) radiation was used, and the tube was operated at 40 kV and 20 mA. Starch crystallinity was measured on the basis of the ratio of the crystalline to amorphous areas (*20*). Each sample was analyzed in duplicate.

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy. ^{1}H NMR analyses of starch samples were performed using a Varian Unity Inova 300 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) according to the method of Gidley (*21*). Samples were first dissolved in 1 mL of deuterium oxide (D_2O), left for 2 h at room temperature, and then lyophilized. The samples were dissolved in 0.6 mL of D_2O again, and ^{1}H NMR spectra were obtained at 80 °C.

Statistical Analysis. Analysis of variance and Tukey's tests ($P < 0.05$) were used to determine significant differences in rapidly digested starch, slowly digested starch, and resistant starch among control and enzyme-treated starches using SAS version 9.1 (SAS Institute Inc., North Carolina).

RESULTS

In Vitro Digestion of Samples with Porcine Pancreatic α-Amylase and a Combination of Pancreatin and Amyloglucosidase. The digestion profiles of samples using porcine pancreatic α-amylase are shown in **Figure 1**. The digestion rates of enzyme-treated starches were slower than that of control normal maize starch. In contrast to the rate of digestion of normal maize starch, the values of β-amylase-, β-amylase- and transglucosidase-, maltogenic α-amylase-, and maltogenic α-amylase- and transglucosidase-treated starches at 10 min were reduced by 67%, 61%, 31%, and 69%, respectively. At 180 min, the digestion values of these four samples were reduced by 49%, 47%, 42%, and 55% of that of the control. Overall, the digestion rates of starches treated with amylase and transglucosidase were lower than those of starches treated with one of the amylases alone. Thus, addition of transglucosidase improved the slow digest property of modified starch by β-amylase and maltogenic α-amylase. Only a minor amount of reducing sugar was found in pullulan treated with α-amylase (**Figure 1**), indicating that the hydrolysis of pullulan was restricted by its structure. The hydrolysis rate of glycogen, with its relatively high proportion of α-1,6 linkages, was lower than that of normal maize starch, but greater than for other samples.

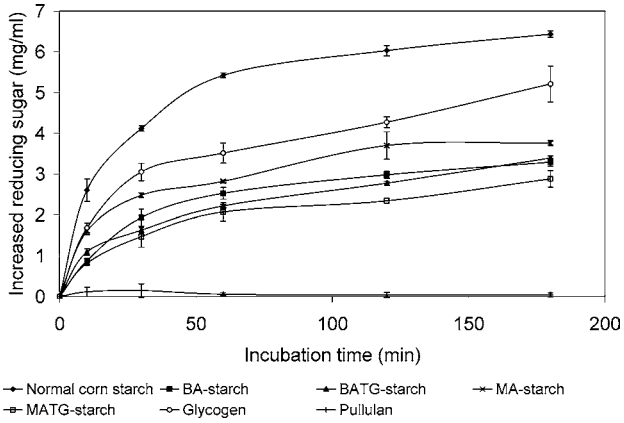

Figure 1. Digestion profiles of the normal maize starch control, enzyme-treated normal maize starches, glycogen, and pullulan incubated with porcine pancreatic α-amylase at different times. BA-starch = β-amylase-treated maize starch, BATG-starch = β-amylase- and transglucosidase-treated maize starch, MA-starch = maltogenic α-amylase-treated maize starch, and MATG-starch = maltogenic α-amylase- and transglucosidase-treated maize starch.

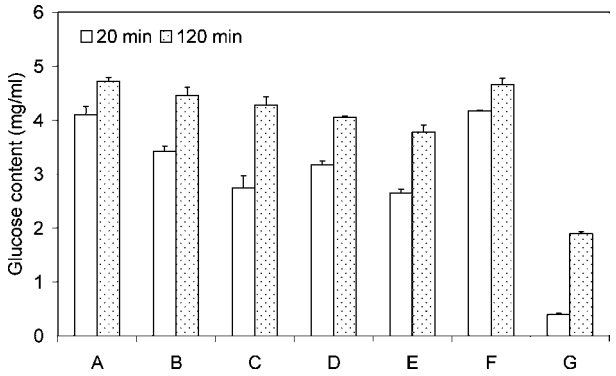

Figure 2. Englyst assay (modified) of glucose contents at 20 and 120 min of the normal maize starch control, enzyme-treated normal maize starches, glycogen, and pullulan: (**A**) normal maize starch control, (**B**) BA-starch, (**C**) BATG-starch, (**D**) MA-starch, (**E**) MATG-starch, (**F**) glycogen, (**G**) pullulan. Abbreviations are the same as in the **Figure 1** caption.

To evaluate further the property of glucose release from the enzyme-treated maize starches, samples were incubated with a combination of pancreatin and amyloglucosidase following a modified Englyst assay procedure (*1*). Digestions at 20 and 120 min are shown in **Figure 2**, and the rapidly digested starch, slowly digested starch, and resistant starch results are summarized in **Table 1**. The data indicate that the four spray-dried enzyme-treated starch samples significantly differed from the control starch and had an increase in the slow digestion property. Normal maize starch had 87.3% rapidly digested starch at 20 min and 13.1% slowly digested starch. Compared to normal maize starch, the contents of rapidly digested starch were reduced by 14.5%, 29.0%, 19.8%, and 31.0% for β-amylase-, β-amylase- and transglucosidase-, maltogenic α-amylase-, and maltogenic α-amylase- and transglucosidase-treated starches, respectively, whereas, the contents of slowly digested starch were 22.1%, 32.8%, 18.8%, and 24.1% for these four samples, respectively (normal maize starch was 13.1%). There were different contents of resistant starch present in spray-dried samples, as measured in this assay. The starch treated with maltogenic α-amylase alone or concurrently with transglucosi-

Table 1. Contents (%, w/w, Dry Weight) (Mean ± Standard Deviation) of Rapidly Digested Starch, Slowly Digested Starch, and Resistant Starch in Normal Maize Starch and Enzyme-Treated Samples[a]

sample	rapidly digested starch	slowly digested starch	resistant starch[b]
normal maize starch	87.3 ± 3.2 a	13.1 ± 1.6 c	–0.4 ± 1.6 b
BA-starch	72.8 ± 2.2 b	22.1 ± 3.2 bc	5.2 ± 3.2 ab
BATG-starch	58.3 ± 4.8 cd	32.8 ± 3.1 ab	5.1 ± 3.2 ab
MA-starch	67.5 ± 1.5 bc	18.8 ± 0.4 bc	13.5 ± 0.4 a
MATG-starch	56.3 ± 1.6 d	24.1 ± 2.7 ab	11.4 ± 2.7 a

[a] Significant differences in each column are expressed as different letters ($P < 0.05$). [b] Resistant starch = total starch – (rapid digested starch + slowly digested starch).

dase had a greater resistant starch content than starch treated with β-amylase or a combination of β-amylase and transglucosidase. The digestion rate of glycogen was similar to that of normal maize starch. However, the amount of digestion of pullulan was much lower than in other samples (**Figure 2**). The results reflect that amyloglucosidase alone can act on molecules such as pullulan that are not well hydrolyzed by α-amylase alone (**Figure 1**), but produce a lesser amount of glucose than structures readily available to α-amylase and amyloglucosidase.

IMO Analysis. Chromatograms of IMOs determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) are illustrated in **Figure 3**, and the computed results are shown in **Table 2**. The total amounts of isomaltose, isomaltotriose, and panose (IMOs) were 2.3% for starch treated with β-amylase and transglucosidase and 5.5% for starch treated with maltogenic α-amylase and transglucosidase. The other two samples treated with only β-amylase and maltogenic α-amylase had 5.0% and 6.8% maltose contents, respectively, but no IMOs. It should be noted that glucose was produced by transglucosidase activity as is confirmed in **Figure 3** and **Table 2**. Transglucosidase catalyzes hydrolytic and transfer reactions to convert maltooligosaccharides to IMOs. The IMO contents can be varied, depending on the enzyme dosage and incubation time used.

Molecular Weight and Chain Length Distributions. Figure 4 shows the molecular weight distributions of normal maize starch and the enzyme-treated samples determined by HPSEC-MALLS-RI analysis. The peaks of amylopectin and amylose of normal maize starch were $M_w = 3.8 \times 10^7$ and 1.1×10^5, respectively (**Figure 4A**). Starches treated with enzymes had lower proportions of larger molecules and greater proportions of smaller molecules than normal maize starch; the molecular weights showed varied distributions (**Figure 4B,C**). Starches treated with the combination of β-amylase and transglucosidase or maltogenic α-amylase and transglucosdase had substantial proportions of lower molecular weight molecules. For instance, the starch treated with β-amylase and transglucosidase contained a very small amount of high molecular weight molecules at the elution volume of 40 mL (position of amylopectin) and had a major peak with a molecular weight of 2.9×10^4, whereas starch treated with β-amylase alone showed a higher amount of molecules in the area of amylopectin and a large proportion of molecules with a peak at $M_w = 1.9 \times 10^5$. The results demonstrated in **Figure 4C** also show that the starch treated with maltogenic α-amylase and transglucosidase had a substantially larger proportion of low molecular weight molecules than the starch treated with maltogenic α-amylase alone. The results indicate that transglucosidase accelerated the hydrolysis of starch with β-amylase or maltogenic α-amylase and created new molecular weight structures.

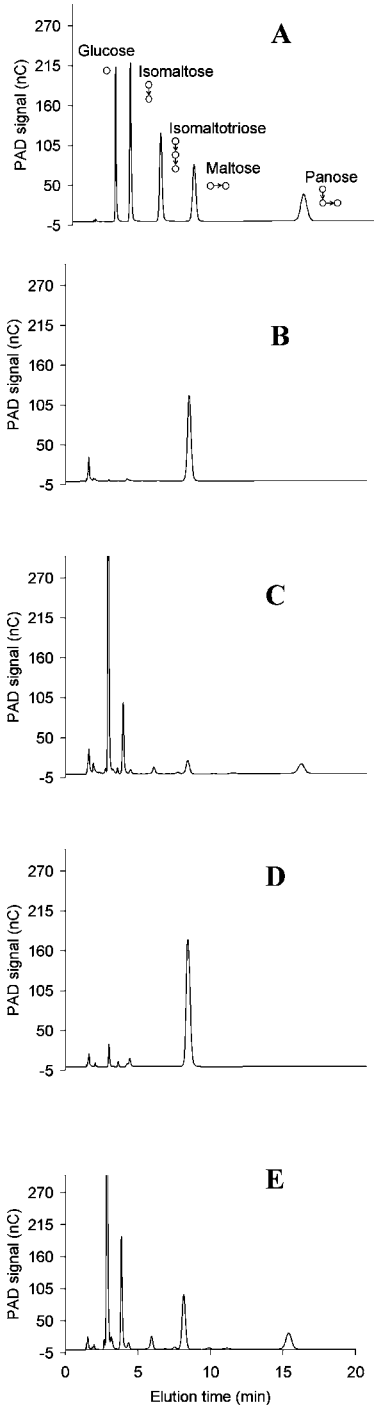

Figure 3. HPAEC chromatograms of standard sugars and enzyme-treated normal maize starches: (**A**) standard sugars (glucose, maltose, isomaltose, isomaltotriose, and panose), (**B**) BA-starch, (**C**) BATG-starch, (**D**) MA-starch, (**E**) MATG-starch, (↓) α-1,6 linkage, (→) α-1,4 linkage. Abbreviations are the same as in the **Figure 1** caption.

Chain length distributions of starch samples debranched using isoamylase and analyzed using HPSEC-RI are shown in **Figure 5**. The graph in **Figure 5A** is a typical profile of debranched whole normal maize starch which has the first peak of amylose overlapped with some of the longest chains of amylopectin with a peak DP at 671 followed by peaks at DP 45, DP 18, and DP 13. The majority of short linear chains (A and B_1 chains) of amylopectin were shortened by the action of the enzymes (**Figure 5B,C**), as evidenced by the substantial reduction in proportion of the low DP peak. The relative proportion of amylopectin branch chains (B_2 or B_3 chains) was reduced, and

Table 2. Contents (%, w/w) (Mean ± Standard Deviation) of Oligosaccharides in Enzyme-Treated Maize Starches

sample	glucose	maltose	isomaltose	isomaltotriose	panose	total IMOs[a]
BA-starch	0.0 ± 0.0	5.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0
BATG-starch	3.6 ± 0.1	0.9 ± 0.0	1.2 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	2.3
MA-starch	0.3 ± 0.0	6.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0
MATG-starch	8.3 ± 0.1	5.4 ± 0.1	3.1 ± 0.1	0.8 ± 0.0	1.6 ± 0.1	5.5

[a] Total amount of isomaltose, isomaltotriose, and panose

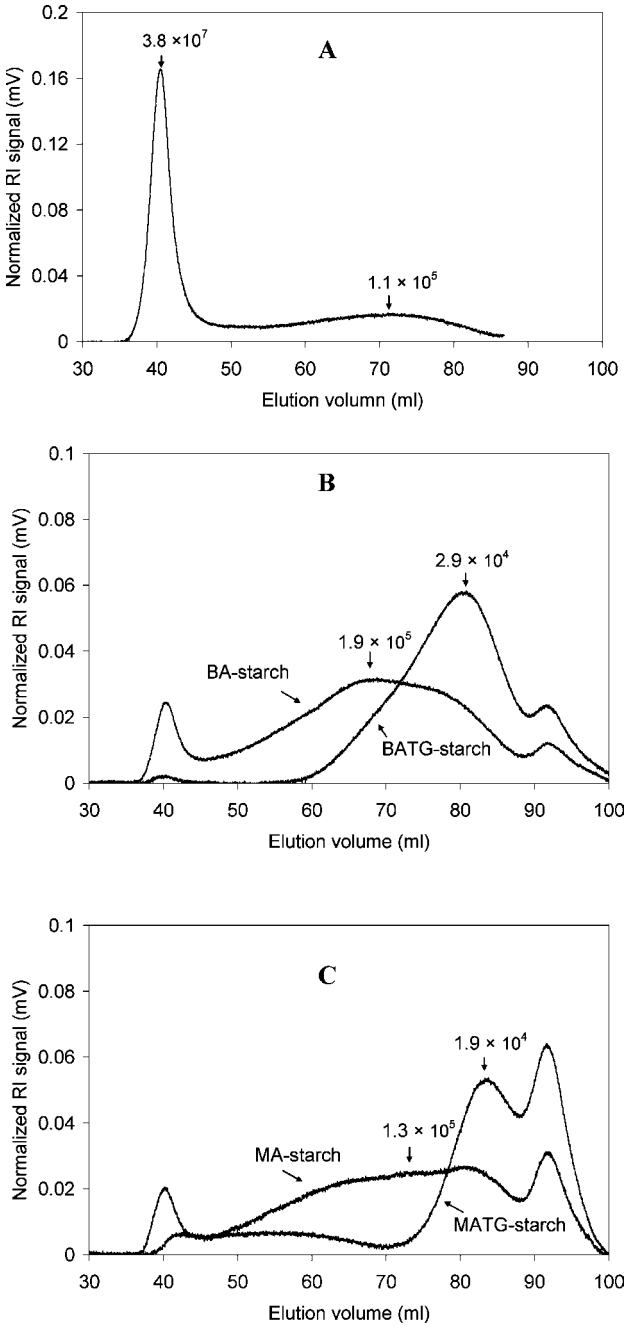

Figure 4. Molecular weight distributions of the normal maize starch control and enzyme-treated normal maize starches analyzed using HPSEC-MALLS-RI: (**A**) normal maize starch, (**B**) BA-starch and BATG-starch, (**C**) MA-starch and MATG-starch. Abbreviations are the same as in the **Figure 1** caption.

the peak was shifted from DP 45 to DP 37, which means that the B_2 or B_3 chains were shortened as well. The graphs also show that there were some oligosaccharides present in the

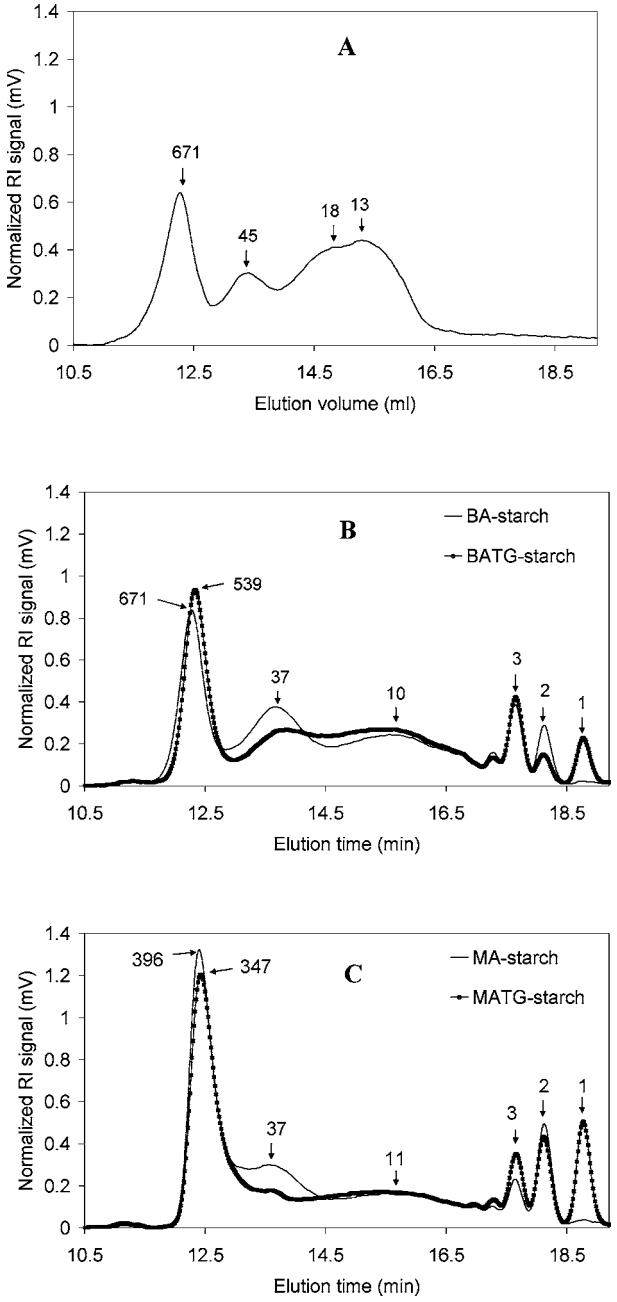

Figure 5. Chain length distributions of the isoamylase-debranched normal maize starch control and enzyme-treated normal maize starches analyzed using HPSEC-RI: (**A**) normal maize starch, (**B**) BA-starch and BATG-starch, (**C**) MA-starch and MATG-starch. Abbreviations are the same as in the **Figure 1** caption. Numbers above the curves are DP (degree of polymerization) values.

hydrolyzed samples, though maltotriose may have originated from debranching of shortened A chains. The peak DPs of linear long chains of enzyme-treated starches were shifted from 671 to 539 (**Figure 5B**) and from 396 to 347 (**Figure 5C**). When starches were prepared with combinations of transglucosidase and β-amylase or maltogenic α-amylase, the B_2 and B_3 chains of amylopectin were of less proportion than starches prepared without transglucosidase.

X-ray Diffraction Patterns. X-ray diffraction patterns are shown in **Figure 6**. Normal maize starch showed a typical A-type X-ray diffraction pattern (main peaks at $2\theta = 14.9°$, 17.0°, 17.9°, and 23.0°). Starches modified by enzymes had a mixture of B-type (main peak at $2\theta = 17.1°$) and V-type (main peaks at $2\theta = 7.6°$, 12.9°, and 19.7°) X-ray patterns. The

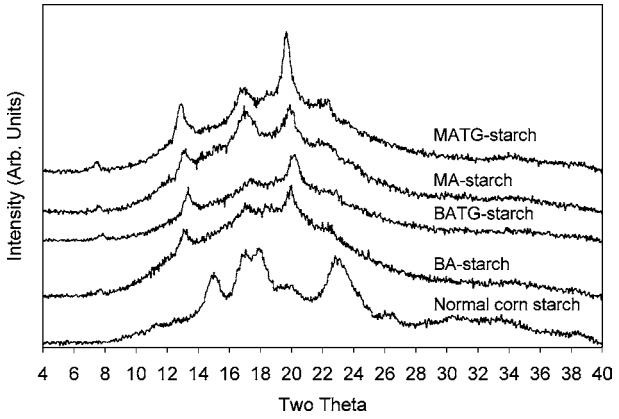

Figure 6. X-ray diffraction patterns of the normal maize starch control and enzyme-treated normal maize starches. Abbreviations are the same as in the **Figure 1** caption.

degrees of crystallinity of starches treated with β-amylase, β-amylase and transglucosidase, maltogenic α-amylase, and maltogenic α-amylase and transglucosidase were 16.9%, 15.6%, 22.2%, and 19.2%, respectively, which were lower than that of the control normal maize starch of 30.1%. The peak at $2\theta = 17.1°$ of starches treated with β-amylase or maltogenic α-amylase alone was higher than that of starches prepared with the combination of transglucosidase and β-amylase or maltogenic α-amylase. This can be attributed to the fact that there were higher proportions of long chains in these samples (**Figure 5B,C**). All enzyme-treated samples had a greater intensity at $2\theta = 19.7°$, which indicates an amylose (or long chain)−lipid complex or an amylose (or long chain)−ethanol complex. Ethanol was used to precipitate enzyme-treated starches, and it forms a complex with amylose or longer chains (*22*, *23*).

^{1}H NMR Spectroscopy. To further characterize the structural properties of the enzyme-modified starches, their ratios of α-1,4 and α-1,6 linkages were analyzed using ^{1}H NMR spectroscopy. This technique can be used to determine the degree of branching of starch amylopectin or glycogen (*21*). α-D-Glucopyranosyl units of starch polysaccharides are mainly linked by α-1,4 bonds, as well as some α-1,6 bonds. In this experiment, panose was used as a standard to verify the positions for H-1 of α-1,4- and α-1,6-linked units since it has 50% α-1,4 and 50% α-1,6 linkages. **Figure 7** shows partial ^{1}H NMR spectra of enzyme-treated starches. Percentages of α-1,4 and α-1,6 linkages were determined using the area ratios from the spectra in which peaks at 5.4 and 5.0 ppm were assigned to H-1 of α-1,4- and α-1,-6-linked units (**Figure 7** and **Table 3**). The results show that all of the enzyme-treated starches had lower ratios of α-1,4 to α-1,6 linkages than waxy maize starch. The starches treated with β-amylase and maltogenic α-amylase had the same ratio of α-1,4 to α-1,6 linkages although they showed different fine structures (**Figures 4** and **5**). The ratios from the combination treatment of transglucosidase with either β-amylase or maltogenic α-amylase were 6.7 (13% α-1,6) and 3.3 (23% α-1,6), respectively, which were lower than those for samples treated without transglucosidase. This confirmed that transglucosidase added more α-1,6 linkages to the products.

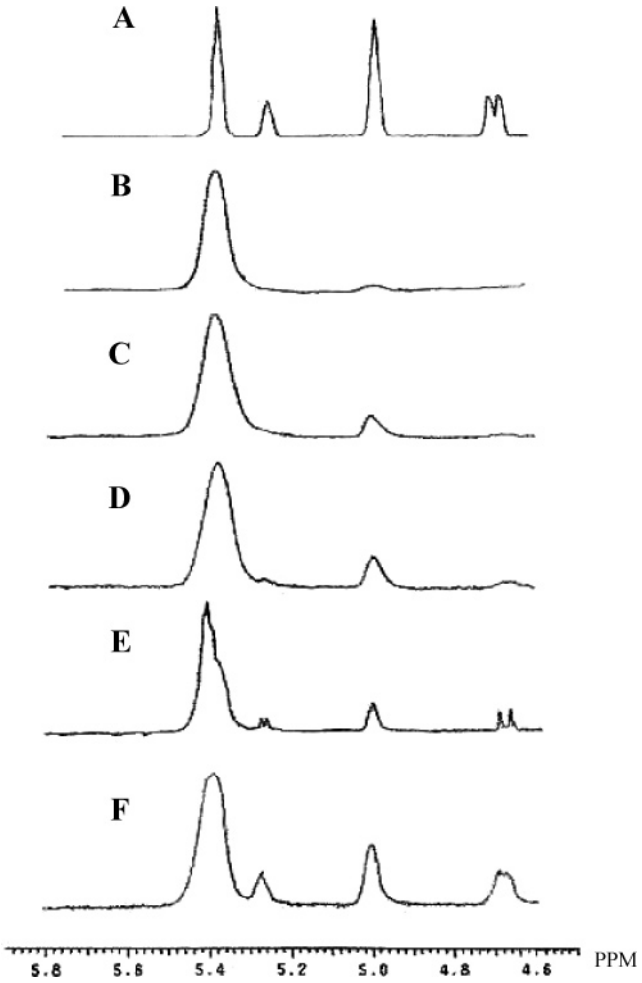

Figure 7. ^{1}H NMR spectra of panose (**A**), waxy maize starch (**B**), and enzyme-treated normal maize starches: BA-starch (**C**), BATG-starch (**D**), MA-starch (**E**), MATG-starch (**F**). Abbreviations are the same as in the **Figure 1** caption.

Table 3. Concentrations (%) of α-1,4 and α-1,6 Linkages in the Samples[a]

sample	α-1,4 linkages	α-1,6 linkages	ratio of α-1,4 to α-1,6 linkages
panose	50	50	1
waxy maize starch	95	5	20
BA-starch	90	10	9
BATG-starch	87	13	6.7
MA-starch	90	10	9
MATG-starch	77	23	3.3

[a] Percentages were determined using the area ratios from ^{1}H NMR (**Figure 7**) in which peaks at 5.4 and 5.0 ppm were assigned to H-1 of α-1,4 and α-1, 6 linked units, respectively.

DISCUSSION

In the Englyst assay (*1*), starch is digested by a combination of α-amylase and amyloglucosidase. The action of α-amylase alone does not produce an appreciable amount of glucose, and that which is produced is a very minor product formed from a very slow secondary reaction of α-amylase on maltotriose and maltotetraose (*24*). Instead glucose is produced from the action of amyloglucosidase on α-amylase degradation products. Amyloglucosidase not only consecutively hydrolyzes α-1,4 linkages, but also hydrolyzes α-1,6 linkages to produce D-glucose from the nonreducing ends of starch and glycogen. However, the hydrolysis of α-1,6 linkages takes place at a slower rate than that of α-1,4 linkages (*14*−*16*). For example, the rate of hydrolysis of the α-1,4 linkage of maltose is ~28-fold the rate of hydrolysis of the α-1,6 linkage of isomaltose (*25*). The K_m (Michaelis constant) values of amyloglucosidase for maltose, isomaltose, and panose are 0.91, 42, and 13 mM, respectively (*26*). Taken together, these reports clearly demonstrate that, in

the hydrolysis of branched starch structures, α-1,6 linkages are the rate-limiting step. Therefore, enriched α-1,6 linkages in starch products should slow the enzyme digestion rate. There are several ways to increase the relative ratio of α-1,6 linkages compared to α-1,4 linkages: use branching enzymes such as glycogen branching enzyme to increase α-1,6 linkages (*27*) or, as in the study reported here, to decrease directly the amount of α-1,4 linkages using enzymes such as *β*-amylase or maltogenic α-amylase, or in combination with transglucosidase to further increase α-1,6 linkages.

β-Amylase hydrolyzes the second α-1,4 linkage from the nonreducing ends of chains in amylose, amylopectin, and glycogen to produce maltose. The rate of *β*-amylase action gradually decreases as the α-1,6 linkages are approached. The average end groups of *β*-limit dextrins cannot be shorter than 2.5 glucose units in length (*28*). Maltogenic α-amylase (glucan 1,4-α-maltohydrolase, EC 3.2.1.133) is defined as an enzyme that hydrolyzes α-1,4-D-glucosidic linkages in polysaccharides to remove successive maltose residues from the nonreducing ends of the chains. This originally led to speculation that maltogenic α-amylase is an exoenzyme with a requirement of nonreducing ends like *β*-amylase. However, maltogenic α-amylase exhibits no specificity or requirement for polymer chain ends since it can catalyze the hydrolysis of terminally modified maltodextrins and cyclodextrins (*29*). It was hypothesized that maltose represents the final reaction product from prolonged hydrolysis with a multiple-attack mechanism (*30*).

Our results indicate that starch modified with *β*-amylase or maltogenic α-amylase had lower ratios of α-1,4 to α-1,6 linkages than waxy starch (**Table 3**). Maltose was the byproduct (**Table 2**). The concentration of maltose increased with the extent of the enzyme hydrolysis of the starch. It is known that the enzyme hydrolysis speed is partially inhibited by its hydrolysis products (*31*). Transglucosidase was chosen to remove maltose and increase α-1,6 linkages through both increasing reaction speed and accumulation of new α-1,6 branch linkages. The commercial enzyme of transglucosidase is a purified D-glucosyltransferase free from glucoamylase activity. Transglucosidase catalyzes the transfer of one glucosyl group from many types of D-glucosyl donor substrates, preferably maltose, to suitable acceptor substrates, forming the new oligosaccharides. The enzyme and D-glucosyl group form a complex as an intermediate followed by displacing the D-glucosyl group from the enzyme by acceptor substrates. Transglucosidase also synthesizes oligosaccharides from maltooligosaccharides of higher molecular weight to yield compounds having α-1,6-linked D-glucosyl groups at the nonreducing end (*32*, *33*). Therefore, transglucosidase catalyzes hydrolytic and transfer reactions to convert maltooligosaccharides to IMOs, as well as to create more α-1,6 linkages on the long chains of starch structures. Consequently, this enzyme increased the reaction speed of both *β*-amylase and maltogenic α-amylase (**Figure 5**), not only decreasing the concentration of maltose (**Table 2**), but also adding more α-1,6 linkages to the products (**Table 3**) that were more slowly hydrolyzed by amyloglucosidase (**Figure 2**). IMOs were also produced, particularly in the maltogenic α-amylase and transglucosidase treatment (**Table 2**). IMOs are used as prebiotics to increase the levels of health-promoting bacteria in the intestinal tract of humans and animals (*34*−*36*).

The slowly digested and resistant properties of the starch modified by the enzyme treatments were attributed to not only an increase in the amount of α-1,6 linkages and shortened chains, but also likely new structures of more exposed long interior chains that were more easily retrograded to reduce enzyme susceptibility. This was evidenced by higher resistant starch levels, though most notably in maltogenic α-amylase-treated starch (**Table 1**). The retrograded fraction formed B- and V-type crystalline structures (**Figure 6**) that are inherently more resistant to enzymatic hydrolysis than A-type structures (*37*).

In conclusion, partial shortening of the outer branch chains of amylopectin, and possibly amylose, by *β*-amylase and maltogenic α-amylase reduced the overall starch digestion rate, which was related to an increase in the amount of α-1,6 linkages and a decrease of α-1,4 linkages. Changes in chain length and their distributions appear to facilitate retrogradation to produce B- and V-type crystalline structures, leading to a more resistant property of the starch. Transglucosidase not only amplified the action of *β*-amylase and maltogenic α-amylase on starch modification, but also increased the content of α-1,6 linkages and produced IMOs with prebiotic functions.

ABBREVIATIONS USED

BA, *β*-amylase; MA, maltogenic α-amylase; TG, transglucosidase; BA-starch, *β*-amylase-treated maize starch; BATG-starch, *β*-amylase- and transglucosidase-treated maize starch; MA-starch, maltogenic α-amylase-treated maize starch; MATG-starch, maltogenic α-amylase- and transglucosidase-treated maize starch; DP, degree of polymerization; IMO, isomaltooligosaccharide.

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