Enzymatic modification of corn starch influences human fecal fermentation profiles

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ABSTRACT

Enzymatically modified starches have been widely used in food applications to develop new products but information regarding digestion and fecal fermentation of these products is sparse. The objective of this study was to determine the fermentation properties of corn starch modified with α -amylase, amyloglucosidase or cyclodextrin glycosyltransferase and the possible role of hydrolysis products. Samples differed in their digestibility and availability to be fermented by the microbiota, resulting in differences in microbial metabolites produced during *in vitro* fermentation. The presence or absence of hydrolysis products and gelatinization affected starch composition and subsequent metabolite production by the microbiota. Amyloglucosidase-treated starch led to the greatest production of short and branched chain fatty acid production by the microbiota. Results from this study could be taken into consideration to confirm the possible nutritional claims and potential health benefits of these starches as raw ingredients for food development.

KEYWORDS: Corn starch, enzymatic modification, short chain fatty acids, digestion, fermentation

INTRODUCTION

Enzymes play important roles in many industrial applications, and can be considered processing aids to improve food quality attributes. Enzymes are widely use in the cereal food industry (reviewed in Rosell and Dura).¹ In these products, starch modification by enzymes provides food ingredients that could not be produced by chemical or physical methods.² Various enzyme modifications have been attempted to modify intrinsic properties of starch, increase water holding capacity or heat resistant behavior, minimize syneresis, improve thickening, and to achieve overall desired functionality of end products.³ Hydrolases like α -amylases (AM) and amyloglucosidases (AMG) have been extensively used as replacements of acid hydrolysis in the production of starch hydrolysates.⁴ Another attractive opportunity is the production of porous starches by enzymatic treatments to extend their absorption capacity. Initially, porous starch was obtained by the joint action of AM and AMG on corn starch.² These changes in the starch structure by the individual action of AM or AMG were also observed by Dura et al.,⁵ increasing the susceptibility of the starch to be digested and modifying hydration properties and pasting parameters. Depending on the type and level of hydrolytic enzyme, it is possible to modulate the number and size of the pores on the surface of the granule, and therefore their adsorption capacity.⁶ Further studies have shown that enzymatic modification with cyclodextrin glycosyltransferase (CGTase) modifies pasting behavior in a pH dependent manner.⁷ Starch modification by CGTase seemed to hydrolyze the amorphous part of the starch granule, which led to small pores on the surface, with a simultaneous release of oligosaccharides and production of cyclodextrins. Other studies have reported that enzymatic modification of starches alters chain length, branch density and crystalline structure.⁸⁻⁹ Those studies confirmed the morphological

changes of the porous starches, but no information exists about the impact of those changes on their digestion performance.

Diverse studies have pointed out the relevance of structural changes in food digestibility. For instance, *in vitro* starch digestion that simulates stomach and small intestinal conditions revealed that the increase in starch branch density and the crystalline structure in treated starches contributed to slow digestion.¹⁰ An increase in resistant starch was obtained when altering the crystallinity of the starch by treatment with α -amylase and pullulanase.¹¹ In addition, *in vitro* fecal fermentation coupled to *in vitro* digestion can provide information about how dietary components, such as resistant starch, are metabolized by gut bacteria. *In vitro* fermentation tests provide an insightful opportunity to study the function of the microbiota, i.e., to determine how the microbiota utilize certain substrates, which is difficult to do *in vivo* in humans because of the complexity of diets.¹² Furthermore, *in vitro* fermentations allow for quantitative measurement of metabolites produced by the microbiota upon fermentation of specific substrates,¹³⁻¹⁴ which have importance in the health of the host.¹⁵⁻¹⁸ The most important end products of fermentation are short chain fatty acid (SCFA), principally acetate, propionate, and butyrate, and branch chain fatty acid (BCFA) that are then absorbed and metabolized by the host. SCFA are produced mainly from carbohydrate fermentation and have health benefits, whereas BCFA are a result of proteolytic fermentation and may have deleterious effects.¹⁹ *In vitro* fermentation and SCFA production have been useful to estimate the impact of dietary carbohydrates on human health.²⁰⁻²³ However, while several studies have reported on the fermentation properties of resistant starches, no studies have reported on the susceptibility of structurally modified starches to be fermented by gut bacteria.²⁴ The objective of this research was to determine the impact of structural modification of starch granules on the *in vitro* digestion and fermentation. For that

purpose different enzymatically modified starches have been used to get a deeper understanding on how diverse porous corn starches and microbes interact with gastrointestinal microbiota and the types of metabolites that are produced upon fermentation.

MATERIALS AND METHODS

Materials and reagents

Corn starch samples were purchased from Daesang Corporation (Korea). Fungal α -amylase (AM, EC 3.2.1.1) (Fungamyl® 2500SG, declared activity 2500 FAU/g product), amyloglucosidase (AMG, EC 3.2.1.3) (amyloglucosidase 1100 L, declared activity 1100 AGU/g product) and cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) from *Thermoanaerobacter* sp (Toruzyme® 3.0 L, declared activity 3KNU/mL product) were kindly provided by Novozyme (Bagsværd, Denmark). Chemical reagents from Sigma-Aldrich (Madrid, Spain) were of analytical grade.

Methods

Samples preparation

Corn starch (10.0 g) was suspended in 50 mL of 20 mM sodium acetate buffer at pH 4.0 or in sodium phosphate buffer at pH 6.0, for enzymatic modification with AMG or AM and CGTase, respectively.^{5,7} Enzymes (4 U of AMG/g starch, 5 U of AM/g starch or 0.32 U of CGTase/g starch) were then added to the starch suspension and suspensions were kept in a shaking water bath (50 rpm) at 50 °C for 48 h. Water (50 mL) was then added to the suspensions and the mixture was homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA Works, Inc. Wilmington, DE, USA) for 1 min at 14,000 rpm. Samples were centrifuged for 15 min at 7,000×g and 4 °C. The starch pellets were washed twice with 50 mL of water and centrifuged

again using the same conditions. Supernatants were pooled and placed in a boiling water bath for 10 min to inactivate enzymes.

For each enzyme, two enzymatically treated corn starches were prepared, the washed enzymatically treated corn starch (AMG-W; AM-W; CGT-W) and the other one containing the hydrolysis products (AMG-NW; AM-NW; CGT-NW) that were added back to assess the role of the water soluble hydrolysis products. Samples were freeze-dried and kept at 4 °C for further analyses.

Samples were also analyzed in the gelatinized (G) and non-gelatinized (NG) forms. To obtain gelatinized samples, treated and untreated corn starch samples (10 g) were suspended in 40 mL of distilled water and incubated 15 min at 100 °C. Samples were then freeze-dried and kept at 4 °C for further analyses. Native corn starch (N) was used as the control sample. Two batches were prepared for each sample.

***In vitro* digestion**

The digestion process consisted of a simulated gastric digestion followed by a small intestinal phase (Figure 1), according to the method described by Yang et al.²⁵ Briefly, samples (25 g) were suspended in 300 mL distilled water. Eight mL of 1 M HCl was added to the sample to reduce the pH to 2.5. Ten milliliters of 100 mg/mL freshly prepared pepsin (P-700; Sigma, St. Louis, MO, USA) dissolved in 50 mM HCl was added and the mixture was placed on an orbital shaker at 250 rpm (MaxQ 7000, 150 rpm; Barnstead, Dubuque, IA, USA) at 37 °C for 30 min to achieve the gastric phase. The small intestinal phase was initiated with the addition of 50 mL of 0.1 M sodium maleate buffer (pH= 6, containing 1 mM CaCl₂) and 20 mL of 1 M NaHCO₃ to bring the pH to 6.9. Fifty milliliters of 125 mg/mL pancreatin (P-7545, Sigma) dissolved in the sodium maleate buffer and 6500 U of amyloglucosidase (0.56 g of 11600U/g; A7255, Sigma)

were then added and samples were incubated in a shaking water bath at 37 °C for 6 h. Digested contents were then poured into dialysis tubing (molecular weight cutoff 12,000-14,000) and dialyzed for three days against distilled water with changing of the water every 2-3 h. The retentate was then frozen (-20 °C) overnight and then freeze dried. Total starch content was determined before and after the *in vitro* digestion following AACC approved method 76-13.01 16 using a kit (K-TSTA, Megazyme, Bray, Ireland). Digestion assays were run in triplicate.

***In vitro* fermentation**

In vitro fecal fermentation was performed according to the method described by Arcila et al.²⁶ with minor modifications (Figure 1). In short, 15 mg of digested, freeze-dried material was suspended in 1 mL of sterile fermentation medium consisting of (per liter) peptone (2 g, BP1420-100; Fisher Scientific, Pittsburgh, PA, USA), yeast extract (2 g; CAS8013-01-2; Alfa Aesar, Ward Hill, MA, USA), bile salts (0.5 g; S71919-1; Fisher Science; Hannover Park, IL, USA), NaHCO₃ (2 g), NaCl (0.1 g), K₂HPO₄ (0.08 g), MgSO₄·7H₂O (0.01 g), CaCl₂·6H₂O (0.01 g), L-cysteine hydrochloride (0.5 g; CAS52-89-1; Sigma), hemin (50 mg; CAS16009-13-5; Sigma), Tween 80 (2 mL; P8074), vitamin K (10 µL; 84-80-0; Sigma), and 0.025% (w/v) resazurin solution (4 mL; CAS62758-13-8; Sigma), hydrated and placed it in the anaerobic hood overnight.

Fresh fecal samples were collected from three healthy individuals with no record of gastrointestinal abnormalities or antibiotic administration in the last 6 months. The fecal slurry was prepared by blending each fecal sample separately with sterile phosphate buffered saline in the ratio of 1:9 (w/v) using a hand blender for 1 min and then were filtered through four layers of cheesecloth. Tubes were then inoculated with 0.1 mL of fecal slurry, capped, placed at a 45 ° angle, and incubated at 37 °C with shaking (MaxQ 7000, 140 rpm). Samples were taken at

specific time points (0, 4, 8, 12 and 24 h) stopping the reaction with 0.4 mL of NaOH 7.5 M mixed with SCFA standard (7 mM 2-ethylbutyric acid). Samples were then transferred to a freezer (-80 °C) until analysis. Total starch content was analyzed after the *in vitro* fecal fermentation as described above. Three replicates were carried out to obtain the average.

Short chain fatty acid determination

Samples for SCFA were quantified by gas chromatography (Clarus 580, PerkinElmer, MA, USA) equipped with a capillary column (Elite-FFAP, 15m × 0.25mm inner diameter × 0.25 µm film thickness, PerkinElmer) and detected with a flame ionization detector, according to Arcila et al.²⁶ SCFA were quantified by calculating response factors for each SCFA relative to 2-ethylbutyric acid using injections of pure standards.

Data analysis

All treatments were performed in triplicate. Compositional data were analyzed using a three-factor (enzyme, washed/unwashed, gelatinization) ANOVA, including the two-way and three-way interactions, using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When ANOVA indicated significant *F* values ($P < 0.05$), multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence. The correlation matrix was also calculated using Statgraphics Centurion XV software. All data were reported on a wet weight basis.

For SCFA analysis, the repeated measure of time was also included in the ANOVA model. For detecting significant effects a four factor ANOVA with interaction was performed. Significant differences among samples were determined using Bonferroni's least significant difference (LSD) test with $\alpha = 0.05$. Data were analyzed using SAS software (version 9.2, SAS Institute, Cary, NC USA).

RESULTS AND DISCUSSION

In vitro digestion of enzymatically treated starches

Modified starches were *in vitro* digested and the impact of gelatinization was also assessed. The starch content was determined before and after the *in vitro* digestion (Figure 2). Significant differences ($P < 0.05$) were found among samples regarding digested starch for non-gelatinized samples (Figure 2A) and gelatinized samples (Figure 2B). When comparing treated starches in granular form, it was observed that the enzymatic modification influenced the action of digestive enzymes. The CGTase-NW sample showed the highest digestibility among all samples (Figure 2A). The presence of hydrolysis products influenced the quantity of starch digested. Although no significant differences were found, lower amount of starch was digested in the samples containing hydrolysis products (AM-NW, AMG-NW), which was expected due to product inhibition. This trend was not observed in the case of CGTase-NW starch, likely due to the low amount of oligosaccharides released during starch treatment⁶, which induced higher digestion of the starch granules. The digestion of this CGTase-NW starch was even higher than the native starch, which could be attributed to the presence of very small surface pores that increase the starch susceptibility to be digested.²⁴ Among washed samples, digestibility of AMG-W starch was higher than CGTase-W, which was ascribed to the large surface holes that are present in this type of porous starch.⁵ AMG, AM and CGTase are all hydrolytic enzymes that affect the amorphous and crystalline domains of starch and increased the exposure of the granule to subsequent digestion.^{5,7}

Differences in the digestion of the starches were intensified after gelatinization (Figure 2B). CGTase-NW sample gave the highest percentage of digestible starch among all samples. The gelatinization process encompassed the rearrangement of the starch granules and loss of

crystallinity, transforming the starch granule into a more amorphous structure and favoring enzymes access to the starch chains.²⁷ Therefore, gelatinization even augmented the susceptibility of CGTase-NW to be digested. Again, non-washed samples (AM-NW, AMG-NW) containing the hydrolysis products displayed lower digestion than their washed counterparts. AMG treated samples underwent higher digestion than those treated with AM. Previous studies showed that AMG action on the starch granules was greater than that induced by AM,⁵ affecting the crystalline structure, which in consequence favored the chain disentanglement during gelatinization and its further hydrolysis by digestive enzymes.

***In vitro* fecal fermentation and SCFA production**

Microbial metabolites produced during fecal fermentation of the modified corn starch samples was affected by several of the treatment variables and their interactions (Table 1). The enzymatic treatment and the granule state (gelatinized or non-gelatinized) affected the production of BCFA and there was also an interaction between those factors. Only the enzymatic treatment provoked a significant effect on the production of propionate. The amount of starch remaining after fecal fermentation was significantly affected by the interaction among enzymatic treatment, hydrolysis products, and gelatinization. In addition, significant effects were observed at specific times of the fermentation process. Total SCFA, acetate and propionate exhibited a rapid increase during the first eight hours of fermentation, and thereafter slowed (Figure 3). Total SCFA were found to be significantly different at the end of the fermentation, showing lower values for those samples treated with AM or CGTase compared with the untreated sample and that treated with AMG. Propionate production showed significant differences from 12 h of fermentation: AMG treated sample resulted in higher propionate production compared with the other samples. Likewise, the

198 AMG-treated sample resulted in significantly more butyrate production compared with the other
 199 samples.
 200 SCFA production, especially butyrate production, is dependent on the presence of fermentable
 201 carbohydrate substrate. The higher SCFA production in the AMG-treated sample suggested that
 202 AMG treatment of starch resulted in more microbial available carbohydrates during
 203 fermentation. Given that Western diets are generally lacking in microbial available
 204 carbohydrates,²⁸ AMG-treated starches may provide an important source of energy for the
 205 microbiota. An adequate carbohydrate supply promotes the growth of beneficial, saccharolytic
 206 members of the microbiota, such as *Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*
 207 that otherwise are outcompeted by more resilient but less beneficial genera such as *Alistipes*,
 208 *Bilophila* and *Bacteroides*.²⁹ *Roseburia* and *Eubacterium rectale* especially are important
 209 butyrate producers in the gut microbiota.³⁰
 210
 211 Butyric acid rich fermentations are generally observed with some types of starch that escape
 212 digestion in the small intestine.³¹ A recent study conducted by Dan et al.³² showed that *in vivo*
 213 colonic fermentation of unripe banana starch resulted in high butyrate production when
 214 compared to the control diet, showing also a good correlation between *in vitro* and *in vivo*
 215 fermentation. Butyrate, in particular, has been targeted as a beneficial SCFA due to its role in the
 216 maintenance of colonic homeostasis and anti-inflammatory properties.³³⁻³⁴
 217 Propionate fermentation has been reported with diets containing soluble substrates.³⁵
 218 Overall, results indicated that AMG-treated starch favored the simultaneous production of
 219 relatively larger quantities of propionic and butyric acids compared with the other starches.
 220 During starch modification, AMG was greatly active breaking the degree of association between

glucose molecules. Likewise, considering that AMG hydrolyses α ,1-4 bond approximately 500 times faster than the α ,1-6 linkage,³⁶ it would be right to suppose that the resulting AMG-treated starches would have a highly branched structure and very accessible to fermentation due to its perforated structure.⁶ Its saccharifying activity resulted in more accessible structure for starch digestion and more apparent starch available for fermentation.³⁷⁻³⁸ Furthermore, AM and CGTase were not as active as expected in hydrolyzing glucose linkages, leading less perforated starch granules. These structural features could explain the higher production of SCFA due to higher fermentable starch when modified with AMG.

The fermentation plots of BCFA released were significantly different for non-gelatinized and gelatinized samples (Figure 4), as indicated the statistical analysis (Table 2). The release of BCFA was rather low during the initial 12 hours of fermentation. Only at longer fermentation time a significant amount of these fatty acids were detected, being higher when samples were previously gelatinized (Figure 4). Regarding the effect of enzymes, AMG treated and native starch, in their non-gelatinized state, showed higher amounts of BCFA (Figure 4A). For gelatinized samples (Figure 4B) at 24 h of fermentation, only a significantly high value was found for the AMG treated starch. Again, results indicated higher metabolic activity in the AMG-treated starches, which agrees with their highest hydrolyzed structure previously mentioned. Moreover, its likely high branched structure would lead higher amount of BCFA.

In contrast to SCFA, BCFA have been associated with undesirable protein fermentation, leading to many metabolites that are potentially toxic to the host and associated with carcinogenic effects in the colon.³⁹ However, in batch *in vitro* fermentation systems such as that employed in this study, high BCFA production can also result from higher total metabolic activity of the microbiota.²² Because AMG also resulted in high butyrate production, which arises from

carbohydrate fermentation and not protein fermentation, it appeared that the high BCFA arising from the AMG-treated starch was due to higher metabolic activity and not due to lack of carbohydrate substrate.

Hence, modifications induced by enzymatic treatment of starch granules had a significant effect on *in vitro* digestion and fermentation pattern. The presence of short chain accessible saccharides besides the starch granules significantly affected the digestion stage, reducing starch digestion. CGTase treated starch was the most hydrolyzed by digestive enzymes in the presence of the sugars released by the enzyme. Conversely, only the structural shape of treated starches affected the fecal fermentation stage, thus the susceptibility of the gut microbiota concerning the balance between SCFA and BCFA production. AMG enzymatically modified starch resulted in enhanced total SCFA production and greater polysaccharide utilization by human fecal microbiota. These results indicate that modification by enzymes of the native starch was accomplished and the possibility to obtain changes in the starch structure leading to a higher butyrate and propionate production improving gut health. Future studies should determine the utilization impact of enzymatically modified samples by AMG as an ingredient in food formulations.

ABBREVIATIONS

AM-NW: unwashed enzymatically modified sample with α -amylase

AM-W: washed enzymatically modified sample with α -amylase

AMG-NW: unwashed enzymatically modified sample with amyloglucosidase

AMG-W: washed enzymatically modified sample with amyloglucosidase

BCFA: Branch chain fatty acid

267 CGT-NW: unwashed enzymatically modified sample with cyclodextrin glycosyltransferase

268 CGT-W: washed enzymatically modified sample with cyclodextrin glycosyltransferase

269 G: gelatinized

270 N: Native corn starch

271 NG: non-gelatinized

272 SCFA: Short chain fatty acid

273 **ACKNOWLEDGEMENT**

274 A. Dura would like to thank predoctoral fellowship from Spanish Ministry of Economy and
275 Competitiveness. This research was performed in part at the Department of Food Science &
276 Technology, University of Nebraska-Lincoln, Lincoln, NE, USA.

277 **Funding**

278 Authors acknowledge the financial support of the Spanish Ministry of Economy and
279 Competitiveness (AGL2014-52928-C2-1-R) and the European Regional Development Fund
280 (FEDER).

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Figure captions

Fig 1 Flow diagram for *in vitro* digestion and fermentation of starch samples.

Fig 2 Digested starch content of enzymatically modified samples with (W) or without hydrolysis products (NW). For non-gelatinized (A) and gelatinized (B) samples. N (native), CGTase (Cyclodextrin glycosyltransferase), AM (α -amylase) and AMG (amyloglucosidase). (*) Denotes significant differences ($P < 0.05$).

Fig 3 Total short chain fatty acids and individual SCFA (butyrate, propionate and acetate) produced during fermentation of enzymatically modified samples that had been subjected to *in vitro* digestion. Values pointed with (*) denote significantly different levels ($P < 0.05$). Native ($\circ$); CGTase ($\blacksquare$); AM ($\blacklozenge$); AMG ($\blacktriangle$).

Fig 4 Total branch chain fatty acids (BCFA) produced during fermentation of enzymatically modified samples without hydrolysis products, that had been subjected to *in vitro* digestion previously not gelatinized (A) or gelatinized (B). Values pointed with (*) denote significantly different levels ($P < 0.05$). Native ($\circ$); CGTase ($\blacksquare$); AM ($\blacklozenge$); AMG ($\blacktriangle$).

Table 1. Effects Interaction of Type of Enzyme, Washed/Unwashed and Gelatinization in Fecal Fermentation Metabolites and Total Starch after Fecal Fermentation^a.

Effect ^a	BCFA	Acetate	Propionate	Butyrate	SCFA	Starch
Washed	8.21	0.83	3.37	3.11	0.31	130**
Gelatinized	19.6*	0.26	12.0	0.11	0.29	119**
Washed*Gelatinized	4.26	2.39	0.52	0.00	0.70	13.3
Enzyme	33.2***	2.31	27.7***	1.64	4.26	203***
Washed*Enzyme	4.80	0.07	0.53	3.09	1.91	47.8**
Gelatinized*Enzyme	19.04**	3.50	4.65	0.79	1.94	8.32*
Washed*Gelatinized*Enzyme	1.81	0.14	3.06	0.03	0.04	10.6*
Time	20.72***	296***	141***	29.1***	193***	329***
Washed*Time	1.91	0.13	1.61	2.83	1.86	2.44
Gelatinized*Time	6.83*	1.84	1.74	0.17	0.49	11**
Washed*Gelatinized*Time	0.89	1.57	0.54	0.10	0.84	1.19
Enzyme*Time	12.08***	1.37	5.16***	2.01	2.19*	3.35**
Washed*Enzyme*Time	1.50	1.45	3.53*	3.05*	3.96**	3*
Gelatinized*Enzyme*Time	6.22***	0.47	0.62	0.48	0.46	2.28*
Washed*Gelatinized*Enzyme*Time	0.90	0.60	1.09	0.02	0.21	0.83

^a Values followed by $P<0.05$ (*) $P<0.01$ (**) $P<0.001$ (***)

Figure 1

Figure 2

A.

B.

Figure 3

Figure 4

A.

B.

TOC Graphic