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Mapping the intestinal alpha-glucogenic enzyme specificities of starch digesting maltase-glucoamylase and sucrase-isomaltase

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ABSTRACT

Inhibition of intestinal α-glucosidases and pancreatic α-amylases is an approach to controlling blood glucose and serum insulin levels in individuals with Type II diabetes. The two human intestinal glucosidases are maltase-glucoamylase and sucrase-isomaltase. Each incorporates two family 31 glycoside hydrolases responsible for the final step of starch hydrolysis. Here we compare the inhibition profiles of the individual N- and C-terminal catalytic subunits of both glucosidases by clinical glucosidase inhibitors, acarbose and miglitol, and newly discovered glucosidase inhibitors from an Ayurvedic remedy used for the treatment of Type II diabetes. We show that features of the compounds introduce selectivity towards the subunits. Together with structural data, the results enhance the understanding of the role of each catalytic subunit in starch digestion, helping to guide the development of new compounds with subunit specific antidiabetic activity. The results may also have relevance to other metabolic diseases such as obesity and cardiovascular disease.

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1. Introduction

Starch comprises a substantial portion of the human diet and plays an integral role in human metabolism, contributing a sizable fraction of total caloric intake. Starch is digested to glucose in the small intestine, contributing to the postprandial rise in blood glucose levels as well as insulin response.[1] Two macromolecules comprise starch, amylose and amylopectin. Amylose is a linear chain polymer of α-D-glucopyranose units linked by α-1,4 bonds, with only minor branching. Amylopectin is a branched polymer of α-D-glucopyranose linked by α-1,4 linkages with branching α-1,6 bonds.

The digestion of starch to glucose requires multiple enzymatic reactions in the human body. Glucose is only a minor product of α-amylase digestion of starch, the major products being soluble glucose oligomers, with both linear and branched structures.[2] The small, linear and branched α-limit oligomers (dextrins) produced by α-amylase hydrolysis cannot be absorbed into the bloodstream and must be further hydrolyzed to free glucose. Maltase-glucoamylase (MGAM) and sucrose-isomaltase (SI) process the small linear oligomers and α-limit dextrins in the small intestine.[3] The apparent redundancy in glucosidase activities in the gut, as described below, is not understood and forms the basis for the present study.

Here, we compare the kinetic effect on the four catalytic subunits by six inhibitors: two of the currently available antidiabetic drugs, acarbose[4] (**1**) and miglitol[5] (**2**), a novel class of sulfonium-ion glucosidase inhibitors, salacinol (**3**), kotalanol (**4**), and de-O-sulfonated kotalanol (**5**), isolated from an Ayurvedic antidiabetic medicinal plant, *Salacia reticulata*, and the selenium analogue of salacinol, namely blintol (**6**) (Fig. 1). The results demonstrate catalytic domain selectivity, helping differentiate the active site requirements of each catalytic subunit. This directly enhances the understanding of the role these enzymes play in glucogenesis of starch in vivo. The future applications of this research include the ability to turn the enzyme activities on and off in animal models individually and in various combinations and eventually modulation of MGAM and SI activity as an effective method to control serum insulin and blood glucose levels.

2. Biology

SI demonstrates hydrolytic activity on branched α-1,6 linkages, which is complemented by the hydrolytic activity of both

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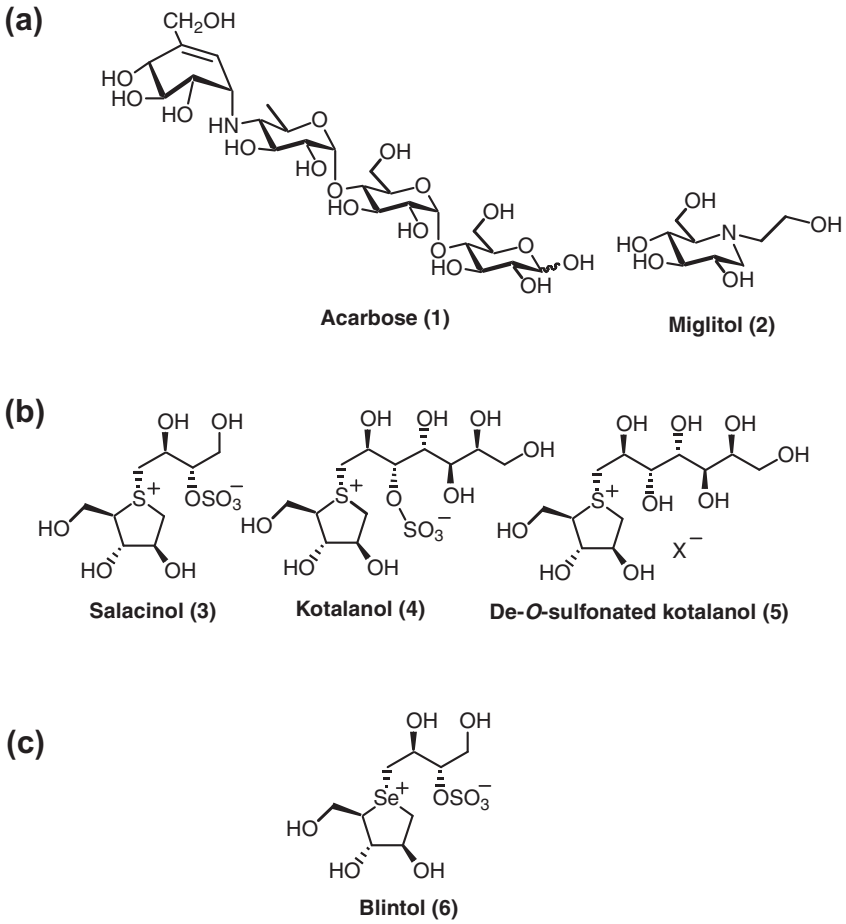

Figure 1. α-Glucosidase inhibitors discussed in this paper. (a) Structures of α-glucosidase inhibitors currently used in the treatment of Type II diabetes, (b) Structures of sulfonium-ion glucosidase inhibitors from *Salacia reticulata* and (c) Blintol, the selenium analogue of salacinol.

SI and MGAM on α-1,4 linkages[6]; however, the main substrate of SI is the α-1,4 linkage.[7] The complementary activities of the human enzymes allow for digestion of the spectrum of food starches from more than 400 plants that comprise two-thirds of most human diets; SI is more abundant but MGAM displays higher hydrolytic activities.[8–10] In studies of severely malnourished infants, it has been demonstrated that the terminal steps of MGAM α1–4 and SI α1–6 starch digestion are decreased. The reduction of SI and MGAM expression and activity is a common alteration secondary to the villous atrophy. The loss of maltase-glucoamylase message parallels the reduction in villin message and degree of villous atrophy. Further, the reduced maltase-glucoamylase message also parallels the sucrase-isomaltase message. It has also been shown that the correlations between the two enzyme activities (SI and MGAM) exist at the mRNA level.[11] Additionally, recent studies demonstrate that feeding rats a high fat/carbohydrate ratio diet reduces the jejunal Sucrase/Isomaltase (S/I) activity ratio. In addition, the SI-linked oligosaccharides show modifications of unsialylated galactose as well as the mannose-linked form when the S/I activity ratio changes, and thus is thought to be regulated by altering the glycosylation of SI.[12–16] These studies emphasize the importance of MGAM and SI activities, regulated both by expression levels and enzyme structure, in human nutrition. Interestingly, protein expression regulated by epigenetic changes resulting from human pre-natal malnutrition has been associated with a number of disease conditions.[17,18] Both MGAM and SI are anchored to the brush-border epithelial cells of the small intestine and each contains two catalytic subunits (Fig. 2), an N-terminal subunit (ntMGAM and ntSI) adjacent to the membrane bound end of the enzyme and a C-terminal luminal subunit (ctMGAM and ctSI). Each of the subunits comprises a glycoside hydrolase enzyme of family 31 (GH31),[19] which operate through a mechanism that results in retention of configuration at the anomeric center. SI and MGAM show 59% amino acid sequence similarity and it has been proposed that the shared domain structures imply that MGAM and SI evolved through duplication of an ancestral gene, which itself had already undergone tandem gene duplication.[7] Further, the catalytic subunits of MGAM and SI are 40–60% identical in amino acid sequence and

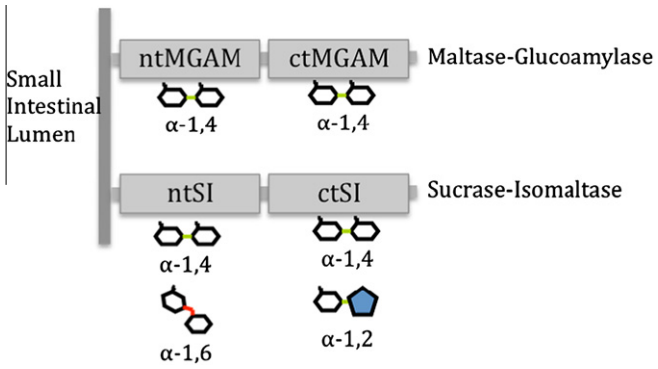

Figure 2. Schematic diagram of MGAM and SI indicating hydrolytic activity.

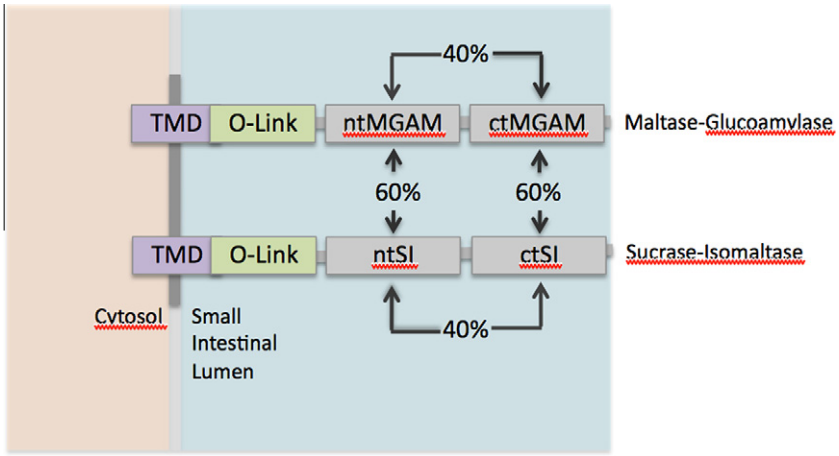

Figure 3. Linear representation of protein organization of SI and MGAM. Percentage of sequence identify of catalytic domains is indicated (adapted from[28]).

contain the catalytic site signature sequence WiDMNE (i indicates a variable residue), characteristic of GH31 subgroup four members (Fig. 3).[20] In summary, each of these proteins include a small cytosolic domain, a transmembrane domain (TMD), an O-glycosylated linker (O-link), and two homologous catalytic subunits (ntMGAM, ctMGAM, ntSI, and ctSI). Additionally, there are various alternative splicing patterns of ctMGAM in mammals; two spliceforms from mice are discussed in this paper: ctMGAM-N2 and ctMGAM-N20 (Fig. 4, Quezada-Calvillo, in preparation).[21]

Until recently, there has been little investigation into the individual roles of the four catalytic subunits comprising the human intestinal MGAM and SI complexes in the digestion of food starches. Currently, inhibitors targeting intestinal α-glucosidases as well as pancreatic α-amylases are used in the treatment of non-insulin-dependent Type II diabetes. Inhibition of these enzymes decreases postprandial hyperglycemia and hyperinsulinemia, reducing the occurrence of insulin resistance as well as reducing the stress on the beta cells of the pancreas, preventing further insulin-dependent disorders.[22–26] Presently, there are two key α-glucosidase inhibitors that have demonstrated value as dietary and treatment supplements for patients with Type II diabetes. These are acarbose and miglitol, which inhibit the enzymes MGAM and SI. These compounds exhibit a broad affinity for α-glucosidases, such as α-amylases as well as the target enzymes, leading to a requirement for relatively large doses, resulting in unpleasant side effects such as gastrointestinal upset. These problems can be mitigated through targeting the core activities that contribute to postprandial glycemic excursions.[27] Novel compounds that can distinguish each of the four enzyme subunits of MGAM and SI will be important tools for understanding their individual roles, but also in limiting side effects resulting from unnecessary inhibitory activities.

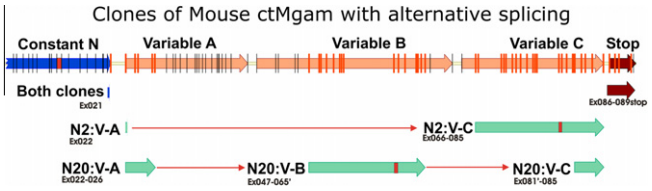

Figure 4. Alternative splicing of cloned ctMGAM subunits of mouse MGAM. Diagram of genomic map at the top. Green arrows: map of transcribed segments present in the individual clones N2 and N20. The included exons are noted in black below the clone segments. Red vertical bars represent the position of the coded GH31 signature sequence WIDMNE.

The crystal structures of human ntMGAM in complex with inhibitors including acarbose revealed the structural basis for its poor inhibitory properties.[28–30] These structural results in conjunction with sequence analysis indicate ctMGAM and ctSI have features that extend their substrate-binding clefts to more subsites than in ntMGAM. Here, we compare the kinetic effect on the four catalytic subunits by six inhibitors: two of the currently available antidiabetic drugs, acarbose (**1**) and miglitol (**2**), a novel class of sulfonium-ion glucosidase inhibitors, salacinol (**3**), kotalanol (**4**), and de-O-sulfonated kotalanol (**5**), isolated from an Ayurvedic antidiabetic medicinal plant, *S. reticulata*, and the selenium analogue of salacinol, namely blintol (**6**) (Fig. 1). The results demonstrate catalytic domain selectivity, helping differentiate the active site requirements of each catalytic subunit. This directly enhances the understanding of the role these enzymes play in glucogenesis of starch in vivo. The future applications of this research include the ability to turn the enzyme activities on and off in animal models individually and in various combinations and eventually modulation of MGAM and SI activity as an effective method to control serum insulin and blood glucose levels.

3. Results and discussion

3.1. Kinetic characterization

Kinetic parameters of the C-terminal catalytic subunits were determined (Table 1). Kinetic analysis revealed that for maltose substrates, ctMGAM-N2 has a K_m of 1.59 ± 0.18 mM, fourfold lower than that of ctMGAM-N20 (K_m of 1.91 ± 0.23 mM), while ctSI has a K_m of 2.5 ± 0.5 mM. Both isoforms of ctMGAM also display strong substrate inhibition by maltotriose (Table 1). Unlike ctMGAM, no substrate inhibition is seen with maltotriose with ctSI (K_m of

Table 1
Kinetic parameters of the C-terminal catalytic subunits

Catalytic subunit	Substrate	K_m (mM)
ctMGAM-N2	Maltose	1.59 ± 0.18
	Maltotriose	Strong substrate inhibition
ctMGAM-N20	Maltose	1.91 ± 0.23
	Maltotriose	Strong substrate inhibition
ctSI	Maltose	2.5 ± 0.5
	Maltotriose	2.8 ± 0.2
	Sucrose	38 ± 10

Table 2
K_i values for each α-glucosidase inhibitor with ntMGAM, ctMGAM, ntSI, and ctSI (μM)

	ctMGAM-N2	ctMGAM-N20	ntMGAM	ctSI	ntSI
K_m (Maltose)	1.59 mM ± 0.18	1.91 mM ± 0.23	4.3 mM ± 1.2[31]	2.5 mM ± 0.5	7.1 mM ± 1.3[29]
Acarbose	0.009 ± 0.002	0.028 ± 0.005	62 ± 13[31]	0.246 ± 0.005	14 ± 1[29]
Salacinol	0.213 ± 0.018	0.058 ± 0.003	0.19 ± 0.02[31]	0.047 ± 0.0077	0.277 ± 0.068
Blintol	0.018 ± 0.004	0.013 ± 0.002	0.49 ± 0.05[32]	0.029 ± 0.005	0.16 ± 0.01
Kotalanol	0.064 ± 0.006	0.092 ± 0.007	0.19 ± 0.03[31]	0.042 ± 0.007	0.60 ± 0.06[29]
De-O-sulfonated kotalanol	0.026 ± 0.004	0.078 ± 0.009	0.03 ± 0.01[31]	0.026 ± 0.008	0.012 ± 0.001
Miglitol	0.211 ± 0.072	0.230 ± 0.076	1.0 ± 0.1[31]	0.130 ± 0.010	0.148 ± 0.011

2.8 ± 0.2 mM). With sucrose as a substrate, ctSI has a K_m of 38 ± 10 mM.

3.2. Kinetic analysis of inhibitors

The inhibition constant (K_i) for each catalytic subunit with each of the five inhibitors studied was determined using maltose as a substrate (Table 2).

3.2.1. Acarbose

The current antidiabetic compound, acarbose, was found to be a micromolar inhibitor of the both ntMGAM (K_i = 62 ± 13 μM)[29] and ntSI (K_i = 14 ± 1 μM).[29] In contrast, acarbose is a 1000-fold better inhibitor of ctMGAM-N2 (K_i = 0.009 ± 0.002 μM), ctMGAM-N20 (K_i = 0.028 ± 0.005 μM) and 100-fold better for ctSI (K_i = 0.246 ± 0.005 μM). This supports the notion that the tetrasaccharide analogue occupies extended binding subsites in the C-terminal catalytic subunits as compared to the N-terminal domains.[28,29,31]

3.2.2. Salacinol

Salacinol is a submicromolar inhibitor of ntMGAM (K_i = 0.19 ± 0.02 μM)[31] and ntSI (K_i = 0.277 ± 0.068 μM). The inhibition is about fivefold better for ctMGAM-N20 (K_i = 0.058 ± 0.003 μM) and ctSI (K_i = 0.047 ± 0.008 μM). Interestingly, salacinol exhibits some selectivity (fourfold) against ct MGAM-N2 as compared to the N20 splice form.

3.2.3. Blintol

Surprisingly, blintol, the Se analogue of salacinol, shows an improved inhibition of ctMGAM-N2 (K_i = 0.018 ± 0.004 μM), with little change against the other enzymes in comparison to salacinol: ctMGAM-N20 (K_i = 0.013 ± 0.002 μM), and ctSI (K_i = 0.029 ± 0.005 μM), ntMGAM (K_i = 0.49 ± 0.05 μM)[32] and ntSI (K_i = 0.16 ± 0.01 μM).

3.2.4. Kotalanol

Kotalanol generally shows some selectivity (4 to 6-fold) for inhibiting C-terminal enzymes, as compared to N-terminal: ctMGAM-N2 (K_i = 0.064 ± 0.006 μM), ctMGAM-N20 (K_i = 0.092 ± 0.007 μM), and ctSI (K_i = 0.042 ± 0.007 μM) over ntMGAM (K_i = 0.19 ± 0.03 μM) and ntSI (K_i = 0.60 ± 0.06 μM).[29]

3.2.5. De-O-sulfonated kotalanol

De-O-sulfonated kotolanol demonstrates very similar inhibitory action against all five enzymatic subunits, ntSI (K_i = 0.012 ± 0.0006 μM), ctMGAM-N2 (K_i = 0.026 ± 0.004 μM), ctMGAM-N20 (K_i = 0.078 ± 0.009 μM), ctSI (K_i = 0.026 ± 0.008 μM), and ntMGAM (K_i = 0.03 ± 0.01 μM).[31]

3.2.6. Miglitol

Miglitol, another compound currently used as an α-glucosidase inhibitor in patients with Type II Diabetes, is a high micromolar inhibitor with little distinction between the catalytic subunits. With respect to ntMGAM, the K_i value is approximately fourfold higher than the other four enzymes.

Mammals have evolved a system of multiple enzymes for the derivation of glucose from various sources of dietary starch. We hypothesize that the four GH31 enzyme units act in a complementary manner depending on the organism's nutritional sources and requirements, as well as their physical and environmental conditions. In order to test this hypothesis, we are developing compounds that can regulate (toggle) the activities of the enzyme units individually. This report builds the foundation for that approach by identifying differential characteristics of the enzyme units, as reflected in the inhibitory properties of glucosidase inhibitors.

While the results demonstrate a wide range of inhibitory activities, this represents the first report of the profile of the effectiveness of this family of inhibitors against the individual mammalian intestinal glucosidase activities. These inhibitors, as a group, show the highest inhibitory activity reported against MGAM and SI.

The results provide support to the notion that the salacinol/kotalanol family will be useful in deriving compounds that have the ability to inhibit selectively each of the glucosidase enzymes units. Relatively small structural changes in a compound can result in significant changes in its ability to selectively inhibit one enzyme unit over the others. For example, salacinol, itself, is a 4 to 5-fold better inhibitor of ctSI as compared to the N-terminal enzymes and ctMGAM-N2. Replacement of the ring S of salacinol by Se, to make blintol, induces a 10-fold improvement in its inhibition of ntMGAM-N2, while having little effect on the other enzyme units. A similar effect is seen upon de-O-sulfonation of kotalanol which results in a 10-fold change in activity against ntMGAM, with little effect on the other enzyme units.

These observations reflect the different biochemical and structural properties of the enzyme units, despite their similarity in overall amino acid sequence and atomic structure. We hypothesize that both the C-terminal subunits have stronger affinity for longer oligomers, and this is reflected in the results as ctMGAM-N2, ctMGAM-N20, and ctSI are more sensitive to the longer inhibitor acarbose than ntSI and ntMGAM. According to previous studies by Sim et al.[28] involving structural analysis of ntMGAM, subsite mapping with acarbose, and sequence alignments, it is likely that the higher affinity seen in the C-terminal subunit is due to an extended substrate-binding site. The structure of ntMGAM indicates that this subunit lacks +2 and +3 subsites. Further, in ctMGAM there is a substitution of an alanine to a phenylalanine as well as a 21-residue extension near the catalytic site in ctMGAM. We speculate that the different hydrolytic properties of the MGAM and SI catalytic subunits and the observed overlapping substrate specificity aid in accommodating the diverse composition of starches in the human diet. Ultimately, compounds that can independently toggle the activities of each enzyme in turn will open the door to understanding their roles in starch digestion individually and in all possible combinations.

4. Methods

4.1. Inhibitors

Compounds from the salacinol class of inhibitors examined in this paper (Fig. 1) were synthesized by the Pinto laboratory.[33–35] Miglitol was purchased from Toronto Research Chemicals Inc. (North York, ON).

4.2. Kinetic properties of NtMGAM, CtMGAM, NtSI, and CtSI

Kinetic parameters of the four domains were determined using a glucose oxidase assay quantifying the amount of glucose produced upon the addition of enzyme at increasing maltose concentrations (from 0.5 to 30 mM). The methods used for kinetic assays were reported previously.[29,36] All reactions were performed in quadruplicate, and absorbance measurements were averaged to give the final value. The program KaleidaGraph4.1 was used to fit the data to the Michaelis–Menten equation and estimate K_m and V_{max} of the catalytic subunits. K_i values for each inhibitor were determined using the equation $K_i = [I]/(K_{mobs}/K_m) - 1)$. The reported K_i values for each inhibitor were determined by averaging the K_i values from three different inhibitor concentrations.

4.3. Protein preparation

4.3.1. ctSI and ctMGAM

The mouse coding sequences have been reported to GenBank for MGAM (EU073529) and for SI (EU937530). Wild-type Mouse jejunal RNA template was used for RT with SuperScript III/RNaseOUT mix (Invitrogen). The RT primer for SI was Oligo-dT-Adaptor–Primer and the reverse primer for MGAM is shown below. The MGAM PCR forward primer was 2432-*ACAACAGCCTGCCACAACCAC* and reverse primer was 6215-*TCCAACTCCTTCCATCAGCTCTCTC*. The SI forward primer was 2645-*ATCTCACATTCAACCTCGGCAAA* and the reverse primer was 5373-*CCAGCTGATTTGTATTGGTTCATCA* (BCM/CHRC Core Lab). A SnaB I restriction site was inserted at the 5′ end of each SI primer. MGAM possessed unique restriction sites (EcoICR I; Taq I) in the sequence. The GeneAmp XL kit (Applied Biosystems) was used for long PCR. Amplicons of the expected size were separated on 1.5% TAE agarose gel. The bands were isolated and purified using QIAquick gel extraction kit (Qiagen), and ligated into pT7-blue T-vector. The constructs were subsequently transformed into NovaBlue *Escherichia coli* (EMD4Biosciences). Clones were again screened by gene specific PCR and gel electrophoresis and harvested using Plasmid Mini-Prep Kit (Qiagen). After digesting with the restriction enzymes indicated above, (Promega) the clones were selected by predicted size and harvested as described above. DNA was sequenced to select error free inserts (Applied Biosystems, 373A automated sequencer).

Inserts were ligated into pAcGP67 baculovirus vectors (BD Biosciences) and named pAcGP67 His-CtSI and pAcGP67 His-CtMGAM. The pAcGP67 vector was modified to code 10 histidines (His-1) at the 3′ end of expressed proteins. Snab I in SI or EcoICR I in MGAM combined with Bgl I double digestion; gel purification; harvesting and sequencing confirmed the quality of the resulting constructs. The inserts were ligated into dephosphorylated pAcGP67B_His-1 plasmid and the constructs were used to transform NovaBlue Singles (EMD4Biosciences) competent cells. Colonies were screened using: gene specific PCR primers; BamHI/HindIII double digestion; isolation from the gel; harvesting and sequencing to confirm orientation. These pAcGP67 His-CtSI and pAcGP67 His-CtMGAM constructs were separately co-transfected with linearized wild type baculovirus DNA (BD Biosciences) into Sf9 insect cells. Isolates from each co-transfection were obtained by plaque purification. Ten isolates from each co-transfection were analyzed for their ability to produce enzyme by infecting small individual T25 flasks of Sf9 cells with the viral isolates and harvesting the supernatant 120 h post-infection.

Secreted proteins from multiple cultures were screened for maltase enzyme activity captured using anti His-Tag monoclonal antibody coated 96-well plates (EMD4Biosciences) with the glucose oxidase real-time assay described previously.[28] SI-C14 and MGAM-N2 viral isolates were selected based on the screening assay results, amplified to titered 500 ml passage 2 viral stocks, and these passage 2 stock were used to infect High Five insect cells grown in 4 l of Express Five serum free media (Invitrogen) in an Applikon stirred tank bioreactor at a MOI of 1.0 for 48 h. SI-C14 and MGAM-N2 were purified from infected cell culture supernatant on His-Select Nickel Affinity columns (Sigma–Aldrich) by elution with imidazole. Purified recombinant mouse ctSI, ctMGAM-N2, and ctMGAM-N20 were then run on an SDS–PAGE gel (Supplementary data, Figs. 2–4).

4.3.2. ntMGAM and ntSI

Methods for cloning, expression, and purification of human NtMGAM from *Drosophila* S2 cells were previously reported.[28,32] The N-terminal domain of MGAM was cloned into a *Drosophila* pMT-BiP-V5-His vector, permitting secretion of NtMGAM into the culture media with a C-terminal hexa-histidine tag for purification. NtMGAM was then purified by copper-chelating and ion-exchange chromatography.[37]

Cloning, expression, and purification of ntSI from *Drosophila* S2 cells were previously reported.[29] Residues 62–931 of full-length human SI (GenBank™ accession number NP_001032) was cloned into a *Drosophila* pMT-TEVA expression vector[37] allowing for secretion of ntSI into the culture medium with an N-terminal hexa-histidine tag for purification. ntSI was expressed in *Drosophila* S2 cells using a similar expression protocol as for ntMGAM.[28,32]

4.4. Kinetic characterization of C-terminal catalytic subunits

4.4.1. ctMGAM-N2, ctMGAM-N20, and ctSI

Kinetic parameters for ctMGAM-N2, ctMGAM-N20, and ctSI were measured. Kinetic assays with maltose and maltotriose as substrates were performed with ctMGAM-N2 and ctMGAM-N20 and glucose end-products were detected using the glucose oxidase assay. Kinetic parameters for mouse ctSI were determined using maltose, maltotriose, and sucrose, also using the glucose oxidase assay method. The final concentrations of ctMGAM-N2 and ctMGAM-N20 used in the assay were ~0.5 nM; these assays were supplemented with 0.3 mg/mL BSA to maintain enzyme stability and reactions were carried out over 45 min. During this time period, the reactions were linear. The final concentration of ctSI used in the assays were ~1 nM for the maltose/maltotriose assays and ~30 nM for the sucrose assays; these assays were supplemented with 0.1 mg/mL BSA to maintain enzyme stability and reactions were carried out over 45 min (maltose/maltotriose) or 90 min (sucrose) and were linear over this time period.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.033.

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