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Evidence of native starch degradation with human small intestinal maltase-glucoamylase (recombinant)

Zihua Ao[a], Roberto Quezada-Calvillo[b], Lyann Sim[c], Buford L. Nichols[d], David R. Rose[c], Erwin E. Sterchi[e], Bruce R. Hamaker[a,*]

[a] *Whistler Center for Carbohydrate Research and Department of Food Science, 745 Agriculture Mall Drive, Purdue University, West Lafayette, IN 47907-2009, United States*
[b] *CIEP-Facultad de Ciencias Quimicas, Universidad Autonoma de San Luis Potosí, Av. M. Nava 6, Zona Universitaria, San Luis Potosí, S.L.P., 78360, Mexico*
[c] *Division of Cancer Genomics and Proteomics, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 101 College Street, Toronto, ON, Canada M5G 1L7*
[d] *US Department of Agriculture, Agricultural Research Service, Children's Nutrition Research Center and Department of Pediatrics, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030-300, United States*
[e] *Institute of Biochemistry and Molecular Medicine, University of Berne, CH-3012, Switzerland*

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Abstract **Action of human small intestinal brush border carbohydrate digesting enzymes is thought to involve only final hydrolysis reactions of oligosaccharides to monosaccharides. In vitro starch digestibility assays use fungal amyloglucosidase to provide this function. In this study, recombinant N-terminal subunit enzyme of human small intestinal maltase-glucoamylase (rhMGAM-N) was used to explore digestion of native starches from different botanical sources. The susceptibilities to enzyme hydrolysis varied among the starches. The rate and extent of hydrolysis of amylomaize-5 and amylomaize-7 into glucose were greater than for other starches. Such was not observed with fungal amyloglucosidase or pancreatic α-amylase. The degradation of native starch granules showed a surface furrowed pattern in random, radial, or tree-like arrangements that differed substantially from the erosion patterns of amyloglucosidase or α-amylase. The evidence of raw starch granule degradation with rhMGAM-N indicates that pancreatic α-amylase hydrolysis is not a requirement for native starch digestion in the human small intestine.**

Keywords: Human; Small intestine; Maltase-glucoamylase; Starch; Granule; Enzyme

1. Introduction

Starch is one of the major digestible carbohydrates in the human diet and contributes a substantial amount of calories for human metabolism. It is mainly digested to glucose in the small intestine. Undigested or resistant starch is fermented in the large intestine producing short chain fatty acids. Foods made with different starch sources can cause considerable difference in the postprandial rise in blood glucose and insulin response. Numerous in vitro and in vivo studies have demonstrated starchy foods with distinctly different digestion rates [1,2].

Starch is composed of two distinct types of macromolecules, amylose and amylopectin, with molecular weights of 10^4–10^6 Da and 10^7–10^8 Da, respectively [3]. Amylose, a polymer of α-D-glucopyranosyl units mainly linked by the α-1,4 bonds, is defined as a linear molecular chain with minor branching (0.2–0.7%). Amylopectin, a branched polymer of α-D-glucopyranosyl units linked by α-1,4 and α-1,6 linkages, has 4.0–5.5% branching [3]. Amylopectin has a cluster-like organization and forms crystalline regions developed from double helices of linear branched chains. That results in the formation of amorphous and crystalline lamellae arranged in an overall semicrystalline structure. In starch granules at a larger scale, semicrystalline regions alternate with amorphous regions revealing ring-like structures. The molecular structures, crystalline structures, and sizes of native starch granules vary among different botanical sources. Starch is easily digested in the human body after its crystalline structure is destroyed with processing, such as cooking. The digestion of starch to glucose in the human body requires several enzymatic degradation steps. The salivary and pancreatic α-amylases (E.C. 3.2.1.1), which are α-1,4 endo-glucosidases, hydrolyze starch to soluble glucose oligomers with linear and branched structures. Glucose is only a very minor product of the α-amylase digestion of starch [4]. These α-limit dextrins and small linear oligomers are not absorbable into the bloodstream without further hydrolysis to glucose. They, and possibly larger α-glucans, are converted to glucose in the human small intestine by the combined action of mucosal maltase-glucoamylase (MGAM, E.C. 3.2.1.20 and 3.2.1.3, encoded by the gene *MGAM*, located on chromosome 7q34) and sucrase-isomaltase (SIM, E.C. 3.2.1.48 and 3.2.1.10, encoded by the gene *SI*, located on chromosome 3q26) [5,6]. Both MGAM and SIM display α-1,4 exo-glucosidic activity on the non-reducing ends of linear chains of glucose oligomers and polymers with release of free glucose and serve as the final step in small intestinal digestion. The hydrolytic activity on branched α-1,6 linkages shown by SIM

*Corresponding author. Fax: +1 765 494 7953.
E-mail address: hamakerb@purdue.edu (B.R. Hamaker).

Abbreviations: rhMGAM-N, recombinant N-terminal subunit of human small intestinal maltase-glucoamylase; MGAM, maltase-glucoamylase; SIM, sucrase-isomaltase; AMG, amyloglucosidase; SBD, starch binding domain; HPAEC, high-performance anion-exchange chromatography

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is complementary to the α-1,4 of MGAM and SIM. MGAM substrate specificity somewhat overlaps with that of SIM. Both enzymes belong to the glucohydrolyase Family 31; their proteins show 59% amino acid sequence homology. Human MGAM with 1857 amino acids has five distinct protein domains: an N-terminal cytoplasmic tail domain (26 amino acids), a transmembrane domain (anchoring domain, 21 amino acids), an O-glycosylated stalk domain (52 amino acids), and two similar catalytic domains (about 900 amino acids) [5–8]. MGAM and SIM are anchored to the brush border epithelial cells of the small intestine. Each of the enzymes has two subunits with complete conservation of the catalytic residues. Recent research shows that substrate affects MGAM activity. At low oligomer concentrations, MGAM was 10 times more active than SIM, but at high concentrations MGAM experienced substrate inhibition while SIM was not affected [9].

It has been hypothesized that human mucosal maltase-glucoamylase exoenzyme activity is an alternate pathway for starch digestion when luminal salivary and pancreatic α-amylase activity is inhibited or reduced because of immaturity and malnutrition [5]. In these situations, MGAM plays a unique role in the digestion of α-glucans contained in foods and beverages. Published kinetic studies of MGAM are based on the reaction of the enzyme and soluble starch (partially hydrolyzed or pregelatinized starch (soluble starch) or maltodextrins with low molecular weight) [6,10]. Our reason for the present study originated with a finding that rhMGAM-N can hydrolyze starch chains without pretreatment using alpha-amylase. It was thought for a long time that the human MGAM can only hydrolyze malto-oligosaccharides with low molecular weights. Also, there was no indication that crystalline structures could be digested by MGAM. We were interested to investigate how human MGAM digests these structures and begin to understand more fully the enzyme action of MGAM.

In the study reported here, 10 native starches were selected from different botanical sources based on their physicochemical properties and commercial importance. Kinetic studies of MGAM-N on the native starches were monitored by the formation of glucose at different incubation times, and compared with fungal amyloglucosidase (AMG, glucoamylase). AMG is widely used for in vitro starch digestion evaluation [1]. AMG not only consecutively hydrolyzes α-1,4 linkages, but also hydrolyzes α-1,6 linkages to produce D-glucose from non-reducing ends of starch and glycogen. We have recently found (data not shown) that rhMGAM-N and AMG displays distinctly different enzyme activities on soluble substrates, and this led us to examine digestion of native starch granules that are considered to be indigestible by MGAM. The objective of this research was to determine the susceptibility of the starches towards MGAM and to establish their patterns of digestion. The study may lead to a better understanding of the wide differences among starchy foodstuffs with different postprandial glycemic and insulinemic indices.

2. Materials and methods

2.1. Starch sources

Normal maize and tapioca starches were gifts from Tate and Lyle (Decatur, IL). Waxy maize and rice starches were purchased from Sigma Chemical Co. (St. Louis, MO). Amylomaize-5 (high-amylose maize starch with 50% of amylose) was obtained from Cargill, Inc. (Cedar Rapids, IA). Wheat starch was obtained from MGP Ingredients, Inc. (Atchinson, KS). Potato starch was obtained from Penford Food Ingredients Co. (Englewood, CO). Amylomaize-7 (high-amylose maize starch with 70% of amylose) was a gift from National Starch and Chemical Co. (Bridgewater, NJ). Banana starch was a gift from Dr. Roy Whistler (Purdue University). Pea starch was isolated from lentils purchased from a local market following the procedure of Hoover and Sosulski with slight modification [11].

Starches were purified using the toluene–water procedure [12]. Purified starches were washed with water and ethanol, and recovered by filtration using Whatman No. 4 filter paper before drying in a hood at room temperature for 48 h.

2.2. Enzymes

Production of rhMGAM-N was performed as described previously [7]. Briefly, rhMGAM-N was expressed and secreted using a DES expression system and Schneider S2 cells. The recombinant pMT-Bip-MGAnt-His6 vector was transfected into S2 cells in combination with the pCoBLAST selection vector containing a blasticidin resistance cassette under the control of the Drosophila copia promoter. Recombinant protein secretion was induced in transiently transfected cells with 10 μmol/L $CdCl_2$ and the cell medium assayed for rhMGAM-N expression by SDS–PAGE and immunoblotting with anti-pentaHis antibodies. Stably transfected cells were obtained by selection in enriched medium containing 16 μg/ml blasticidin (Invitrogen). The resulting cells were grown in enriched medium mixed with non-transfected S2 cells (to serve as a feeder layer) in a 96 well tissue culture plate and selected for their ability to hydrolyze pNP-glucose. Cell clones expressing rhMGAM-N were adapted to Ex-Cell 420 Insect Serum Free Media (JRH Biosciences, Lenexa, KS, USA) and then scaled up to 3200 ml in shaker flasks inducing the secretion of protein with 2 μmol/L $CdCl_2$ for 3 days. rhMGAM-N was purified from the media by chelating chromatography in a Sepharose resin (GE Healthcare, Montreal, Quebec, Canada), washed with 20 mmol/L Tris pH 8.5 containing 300 mmol/L NaCl, and the recombinant protein was eluted step-wise with 2, 6, 10, 20, 30, and 50 mmol/L imidazole. Eluted fractions containing rhMGAM-N were identified by SDS–PAGE and pNP-glucose hydrolytic activity, pooled, concentrated, and dialyzed against 20 mmol/L Tris pH 8.5 containing 100 mmol/L NaCl. Anion exchange chromatography was used to further purify rhMGAM-N in a BioCAD Poros-HQ anion exchange column (PerSeptive Biosystems, Framingham, MA, USA) at pH 7.0. The column was washed with 100 mmol/L BIS-Tris Propane pH 7.0, and then the protein was eluted with a linear gradient of 0–1 mol/L NaCl. Fractions containing pure, active rhMGAM-N were pooled, dialyzed against 10 mmol/L phosphates buffer containing 150 mmol/L NaCl and 2.5 mmol/L EDTA, and the protein concentration adjusted to 1 mg/ml.

Amyloglucosidase (AMG) from *Rhizopus* sp. (electrophoretically homogeneous, freeze-dried powder) was obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland). Human pancreatic α-amylase was obtained in pure-grade from Meridian Life Science, Inc. (Saco, Maine).

The activity of rhMGAM-N was determined using maltose (50 mmol/L) as substrate in 10 mmol/L phosphate buffer with 150 mmol/L sodium chloride at 37 °C, pH 7.0. The glucose released was determined using the glucose oxidase-peroxidase assay. One unit of activity is defined as the amount of the enzyme that released 1 μmol of glucose per min. The activity of amyloglucosidase from *Rhizopus* sp. was determined using maltose (50 mmol/L) as substrate in 0.1 mol/L sodium acetate buffer at 37 °C, pH 4.5. The glucose released was analyzed based on the same method for rhMGAM-N. One unit of activity is defined as the amount of the enzyme that released 1 μmol of glucose per min. The activity of human pancreatic α-amylase was determined using soluble starch (1%) as substrate in 10 mmol/L phosphate buffer with 150 mmol/L sodium chloride at 37 °C, pH 7.0. The amount of reducing sugar formed was determined by the Somogy–Nelson method [13]. One unit of the activity is defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent glucose per min.

2.3. Native starches hydrolyzed with rhMGAM-N and AMG

For rhMGAM-N hydrolysis, starch granules (5 mg) were suspended in 0.1 ml of 10 mmol/L phosphate buffer (pH 7.0) with 150 mmol/L sodium chloride and 0.02% sodium azide. rhMGAM-N (1.44 U per 100 mg starch) was added to the starch slurry and incubated at

37 °C for 48 h with rotation. For AMG hydrolysis, the starch suspensions were prepared with 0.1 mol/L sodium acetate buffer (pH 4.5) with 0.02% of sodium azide. AMG from *Rhizopus* sp. (1.60 U per 100 mg starch) was used for the starch digestion.

The suspensions were centrifuged at 1000 rpm for 1 min in a microcentrifuge at 4, 8, 12, 24 and 48 h, respectively. Supernatant (10 μl) was taken at each time interval and mixed with 20 μl of sodium hydroxide (0.1 mol/L), and then kept at −20 °C for glucose content analysis. The residue after 48 h incubation was washed twice with ethanol, and then dried at 37 °C. The dried samples were used for scanning electron microscopy analysis.

2.4. Human pancreatic α-amylase hydrolysis of native starches

Suspensions of normal corn, amylomaize-5 and amylomaize-7 were prepared as for rhMGAM hydrolysis in Section 2.3. Human pancreatic α-amylase (0.04 units) was added to the starch slurry and incubated at 37 °C for 48 h with rotation. The dried samples, prepared as in Section 2.3, were used for scanning electron microscopy analysis.

2.5. Human pancreatic α-amylase and rhMGAM-N hydrolysis of native starches

Starch granules (5 mg) were suspended in 0.05 ml of 10 mmol/L phosphate buffer (pH 7.0) with 150 mmol/L sodium chloride and 0.02% sodium azide. Human pancreatic α-amylase (0.04 U or 4 U) was added to the starch slurry and incubated at 37 °C for 2 h. Then another 0.05 ml of phosphate buffer (10 mmol/L, pH 7.0) with 2.5 mmol/L EDTA, 150 mmol/L sodium chloride and 0.02% sodium azide was added to the starch slurry, followed by adding rhMGAM-N (1.44 U per 100 mg starch). The incubation was further carried out at 37 °C for 8 or 48 h with rotation. Glucose content was analyzed at different time intervals on supernatants as prepared in Section 2.3.

2.6. Measurement of glucose released from native starches hydrolyzed with rhMGAM-N and AMG

Glucose contents in hydrolyzed starch suspensions were determined by using high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD) (Dionex, Sunnyvale, CA). A PA-100 anion-exchange analytical column and a guard column (Dionex, Sunnyvale, CA, USA) with an eluent of 100 mmol/L sodium hydroxide were used for sample separation. A linear regression equation was setup with different glucose concentrations (2.5, 5, 10, 20, 40, 60 μg/ml) and corresponding peak areas.

2.7. Scanning electron microscopy of starch granules

Starch samples were analyzed on a scanning electron microscope (JEOL JSM-840, Tokyo, Japan) at the Life Science Microscopy Facility, Purdue University. Samples were mounted on SEM stubs with double-sided adhesive tape. The specimens were coated with gold-palladium alloy and images taken at 5 kV accelerating voltage.

3. Results

3.1. Degradation of native starch by rhMGAM-N

The production of glucose from native starches hydrolyzed with rhMGAM-N, and determined using HPAEC, is shown in Fig. 1. The contamination of free glucose from native starches was negligible ($0.6–2.2 \times 10^{-3}$ μg/mg native starch) since the starches from all sources were again purified in our laboratory. Upon addition of rhMGAM-N to the native granules, amylomaize-5 starch displayed the most rapid and highest extent of degradation compared to other starches, followed by amylomaize-7 starch. Other starches tested were much less digested to glucose. This was a surprising finding since high-amylose maize starches are generally considered as resistant starches, both in vivo and in vitro [1,2]. At the initial stage of hydrolysis (up to 4 h), wheat and amylomaize-7 starches displayed similar rates, and were greater than other starches, except amylomaize-5. The extent of hydrolysis among starches at

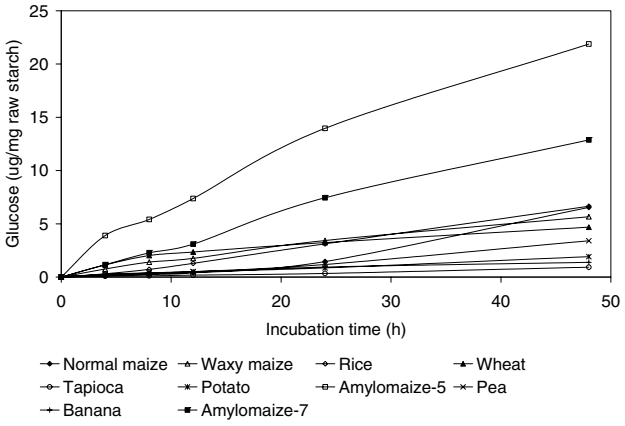

Fig. 1. Kinetics of rhMGAM-N hydrolysis of native starch granules.

4 h followed the order: amylomaize-5 > amylomaize-7 > wheat > waxy maize > rice > potato > pea > banana > normal maize > tapioca. The same trends were found for hydrolyzed starches at 8 and 12 h. Based on the hydrolysis extent of native starch at 48 h, starch could be classified into three general groups (from highest to lowest conversion): amylomaize-5 and amylomaize-7 as the first group; rice, waxy maize, normal maize, and wheat starches as the second group; and pea, potato, banana, and tapioca starches as the third group. There was a linear production of glucose from the native starches. Generally, rhMGAM-N produced relatively low glucose yields over the entire digestion period. Maltodextrins were hydrolyzed at a much greater rate than native starches (data not shown), indicating that formation of enzyme–substrate complexes is more favorable for soluble substrates. The relatively low digestion rate of native starches is likely in part due to the inaccessibility of non-reducing ends to the catalytic site of rhMGAM-N.

3.2. Degradation of native starch by AMG

For purpose of comparison, hydrolysis of native starches was carried out using AMG with results shown in Fig. 2. Enzyme activities of rhMGAM-N and AMG were measured using both soluble starch and maltose as substrates at their optimum pH's. For soluble starch, AMG was 524 times more active than rhMGAM-N, however for maltose, rhMGAM-N was about 4.5 times more active. In these experiments,

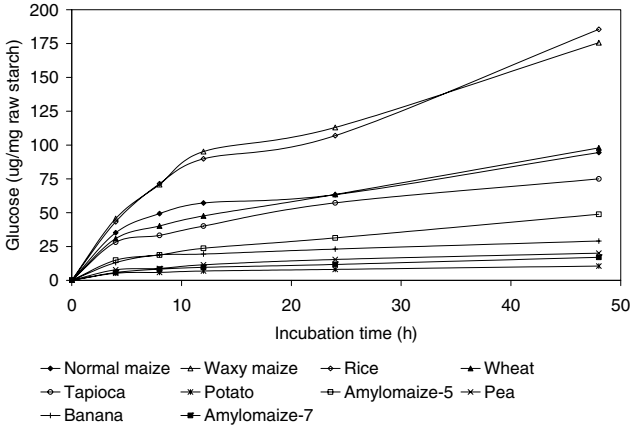

Fig. 2. Kinetics of AMG hydrolysis of native starch granules.

activities based on maltose were approximately the same. Relative rankings of hydrolysis of starches were different from rhMGAM-N. Using AMG, waxy maize and rice starches gave the highest production of glucose after 4 h incubation (45.7 and 43.2 μg per mg starch), whereas potato starch showed the lowest rate among the native starches (5.2 μg per mg starch). Starches from wheat, normal maize, and tapioca revealed slower hydrolysis rates compared to rice and waxy maize, but showed a relatively higher glucose production than amylomaize-5, banana, lentil pea, amylomaize-7, and potato starches. According to their susceptibility to AMG hydrolysis and the rate of glucose production, the starches may be classified into three groups as well: the first most susceptible group being rice and waxy maize starches; the intermediate as wheat, normal maize, and tapioca starches; and the least susceptible group as amylomaize-5, banana, lentil pea, amylomaize-7, and potato starches. These results agree with the report of Kimura and Robyt [14]. The authors also found that the starches displayed similar hydrolysis trends in three enzyme concentrations (1×, 10×, and 100×).

3.3. Synergistic effect of native starch degradation with human α-amylase and rhMGAM-N

To elucidate the effect of α-amylase on the degradation of native starch with rhMGAM-N, the starches were first hydrolyzed by human α-amylase for 2 h followed by incubating with rhMGAM for 8 h, and glucose yields were determined by HPAEC (Fig. 3). The profiles show that pretreatment of native starch granules with the α-amylase greatly facilitates glucose production by the action of rhMGAM-N. After adding rhMGAM-N, glucose yields from all tested starches at 30 min substantially increased. At 4 h, the extent of hydrolysis among starch followed the order: wheat ≫ rice > amylomaize-7 > waxy maize = normal maize > amylomaize-5 > tapioca > pea > banana > potato. Based on glucose production at 2, 4, and 8 h, the starches may be classified into three groups: the first group composed of wheat and rice with much higher hydrolysis extent; amylomaize-7, waxy maize, maize, amylomaize-5, and tapioca as the second group; and pea, potato, and banana as the third group. Tapioca was no longer the least digestible starch as with rhMGAM-N alone; and pea, potato, and banana starches still displayed low digestion rates (Figs. 1 and 3). In general, glucose yields were greatly improved by pretreatment with human α-amylase. Amylomaize-5 and amylomaize-7 appeared to be less resistant than native tapioca, pea, banana, and potato starches. Glucose yield from amylomaize-7 was slightly higher than for amylomaize-5. Glucose production from wheat starch at 30 min and 8 h was 5.1 and 5.9 times that of potato starch, respectively. Each starch displayed a different extent of amplification of hydrolysis rate by human α-amylase. For example, glucose release from tapioca starch at 8 h (Fig. 3) was 454-fold that of the starch hydrolyzed with rhMGAM-N alone at the same incubation time (Fig. 1), whereas, glucose release of amylomaize-5 and amylomaize-7 at 8 h was only 10.9-fold and 26.2-fold (Figs. 1 and 3). The data indicate that different native starches have varied susceptibility to α-amylase.

To further elucidate the effect of α-amylase on glucose production from native starch granules hydrolyzed with rhMGAM-N, three starches (normal maize, amylomaize-7, and pea) were pretreated with 100-fold the α-amylase units used above and followed by rhMGAM-N treatment. At the initial hydrolysis stage (2 h), all starches displayed similar degradation rates (Fig. 4). However, after 4 h incubation, glucose production and hydrolysis rate of normal corn starch were greater than pea and amylomaize-7 starches. At 48 h, raw starches converted into glucose were 73.0%, 66.8%, and 36.3% for normal maize, pea, and amylomaize-7, respectively. The hydrolysis order for these three starches with the high amount of α-amylase treatment differed from the order from the starches treated with lower concentration of the enzyme (Figs. 3 and 4).

3.4. Morphology of native and hydrolyzed starch granules

Scanning electron micrographs of native normal maize, potato, and amylomaize-5 and -7 starches and these samples after hydrolysis by rhMGAM-N are presented in Fig. 5. As a comparison, normal maize, amylomaize-5, and amylomaize-7 starches treated with human pancreatic α-amylase or AMG are shown in Fig. 6. Morphologies of the partially hydrolyzed starch granules residues showed remarkable differences between treatments of rhMGAM-N, human α-amylase, and AMG (Figs. 5 and 6).

Unusual degradation patterns of rhMGAM-N on starch granules were observed. The degradation of normal maize and potato starches showed a surface furrowed pattern in ran-

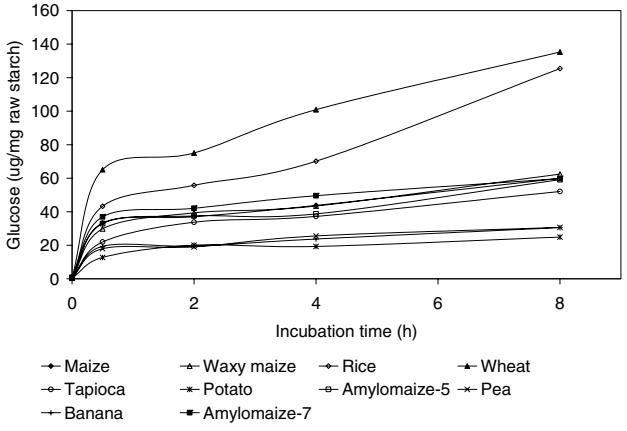

Fig. 3. Profiles of native starch granules hydrolyzed using rhMGAM-N after pretreatment with human α-amylase (0.04 U/5 mg starch).

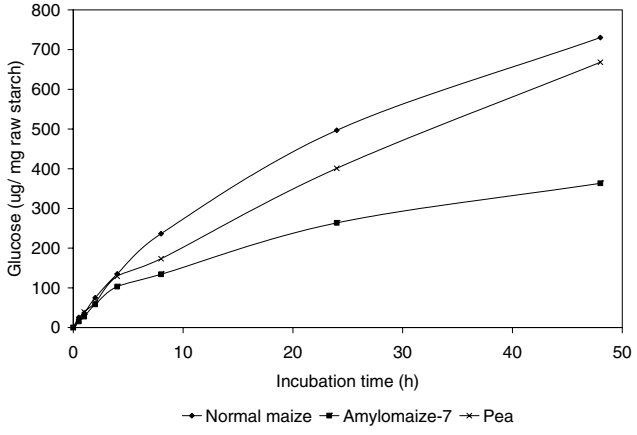

Fig. 4. Profiles of normal maize, amylomaize-7, and pea starch granules hydrolyzed using rhMGAM-N after pretreatment with human α-amylase (4 U/5 mg starch).

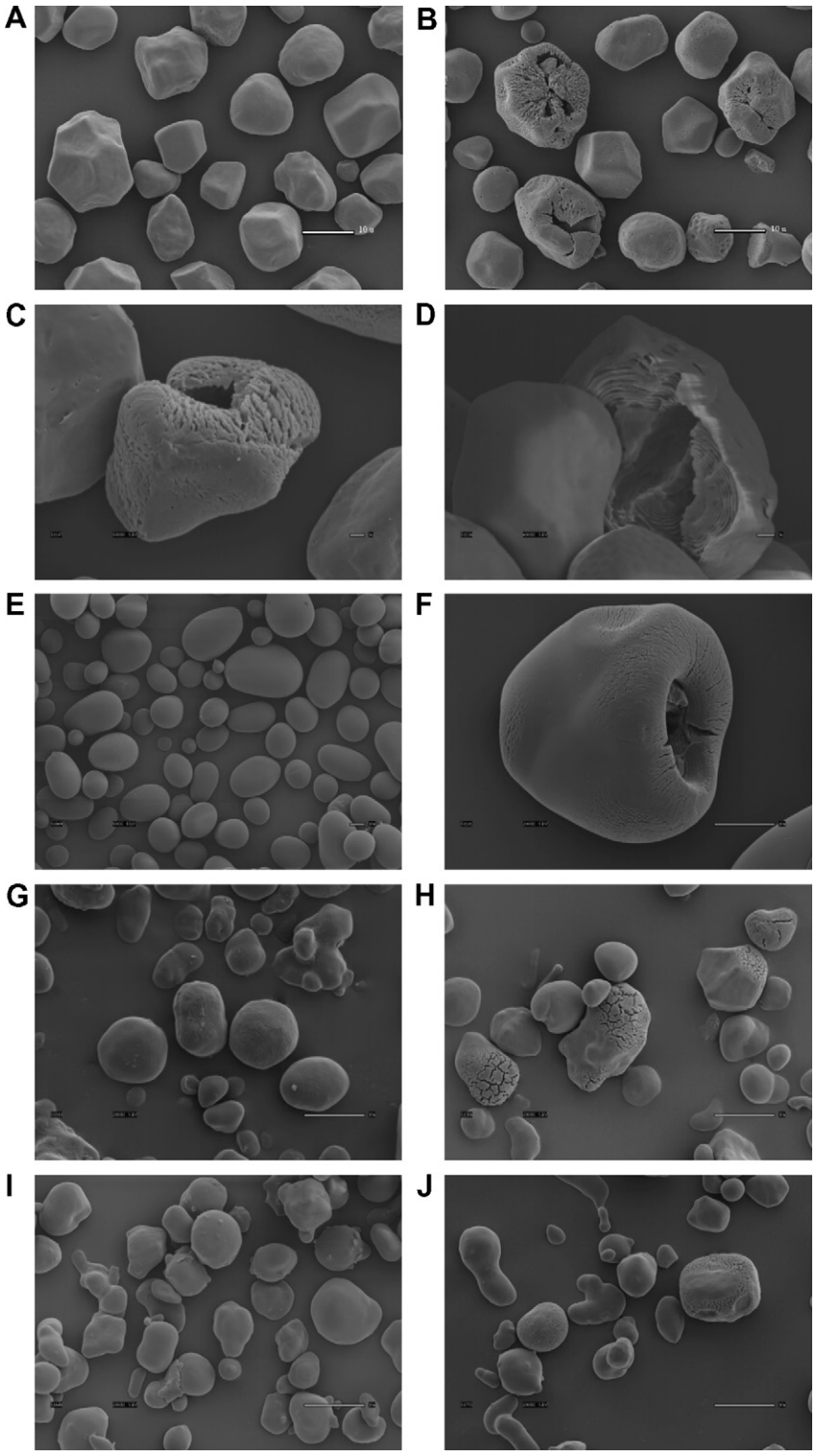

Fig. 5. Scanning electron micrographs of normal maize, potato, amylomaize-5, and amylomaize-7 starches: (A) Normal maize starch control (2000×); (B) (2000×), (C) (5000×), (D) (6000×), rhMGAM-N-treated normal maize starch; (E) potato starch control (500×); (F) rhMGAM-N-treated potato starch (2000×); (G) amylomaize-5 starch control (2000×); (H) rhMGAM-N-treated amylomaize-5 starch (2000×); (I) amylomaize-7 starch control (2000×); and (J) rhMGAM-N-treated amylomaize-7 starch (2000×).

dom, radial, or tree-like arrangements (Fig. 5B, C, F, H and J). Numerous cracks or erosion channels were found on granule surfaces. Hydrolysis appeared to occur from the inside-out in some granules indicating that the internal amorphous lamella of starch granules is hydrolyzed first (Fig. 5D). The remaining shell structure, representing the peripheral regions of hydrolyzed normal maize starch granules, showed this region to be more resistant to rhMGAM-N hydrolysis. Native potato

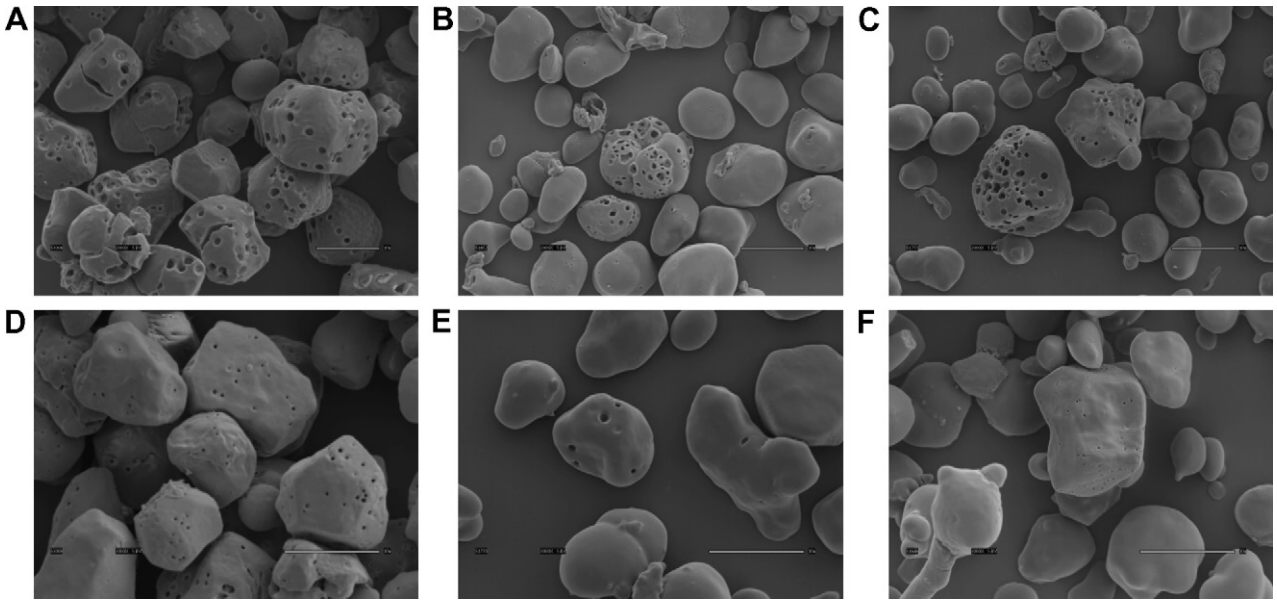

Fig. 6. Scanning electron micrographs of starches hydrolyzed with human α-amylase or amyloglucosidase from *Rhizopus* sp. Starches hydrolyzed by human α-amylase: (A) normal maize (2000×), (B) amylomaize-5 (2000×), (C) amylomaize-7 (2000×). Starches hydrolyzed by amyloglucosidase: (D) normal maize (3000×), (E) amylomaize-5 (3000×), (F) amylomaize-7 (3000×).

starch granule surfaces are smooth, and even after 48 h incubation, the major population of granules was still intact with only few showing digestion patterns (Fig. 5F). The least rhMGAM-N susceptible sample, tapioca starch, in the third group (Fig. 1) did not show visible change in morphology (data not shown).

α-Amylase is an endo-glucosidase that hydrolyzes α-1,4 glucosidic linkages and produces oligomers or α-limit dextrins. AMG, on the other hand, hydrolyzes α-1,4 glucosidic linkages from non-reducing ends of starch chains and produces glucose. It can also hydrolyze α-1,6 linkages in starch at a slower rate. α-Amylase and AMG-treated normal maize starch showed randomly distributed pinholes, differing substantially from digestion patterns of rhMGAM-N-treated normal maize starch (Figs. 5A–D and 6A,D). The holes in α-amylase-treated starches were deeper and wider than using AMG. AMG and α-amylase initially adsorb onto the surface of normal maize granules, and then penetrate through existing channels into the granule interior. α-Amylase and AMG acted differently than rhMGAM-N on amylomaize-5 and amylomaize-7 starches showing different patterns and varying, non-uniform susceptibility among starch granules (Fig. 6). The extent of digestion by α-amylase was visibly higher in normal maize starch with more pronounced eroded areas, contrary to an increase in number of pinholes with AMG.

4. Discussion

This is the first report showing that MGAM can hydrolyze native starch granules. It has generally been thought that human glucosidases, such as MGAM and SIM, act on small dextrins produced by α-amylase digestion, and not on undigested larger starch chains [15]. Additionally, it is clear in this study that raw starches from different botanical sources display remarkably different susceptibilities to the two glucoamylases, human small intestinal rhMGAM-N and fungal AMG. For example among a range of native starch types, resistant starches amylomaize-5 and -7 were the most easily hydrolyzed by rhMGAM-N and were among the least hydrolyzed by AMG. While human α-amylase was not required for native starch digestion, it both increased digestion rate and changed susceptibility of the different test starches to rhMGAM-N.

A comparison of activity levels of rhMGAM-N, AMG, and α-amylase used to digest raw starches showed that rhMGAM-N has the lowest hydrolysis rate of intact starch granules, yet digestion still proceeded to degradation of some granules to near completion or to thin shell remnants. Hydrolysis of starches with rhMGAM-N occurred granule by granule showing that the enzyme action was not uniform amongst the granule population. The same occurred with AMG and α-amylase. This contrasts with acid hydrolysis which attacks starch granules evenly with degradation occurring in amorphous regions [16].

Starch granules hydrolyzed by rhMGAM-N showed distinct surface furrowed patterns in random, radial, or tree-like arrangements (Fig. 5). This digestion pattern was uniquely different from digestion of native granules by either AMG or α-amylase which showed typical pitting and enlarged holes (Fig. 6). These differences in visual patterns of digestion are likely related to different actions of rhMGAM-N on raw starch substrates compared to the fungal glucoamylase, AMG (Figs. 1 and 2).

Pretreatment of starch granules with α-amylase resulted in an expected amplification of rhMGAM-N on glucose production and changed the ranking of hydrolysis of the samples (Figs. 3 and 4). Oligosaccharides resulting from the action of α-amylase on native starches are good substrates for MGAM-N and contributed to the large increase in glucose at the initial hydrolysis stage (Figs. 3 and 4). The extent of amplification of overall starch digestion due to α-amylase var-

ied with starches, a consequence of starch fine structures that also lead to different granule organizations and degrees of crystallinity.

Regarding a mechanism or action pattern of rhMGAM-N on native starches, the amino-acid sequence of MGAM has failed to reveal the presence of a classical starch binding domain (SBD) with similarity to those found in fungal glucoamylases [17]. This may be a distinctive feature of proteins belonging to family 31 glucohydrolases since none of the enzymes of this family with known amino-acid sequences, including SIM or the *Escherichia coli* Yic putative proteins, appear to contain known binding domains. This type of domain is found in about 100 amino acid stretches in the vicinity of the catalytic site and contains conserved hydrophobic residues, generally Trp, assumed to be involved in the binding process of the enzyme molecules to the lineal α-1,4 chains of starch molecules. The binding process enhances the efficiency with which the active site performs its hydrolytic function. The lack of SBD in rhMGAM-N likely accounts for its lower amylolytic activity compared to AMG that contains a SBD domain. The observed activity of rhMGAM-N apparently is related to its ability to attach at the terminal non-reducing residues of starch molecules.

Molecular structural characters of the starches, and their resulting crystalline and granular structures, are responsible for the observed difference in the hydrolysis rates among samples tested. The differential molecular organization of granules is related to the proportion of amylose and amylopectin, chain-lengths and branching patterns of amylopectin, and crystallinity [18,19]. SEM observation of the less susceptible structures emerging from layered internal regions of normal maize granules (Fig. 5D) indicated that amorphous regions are more susceptible to rhMGAM-N than crystalline regions. It should be noted that rhMGAM-N can hydrolyze α-1,6 linkages to some extent [5], which may allow for its action on crystalline regions. In terms of mode of action on the macrostructure, rhMGAM-N may penetrate into microcracks, attack in the many crevices and then disrupt the crystallites. Therefore, the degradation results in an observed deepening of grooves.

In summary, based on the kinetic profiles of native starch degradation and observations by SEM, results demonstrate that rhMGAM-N can hydrolyze native starch granules. rhMGAM-N hydrolyzes both at the granule surface and, when pores and channels are present, within the granules from the inside to out. Unexpectedly, high-amylose maize starches, which are considered as resistant starch from the action of α-amylases and amyloglucosidase, showed a higher glucose yield with rhMGAM-N than other starches. The results showed that pancreatic α-amylase is not an absolute requirement for native starch degradation in the human small intestine, though certainly it dramatically increases hydrolysis rate to glucose in combination with MGAM. It is known that native starch can be hydrolyzed to oligosaccharides by pancreatic α-amylase and, based on our finding that rhMGAM-N shows comparably poor hydrolysis of native starch, α-amylase is the amplifying factor in human enzymatic hydrolysis of native starch. Since clinical data show that the high-amylose maize resistant starches are resistant in the small intestine [2], they are likely resistant to pancreatic α-amylase, even though they (amylomaize-5 and -7) were the most susceptible of the starches tested to rhMGAM-N.

Lastly, the above findings should be helpful in developing a better methodology to evaluate the digestibility of starchy foods in the human body. Currently, AMG is widely used for analysis of starch digestibility which, in the present study, had distinctly different properties from human small intestinal MGAM. Use of human enzymes gives the promise of finding starches that have special structures with slow digestion or resistant properties for health benefit.

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