Short communication: In vitro ruminal fermentability of a modified corn cultivar expressing a thermotolerant α -amylase

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ABSTRACT

The fermentability of a corn cultivar that expresses a thermostable α -amylase (CA3272) was evaluated under various in vitro conditions. The CA3272 corn was developed as a replacement to microbial enzyme additions during the high-temperature processing of corn to produce ethanol. The α -amylase activity in the corn might have the potential for positive effects on ruminant performance if incorporated into the ration. Four corn cultivars were evaluated in an in vitro ruminal fermentation where the digestion of starch was measured after 6 h. The cultivars included a flint corn, an opaque corn, CA3272, and its near-isogenic counterpart (IC). The flint corn produced less total volatile fatty acids (18.4 mM) than the other 3 corns (average of 25.3 mM), supporting the fact that it had the highest concentration of prolamins, which are negatively associated with starch availability. A second 6-h in vitro ruminal fermentation evaluated mixtures of the CA3272 and IC corns (0, 25, 50, 75, and 100% concentrations of CA3272). Total volatile fatty acid production was not different among treatments for any proportions of CA3272. In a third in vitro experiment, there was a small but significant difference in starch degradation of CA3272 compared with IC (90.6 vs. 89.7%) but this difference is most likely not biologically relevant. In a fourth in vitro experiment, CA3272 and IC were incubated in water at 40 and 65°C for 24 h. Degradation of starch from native amylase activity at 40°C was 1.99 and 1.60% for CA3272 and IC, respectively, but when they were incubated at 65°C, starch degradation was 10.56 and 0.85% for CA3272 and IC, respectively. These data demonstrate that amylase activity in CA3272 is expressed at a high temperature (65°C) but at the physiological temperature expected in a rumen of a cow (39–40°C), expression of amylase

activity does not appear to be sufficient to have any positive (or negative) effects on ruminal metabolism.

Key words: corn, amylase, starch, rumen

The majority of ethanol in the United States is produced from corn (*Zea mays* L.) through wet- or dry-milling processes. In both cases, starches in the corn are enzymatically hydrolyzed to sugars that are fed into fermenters containing yeasts for conversion into ethanol (Vermerris et al., 2007). Currently, α -amylases produced from microbial fermentations are used commercially in the starch processing. Syngenta Biotechnology Inc. (Research Triangle Park, NC) has developed a thermostable α -amylase enzyme (**AMY797E**) expressed in a corn cultivar (**CA3272**) with the ability to resist high temperatures associated with dry-grind ethanol production. The CA3272 cultivar could serve as the source of α -amylase enzyme in the dry-grind ethanol process, replacing the need for addition of microbial-produced enzymes. The enzyme AMY797E is functionally similar to the thermostable, genetically engineered α -amylases (e.g., *Bacillus* spp.) currently used and has a history of safe use in food and feed processing (Pariza and Johnson, 2001; Olempska-Beer et al., 2006).

To date, there have been no reported studies evaluating the potential of CA3272 as a feedstock for ruminants. Therefore, the objective of this study was to investigate the influence of genotype; that is, the genetically modified corn CA3272 and its near-isogenic corn counterpart (**IC**) on the digestion characteristics by ruminal microorganisms under in vitro conditions.

In experiment 1, the fermentation potential of various corn hybrids on production of VFA from in vitro ruminal fermentation was evaluated. The hybrids included a flint and an opaque corn (described by Blasel et al., 2006) and CA3272 and IC corns (Syngenta Biotechnology Inc.). Samples were analyzed by the Soil and Forage Analysis Laboratory, University of Wisconsin-Department of Soil Sciences (Madison, WI) for DM, CP, ash, and fat (AOAC, 1995). Samples were also analyzed for soluble protein (Krishnamoorthy et al., 1983), starch (Ehrman, 1996), amylase-treated NDF (**aNDF**; Van Soest et al., 1991), baseline shift (Hoffman et al.,

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Table 1. Characteristics of corn grains used in the study

Item	Corn hybrid ¹			
	CA3272	IC	Flint	Opaque
DM, %	93.46	93.35	93.58	94.23
CP, % of DM	9.28	9.29	12.44	12.64
Soluble CP, % of CP	14.33	13.34	14.14	26.50
Prolamin, % of DM	4.69	4.39	6.19	2.17
Baseline shift ²	4.4818	3.6398	5.2550	2.8758
Starch, % of DM	69.59	64.91	63.80	65.89
aNDF, ³ % of DM	8.99	12.64	11.75	12.52
Fat, % of DM	2.74	2.75	3.92	3.58
Ash, % of DM	1.29	1.20	1.47	1.71

¹CA3272 = corn with a thermostable α -amylase gene; IC = a near-isogenic line of CA3272 corn; Flint = a normal flinty corn; Opaque = a normal opaque corn.

²Near-infrared spectroscopy baseline shift (1040–1140 nm).

³Amylase-treated NDF.

2010), and prolamin content (Larson and Hoffman, 2008). The chemical composition and characteristics of the corns used are shown in Table 1. All grains were ground through an Udy mill (2-mm screen; Cyclone Sample Mill, Udy Corp., Fort Collins, CO). Two hundred fifty milligrams of corn sample was weighed into replicate 60-mL serum bottles ($n = 6$ per corn sample). Ruminal fluid was collected approximately 3 h post-feeding from a ruminally fistulated Holstein steer fed a TMR consisting of about 45% concentrate and 55% forage on a DM basis. The diet was formulated to meet or exceed the dietary requirements for a cow weighing 650 kg and producing 35 kg of milk/d (NRC, 2001). Ruminal fluid was maintained at 40°C under anaerobic conditions before use. The fluid and contents were strained through 8 layers of cheesecloth under anaerobic conditions. Within 45 min of sampling, 12 mL of strained ruminal fluid and 18 mL of prewarmed (40°C) buffer solution (Kung and Hession, 1995) were added to 60-mL serum bottles. Bottles without corn were used as blanks. Bottles containing all experimental ingredients were immediately gassed for 10 s with anaerobic-grade CO₂ and crimp sealed with butyl rubber-lined stoppers. Bottles were incubated in a water bath maintained at 40°C. Initial samples of ruminal fluid and buffer were sampled at time 0, and all experimental bottles were sampled after 6 h of incubation (the fermentation was stopped immediately by addition of 0.1 mL of 50% H₂SO₄). Once the fermentations were stopped, 5 mL of sample was acidified with 25% meta-phosphoric acid (1 part of acid to 5 parts of sample) and centrifuged at $10,000 \times g$ for 10 min before being analyzed for VFA by gas chromatography (model 5890, Series II; Agilent Technologies Inc., Santa Clara, CA) with a 530- μ m Carbowax 20M column and flame-ionization detector. The chromatograph oven was programmed as follows: 70°C for 1 min, 5°C increase/min to 100°C,

45°C increase/min to 170°C, and a final holding time of 5 min. Production of total VFA was calculated by subtracting the initial concentrations of total VFA from the final concentrations of total VFA after 6 h of incubation. Molar proportions of the VFA were calculated by dividing the concentration of individual acids by the total concentrations of VFA. Minor acids (isobutyric, butyric, isovaleric, and valeric) are not shown but were included in the calculation of total VFA.

In experiment 2, the in vitro VFA production potential of CA3272 and IC were evaluated by incubating a set of mixtures of CA3272 and IC in rumen fluid and buffer for 6 h. The mixtures were developed by mixing 100, 75, 50, 25, and 0% CA3272 with 0, 25, 50, 75, and 100% IC on a DM basis, respectively. The experimental methods were the same as described for experiment 1.

In experiment 3, the in vitro ruminal starch degradability of CA3272 and IC was directly assessed. Samples of CA3272 and IC were ground as described in experiment 1, but via a 1-mm screen. Samples were analyzed in 5 replicates for starch content, using the amyloglucosidase/ α -amylase method (total starch assay kit, Megazyme International, Wicklow, Ireland). Starch content was determined based on the measured glucose concentration and expressed as a percentage of DM. In vitro starch degradability procedures were as described previously with the following exceptions. Ruminal fluid was strained through 8 layers of cheesecloth into a 500-mL bottle gassed with CO₂ and placed in a 40°C water bath for 20 min. Settled and floating materials were discarded after separation. Six 60-mL serum bottles containing 12 mL of this strained and separated ruminal fluid and 18 mL of buffer solution were prepared for each sample. Moreover, fermentation was stopped after 6 h by addition of 10 mL of cold 0.2 M acetate buffer (pH 4.5) and samples were stored at –20°C until further analysis. Starch digestion was

calculated by subtracting residual starch at time 6 h from the starting concentration of starch in the tube at time 0.

In experiment 4, the native amylase activity in CA3272 and IC corn was evaluated when the corns were incubated in water at various temperatures. One hundred milligrams of CA3272 and IC (ground via a 1-mm screen) were weighed into tubes for each of 5 replicate tubes per grain. Four milliliters of distilled water was added to each tube and all treatments were incubated at 40 and 65°C for 24 h. Glucose contents of samples in the tubes at time 0 and 24 h after incubation in water were measured and starch was determined as described in experiment 3.

Data were analyzed using the GLM procedure of SAS (SAS Institute, 2004). The treatment effect in experiments 1, 2, and 3 was tested with the model

$$Y_i = \mu + T_i + e_i,$$

where μ = overall mean; T_i = effect of treatment (in experiment 1, treatment was corn hybrid including CA3272, IC, flint, and opaque corns, $i = 1, 2, 3, 4$; in experiment 2, treatment was CA3272, IC, and their combination of various proportions, $i = 1, 2, 3, 4, 5$; in experiment 3, treatment was CA3272 and IC, $i = 1, 2$); and e_i = error term. The multiple comparisons among treatment means in experiments 1 and 2 were conducted using Tukey's test. The effects of corn hybrid and incubating temperature, and their interaction in experiment 4 were tested with the model

$$Y_i = \mu + C_i + A_j + (C \times A)_{ij} + e_{ij},$$

where μ = overall mean; C_i = effect of corn genotype ($i = 1, 2$); A_j = effect of temperature ($j = 1, 2$); $(C \times A)_{ij}$ = effect of interaction of corn genotype and tem-

perature; and e_{ij} = error term. Significance for all 4 experiments was defined as $P < 0.05$.

The effects of in vitro ruminal fermentation of various corn hybrids on VFA profiles in experiment 1 are shown in Table 2. Production of total VFA was greatest from opaque corn. Production of total VFA from CA3272 was similar to that from IC and both were intermediate to the total VFA production of the opaque and flint corns. Production of acetic acid from CA3272 was reduced compared with that from IC ($P < 0.05$), but no difference in production of propionic acid was observed between CA3272 and IC. The molar ratio of acetic to propionic acid was reduced for CA3272 compared with that from IC ($P < 0.05$). In experiment 2, there were no significant differences in VFA production between various mixtures of CA3272 and IC (data not shown), which was consistent with those of experiment 1. The production of VFA was used as an indicator of total starch fermentability because starch is the primary fermentable component in corn that would yield VFA. Sveinbjornsson et al. (2007) found that the amount of VFA produced after an 8-h in vitro incubation was closely correlated to the amount of starch degraded. The flint and opaque corns were included in experiment 1 as references to access in vitro starch fermentability via the production of VFA. The endosperm texture of the grain appears to play an important role in ruminal VFA production and starch degradation (Philippeau et al., 1999). The flint corn used in our study had a higher concentration of prolamins and greater baseline shift than the opaque corn and thus it had lower ruminal starch degradation than the opaque and other corns. The CA3272 and IC corns had intermediate amounts of prolamins and thus VFA production was between that of the flint and opaque corns. Overall, results suggest that the amylase enzyme in CA3272 had little effect on starch degradation as measured by VFA production under in vitro ruminal conditions.

Table 2. Production and profiles of VFA from corn hybrids incubated in ruminal fluid and buffer (40°C) for 6 h (experiment 1)

Item	Corn hybrid ¹				SEM
	CA3272	IC	Flint	Opaque	
Total VFA, ² mM	23.28 ^b	25.52 ^{ab}	18.37 ^c	27.18 ^a	0.74
Acetic acid, mM	12.99 ^b	14.82 ^a	10.57 ^c	16.18 ^a	0.48
Propionic acid, mM	7.54 ^b	7.62 ^b	6.18 ^c	8.79 ^a	0.22
Acetic acid, molar %	0.56 ^b	0.58 ^{ab}	0.58 ^{ab}	0.60 ^a	0.008
Propionic acid, molar %	0.32 ^a	0.30 ^b	0.34 ^a	0.32 ^a	0.004
Ratio of acetic to propionic acid	1.72 ^c	1.95 ^a	1.71 ^c	1.84 ^b	0.028

^{a-c}Means within rows with unlike superscripts differ ($P < 0.05$).

¹CA3272 = corn with a thermostable α -amylase gene; IC = a near-isogenic line of CA3272 corn; Flint = a normal flinty corn; Opaque = a normal opaque corn.

²Isobutyric, butyric, isovaleric, and valeric acids were not reported but were included in the calculation of total VFA.

Table 3. Starch degradability of CA3272 and IC corn hybrids¹ incubated in water at 40 and 65°C (Temp.) for 24 h (experiment 4)

Item	40°C		65°C		SEM	Effect ($P < $)		
	CA3272	IC	CA3272	IC		Corn	Temp.	Corn $\times$ temp.
Starch degradability	1.99	1.60	10.56	0.85	0.08	<0.01	<0.01	<0.01

¹CA3272 = corn with a thermostable α -amylase gene; IC = a near-isogenic line of CA3272 corn.

A third experiment was conducted to directly measure potential differences in ruminal in vitro starch degradation between CA3272 and IC. A single time point determination of starch degradation in cereal grain has been employed and proved feasible in an in vitro system to evaluate starch disappearance (Wester et al., 1992; Richards et al., 1995). We chose a 6-h incubation time because it was used successfully to show differences in in vitro starch digestion in a previous study from our laboratory (Klingerman et al., 2009). In the present study we found that in vitro degradation of starch from CA3272 after 6 h was greater than that from IC, even though the actual difference was very small and probably not biologically relevant (90.6 vs. 89.7%, $P < 0.01$; data not shown).

The degradation of starch by amylase activity in CA3272 and IC in water at 40 and 65°C for 24 h (experiment 4) is shown in Table 3. Differences existed in degradation of starch between corn hybrids (CA3272 vs. IC; $P < 0.01$) and between incubation temperatures (40 vs. 65°C; $P < 0.01$). The significant interaction effect between corn hybrid and incubation temperature ($P < 0.01$) was due to the relatively small difference in degradation of starch at 40°C and the large increase in degradation of starch with the CA3272 at 65°C. This difference in temperature-dependent enzyme activity was expected because this enzyme was developed to have maximum activity at the high temperatures utilized in the corn to ethanol production process. This temperature-dependent activation readily explains why the enzyme has no effect or a very limited effect at rumen physiologic temperatures in our experiments. Because the expression of the amylase activity in CA3272 is dependent on a high temperature, feeding CA3272 to ruminants would not appear to be beneficial and we observed no detrimental effects of having this activity in corn.

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