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Effect of glucoamylase enzyme extract on *in vitro* gas production and degradability of two diets with 25% of corn or sorghum grains

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ABSTRACT

The objective of this study was to evaluate the effect of glucoamylase enzyme (GEZ) extract on the *in vitro* ruminal gas production (GP) and degradability of 2 total mixed rations (TMR) of 25% of corn and other of 25% of sorghum grains. The 2 diets were treated with 0, 1.5 and 3 g of GEZ protein (65% of protein) per kg of grain in diet. Diets GP were measured at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of incubation. Incubations were stopped after 72 h where pH was measured and supernatant was filtered to determine *in vitro* dry matter (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) degradabilities. Addition of GEZ to corn diet had no effect on kinetics of GP, whereas GEZ added to sorghum diet, at the high dose of the enzyme (3 g/kg DM), was traduced by an increase of the rhythm of GP (*c*) and the volume of GP at 2, 4 and 6 h of incubation. Likewise, effect of GEZ was not affected either on the DMD or cell wall (NDFD and ADFD) of both diets (sorghum or corn). Irrespective to enzyme supply, kinetics of GP and pattern of degradation of corn were generally higher than those of sorghum. A net effect of the diet and the interactions between diet and enzyme were recorded for the volume of GP at different incubation times. The use of high doses of GEZ should be tested on the pattern rumen fermentation.

Key words: Corn, Glucoamylase, *In vitro* fermentation, Sorghum

The optimal use of starch is fundamental in improving performance of ruminants fed high grain diets (Huntington 1997, Rojo *et al.* 2000). Many strategies were developed to increase starch digestion rate and grain energetic value such as ground, dry rolled and steamed, and harvest of grains with high moisture content (Owens *et al.* 1997). Amylolytic enzymes in the rumen are extracellular or cell-bound (Thurn and Kotarsky 1987), and the extracellular enzymes are the most important in the group of amylolytic bacteria (Cotta 1988). Amylases are present in protozoa (Mendoza *et al.* 1993 1995) and ruminal fungi (Yanke *et al.* 1993). Exogenous amylolytic enzymes are obtained from controlled fermentation of bacteria or fungi (Declerk *et al.* 1997) and they are used in the food industry for starch hydrolysis (Reilly 1985).

In Mexico, sorghum and corn are the major grains used in the cattle feeding (Mendoza and Ricalde1993). Based on the low ruminal degradability of grains, which is estimated to be only 50% (Britton and Stock 1986), many studies revealed the necessity to use exogenous amylolytic enzymes to increase ruminal starch digestion (Rojo *et al.* 2000*,* Gutiérrez *et al.* 2005) and to improve performance of ruminants fed on grain based diets (Rojo *et al.* 2001a*,* Mora *et al.* 2002*,* Buendía *et al.* 2003). Studies carried out *in vitro* (Mendoza *et al.* 1998) and *in situ* (Rojo *et al.* 2005) recorded an increase of 20 and 10%, respectively, in ruminal degradation of starch, and supposed that such response is attributed to the ruminal conditions (temperature and pH) favorable to a synergetic action between exogenous amylases and enzymes produced by ruminal microorganisms. Even though improvement of feed efficiency due to exogenous amylolytic enzymes was only shown in few experiments (Rojo *et al.* 2005) yet use of amylolytic enzymes as a treatment for grain or food additive in ruminants has been given little attention (Frumholtz and Beauchemin 1999*,* Kung 1999).

The enzyme dose is considered to be one of the major factors that can modify the response of animals fed on grain based diet. Therefore, the objective of this study was to characterize the GEZ dose response in kinetics of gas production (GP) and pattern of *in vitro* ruminal fermentation of total mixed rations with either 25% corn or 25% sorghum.

MATERIALS AND METHODS

Substrate and treatments: Samples of 2 total mixed rations, of 25% corn and other of 25% of sorghum grains were prepared using ingredients (Table 1) collected from the state of Mexico in Mexico. Samples of diets were dried at 60ºC for 48 h in a forced air oven to constant weight, ground in a Wiley mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical

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composition and *in vitro* GP. The two diets were treated with 0, 1.5 and 3 g of enzyme protein (65% of protein) / kg of grain in diet as exogenous commercial enzymes of glucoamylase (GNZ) was produced from *Aspergillus niger*, in liquid form.

In vitro *incubations:* Effects of enzymes on ruminal fermentation of forages are widely determined using the *in vitro* GP technique (Eun *et al.* 2006). Rumen inoculum was collected from two Brown Swiss cows (450±20 kg body weight) fitted with permanent rumen cannula and fed *ad lib.* a total mixed ration made up of 1:1 commercial concentrate and alfalfa hay formulated to meet all of their nutrient requirements (NRC 2001). Freshwater was available to cows at all times during the rumen inoculum collection phase.

Ruminal contents from each cow were obtained before the morning feeding, mixed and strained through four layers of cheesecloth into a flask with O_2 free headspace. Samples of each diet were weighed into 120 ml serum bottles with appropriate addition of ENZ doses/g dry matter (DM). Consequently, 10 ml of particle free ruminal fluid was added to each bottle followed by 40 ml of the buffer solution according to Goering and Van Soest (1970), with no trypticase added, in a 1:4 (v/v) proportion. Exogenous enzymes of GEZ were added on bottle contents (i.e. the substrate and buffered rumen fluid) immediately before closing.

Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in an incubator at 39 ºC. The pressure of gas produced was recorded after 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h of incubation using the GP technique of Theodorou *et al.* (1994). At the end of incubation at 72 h, bottles were uncapped, pH was measured using a pH meter and the contents of each bottle were filtered to obtain the non-fermented residue for determination of degraded substrate.

Dry matter, NDF and ADF degradability: Degradability of DM and cell wall fractions (neutral detergent fiber; NDF and acid detergent fiber; ADF) were determined at the end of incubation according to Ørskov and McDonald (1979). The contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter. Fermentation residues were dried at 105ºC overnight to determine DM disappearance, with loss in weight after drying being the measure of undegradable DM. the degradability of NDF and ADF were also determined.

Chemical analyses and assay of enzymatic activity: Samples of each TMR were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and ether extract (EE, #920.39) according to AOAC (1997). The neutral detergent fiber (NDFom, Van Soest *et al.* 1991), acid detergent fiber (ADFom) and lignin (sa) (AOAC 1997, method 973.18) analyses were carried out using an fiber analyzer unit. The content of NDFom was assayed without use of an alpha amylase but with sodium sulfite. Both NDFom and ADFom were expressed without residual ash.

Calculations and statistical analyses: To estimate kinetic parameters of gas production (GP), results (ml/g DM) were fitted using the NLIN option of SAS (2002) according to France *et al.* (2000) model as:

$$A = b \times (1 - e^{-c(-L)})$$

where: A is the volume of GP at time t; *b* is the asymptotic GP (ml/g DM); *c* is the rate of GP (/h), and *L* (h) is the discrete lag time prior to initiation of gas production.

Data of each of the 3 runs within the same sample of each of the 3 individual samples of each TMR were averaged prior to statistical analysis. Mean values of each individual sample were used as the experimental unit. Results of *in vitro* GP and ruminal fermentation parameters were analyzed as a 2 × 3 factorial experiment (*i.e.* 2 total mixed rations) with 3 exogenous enzymes doses (i.e. 0. 1.5 and 3 g/kg grains), using the PROC GLM option of SAS (2002) as:

$$Yijk = \mu + Di + EZi + (Di\ ´EZi) + ij$$

where: Y_{ijk}, every observation of the ith Diet (D_i) when incubated with j^{th} EZ doses (EZ_j; doses of enzyme); µ, the general mean; SB_i (i=1–2), the diet effect; EZ_j, enzyme dose effect (j=1–3); $(D*EZ)_{ij}$, interaction between diet and enzyme dose.

RESULTS AND DISCUSSION

Addition of GEZ to corn diet had no significant effect (P>0.05) on kinetics of GP. However, when added to sorghum diet, the high dose of the enzyme (3 g/kg DM), was traduced by an increased (P<0.05) rhythm of GP (*c*) and the volume of GP at 2, 4 and 6 h of incubation, with no statistical differences (P>0.05) between the control and the lowest dose of enzyme (i.e., 1.5 g/kg DM) (Tables 2, 3). Likewise, effect of GEZ was insignificant (P>0.05) either on the *in vitro* degradation of DM or cell wall (NDFD and ADFD) of both diets (Table 4). Effect of enzyme supply on pH values was statistically increased (P<0.05) only with

Table 1. Ingredients and chemical composition (g/kg DM) of the two total mixed rations used as substrates

	Corn	Sorghum
Ingredients		
Corn stover	70	70
Corn grain	250	0
Sorghum grain	0	250
Canola meal	610	610
Sugarcane molasses	50	50
Minerals mixture[1]	20	20
Chemical composition		
Organic matter	943.8	936.7
Crude protein	154.3	158.2
Ether extract	53.1	63.2
Neutral detergent fiber	114.6	141.7
Acid detergent fiber	82.7	89.7
Soluble carbohydrates	636.0	639.8

[1]Contained per kg: Fe (853 mg), Zn (2,000 mg), Mn (14,48 mg), Cu (60 mg), I (19.8 mg), Se (3.6 mg), Co (3.7 mg), Mg (6,479.8mg), $CaCO_3$ (499 g), NaCl (180 g), $NaHCO_3$ (250 g), Na (6,894 mg), K (5,540 mg).

Table 2. *In vitro* rumen gas kinetics[1] of the total mixed rations of 25% corn or sorghum as affected by different levels of GEZ enzyme protein (g/kg grains)

Diet	Enzyme	pH	*b*	*c*	*Lag*
Corn	0	5.86	316.9	0.085	4.94
	1.5	5.86	346.1	0.076	4.58
	3	5.78	311.5	0.092	4.84
	SEM	0.0203	7.57	0.004	0.281
	P				
	Linear	0.133	0.778	0.504	0.889
	Quadratic	0.423	0.079	0.182	0.622
Sorghum	0	5.6^{b}	310.1	0.068^{b}	5.45
	1.5	5.7^{a}	319.5	0.0730^{ab}	6.37
	3	5.7^{a}	314.6	0.0753^{a}	6.24
	SEM	0.008	4.26	0.0007	0.223
	P				
	Linear	0.016	0.6728	0.0053	0.1798
	Quadratic	0.076	0.4457	0.6395	0.2952
SEM pooled		0.011	4.3442	0.002	0.1793
P					
Diet		<0.0001	0.2614	0.0071	0.0029
Enzyme		0.3103	0.1344	0.1912	0.7114
Diet × enzyme		<0.0001	0.2187	0.0372	0.0402

[1] *b* is the asymptotic gas production (ml/g DM); *c* is the rate of gas production (/h); *L* is the initial delay before gas production begins (h). [a,b]Different superscripts following means among enzymes doses in the column within each diet indicate differences at *P*<0.05.

sorghum diet, and no significant differences (P>0.05) between both doses were recorded (Table 2). Irrespectively of enzyme supply, kinetics of GP and pattern of degradation of corn diet were generally higher than those of sorghum diet. A net significant effect (*P*<0.0001) of both diets and the interaction between diet and enzyme were recorded for the volume of GP at different incubation times.

The *in vitro* digestibility of NDFD, ADFD and DMD of both TMR was not affected by the enzyme treatment even when used at the highest dose (3 g GEZ /kg diet). Similar results were reported by Buendía *et al.* (2003). Rojo *et al.* (2001b) found that when the enzyme was added to the substrate at 24 h before mixing with other ingredients, the GEZ can act partially before entering the rumen, pre-digesting the substrate and facilitating the hydrolysis of its components. In our present study, the enzyme was added immediately to the diet before incubation. In experiments conducted *in vivo*, Mora *et al.* (2002) recorded an improvement of DMD of diet based on 50% sorghum. However, increasing doses of GEZ in diets with 50% (Mora *et al.* 2002) and 70% (Buendía *et al.* 2003) of sorghum, no quadratic or linear response was detected neither on dry matter intake nor on animal performance. However, a clear response was observed *in vitro*, which cannot detected *in vivo*, suggesting that animal related factors are associated with response to amylase. It is also believed that activity of amylase enzymes is affected by some external factors (Rojo *et al.* 2001a). GEZ from *Aspergillus niger* requires a pH of 4.5 and a temperature of 50ºC to reach its maximum potential for degrading starch (Slovay 1991, Rojo *et al.* 2001a); conditions which are not present in *in vitro* digestibility (Bahar and Celebi 1998). This can explain, in part, the findings of this *in vitro* trial. It is pertinent to mention that the most reported data dealing with effect of amylase enzymes were carried out *in vivo* with animals fed on high diet grains of 50% (Mora *et al.* 2002) or 70% (Rojo *et al.* 2005*,* Crosby *et al.* 2006); and animals were in most cases adapted to the ingestion of such diets (Crosby *et al.*

Table 3. *In vitro* rumen cumulative gas production after 72 h of incubation of the total mixed rations of 25% of corn or sorghum as affected by different levels of GEZ enzyme protein (g/kg grains)

Diet	Enzyme	2 h	4 h	6 h	8 h	10 h	12 h	24 h	36 h	48 h	72 h
Corn	0	18.8	39.2	62.6	91.7	132.5	180.2	284.3	320.4	335.7	346.9
	1.5	20.1	41.1	65.6	95.7	138.0	187.3	297.5	340.1	360.2	371.8
	3	20.0	43.4	67.4	98.4	141.6	192.2	285.9	320.5	334.4	345.4
	SEM	0.66	1.09	1.29	1.72	2.45	2.99	5.87	6.18	7.06	7.06
	P										
	Linear	0.502	0.081	0.165	0.1417	0.164	0.136	0.913	0.994	0.938	0.935
	Quadratic	0.629	0.925	0.840	0.856	0.856	0.871	0.346	0.167	0.128	0.121
Sorghum	0	14.1 b	29.3 b	44.9^{ab}	68.3	98.9	138.2	248.8	293.5	316.7	329.9
	1.5	13.4 b	28.0 b	43.3 b	66.4	97.0	137.8	261.2	308.0	332.2	345.9
	3	15.0 a	31.0 a	47.2^{a}	71.5	103.8	145.2	264.9	308.7	331.9	345.0
	SEM	0.13	0.24	0.51	1.14	1.71	2.02	3.75	4.53	4.85	
	P										
	Linear	0.019	0.018	0.0912	0.291	0.279	0.194	0.216	0.203	0.233	0.245
	Quadratic	0.003	0.002	0.034	0.185	0.259	0.385	0.727	0.490	0.459	0.356
SEM pooled		0.203	0.365	0.493	0.800	1.149	1.448	2.780	2.928	3.084	3.123
P											
Diet		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Enzyme		0.8811	0.8528	0.5452	0.3154	0.088	0.0543	0.3834	0.2775	0.1555	0.1716
Diet × enzyme		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0007	0.0015

[a,b] Different superscripts following means among enzymes doses in the column within each diet indicate differences at P<0.05.

Table 4. *In vitro* rumen degradability[1] (%) of NDF, ADF and DM after 72 h of incubation of the total mixed rations of 25% of corn or sorghum as affected by different levels of GEZ enzyme protein (g/kg grains)

Diet	Enzyme	NDFD	ADFD	DMD
Corn	0	16.38	9.94	72.78
	1.5	15.16	9.30	73.34
	3	15.34	8.93	72.60
	SEM	0.593	0.3022	0.7513
	P			
	Lineal	0.500	0.219	0.928
	Quadratic	0.598	0.842	0.696
Sorghum	0	13.29	11.74	74.53
	1.5	14.12	8.68	72.33
	3	13.10	7.88	72.29
	SEM	0.307	1.226	0.5177
	P			
	Linear	0.810	0.246	0.128
	Quadratic	0.207	0.679	0.361
SEM pooled		0.334	0.632	0.456
P				
Diet		0.0078	0.9736	0.8783
Enzyme		0.7498	0.2946	0.5618
Diet × enzyme		0.0932	0.6083	0.7158

[1] DMD is dry matter degradability; NDFD is *in vitro* neutral detergent degradability; ADFD is the *in vitro* acid detergent fiber degradability.

2006). In the present study, the donor animals of ruminal liquid were fed on diet contained only 25% grains. When increasing the amount of enzyme, digestibility of corn and sorghum grain was increased (Buendía *et al.* 2003). Rojo *et al.* (2001b) and Mendoza *et al.* (1999) sprayed amylase from *B. licheniformis* on corn grain (350 mL/kg) and found an increased *in vitro* DMD (from 60.36 to 73.08%). These results do not agree with those reported herein since effect of GEZ was tested on the total mixed ration which includes only 25% of corn or sorghum and not on the grain. Moreover, the *in vitro* results are not consistent with the *in vivo* results, due to several factors, but the treatment with enzymes has increased *in vivo* digestibility of flaked sorghum (Chen *et al.* 1995). It is believed that ruminal microorganisms act synergically with exogenous enzymes, conditions which are not completed *in vitro* (Rojo *et al.* 2001a). Factors such as the type or source of starch chemistry and nutrient composition of the diet, type of enzymes, method of application, complement of enzyme activities, pH as well as the amount of feed consumed per unit time, mechanical alterations (grade processing and chewing) and physicochemical (degree of hydration and gelatinization) may explain the variability of the results (Kaiser 1999). The ability of the rumen microorganisms to use efficiently the energy contained in the grains is another determinant factor of ruminal starch degradation since bacteria are thought to be the main microorganisms responsible for starch degradation (Mendoza *et al.* 1993). The differences between enzymes and their efficiency on starch degradation were reported by Rojo *et al.* (2001a) and was explained by their activity and the effect of pH. Enzyme activity reported at pH 7.0 and 39ºC was 4.19 units (mmol of glucose formed / min/ mg of protein enzyme) for amylase from *Bacillus licheniformis* and 1.95 from *Aspergillus niger* (vice only 0.062 from ruminal fluid) (Rojo *et al.* 2001a).

Irrespectively of enzyme dose, there are differences in the intensity and rate of ruminal starch degradation between both TMRs. All parameters were generally higher for corn than for sorghum (Table 1). This result is in accordance with the most reported studies which revealed that digestibility of corn starch is usually higher than that of sorghum (Britton and Stock 1986). From the interaction between starch properties (grain type) and amylolytic enzymes resulted bacterial limited ruminal degradation of starch grains, particularly sorghum (Stock *et al.* 1987), marking the possibilities of use of different kinds of treatments, especially the enzyme to enhance ruminal starch degradation. In this context, Duran *et al.* (1999) showed that ruminal digestion of sorghum ranged between 40 and 75%, whereas that of corn starch was between 51 and 93% (Ortega and Mendoza 2003). In our present study, DMD of TMRs ranged in a very narrow interval (72–74%). Moreover, rate of GP (*c*) and volume of gas produced were enhanced for sorghum diet as response to enzyme treatment till 6 h of incubation ($P<0.05$). It is possible therefore that use of amylolytic enzymes could increase the rate of starch digestion in some slowly digested grains, such as sorghum (Britton and Stock 1986, Huntington 1997). It is likely also that the answer depends on the time half-life that may have the exogenous enzyme in the rumen, since there is the possibility to be inactivated by the ruminal protease which hydrolyzes them (Rojo *et al.* 2007). Morgavi *et al.* (2000) found that amylase activity from *A. niger* was not affected after 6 h of ruminal incubation (95% of initial activity), but activity of amylases from *Irpex lacteus* and *Trichoderma viride* was reduced with 50 and 10%, respectively. Our results indicate that amylolytic enzyme was degraded slowly, suggesting that it was protected, presumably by glycosylation, which has been reported in several bacterial cellulase systems (Mackie and White 1990). Many enzymes produced by fungal systems are glycosylated (Matsuo and Yasui 1988). Effects of enzymes on rumen starch digestion in metabolic studies confirm that they are active before rumen proteolysis, as observed by Mora *et al.* (2002). Actually there are biotechnological techniques (Klibanov 1983) that may alter the configuration of the enzyme to increase its resistance to degradation by ruminal proteases.

The acidic characteristics of the enzyme and the low enzyme activity also may explain the absence of effects on cell wall digestion of both TMRs. Other reports have indicated little response on *in situ* (Gutiérrez *et al.* 2005) and *in vivo* DM and starch digestion of grains treated with these amylolytic enzymes (Mora *et al.* 2002).

The application of fibrolytic enzymes may improve the digestibility of the diet. The knowledge of the structure of

the cell wall of grain based diet and industrial enzymes make possible to develop new alternative treatments to improve the use of cellulose and hemicelluloses by ruminants synergically with starch. In some studies of feedlot, positive results were observed with a combination of fibrolytic and amylolytic enzymes (Romero *et al.* 1992).

Likewise, ruminal pH did not change with enzyme treatment of corn TMR. Similar results were reported earlier by Rojo *et al.* (2001a) and Lee-Rangél *et al.* (2006).

Linear or quadratic response in *in vitro* digestibilities (DM, NDF and ADF), pH and kinetics of GP as a response to the dose of enzyme was observed generally when sorghum diet was incubated. Crosby *et al.* (2006) and Rojo *et al.* (2001b) recorded similar result, and reported that addition of alpha-amylase of *Bacillus licheniformis* increased (P<0.0001) *in vitro* starch digestion of the grains and there was no substrate by enzyme interaction.

The general lack of response to GEZ treatment could be primarily the result of acidic characteristics of the enzyme, the low enzyme activity and the low grain content in the diet. This exogenous enzyme might be considered as an alternative to improve ruminal starch digestion by ruminants fed high grain diets. The use of fibrolytic and amylolytic exogenous enzymes constitute a short term viable alternative in order to increase the use of energy contained in the cereal grains by ruminants.

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