

## Slow-release amylase increases *in vitro* ruminal digestion of maize and sorghum grain

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### Abstract

The objective of this study was to evaluate the effects of slow-release  $\alpha$ -amylase in ruminal *in vitro* digestion of maize and sorghum grains. Digestibility was measured using an *in vitro* procedure with 40 mL of buffer and 10 mL of ruminal fluid, flushed with CO<sub>2</sub> and incubated at 39 °C. The digestibility of sorghum and maize grain was measured after 6 and 12 hours of fermentation with or without exogenous  $\alpha$ -amylase in powder form or dispersed in a matrix system for slow release by a diffusional mechanism. Tablets were used as the drug release matrix system, and were formulated with barium sulphate and ethylcellulose as the core of the final tablet. Treatments consisted of incubation of sorghum or maize grains with four doses of enzyme, using  $\alpha$ -amylase in powder or in the press-coated tablet (16 treatments). The results showed that with a higher dose of exogenous enzyme, the digestibility of the grains was improved. Sorghum and maize digestion with tablets were improved compared with  $\alpha$ -amylase in powder form. Releasing  $\alpha$ -amylase from matrix tablets represents a potential technology to improve grain digestion in ruminants.

**Keywords:** Enzymes, slow-release, grains, digestibility, rumen

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### Introduction

The finishing of sheep can be carried out on high grain diets (Mendoza *et al.*, 2007), where sorghum and maize are commonly used in the diet. However, both grains are usually processed to increase their digestibility (Ortega & Mendoza, 2003). *In vitro* and *in situ* studies using these grains as substrates have shown that the addition of the enzyme,  $\alpha$ -amylase from *Bacillus licheniformis*, or glucoamylase from *Aspergillus niger* in liquid form, increases grain digestion as a function of dose (Gutierrez *et al.*, 2005; Rojo *et al.*, 2007).

Even when amylolytic enzymes have been shown to increase ruminal starch digestion, performance is not always improved (Rojo *et al.*, 2005), because the addition of these enzymes increases the rate of starch digestion, causing subacute acidosis. Crosby *et al.* (2006) did not find a response in terms of gain, but observed a great variation in intake at different doses of glucoamylase in sheep. Therefore, modulation of the rate of starch digestion may be needed in systems based on grains to improve animal performance.

Controlled release tablets may be a potential technology to administer exogenous enzymes because these systems are formulated in the dry state, a stable form that can be released slowly (Melgoza *et al.*, 2002). These kind of tablets can be designed to reduce the frequency of dosage because the polymer in the matrix increases the duration of the release process of the enzyme and can be designed to remain unalterable in ruminal fluid (Melgoza *et al.*, 2007). Matrix tablets are a type of controlled drug delivery system that releases a drug in a continuous manner. These tablets are in solid dosage forms, containing one or more active ingredients, which are prepared by compression and may be left uncoated or coated with different polymers; they contain an inert nucleus or core, which modifies the release platform's density (Mathiowitz, 1999).

Ruminal drug delivery systems have been developed for ruminants to provide solutions to a variety of animal health problems and to promote growth and efficient metabolism. These include antihelminthic

compounds, ionophores and minerals (Vandame & Ellis, 2004). Slow-release systems have also been used for urea (Huntington *et al.*, 2006), but information on incorporating enzymes into these formulations is scarce. Because of the potential benefits of reducing the rate of starch digestion in ruminants, the objective of the present study was to develop a press-coated tablet with a core of BaSO<sub>4</sub> and ethylcellulose/ $\alpha$ -amylase as the matrix system, with prolonged release in a ruminal *in vitro* system, using maize and sorghum as substrates, with the goal of improving grain digestion. The hypothesis of this study was that slowly providing exogenous amylases could improve grain digestion, compared with a powder formulation.

## Materials and Methods

The materials used in the production of press-coated tablets were barium sulphate (synthesized in the laboratory); ethylcellulose (Dow Chemical Company, Midland, MI, USA) as the core of the final tablet and  $\alpha$ -amylase from *Aspergillus niger* (Nutrer S.A., Naucalpan, Edo. de Mexico, Mexico) as the matrix-forming polymer. Barium sulphate was obtained from the equimolar reaction between barium chloride and sulphuric acid. Both solutions were mixed, allowed to react until a white precipitate was formed, and then the phases were separated by decantation, and the precipitate was washed with water until the pH was neutral. Finally, the precipitate was dried in an oven (Riossa HS, Mexico) at 60 °C, and the weight and yield were recorded.

Ethylcellulose (Ethocel std 100 premium Dow Chemical Company, Midland, MI, USA) was used to provide a porous and coherent structure in the matrix system. To determine the mean particle size, the powder was passed through normalized ASTM E-11 sieves with the following screens: 20, 30, 40, 50, 60, 70, 80 (0.178 and 0.853 mm), using an electric sieve (Rotap Rx-29, Mentor, OH, USA) with continuous vibration for 15 minutes. The ethylcellulose was then ground and sieved again with 270 and 140 screens to select a granulometric fraction between 53 and 104  $\mu$ m.

In order to characterize the  $\alpha$ -amylase from *Aspergillus niger*, three solutions were prepared with the enzyme powder (150, 250 and 500  $\mu$ g) and the amount of protein was quantified with a spectrophotometer (Varian Cary 100) at 750 nm, using the Lowry's technique (1951). The enzymatic activity of the amylase was determined by measuring reducing sugars released by starch digestion with di-nitro-salicylic acid as the reagent on a Vary Cary 100 spectrophotometer at 550 nm (Dubois *et al.*, 1956). The substrate was a 1% potato starch solution prepared at different pH at rumen temperature (40 °C), containing 0.06% CaCl<sub>2</sub>. The enzyme was incubated with a phosphate buffer (100 mM) at pH 6.8. The enzyme concentration was 42  $\mu$ g/mL and the incubation time with substrate was 15 minutes. The enzymatic units (micromoles of reducing sugars produced per minute) per milligram of protein (IU/mg) were determined.

The press-coated tablets were prepared in two steps. First, cores of barium sulphate were designed to be 740 mg in weight, 7 mm in diameter and with a density above 3 g/mL by direct compaction in a rotary tablet machine (Riva piccola B/10, Ciudadela-BS.AS, Argentina). In the second step, the matrix systems were elaborated with amylase, Ethocel std 100 premium and barium sulphate in the proportion 30 : 60 : 10. Barium sulphate was passed through a screen 30 (0.599 mm), but the enzyme was not sieved. The core or "sinker" was placed in the centre of the matrix system. Tablets were produced by direct compression at 1500 psi in a hydraulic press (Carver C, Wabash, IN, USA). The weight of the final tablets (press-coated tablets) was 1.2 g with a diameter of 10 mm.

The hardness of six press-coated tablets was measured with a hardness tester (Vankel 2000, Cary, N.C., USA), and friability (property related to tablet hardness) was tested with the equivalent of 6 g of tablets in a friabilator (Elecsa FE30, Mexico) for 4 min at 25 rpm. Enzyme release from the press-coated tablets was performed *in vitro*, using a dissolutor (Hanson Research 72RL, Chatsworth, CA, USA), employing apparatus 2 (USP 32) for 26 hours. The dissolution medium was 500 mL of NaCl (0.85%) at 39  $\pm$  0.5 °C and 50 rpm. The amount of enzyme released was quantified by the Lowry method (1951) and the enzymatic activity was assessed by the release of reducing sugars with the DNS reactive and potato starch as the substrate dissolved in phosphate buffer with pH 6 at 40 °C (Dubois *et al.*, 1956). All analyses were done in triplicate. The release data was analyzed using the first-order Higuchi model to determine the mechanism of drug release from the matrices (Higuchi, 1963).

Digestibility was measured with the *in vitro* procedure of Tilley & Terry (1963), modified for grains with 40 mL of buffer and 10 mL of ruminal fluid, flushed with CO<sub>2</sub> and incubated at 39 °C (Mendoza *et al.*, 1995). Incubation was replicated three times. The ruminal inoculum was collected from a Holstein steer (450 kg of BW), fitted with a ruminal cannula, which was fed a diet consisting of forage (lucerne hay, maize silage) and concentrate (sorghum grain, soybean meal, cane molasses) in a 50 : 50 ratio. Samples of maize

and sorghum were incubated with different doses of  $\alpha$ -amylase. Fermentation was carried out for 6 and 12 hours to determine *in vitro* grain digestibility (Mendoza *et al.*, 1995). The sorghum and maize grains were ground in a Thomas Willey model 4 mill (Philadelphia, USA) and passed through a mesh with an opening of 1 mm. Three incubations were conducted with 16 treatments, and each treatment was incubated in triplicate, using  $\alpha$ -amylase in powder and dispersed in the matrix system of the press-coated tablets. The  $\alpha$ -amylase in powder was incubated in digestibility tubes at doses of 150, 300 and 450 mg, equivalent to the amount of enzyme in one, two and three tablets, respectively, with 500 mg of maize or sorghum grains, 40 mL of McDougall's buffer, and 10 mL of ruminal liquid, and flushed with CO<sub>2</sub>. Fermentation was stopped by freezing. Digestibility data were analyzed with a completely randomized block design with incubation as the blocking criteria, using the GLM procedure of SAS (1996). The effects of the addition of the enzyme as a powder versus the matrix system on digestibility were compared by orthogonal contrasts within doses for both grains. The linear and quadratic effects of the dose of amylase in powder or in the matrix system were tested (SAS, 2002).

## Results and Discussion

The average hardness of six press-coated tablets was 15.45 kp. The enzymatic activity at pH 6.8 was  $1.41 \pm 0.21$  IU/mg protein. This amylase has shown activity under ruminal conditions (Gutierrez *et al.*, 2005; Rojo *et al.*, 2005). The dissolution test showed that the enzyme was released slowly by the tablet, with a percentage of enzyme release of 63.7% at 12 hours and 77.3% at 24 hours. Higuchi's model (1963) confirmed diffusional release ( $r = 0.98$ ), which could help to prevent the inactivation of the enzyme by rumen proteases, and also reduce the potential problem of acidosis.

Although barium is considered a heavy metal and a dangerous toxin, barium sulphate is less soluble, making it nominally safer and practically non-absorbable by organisms that ingest it. In fact, this compound has been used as a faecal marker (Figuroa *et al.*, 1968). Its possible use as an additive in feed for ruminants raises the possibility of considering the potential of pollution of the environment. However, there are no environmental regulations for the use of barium sulphate, and the Texas Commission on Environmental Quality recently confirmed that the insoluble barium compound barium sulphate is not of concern in human health, thereby eliminating the need for clean-up standards or site remediation for this compound (McGinty *et al.*, 2007).

Increasing the amylase dose linearly increased the digestibility of sorghum and maize ( $P < 0.01$ ) at 6 and 12 hours, independent of the powder versus matrix formulation (Tables 2 and 3). Grain digestion was improved when the enzyme was administered in a matrix tablet, compared with the powder (52.6% vs. 45.9%,  $P < 0.01$ ). Our hypothesis was that the enzyme powder would promote a rapid release of glucose from grains, which acidifies the pH and inhibits the action of amylolytic bacteria. This explains why increases in

**Table 1** Effect of  $\alpha$ -amylase in powder or dispersed into a matrix system and dose on *in vitro* dry matter digestibility of maize and sorghum after 6 hours of incubation

Dose, mg	Maize <sup>2</sup>		Sorghum <sup>2</sup>	
	Powder	Matrix system	Powder	Matrix system
0	31.27 <sup>b</sup> $\pm$ 2.30	28.46 <sup>b</sup> $\pm$ 3.76	23.50 <sup>a</sup> $\pm$ 2.90	23.45 <sup>c</sup> $\pm$ 2.24
150	33.17 <sup>b</sup> $\pm$ 2.82	36.61 <sup>a</sup> $\pm$ 3.84	31.27 <sup>a</sup> $\pm$ 0.54	38.67 <sup>b</sup> $\pm$ 5.41
300	38.45 <sup>a</sup> $\pm$ 1.64	38.76 <sup>a</sup> $\pm$ 4.67	32.39 <sup>a</sup> $\pm$ 2.12	41.87 <sup>a</sup> $\pm$ 3.47
450	38.12 <sup>a</sup> $\pm$ 1.41	38.45 <sup>a</sup> $\pm$ 3.20	33.31 <sup>a</sup> $\pm$ 1.26	43.09 <sup>a</sup> $\pm$ 4.85
Linear <sup>1</sup>	0.0001	0.0001	0.02	0.001
Quadratic <sup>1</sup>	0.09	0.003	0.21	0.001

<sup>1</sup>Probability of a significant effect of enzyme dose (linear or quadratic effect).

<sup>2</sup>The difference between the digestibility of the powder vs. the matrix system was different ( $P < 0.001$ ) within doses (150, 300 and 450 mg) for both grains.

<sup>abc</sup>Means with different superscript within columns differ ( $P < 0.05$ ).

**Table 2** Effect of  $\alpha$ -amylase in powder or dispersed into a matrix system and dose on *in vitro* dry matter digestibility of maize and sorghum after 12 hours of incubation

Dose, mg	Maize <sup>2</sup>		Sorghum <sup>2</sup>	
	Powder	Matrix system	Powder	Matrix system
0	40.57 <sup>d</sup> ± 3.76	39.22 <sup>c</sup> ± 3.55	36.84 <sup>b</sup> ± 2.30	34.67 <sup>b</sup> ± 3.76
150	55.40 <sup>c</sup> ± 1.72	62.47 <sup>b</sup> ± 3.84	52.53 <sup>a</sup> ± 3.84	62.88 <sup>a</sup> ± 2.72
300	58.09 <sup>b</sup> ± 4.67	67.09 <sup>a</sup> ± 3.93	55.81 <sup>a</sup> ± 1.64	64.54 <sup>a</sup> ± 4.67
450	63.99 <sup>a</sup> ± 3.20	70.06 <sup>a</sup> ± 2.96	58.73 <sup>a</sup> ± 1.41	66.24 <sup>a</sup> ± 3.20
Linear <sup>1</sup>	0.0001	0.0001	0.0001	0.0001
Quadratic <sup>1</sup>	0.0001	0.0001	0.0001	0.001

<sup>1</sup>Probability of a significant effect of enzyme dose (linear or quadratic effect).

<sup>2</sup>The difference between the digestibility of the powder vs. the matrix system was different ( $P < 0.001$ ) within doses (150, 300 and 450 mg) for both grains.

<sup>abc</sup>Means with different superscript within columns differ ( $P < 0.05$ ).

digestibility are highest when the enzyme was supplied slowly by the tablet formulation. It has been demonstrated that a high glucose concentration in the environment inhibits amylolytic activity (Cotta, 1988). In this experiment, the tablet released the enzyme slowly and may avoid the suppression of ruminal bacteria and the increase in acidity, promoting greater digestibility of starch. Even though this was an *in vitro* assay, it is possible to expect additional benefits *in vivo* by reducing the rate of starch digestion with this technology compared with technology where the enzyme is released rapidly (Mendoza *et al.*, 2000).

Controlled release systems could also reduce the degradation of amylolytic enzymes by ruminal microbes. Rojo *et al.* (2005) evaluated the half-life of exogenous amylolytic enzymes in ruminal conditions and found values between 4 and 6 hours, which could explain the positive effects on grain digestion observed with tablets versus powder. The optimum pH for the action of exogenous amylase is between 5.8 and 6.6 and in a temperature range between 70 and 72 °C (Naz, 2002). Therefore, the physiological conditions of the rumen in a grain-based diet meet the pH, but not the temperature requirements. However, the amylase activity of *Aspergillus niger* is so high that it may have a synergistic effect on the digestion of grains. Enzyme tablet design is a challenge for ruminant nutrition because the conditions for a ruminal device that will remain in the reticulum or the front part of the rumino-reticulum for extended periods requires a specific density to disperse the pharmacologically inactive excipient (the matrix) and the proper physical and chemical properties to control the slow release of the drug. A ruminal device must function in different conditions of the rumen environment, such as changes in pH, motility and abrasion, as well as enzymatic and bacterial activity (Vandame & Ellis, 2004), which require further evaluation.

It was interesting to observe that after 12 h of incubation, the addition of amylase in tablet form at the highest dose with sorghum resulted in similar digestion to that of maize (Table 2). This is important, considering that grains with a poor ruminal digestion such as sorghum have less energy than maize (Ortega & Mendoza, 2003) and may require higher doses of the enzyme if there is enough potentially digestible starch in the rumen.

## Conclusions

The use of  $\alpha$ -amylase from *Aspergillus niger* as an exogenous enzyme increased *in vitro* sorghum and maize grain digestion. Its effect was improved when the enzyme was administered slowly from tablets. Further research is needed to evaluate this formulation *in vivo* and to assess the possibilities of using slow-release enzymes under practical feeding conditions.

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