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# Branch pattern of starch internal structure influences the glucogenesis by mucosal Nt-maltase-glucoamylase

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ABSTRACT

To produce sufficient amounts of glucose from food starch, both α-amylase and mucosal α-glucosidases are required. We found previously that the digestion rate of starch is influenced by its susceptibility to mucosal α-glucosidases. In the present study, six starches and one glycogen were pre-hydrolyzed by α-amylase for various time periods, and then further hydrolyzed with the mucosal α-glucosidase, the N-terminal subunit of maltase-glucoamylase (Nt-MGAM), to generate free glucose. Results showed that α-amylase amplified the Nt-MGAM glucogenesis, and that the amplifications differed in various substrates. The amount of branches within α-amylase hydrolysate substrates was highly related to the rate of Nt-MGAM glucogenesis. After de-branching, the hydrolysates showed three fractions, Fraction 1, 2, and 3, in size exclusion chromatographs. We found that the α-amylase hydrolysates with higher quantity of the Fraction 3 (molecules with relatively short chain-length) and shorter average chain-length of this fraction had lower rates of Nt-MGAM glucogenesis. This study revealed that the branch pattern of α-amylase hydrolysates modulates glucose release by Nt-MGAM. It further supported the hypothesis that the internal structure of starch affects its digestibility at the mucosal α-glucosidase level.

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## 1. Introduction

Starch is the major food source of glycemic carbohydrate for humans. It is composed of two glucans, amylose and amylopectin, that are complexes of linear D-glucose chains joined by α-(1→4) glycosidic linkages and branched through α-(1→6) linkages. Amylose has long linear chains with few branches (0.3–0.5% of total glycosidic linkages), whereas amylopectin has shorter linear chains and is highly branched (4–5% of total glycosidic linkages) (Hizukuri, Abe, & Hanashiro, 1996). In starch granules, amylopectin is a major component of the semi-crystalline growth rings that are formed with stacks of alternating crystalline and amorphous lamellae. The crystalline lamellae consist of short A-chains (without substitution with other molecular chains) and external segments of B-chains (substituted with other molecular chains through α-1→6 linkages) with a double helix structure. The amorphous lamellae contain branches and internal chains. Thus, branches are not evenly distributed. After cooking (gelatinization), starch molecules leach out and disperse in suspension or solution. α-Amylase can easily hydrolyze external chains and break some internal chains of starch molecules but not the highly branched internal fractions.

The nutritional quality of starch depends on the rate and location of glucose release (Lee, Bello-Pérez, Lin, Kim, & Hamaker, 2013). Both α-amylase and mucosal α-glucosidase activities are required to produce glucose from starch (Quezada-Calvillo, Robayo-Torres, Ao, et al., 2007). The lack of either one may result in diet related severe diarrhea, abdominal pain, malnutrition, and other symptoms (Diaz-Sotomayor et al., 2013; Lin, Hamaker, & Nichols, 2012).

*Abbreviations:* Ct-MGAM, the C-terminal subunit of maltase-glucoamylase; Ct-SI, the C-terminal subunit of sucrase-isomaltase; Nt-MGAM, the N-terminal subunit of maltase-glucoamylase; Nt-SI, the N-terminal subunit of sucrase-isomaltase.

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The rate of glucose release depends on the susceptibility of starch to the digestive enzymes. The susceptibility of starch to α-amylase has been extensively investigated. However, relatively little attention has been paid to mucosal α-glucosidases. Accordingly, the conventional *in vitro* digestibility studies are based on the susceptibility of starch to α-amylase and fungal glucoamylase (Akerberg, Liljeberg, Granfeldt, Drews, & Bjorck, 1998; Englyst, Kingman, & Cummings, 1992; Muir & O'Dea, 1992).

In humans, the major tissues producing α-amylases are pancreas and salivary glands (Merritt & Karn, 1977); the action patterns of these enzymes are very similar (Hagele et al., 1982), and their amino acid sequences differ by only 3% (Takahiro, Mitsuru, Yusuke, & Kenichi, 1986). Since porcine pancreatic α-amylase is very similar to that of humans, it is commonly used as a surrogate for human α-amylase (Hagele et al., 1982; Robyt & French, 1967). The hydrolytic properties of α-amylase discussed below are mainly from the studies of the porcine enzyme. Robyt and French (1963, 1970a,b) reported that the α-amylases from porcine and *Bacillus Subtillis* hydrolyze inner α-(1→4) glycosidic linkages, but cannot split α-(1→6) linkages and some neighboring α-(1→4) linkages (Robyt & French, 1970a). α-Amylases digest the non-reducing and reducing sides of branched glucans differently. When α-amylases act on the non-reducing side of a branch, they hydrolyze adjacent and penultimate α-(1→4) linkages of the branched glucosyl residues. On the reducing side, α-amylases hydrolyze glycosidic linkages until the third linkage of the branched residues (Hizukuri et al., 1996). Thus, all the branch linkages remain in the residual hydrolysate. α-Amylases mainly hydrolyze the external molecular chains of starch; Bertoft reported that the α-amylase of *Bacillus amyloliquefaciens* can attack both external and internal chains and has been used for the isolation of the clusters of amylopectin (Bertoft, 2013).

The susceptibility of starch to α-amylases is highly associated with the chain-length of glucans because α-amylases hydrolyze substrates more efficiently when all of their binding sites are bound (Robyt & French, 1963, 1970a); Robyt and French proposed that pancreatic α-amylase has five binding sites, and the cleavage happens between the second and the third binding sites (Robyt, 1989); thus, the major products of α-amylolysis are maltose and maltotriose. Glucose is a very minor product and is produced by a slow secondary hydrolysis of the primary maltodextrin products in α-amylase hydrolysates (Robyt, 2008; Robyt & French, 1970b).

Mucosal α-glucosidases have been thought to simply and passively convert the α-amylase hydrolysates to glucose. We have recently found that mucosal α-glucosidases also directly digest starch (both granules and gelatinized molecules) to freely absorbable glucose, and may act synergistically with α-amylases at the initial stage of the digestion of starch granules (Dhital, Lin, Hamaker, Gidley, & Muniandy, 2013; Lin, Nichols, et al., 2012; Lin, Hamaker, et al., 2012). Mucosal α-glucosidases comprise two brush border-anchored multifunctional enzymes of N- and C-terminal subunits of maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI). All four catalytic subunits of mucosal α-glucosidases belong to the glycosyl hydrolysate Family 31 (GH 31) and are similar in their amino acid sequences (Nichols et al., 2003, 1998). They all cleave α-(1→4) glycosidic linkages from the non-reducing ends of glucans (Dahlqvist & Telenius, 1969; Van Beers, Buller, Grand, Einerhand, & Dekker, 1995); some of them carry additional activities such as isomaltase and sucrase, but the predominant activity is maltase. The C-terminal subunit of MGAM (Ct-MGAM) is considered a *glucoamylase* due to its activity on longer chain glucans. The N-terminal subunit of MGAM (Nt-MGAM) is recognized as a *maltase* because it mainly hydrolyzes small linear α-glucans. The C-terminal SI (Ct-SI) is responsible for sucrose hydrolysis and is the only *sucrase* in humans (Van Beers et al., 1995). The N-terminal SI (Nt-SI) is known as an *isomaltase* because of its activity on α-(1→6) linkage of isomaltose (Dahlqvist, Auricchio, Prader, & Semenza, 1963). In addition to Nt-SI, we have recently found that Nt-MGAM can also hydrolyze branched sugars such as panose, isomaltose, and $6^3$-glucosylmaltotriose (Lin, Lee, et al., 2012). However, the debranching activity of Nt-MGAM is much lower than Nt-SI on these sugars (Lin, Lee, et al., 2012).

We have reported that different kinds of starches have different susceptibility to mucosal α-glucosidases (Ao, Quezada-Calvillo, et al., 2007; Ao, Simsek, et al., 2007). We have also found that mucosal α-glucosidases digest the α-amylase hydrolysate substrates, derived from various maize starch mutants, to glucose at different rates (Lin, Lee, et al., 2012). α-Amylase, reacting with starches, produces maltodextrin products (maltose and maltotriose) and branched dextrins (α-limit dextrins). Maltose is resistant to α-amylase, and the hydrolysis of maltotriose by α-amylase is a very slow process (Robyt, 2008). The residual branched dextrins are composed of at least one α-(1→6) linkage and two α-(1→4) linkages; they are either resistant to or very slowly hydrolyzed by α-amylase (Robyt, 1998). Mucosal α-glucosidases can rapidly convert maltodextrin products (maltose and maltotriose) to glucose. For branched dextrins, three of the mucosal α-glucosidases, Nt-SI, Ct-SI, and Ct-MGAM, surprisingly digest them to glucose, but only Nt-SI was found to have significant activity on α-(1→6) linkages of isomaltose. Nt-MGAM is the only mucosal α-glucosidase that does not hydrolyze the branched dextrins of α-amylase hydrolysates although it has minor activity on panose and $6^3$-glucosylmaltotriose (Lin, Lee, et al., 2012).

Because different kinds of starch hydrolysate substrates have different susceptibilities to mucosal α-glucosidases, we hypothesized that the branch pattern (amount and the distance between branches) of the internal structure of starch modulates its digestibility at the mucosal level. In the present study, we investigated α-amylase hydrolysates from seven substrates (six starches and one glycogen) to study its relationship between branch pattern and Nt-MGAM glucogenesis. Substrates were pre-hydrolyzed by the human pancreatic α-amylase for various time periods (1–3 h), and then treated with recombinant human Nt-MGAM for 2 h to generate free glucose. Kinetics of glucogenesis, the quantity of α-(1→6) linkages of α-amylase hydrolysates, and the chain-length distribution of α-amylase hydrolysates were then investigated. This study importantly revealed how the starch internal structure affects starch digestibility at the mucosal α-glucosidase level.

## 2. Materials and methods

### 2.1. Materials

Normal maize, *aewx* and *su2wx* maize, and tapioca starches were gifts from Tate and Lyle (Decatur, IL, USA). Waxy maize starch and rabbit liver glycogen were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potato starch was obtained from Penford Food Ingredients Co. (Englewood, CO, USA). Human pancreatic α-amylase (213.7 units per mg of protein) was purchased from Meridian Life Science, Inc. (Saco, ME, USA); one unit of α-amylase activity is the amount of enzyme required to release one micromole of maltopentaose, which through coupled reactions results in the formation of five micromoles of glucose-6-phosphate per minute at 37 °C; containing less than 0.0003% of protease and 0.003% of lipase. Isoamylase (91 units per mg of protein based on oyster glycogen) was obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland); one unit of isoamylase activity is the amount

of the protein required to release one micromole of reducing sugar equivalent from the oyster glycogen per min at pH 4.0 and 40 °C; the purity of isoamylase was presenting as a single major band on SDS-gel electrophoresis (71,500 Da) and a single major band on Isoelectric focusing ($pI$ = 5.0). Purified recombinant human N-terminal subunit of maltase-glucoamylase (Nt-MGAM) was a gift of D. Rose (Waterloo University; Ontario, Canada); the Nt-MGAM protein preparation, crystallization, activities and detailed properties were reported previously (Sim, Quezada-Calvillo, Sterchi, Nichols, & Rose, 2008).

### 2.2. Glucogenesis of starches and glycogen

Glycogen and starches (100 mg dry weight) were dispersed in 90% (v/v) dimethyl sulfoxide (5 mL) followed by heating in a boiling water bath for 1 h with stirring. Starches and glycogen molecules were precipitated with ethanol (15 mL), and then re-dispersed in 10 mmol/L phosphate saline buffer (PBS) containing 150 mmol/L sodium chloride (total 10 mL) and 0.001 mmol/L calcium chloride. The solutions were heated in a boiling water bath for 30 min and kept at 37 °C for 5 min before addition of human pancreatic α-amylase (1.5 units). Enzymatic hydrolysis by human pancreatic α-amylase was carried out for 3 h, and aliquots were taken at different time intervals. The human pancreatic α-amylase was inactivated by heating in a boiling water bath for 10 min. The aliquots (10 μL) were further treated with Nt-MGAM (10 μL; 20 μg protein per mL in PBS buffer) for 2 h at 37 °C in a 96-well plate. Glucose production was determined by glucose oxidase and peroxidase assays as described previously (Quezada-Calvillo, Robayo-Torres, Opekun, et al., 2007). Aliquots of α-amylase hydrolysate substrates were also collected for studying their chain-length distribution and the quantity of α-(1→6) linkages.

Glucogenesis kinetics was presented in two ways: glucose amount against the α-amylase hydrolytic time and the logarithm of rate of the change in glucose concentration against the α-amylase hydrolytic time. When starch or starchy foods are hydrolyzed *in vitro* with relatively high concentration of α-amylase for a long period of time, the rate of product concentration increment over time is represented by the exponential decay equation below: (Butterworth, Warren, Grassby, Patel, & Ellis, 2012; Goni, Garcia-Alonso, & Saura-Calixto, 1997).

$$C_t = C_\infty(1 - e^{-kt}) \quad (1)$$

where $C_t$ is the concentration of product (glucose in this study) at time $t$. $C_\infty$ is the theoretical glucose concentration attained after full hydrolysis of the corresponding starch substrates, and $k$ is a first order rate constant. Differentiating the Eq. (1) gives:

$$\frac{dC_t}{dt} = C_\infty k e^{-kt} \quad (2)$$

Taking logarithm on both sides of the Eq. (2) gives:

$$\ln\left[\frac{dC_t}{dt}\right] = \ln(C_\infty k) - kt \quad (3)$$

A plot of $\ln[(dC_t)/dt]$ against $t$, so called "log of slope" (LOS) plot (Poulsen, Ruiter, Visser, & Iversen, 2003), allows the calculation of $k$ as the slope of the resulting straight line. It was applied in this study to determine whether the rate of reaction changes over time.

### 2.3. The quantity of α-(1→6) linkages in α-amylase hydrolysate substrates

The relative quantities of α-(1→4) and α-(1→6) linkages in the α-amylase hydrolysates, the substrates of Nt-MGAM glucogenesis, were analyzed by a Varian Unity Inova 300 MHz NMR spectrometer (Varian INC., Palo Alto, CA, USA) as described previously (Ao, Simsek, et al., 2007).

### 2.4. Chain-length distribution of α-amylase hydrolysate substrates

α-Amylase hydrolysates, the substrate of Nt-MGAM glucogenesis, were de-branched by isoamylase (Lin et al., 2006), and the chain-length distribution of the debranched hydrolysates were analyzed using a high performance size-exclusion chromatography (HPSEC) eluted with dimethyl sulfoxide as described previously (Ao, Simsek, et al., 2007). The chromatographs were divided into three fractions, and the mass ratio of each fraction was its area

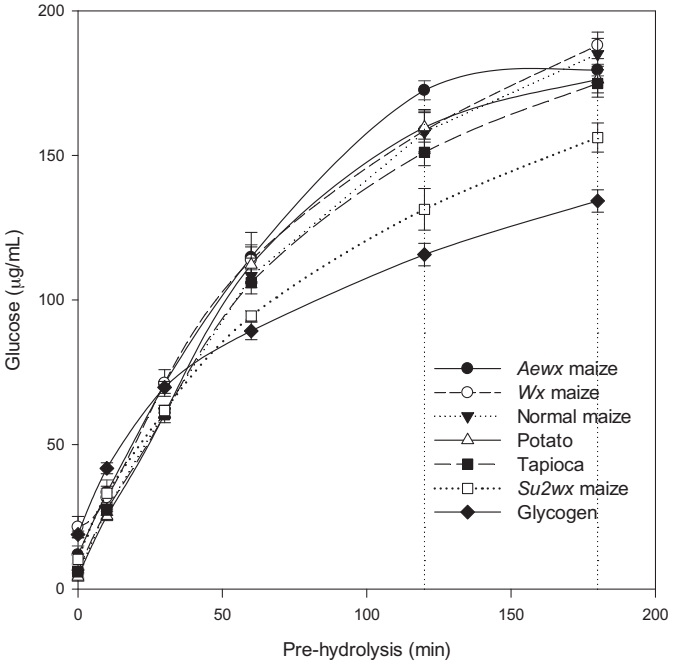


**Fig. 1.** Glucogenesis of α-amylolytic hydrolysate substrates. *Aewx* maize (●), *wx* maize (○), normal maize (▼), potato (△), tapioca (■), *su2wx* maize (□) starches and glycogen (♦) were pre-hydrolyzed with the human pancreatic α-amylase for 0, 10, 30, 60, 120 and 180 min, and then treated with human recombinant Nt-MGAM for 2 h.

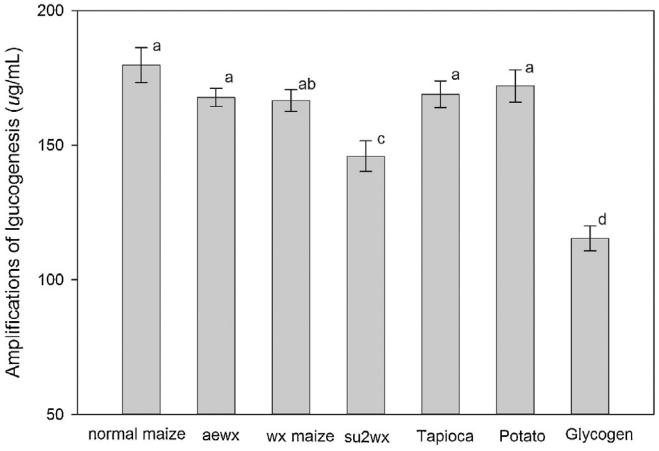


**Fig. 2.** The amplifications of glucogenesis by α-amylolysis. Substrates were pre-hydrolyzed with α-amylase for 3 h and then treated with Nt-MGAM for 2 h. The amplification was the difference in glucose amounts determined at 0 h and 3 h. Data are means of three measurements, and the error bars indicate standard deviations. Data denoted with the same letters (a, b, c, d, or e) had no significant difference ($p > 0.05$).

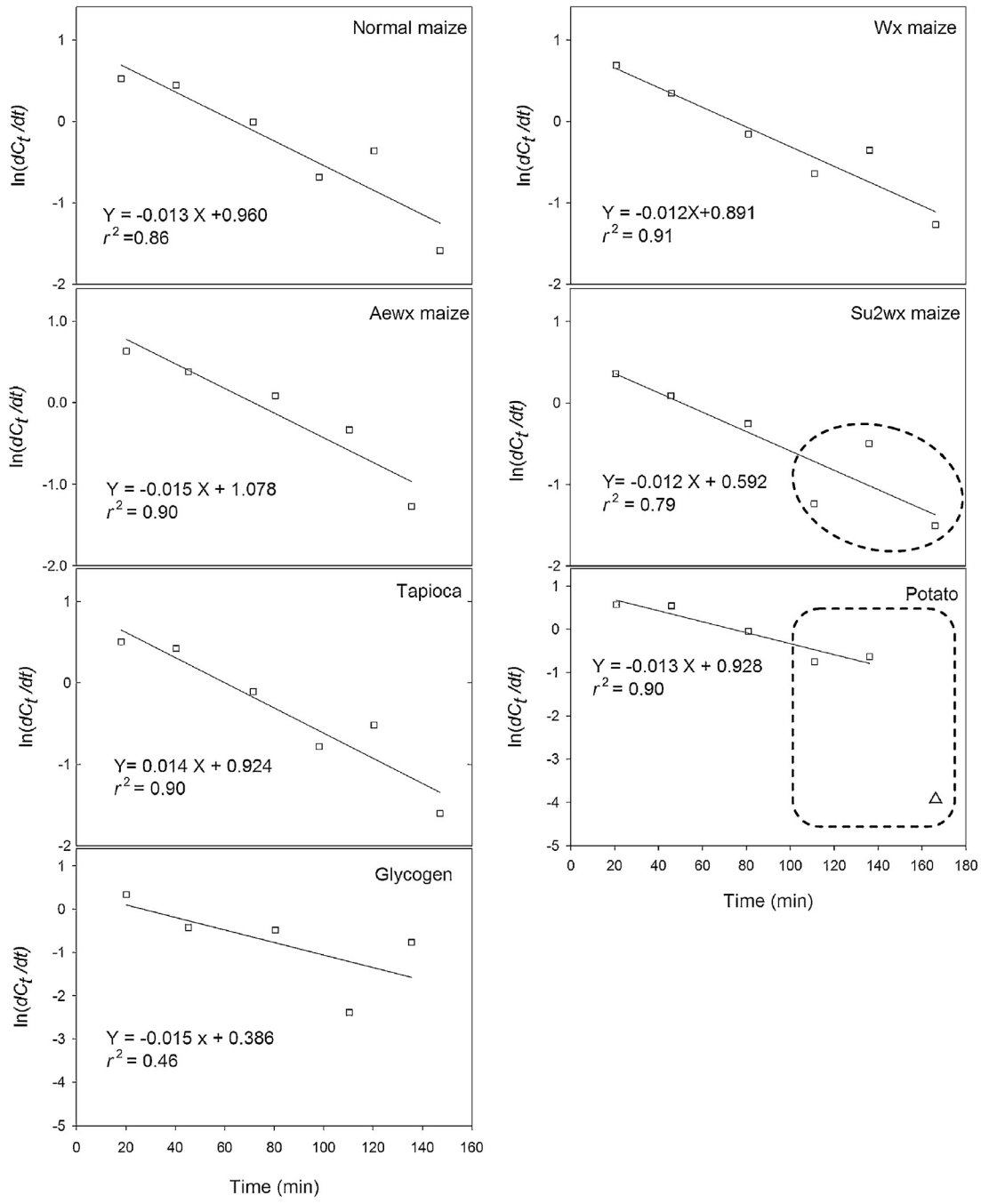


**Fig. 3.** First order kinetic constants of glucogenesis as function of the hydrolytic time of α-amylolysis. Substrates were pre-hydrolyzed with α-amylase for various time periods and then hydrolyzed with Nt-MGAM for 2 h. *X*-axis represents time increment of pre-hydrolysis; *Y*-axis, ln[$(dC_t)/dt$], is the logarithm of the change in glucose concentrations occurring between time intervals.

under the chromatographic curve to the sum of the areas of three fractions.

### 2.5. Statistical analysis

Data were presented as means with standard deviations. Linear regression analysis was performed to correlate branches with glucose amounts, and the degree of correlation was presented by linear correlation coefficient, denoted as $r^2$. Statistical analyses of other data were performed using one-way ANOVA and Tukey's multiple comparison tests. A $p < 0.05$ was considered as significant difference. Data were analyzed using SAS® Proprietary software 9.3 (SAS Institute Inc., Cary, NC).

## 3. Results

### 3.1. Glucogenesis of starches and glycogen

Starches and glycogen were pre-hydrolyzed with human pancreatic α-amylase for various time periods and then treated with Nt-MGAM for 2 h to generate free glucose. The glucogenesis kinetic profiles are shown in Fig. 1. Without pre-hydrolysis (0 min in Fig. 1), Nt-MGAM alone produced approximately 20 μg of glucose from waxy maize and glycogen; 7–10 μg from *su2wx* and *aewx* maize; and 5 μg from potato, tapioca, and normal maize ($p < 0.05$). The pre-hydrolysis with α-amylase amplified the glucogenesis. The amplifications of glucogenesis by Nt-MGAM (difference in glucose

**Table 1**
Maximum glucose yield of various substrates.

| | Glucose (μg/mL)[a] |
|---|---|
| Normal maize | 185.08 ± 5.41ab |
| *aewx* maize | 179.53 ± 1.98a |
| *wx* maize | 188.06 ± 4.58ab |
| *su2wx* maize | 156.19 ± 5.03c |
| Tapioca | 174.89 ± 4.71b |
| Potato | 176.25 ± 4.59ab |
| Glycogen | 134.24 ± 3.85d |

[a] The values are means ± standard deviations of three measurements. The statistical analysis was performed using one-way ANOVA and Tukey's test; the same letter (a, b, or c) associated with different values indicates no significant difference ($p > 0.05$).

amount between 0 and 3 h of α-amylolysis) of various substrates were different; *su2wx* maize and glycogen were significantly lower than those of other substrates (Fig. 2). The amount of total available glucose (glucose amount after 3 h α-amylolysis followed by 2 h Nt-MGAM hydrolysis) from various substrates were also different; *wx*, *aewx*, and normal maize and potato had higher amount of total available glucose, followed by tapioca, *su2wx* maize, and glycogen (Table 1; the results of statistic assay are shown in Table 1).

There is a linear relationship between the rate of the change in glucose and the hydrolytic time of α-amylolysis except glycogen (Fig. 3). The *Y*-axis, $\ln[(dC_t)/dt]$ presents the change in glucose concentrations occurring between time intervals. With increasing reaction time, the rate of change in glucose concentration decreased. It can be referred to a decreasing rate of appearing of digestive products. Normal, *aewx* and *wx* maize and tapioca had a constant decrease in the rate of glucose release (a constant slope of $\ln[(dC_t)/dt]$ over time (Fig. 3). For *su2wx* maize and potato (as circled in Fig. 3), the rate of decrease in glucose concentration differed after 120 min of reaction.

### 3.2. The quantity of α-(1→6) linkages in α-amylase hydrolysate substrates

The quantity of α-(1→6) linkages within α-amylase hydrolysates, the substrates of Nt-MGAM glucogenesis, is presented as the ratio of α-(1→6) to total glycosidic linkages [sum of α-(1→4) and α-(1→6) linkages] determined by NMR. The quantity of α-(1→6) linkages in the hydrolysates increased when the substrates were pre-hydrolyzed with α-amylase, and the α-amylase hydrolysates of *su2wx* and *wx* maize, and glycogen had more α-(1→6) linkages than those of other substrates (Fig. 4).

There was also an inverse linear relationship between the quantity of α-(1→6) linkages and glucose amount ($r^2 = 0.77$, Fig. 4), indicating that a higher quantity of α-(1→6) linkages had a negative impact on Nt-MGAM glucogenesis. However, *aewx* and normal maize and tapioca with similar quantity of α-(1→6) linkages were not converted to glucose at the same rate (bracketed in Fig. 4), suggesting that other factors may also affect glucogenesis. Thus, more structural studies were performed as described below.

The α-amylase hydrolysate substrates (3 h) were de-branched, and the chain-length distribution was examined by HPSEC. The *su2wx* maize hydrolysates showed four fractions, but all of other hydrolysates showed three fractions in the chromatographs; the ranges of the degree of polymerization (DP) of each fraction varied slightly among different substrates and were labeled in Fig. 5. For *su2wx* maize hydrolysate, the two short-chain fractions were combined as Fr. 3 (Fig. 5). The quantity (mass ratio) of Fr. 3 had a positive linear relationship with the glucose amount ($r^2 = 0.51$, Fig. 6). This linear relationship became stronger when *su2wx* maize is excluded. This trend agrees with the degree of glucogenesis shown in Figs. 1 and 4. The average chain-length, presenting as average DP, of the Fr. 3 from different substrates were plotted in Fig. 7. No such linear relationship was observed in Fr. 1 and 2 (plots not shown). Glycogen was excluded in Figs. 5–7 due to the different molecular structure from amylopectin as discussed later.

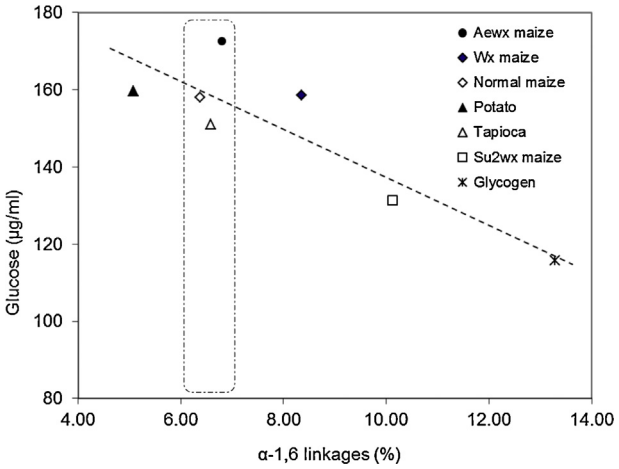


**Fig. 4.** The relationship between glucose amount and the quantity of α-(1→6) linkages within α-amylolytic hydrolysate substrates. *Aewx* maize (●), *wx* maize (♦), normal maize (◇), potato (▲), tapioca (△), *su2wx* maize (□) starches and glycogen (*) were pre-hydrolyzed with α-amylase for 2 h and then treated with Nt-MGAM for 2 h. The linear correlation coefficient, $r^2$, of the seven hydrolysate substrates was 0.77. The three α-amylase hydrolysate substrates (as bracketed), from *aewx* maize, normal maize, and tapioca (as bracketed), had similar quantity of branches but differed in glucose generation.

## 4. Discussion

In our previous studies, we found that the four mucosal α-glucosidases, N- and C-terminal subunits of MGAM and SI, digest α-amylase hydrolysate substrates to glucose at different rates (Lin, Lee, et al., 2012). It was found for the first time that the four α-glucosidases do not equally convert α-amylase hydrolysates to free glucose but produce glucose differently depending on the structure of α-amylase hydrolysates. In the present study, we found that the quantity of α-(1→6) glycosidic linkages in the α-amylase hydrolysates have a negative impact on glucose generation by Nt-MGAM. The higher quantity of branches within α-amylase hydrolysates corresponded to less glucose produced by Nt-MGAM. It was noted that three α-amylase hydrolysate substrates [*aewx* maize, normal maize, and tapioca (as bracketed in Fig. 4)] had a similar quantity of branches but differed in glucose generation. We debranched these α-amylase hydrolysate substrates to explore another structural factor that may affect the Nt-MGAM glucogenesis. We found the quantity of Fr. 3, the relatively short chain-length fraction, was proportional to the glucose generation. In addition, the average chain-length of the Fr. 3 had a negative impact on glucogenesis suggesting that shorter distance between the branches of Fr. 3 has less glucose production.

In our previous studies, Nt-MGAM did not digest the branched dextrins of α-amylase hydrolysates but hydrolyzes panose and $6^3$-glucosylmaltotriose containing α-(1→6) linkage. It is likely that the capability of Nt-MGAM for digesting branched molecules is modulated by the branch pattern of α-amylase hydrolysate substrates. The effect of branch pattern, referring to the branch amount and the distance between branches, on glucogenesis may be associated with the structural conformation of the Nt-MGAM active site which is a pocket containing two primary sugar subsites (−1, +1) (Sim et al., 2008). When Nt-MGAM forms a complex with acarbose (pseudo-tetrasaccharide), only the first two rings of the non-reducing end insert into the pocket and occupy the −1 and

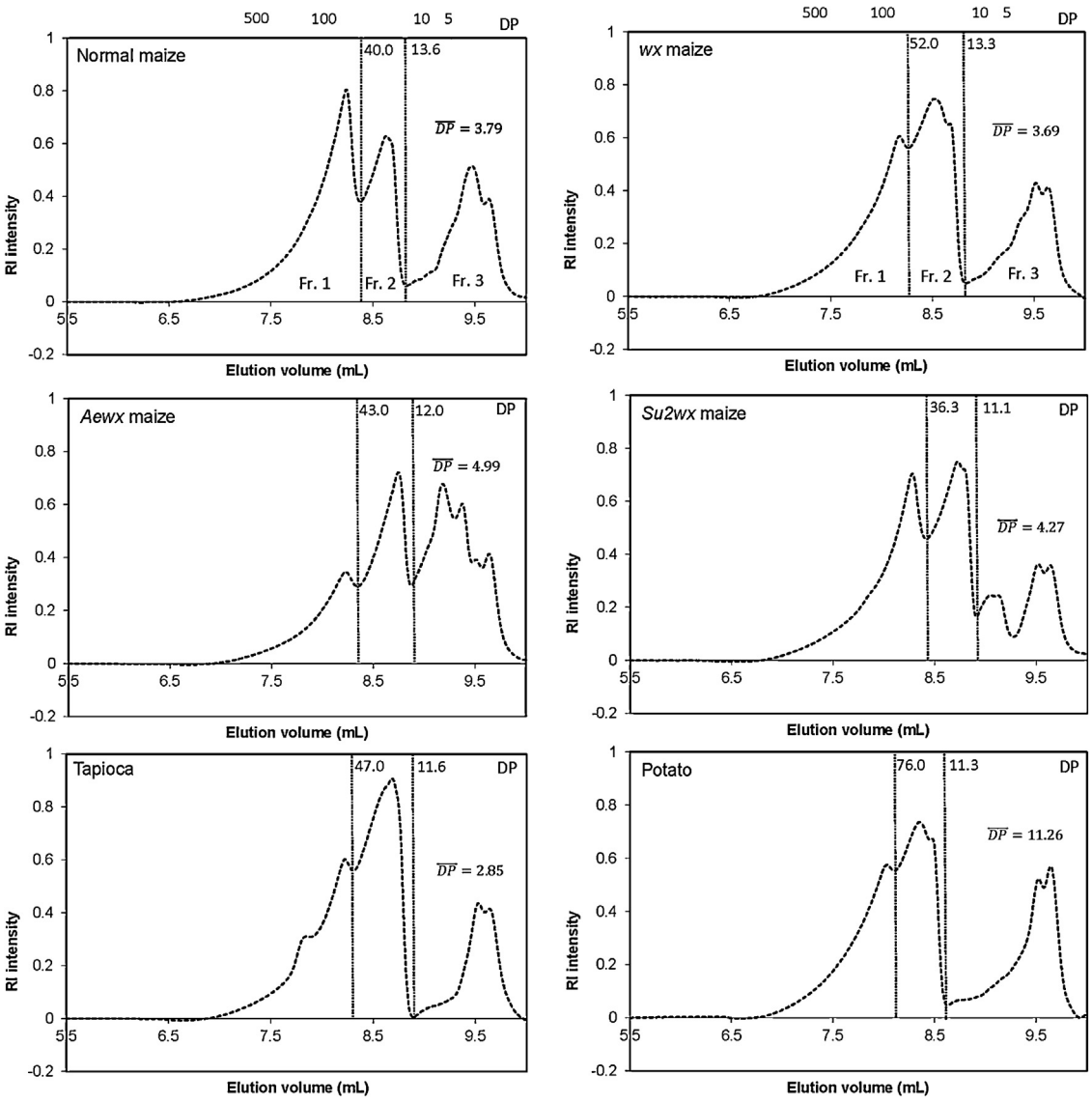


**Fig. 5.** Size-exclusion chromatographs of de-branched α-amylolytic hydrolysate substrates. Six different starches were pre-hydrolyzed with α-amylase for 2 h, and then de-branched with isoamylase. A calibration curve of elution time and chain-length (presenting as degree of polymerization, DP) made by standard pullulans is shown on top *X*-axis of the first chromatograph (normal and *wx* maize). The vertical dotted lines show the ranges of each fraction. The average DP ($\overline{DP}$) of Fraction 3 (Fr. 3) of each α-amylase hydrolysate substrates is labeled in figures.

+1 sugar subsites. The de-branching activity of Nt-MGAM on various sugars is different (Lin, Lee, et al., 2012). Its activity on panose [trisaccharide with one α-(1→4) and one α-(1→6) linkage] and $6^3$-glucosymaltotriose [tetrasaccharide with two α-(1→4) and one α-(1→6) linkages] is higher than on isomaltose [disaccharide with one α-(1→6) linkage] (Lin, Lee, et al., 2012). It is possible that the structural conformation of panose and $6^3$-glucosylmaltotriose fits in the Nt-MGAM active site better than isomaltose. Therefore, we suggest that glucans with different branch patterns have different susceptibility to Nt-MGAM.

In this study, the debranched α-amylase hydrolysates had three to four fractions (Fig. 5). We did not separate the linear from the branched dextrins in order to mimic *in vivo* conditions because they are not separated in lumen. The quantity of Fr. 3 (relatively short chain-length fraction) showed an inverse linear relationship with glucose generation (Fig. 6). The Fr. 3 contained both linear (DP 2 and 3) and small branched molecules with a chain-length less than DP 13. The higher quantity of Fr. 3 indicates higher amount of linear molecules and/or shorter distances between the branches in the branched dextrins. Thus, the select group of α-amylase hydrolysate substrates (potato and *su2wx* maize are excluded) exhibited a linear relationship between the average chain-length of Fr. 3 and Nt-MGAM glucogenesis (Fig. 7). Note that potato and *su2wx* maize hydrolysates have unique amylopectin chain profiles, with relatively long and short chain-length in Fr. 3, respectively (Fig. 5). These two substrates also had different digestion kinetics from those of other substrates (Fig. 3). Their rate of decrease in glucogenesis differed from that of the initial stage of digestion after 120 min. The change of the rate was not previously reported in the literature studying the starch digestion with porcine α-amylase and fungal glucoamylase (Butterworth et al., 2012), which differs from this study using human pancreatic α-amylase and mucosal α-glucosidase. More studies with extensive reaction time and amylopectin molecules with extra-long as well as extra-short chain-length are needed to confirm the change of the decrease rate in glucogenesis.

Both *su2wx* maize starch and glycogen are highly branched with short distances between branches, and it was unknown whether they have similar structures and responses to digestive enzymes. The *su2wx* maize is a double mutant of the *wx* and *sugary-2* (*su2*)

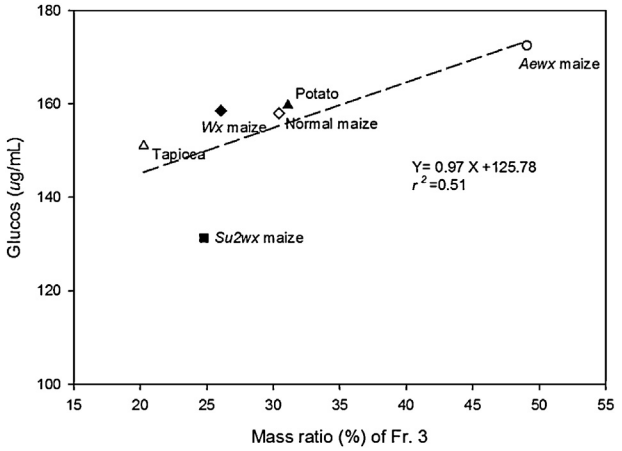


**Fig. 6.** The relationship of glucose production and the quantity of the Fraction 3 of α-amylase hydrolysate substrates. *Aewx* maize (○), *wx* maize (♦), normal maize (◊), potato (▲), tapioca (△), and *su2wx* maize (■) starches were pre-hydrolyzed with α-amylase for 2 h and then further digested to glucose by Nt-MGAM. The quantity of Fr. 3 is presented as mass ratio (*X*-axis), which was calculated from SEC chromatographs of the debranched α-amylase hydrolysates. It is the ratio of the area under the chromatographic curve of Fr. 3 and the sum of the areas of Fr. 1, 2 and 3. The linear correlation coefficient, $r^2$, of the mass ratio (%) of Fr. 3 and glucose generation was 0.51.

genes in the inbred Oh43 maize; it has higher quantity of short B chains and A chains than *wx* maize (Fuwa et al., 1987). Its ratio of the short chains (sum of short B and A chains) to the long B chains is also higher than *wx* maize; but the ratio of A and short B chains is similar to *wx* maize (1.3:1) (Fuwa et al., 1987). Glycogen has an average chain-length of ~12 glucosyl residues with individual chains ranging from 6 to more than 50 glucosyl residues (Manners, 1991). It has equal numbers of exterior and interior chains; the ratio of A to B chains is in the range of 0.7:1 to 1:1 (Manners, 1991). Manners (1991) reported that the distance between branches of the rabbit liver glycogen is about 2 glucosyl residues; and the quantity of branched dextrins is about 2–4% of α-amylase hydrolysates. In the present study, both the α-amylase hydrolysates of *su2wx* maize and glycogen were found to have higher quantity of branches and lower production of glucose. However, glycogen and *su2wx* maize starch are quite different, because glycogen molecules differ from amylopectin in its irregular distribution of branch points

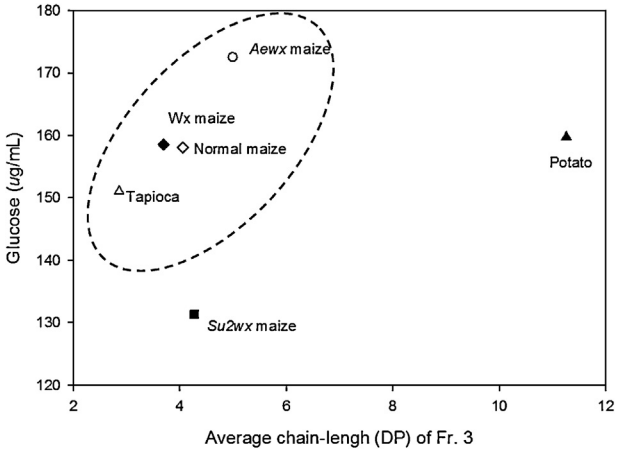


**Fig. 7.** The relationship between glucose production and the average chain-length of the Fraction 3 of α-amylase hydrolysate substrates. *Aewx* maize (○), *wx* maize (♦), normal maize (◊), potato (▲), tapioca (△), and *su2wx* maize (■) starches were pre-hydrolyzed with α-amylase for 2 h and then digested to glucose by Nt-MGAM. The chain-length was calculated from SEC chromatograph of the debranched α-amylase hydrolysate substrates based on the calibration curves shown in Fig. 5.

and molecular arrangement. The glycogen molecules are compact or spherical with irregular and random distribution of branches (Manners, 1991). In contrast, amylopectin molecules have a regular alternation of elongated α-(1→4) linked chains and clustered α-(1→6) branch linkages that give the 70 Å periodicity of the clustered branch linkages (Robyt, 2008; Yamaguchi, Kainuma, & French, 1979). In addition to the difference in molecular organization, glycogen also contains small quantity of 1→2 and 1→3 linkages (less than 0.6% of other types of linkages) (Afanas'eva & Stepanenko, 1956). These structural differences may contribute to the different digestion pattern of glycogen (Fig. 3).

We have found that Nt-MGAM has a digestion role beyond its conventional role as a maltase (Ao, Quezada-Calvillo, et al., 2007; Ao, Simsek, et al., 2007; Lin, Nichols, et al., 2012; Lin, Lee, et al., 2012). The susceptibility of various starches to Nt-MGAM is different from α-amylase (Ao, Quezada-Calvillo, et al., 2007; Ao, Simsek, et al., 2007). For example, in the granular form, high-amylose maize starch is digested faster than waxy maize starch by Nt-MGAM (Ao, Quezada-Calvillo, et al., 2007; Ao, Simsek, et al., 2007); this is opposite to their susceptibilities to α-amylase. In this study, using starch in a dispersed form, we also found that various starches were digested by Nt-MGAM at different rates (without pre-hydrolysis). In addition, we found that α-amylase hydrolysis amplified the Nt-MGAM glucogenesis, which agrees with previous report (Quezada-Calvillo et al., 2008). In the present study, we further discovered that the amplification of various starches in glucogenesis by α-amylase hydrolysis was different (Fig. 2). The following factors were found to affect the amplification: (1) the susceptibility of starch to α-amylase, (2) the susceptibility of the linear degradation products of α-amylolysis to Nt-MGAM, and (3) the susceptibility of the branched residues to Nt-MGAM. The first and second factors are associated with amylose and external amylopectin chains; the third factor is related to the internal structure of starch, highly branched amylopectin fraction.

## 5. Conclusions

Starch digestion rate greatly affects post-prandial glycemic response, and is influenced by its susceptibility to both α-amylases and mucosal α-glucosidases. This study revealed that the branch pattern of α-amylase hydrolysates modulates the glucogenesis by Nt-MGAM. The quantity of α-(1→6) glycosidic linkages determined the glucogenic rate of Nt-MGAM; the distance between branches may also affect the digestion. These findings suggest the crucial role of mucosal α-glucosidases in regulating the glucose release rate at the brush border area of the small intestine. Importantly, the regulation of starch digestion rate at the level of mucosal α-glucosidase is related to food starch sources that determine the nature of starch internal structure. While it is well known that the susceptibility of starch to α-amylase can be changed through chemical, physical, or enzymatical modifications; we show that starch internal structure is a key factor to affect its susceptibility to mucosal α-glucosidases. A new strategy to affect the susceptibility of starch to both types of digestive enzymes is to consider starch sources that determines the nature of their internal structures and the modifications that change the susceptibility to both α-amylase and α-glucosidases.

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