Modulation of Starch Digestion for Slow Glucose Release through “Toggling” of Activities of Mucosal α-Glucosidases*

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Background: Proper breakdown of starch by hydrolytic enzymes to yield glucose has profound implications for avoiding type 2 diabetes and obesity.

Results: Starch digestion by the different human enzymes is controlled using a panel of compounds.

Conclusion: Inhibitors can be used to switch off selectively the different enzyme activities.

Significance: More refined control of starch hydrolysis with the aim of slow glucose delivery is possible.

Starch digestion involves the breakdown by α-amylase to small linear and branched malto-oligosaccharides, which are in turn hydrolyzed to glucose by the mucosal α-glucosidases, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI). MGAM and SI are anchored to the small intestinal brush-border epithelial cells, and each contains a catalytic N- and C-terminal subunit. All four subunits have α-1,4-endoglucosidase activity, and the SI N-terminal subunit has an additional exo-α-glucosidase activity on the α-1,4-linkage. Inhibition of α-amylase and/or α-glucosidases is a strategy for treatment of type 2 diabetes. We illustrate here the concept of “toggling”: differential inhibition of subunits to examine more refined control of glucogenesis of the α-amylolyzed starch malto-oligosaccharides with the aim of slow glucose delivery. Recombinant MGAM and SI subunits were individually assayed with α-amylolyzed waxy corn starch, consisting mainly of maltose, maltotriose, and branched α-limit dextrins, as substrate in the presence of four different inhibitors: acarbose and three sulfonium ion compounds. The IC50 values show that the four α-glucosidase subunits could be differentially inhibited. The results support the prospect of controlling starch digestion rates to induce slow glucose release through the toggling of activities of the mucosal α-glucosidases by selective enzyme inhibition. This approach could also be used to probe associated metabolic diseases.

Starch digestion by the different human enzymes is controlled using a panel of compounds. Recombinant MGAM and SI subunits were individually assayed with α-amylolyzed waxy corn starch, consisting mainly of maltose, maltotriose, and branched α-limit dextrins, as substrate in the presence of four different inhibitors: acarbose and three sulfonium ion compounds. The IC50 values show that the four α-glucosidase subunits could be differentially inhibited. The results support the prospect of controlling starch digestion rates to induce slow glucose release through the toggling of activities of the mucosal α-glucosidases by selective enzyme inhibition. This approach could also be used to probe associated metabolic diseases.

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5 The abbreviations used are: LM/αLDx, linear malto-oligosaccharide/branched α-limit dextrin mixture; MGAM, maltase-glucoamylase; SI, sucrase-isomaltase; nMGAM and nSI subunits; cMGAM and cSI subunits; WC, waxy corn starch; HPAEC, high-performance anion-exchange chromatography.

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Toggling of Mucosal α-Glucosidase Activities

sucrose digestion (8). The individual physiological contributions of the four subunits and the extent to which they act in cooperation under different physical or dietary conditions are unknown.

Inhibition of pancreatic α-amylase and/or intestinal α-glucosidase activity is currently applied to the treatment of type 2 diabetics (non-insulin-dependent) to adjust glucose levels in the blood stream (9, 10). As MGAM and SI are involved in producing glucose from LM/αLDx, regulation of individual mucosal α-glucosidase activities is considered to be one of the effective approaches for regulating blood glucose levels (11, 12). Recent studies by our consortium showed that different mucosal α-glucosidases can be inhibited differently by the same inhibitor, e.g. acarbose (1) (see Fig. 1A) (13), leading to the hypothesis that one might regulate individual α-glucosidase activities with certain inhibitors by partially or wholly inhibiting one, two, or three of the four α-glucosidase activities while leaving the remaining one or more subunits active, a concept we advanced as “toggling” (14, 15). That is, through the judicious action of selective inhibitors, a fast digesting subunit or subunits can be inhibited to place the onus of digestion on slower digesting subunits. It follows that the rate of starch digestion and glucose release to the body could be controlled.

Recently, a relatively new class of sulfonium ion glucosidase inhibitors, including salaprinol (2) (17), salacinol (3) (16, 46, 47), ponkoranol (4) (17, 48), kotalanol (5) (18, 49, 50), de-O-sulfonated kotalanol (6) (19, 49, 51, 52), de-O-sulfonated salacinol (7) (20, 53), and de-O-sulfonated ponkoranol (8) (21, 54) (see Fig. 1A), has been isolated from the roots and stems of the Salacia species, a plant that is widespread in Sri Lanka and Southern India and that used in traditional ayurvedic treatment of type 2 diabetes (22, 55, 56). The compounds were shown to be inhibitors of intestinal α-glucosidase enzymes that attenuate the undesirable spike in blood glucose levels that is experienced by diabetics after consuming a meal rich in carbohydrates. Previously, the compounds have been shown to be stronger inhibitors of ntMGAM, with $K_i$ values in the low-micromolar range (i.e. 0.03–0.19 μM) compared with acarbose (1) ($K_i = 62 ± 13 \mu M$) (23–28). In addition, the de-O-sulfonated compounds (6–9) were either equivalent or better inhibitors than the parent sulfonated compounds (2–5) (14, 29, 54). We have also synthesized the C-3′-β-maltose-extended de-O-sulfonated ponkoranol analog (10) and C-5′-β-maltose-extended de-O-sulfonated ponkoranol (11) (Fig. 1B) (15). The effectiveness of these compounds and acarbose (1) on inhibition of recombinant mammalian MGAM (ntMGAM and ctMGAM) and SI (ntSI and ctSI) using maltose as a substrate has shown some striking selectivities (15).

In the small intestine, SI and MGAM are exposed to LM/αLDx, rather than maltose, as substrates. Therefore, an objective of this work was to explore the role of different inhibitors in MGAM and SI enzyme activities against LM/αLDx structures. Furthermore, we wanted to elucidate whether specific inhibitors could affect glucogenesis by selective inhibition of the different enzymes, having shown this property with maltose as a substrate (14, 15), and to investigate how slow starch digestion or slow glucose delivery to the body might be achieved using this concept. We have chosen, as representative inhibitors, acarbose (1), de-O-sulfonated kotalanol (6), C-3′-β-maltose-extended de-O-sulfonated ponkoranol analog (10), and C-5′-β-maltose-extended de-O-sulfonated ponkoranol (11), and we report here their inhibitory effects on recombinant human MGAM and SI using a mixture of the α-amylase degradation products (LM/αLDx) as a substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Waxy corn starch (WCS) genetically depleted of amylose (Tate and Lyle, Inc., Decatur, IL) was used as the substrate for producing LM/αLDx. Acarbose was purchased from Sigma-Aldrich and other inhibitors (6, 10, and 11) were synthesized as described previously (15, 49). Human pancreatic α-amylase was purchased from Meridian Life Science, Inc. (Saco, ME). The glucose assay kit was purchased from Megazyme (Wicklow, Ireland). All chemicals used in this study were of analytical grade.

**Enzyme Preparation**—Methods for cloning, expression, and purification of human ntMGAM (7) and ntSI (30) from *Drosophila* S2 cells, as well as mouse ctMGAM, and ctSI, were reported previously (14).

**Determination of Protein Concentration**—The protein concentration in the enzyme solution was determined using a Bio-Rad protein assay kit according following the Bradford method (31). Enzyme solution (20 μl) was mixed with 1.0 ml of diluted dye reagent and incubated at room temperature for at least 5 min, and then enzyme activity was measured by the absorbance change at 595 nm using a Beckman DU530 Life Science UV/VIS spectrophotometer.

**Production of LM/αLDx**—WCS was mixed in 10 mM phosphate buffer (pH 6.9) at 10 mg/ml (w/v) and reacted with human pancreatic α-amylase (0.24 units; 1 unit of activity was defined as the amount of enzyme that produced 1 μg 2-chloro-4-nitrophenyl-α-D-maltotrioside in 1 min at 37 °C) at 37 °C for 24 h to produce the LM/αLDx mixture. α-Amylase was inactivated by boiling, and the α-amylolysis product was utilized as a substrate for inhibition testing with recombinant mucosal MGAM and SI subunits.

**Structural Analysis of LM/αLDx by High-performance Anion-exchange Chromatography (HPAEC)—**The size distribution of the LM/αLDx from WCS was characterized by HPAEC (fitted with an ED40 electrochemical detector (Dionex, Sunnyvale, CA)). A filtered (0.22-μm syringe filter) α-amylase-treated WCS sample (25 μl) was injected onto a CarboPac PA1 palli- cular anion-exchange column (Dionex), previously equilibrated with 150 mM NaOH at a flow rate of 1 ml/min. Separation of the LM/αLDx structures was achieved using the linear gradient mode with 600 mM sodium acetate (in 150 mM NaOH).

**Effect of Different Inhibitors on Individual Mucosal α-Glucosidases**—Recombinant α-glucosidases were preincubated with different concentrations of inhibitors (range of 0.5 × 10⁻³ to 500 nM) for 30 min before reacting with substrates. A fixed protein amount of each α-glucosidase (30 μg/ml) was incubated with substrate (LM/αLDx or maltose) in 10 mM PBS (pH 6.9) at 1 mg/ml (w/v) at 37 °C for 1 h. The amount of glucose released from substrate was analyzed by the glucose oxidase/peroxidase method (32). Samples without inhibitors (designated as “blank”) were assumed to be 100% hydrolyzed.
IC₅₀ Calculation—IC₅₀ values were calculated using a quadratic polynomial equation with inhibitor concentrations against 50% of released glucose compared with a control sample without inhibitors (33). IC₅₀ values were determined based on the same protein amount of recombinant C- and N-terminal MGAM and SI subunits. All analyses were performed in duplicate.

RESULTS AND DISCUSSION

Specific Activity of Purified Recombinant Mucosal α-Glucosidases—Purified recombinant C- and N-terminal α-glucosidase solutions were applied to assay enzyme activity. Table 1 shows the specific activities (units/mg) of the individual mucosal α-glucosidases (based on glucose release) upon maltose and LM/αLDx hydrolysis. One unit of enzyme activity was defined as 1 μg glucose released from 1% (w/v) maltose or LM/αLDx in 1 min. ctMGAM had 1.8–3.8 and 2–3.6 times higher hydrolytic activity with maltose and LM/αLDx, respectively, compared with the other α-glucosidases (based on protein amounts). ntSI had the lowest specific activity for both maltose and LM/αLDx hydrolysis among the four mucosal α-glucosidases. Previous enzyme kinetic research using maltose as a substrate showed that ctMGAM had the lowest $K_m$ value (1.9 mM)
(14) and ntSI had the highest $K_m$ value (7.1 mM) (30) among the four $\alpha$-glucosidases.

**Structural Analysis of $\alpha$-Amylase-treated WCS**—The LM/\(\alpha\)LDx mixture, produced by $\alpha$-amylase reaction on WCS and intended to simulate actual starch digestion products that are generated in the small intestinal lumen, was utilized as the substrate for analyzing the inhibitory effects of mucosal $\alpha$-glucosidases. Structural analysis of post-amylase small malto-oligosaccharides obtained from WCS was performed by HPAEC. The chromatographic profiles (Fig. 2) show two types of oligosaccharides, linear and branched, as described previously (4). Peaks of linear oligosaccharides were characterized using standard malto-oligosaccharide solutions. Based on previous investigations, branched oligosaccharides were identified by the peaks present in between the linear oligosaccharide peaks (34, 35). Maltose (G2) and maltotriose (G3) were the major linear products of the $\alpha$-amylase reaction on WCS (Fig. 2A), whereas higher molecular weight branched oligosaccharide

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**TABLE 1**

| Specific activities of the four recombinant mucosal $\alpha$-glucosidases upon maltose and LM/\(\alpha\)LDx hydrolysis |
|---------------------------------------------------------------|
| One unit of enzyme activity was defined as 1 $\mu$M glucose released from 1% (w/v) maltose or 1% (w/v) LM/\(\alpha\)LDx in 1 min. Data are means $\pm$ S.E. |
| Specific activity | ctMGAM | ntMGAM | ctSI | ntSI |
|-------------------|--------|--------|------|------|
| Maltose           | $973.1 \pm 28.9$ | $337.1 \pm 52.9$ | $537.7 \pm 50.1$ | $250.4 \pm 23.8$ |
| LM/\(\alpha\)LDx   | $660.1 \pm 22.8$ | $342.9 \pm 11.3$ | $209.3 \pm 24.8$ | $183.6 \pm 23.4$ |

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**Figure 2.** Structural analysis of LM/\(\alpha\)LDx from human $\alpha$-amylase-treated WCS by HPAEC. A, linear malto-oligosaccharides. B, branched malto-oligosaccharides (magnified from A). Asterisks indicate branched structures. DP, degrees of polymerization. nC, nanoCoulomb.
peaks represent malto-oligosaccharides of DP>4 (Fig. 2B). It should be noted that glucose intensity on the electrochemical detector is not proportional to its actual amount (36). The main products are hydrolyzed with different efficiencies by the four enzyme subunits (37).

**Toggling of Mucosal \(\alpha\)-Glucosidase Activities**

A combination of the four mucosal MGAM and SI enzyme subunits is required for effective digestion of the LM/\(\alpha\)LDx mixture to glucose; therefore, differential inhibition of individual mucosal \(\alpha\)-glucosidase activities, or toggling of the enzymes, is hypothesized to be one of the approaches for moderating blood glucose excursions, which would be desirable for type 2 diabetics. As discussed above, inhibiting \(\alpha\)-glucosidases in the small intestine to regulate carbohydrate digestion is currently applied to treating type 2 diabetes (10). However, partial inhibition to provide a slow and more precisely controlled digestion of starch would be preferable to high-level inhibition, where the bulk of starch goes undigested to the large intestine and can cause discomfort and diarrhea (38). Previous investigations have shown that mucosal \(\alpha\)-glucosidase inhibitors such as acarbose and salacinol-based compounds differentially inhibit the four enzymes, using maltose as the substrate (9, 14). In this study, we investigated the control of starch digestion through the concept of toggling or differential inhibition of subunits.

Acarbose (1) demonstrated the toggling principle of selective inhibition of the four mucosal \(\alpha\)-glucosidases. Fig. 3 shows acarbose inhibition of the \(\alpha\)-glucosidase enzymes with LM/\(\alpha\)LDx as the substrate. Treatment with different acarbose concentrations and the same amount of each \(\alpha\)-glucosidase subunit showed that different concentrations were required to inhibit each enzyme. As an example of the toggling effect, 5 nM acarbose effectively inhibited ctMGAM and ctSI, whereas ntMGAM and ntSI were virtually uninhibited. ctMGAM has the highest activity of the four \(\alpha\)-glucosidases and has high-binding and high-hydrolytic properties for larger malto-oligosaccharides (7, 39, 40). Thus, it is possible that selective inhibition of C-terminal domains could provide slow glucose release by placing the onus of digestion on the slower or more specific N-terminal subunits. We propose that compounds that affect only one or two subunits could be used to control starch digestion and glucose delivery *in vivo*.

Fig. 4 illustrates inhibition of the mucosal \(\alpha\)-glucosidases upon LM/\(\alpha\)LDx hydrolysis by de-\(\text{O}\)-sulfonated kotalanol (6). At a concentration of only 50 pmoles, this compound showed...
20 and 40% inhibition of ctMGAM and ctSI activity, respectively, whereas N-terminal subunits were not inhibited. Thus, de-O-sulfonated kotalanol (6) can toggle off the C-terminal subunits, particularly ctSI, which has sucrase activity, while having little effect on the N-terminal subunits. It follows that de-O-sulfonated kotalanol (6) might be applicable as a blood glucose regulator for sucrose digestion as well as starch hydrolysis.

The toggling effect by C-3′-H11032/H9252-maltose-extended de-O-sulfonated ponkoranol (10) upon LM/H9251/LDx hydrolysis showed that there were almost no inhibitory effects below 50 pmol (Fig. 5). Each α-glucosidase showed a different inhibitor susceptibility at 500 pmol of C-3′-β-maltose-extended de-O-sulfonated ponkoranol (10). Whereas ctMGAM and ntMGAM were inhibited by ~50% at this concentration, the SI subunits showed a different inhibitory pattern: 70% inhibition for ctSI and 20% for ntSI.

C-5′-β-Maltose-extended de-O-sulfonated ponkoranol (11) also did not inhibit any of the α-glucosidases at concentrations below 50 pmol (Fig. 6). In this case, all four subunit activities were simultaneously and abruptly decreased at concentrations over 500 pmol. Because of this similar inhibitory property for all of the subunits at the same concentration of inhibitor, C-5′-β-maltose-extended de-O-sulfonated ponkoranol (11) would not be applicable as an inhibitor for differential inhibition of the mucosal α-glucosidases.

IC_{50} Values of Inhibitors for Mucosal α-Glucosidases with α-Amyloyzed Starch—As the IC_{50} value is variable and depends on the amount of enzyme, type of inhibitor, substrate concentration, and reaction conditions (41), we used a common concentration of protein (30 μg/ml) of each mucosal α-glucosidase, all of which have roughly the same molecular weight. IC_{50} values with four different inhibitors (1, 6, 10, and 11) were determined using LM/αLDx derived from pancreatic α-amylase-treated WCS as well as maltose for purposes of comparison.

Table 2 shows that a relatively similar pattern of IC_{50} values was obtained using LM/αLDx and maltose, although the absolute values were different. The likely explanation is that the LM/αLDx mixture contains a significant fraction of maltose, as well as maltotriose, maltotetraose, and small branched glucan (αLDx) structures (Fig. 2, A and B). The longer linear maltoligosaccharides or branched oligosaccharides would generate different IC_{50} values because MGAM and SI have different hydrolytic activities depending on chain length and linkages (5, 42, 43).
The IC$_{50}$ value of acarbose (1) for ctMGAM (0.12 ± 0.01 nM) was ~500 times lower than that for ntMGAM (62 ± 14 nM). Similarly, the IC$_{50}$ for ctSI (0.4 ± 0.4 nM) was ~300 times lower than that for ntSI (135 ± 18 nM). Therefore, acarbose shows selectivity for inhibition of ctMGAM and ctSI over the N-terminal subunits. The inhibition constant (K$_i$) values previously reported for the N-terminal enzymes also were higher than those for ctMGAM and ctSI (14). The structural basis for this preference for the C-terminal subunits was proposed to be the presence of additional saccharide subsites in the ctMGAM structure compared with ntMGAM (7, 14). This was subsequently supported by the crystal structure of the complex of ctMGAM with acarbose (44).

The IC$_{50}$ values for different α-glucosidases in the presence of de-O-sulfonated kotalanol (6) on LM/αLDx were as follows: ctMGAM, 0.28 ± 0.02 nM; ntMGAM, 0.13 ± 0.01 nM; ctSI, 0.22 ± 0.13 nM; and ntSI, 0.98 ± 0.04 nM. Thus, de-O-sulfonated kotalanol at relatively low concentration effectively inhibits three of the mucosal α-glucosidases and shows limited selectivity over ntSI. Mucosal α-glucosidase reaction on LM/αLDx in the presence of C-3’-β-maltose-extended de-O-sulfonated ponkoranol (10) showed a nearly similar range of IC$_{50}$ values for ctMGAM (0.58 ± 0.01 nM), ntMGAM (0.69 ± 0.01 nM), and ctSI (0.17 ± 0.02 nM). ntSI had a 3–15 times higher IC$_{50}$ value (2.69 ± 0.29 nM). Thus, this compound shows potential for selective inhibition of ctSI for hydrolyzing α-amylolyzed starch molecules. Alternatively, at a moderate concentration, it could be used to toggle off the three former subunits, leaving only ntSI active.

As shown above, C-5’-β-maltose-extended de-O-sulfonated ponkoranol (11) exhibited similar IC$_{50}$ values for all four mucosal subunits: ctMGAM, 0.23 ± 0.02 nM; ntMGAM, 0.12 ± 0.01 nM; ctSI, 0.54 ± 0.01 nM; and ntSI, 0.23 ± 0.01 nM. As in previous studies with maltose hydrolysis (14, 23), we observed that small changes in inhibitor structure can lead to different hydrolytic activity against LM/αLDx (Table 2).

**Conclusions**—The results support the idea that the sulfonium ion-based inhibitors will be useful in deriving compounds that have the ability to inhibit each of the α-glucosidase enzyme units in real starch digestion. Furthermore, the data presented support the concept of controlling starch digestion rate through the toggling of activities of the mucosal α-glucosidases by selective enzyme inhibition. We propose, through the approach of differential inhibition by toggling of specific mucosal enzymes with inhibitors, that the starch digestion rate may be modulated to attain similar effects as observed with slowly digestible starch, which has the property of being digested throughout the small intestine.
Decreasing initial peak glucose levels and extending postprandial blood glucose delivery to the body can be desirable for diabetics and possibly to other groups vulnerable to metabolic syndrome-associated diseases. These candidates thus show promise as oral agents to moderate glucose release for the treatment of type 2 diabetes. Ultimately, an assessment of compounds that can inhibit the activities of each enzyme in actual starch digestion will provide a better understanding of their roles in starch digestion individually and in combination.

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