PAPER

Influence of exogenous fibrolytic enzymes on \textit{in vitro} and \textit{in sacco} degradation of forages for ruminants

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Abstract

An \textit{in vitro} assay was carried out to evaluate the effects of exogenous fibrolytic enzymes (1, 2, 3 and 4 g/kg DM) powder preparation containing xylanase and cellulase from \textit{Aspergillus niger} and \textit{Trichoderma viride} on DM, NDF and ADF degradation of alfalfa hay, corn silage, corn stover, elephant grass, Guinea grass and oat straw. Kinetics data of \textit{in vitro} degradations were analyzed. The potentially degradable fraction and degradation rate of NDF and ADF of alfalfa increased quadratically (P<0.05) as the inclusion level of enzyme increased up to 3 g. The others forages were not affected by the enzyme. An \textit{in sacco} trail was performed using four Holstein steers fitted with ruminal cannulas to evaluate the effects of the exogenous fibrolytic enzymes (3 g/kg DM) on DM, NDF and ADF degradation of alfalfa hay and corn stover. Kinetics data were also analyzed. The potentially degradable fraction degradation of NDF (62.0\% at 65.7\%) and ADF (55.8\% at 56.9\%), of alfalfa hay were increased (P<0.05) by the exogenous fibrolytic enzymes, but no differences were found for corn stover. These results suggest that the enzymes increased \textit{in vitro} and \textit{in sacco} fibre degradation only for alfalfa hay.

Materials and methods

Samples of alfalfa hay, corn silage, corn stover and oat straw from temperate areas, as well as elephant grass and Guinea grass from tropical areas of Mexico, were ground through a 1 mm screen (Wiley mill, Arthur H. Co., Philadelphia, PA, USA) for chemical analysis or through a 2 mm screen for \textit{in sacco} degradation determination. The dry matter (DM) was determined by oven drying at 65°C to a constant weight; ash contents were determined in a muffle furnace at 550°C for 8 h. Crude protein (CP) and acid detergent fibre (ADF) method 973.18 was determined according to AOAC (1995) and neutral detergent fibre (NDF) was determined according to Van Soest et al. (1991) with heat-stable alpha amylase and sodium sulfite. The NDF contents contained insoluble ashes.

The first phase of the \textit{in vitro} degradation technique (Tilley and Terry, 1963) was performed collecting ruminal fluid from three lambs fitted with ruminal cannulas. Lambs had free access to alfalfa hay and oat straw, mineral premix and water, plus 500 g/day of concentrate (CP: 16\%, DM). A 1 kg sample (as DM) of ground forage was mixed with 0, 1, 2, 3 or 4 g of an enzyme product (according to the manufacturer; Fibrozyme, Alltech Inc., Nicholasville, KY, USA). This product was a powder preparation containing xylanase and cellulose from \textit{Aspergillus niger} and \textit{Trichoderma viride} fermentation extract with a cellulase and xylanase activity of 31.0 and 43.4 UI.

A 500 mL sample of ruminal fluid was collected 3 h after the morning feeding and squeezed through two layers of cheesecloth into a 500 mL Erlenmeyer flask with an O2-free CO2 headspace. Samples (500 g) of ground forages were weighed in roll polypropylene tubes equipped with butyl runner stopper. Then, a mixture of 40 mL McDougall saliva (1948) and 10 mL of strained ruminal fluid (4:1) was added. Roll tubes containing 500 mg of DM samples were incubated in a water bath with agitation at 39°C for 6, 12, 24, 48 and 72 h. Residuals were recovered by filtration (Whatman 541), dried at 65°C for 24 h and weighed. A sample of the residual (100 mg) was recovered to quantify NDF and ADF contents as described above. The \textit{in vitro} degradation of DM, NDF and ADF of the forages was determined from the DM, NDF and ADF remaining in the roll tubes after incubation. Three roll tubes per treatment and incubation time were used. The assay was carried out in two runs with two weeks of difference in between. The filter and undigested residues were oven-dried at 105°C for 24 h to remove excess moisture and weighed.

For the \textit{in sacco} assay, four Holstein steers fitted with ruminal cannulas were randomly assigned to one of two diets (treatments): i)
control, without enzyme; and ii) diet with the same enzyme product used in the in vitro assay. The diets, similar to those used in small cattle farms of the State of Mexico, contained alfalfa hay (25%), corn stover (25%), ground sorghum (36.6%), soybean meal (8.8%), poultry litter (2.4%), and minerals (2.2%).

A 1 kg sample of DM ground forage (500 g alfalfa hay plus 500 g corn stover) was sprayed with enzyme preparation as follows: 3 g of enzyme powder were dissolved in 300 mL of distilled water and applied in a fine spray to the alfalfa-corn stover mixture 24 h before the morning feeding. The same amount of water was sprayed daily on the control diet. The forage mixtures were added to the total mixed rations. Diets and water were offered at 07:30 and 19:30 h. Steers had free choice access to diets and fresh water. Each period lasted 18 d, with 15 d for adaptation followed by 3 d for ruminal incubation.

To calculate in sacco degradations, on d 16 of each sampling period, 18 bags (10x15 cm; pore size 52±10 µm; 5 g diet DM basis) for each forage were placed in the rumen at 07:30 h and removed at 0, 6, 12, 24, 48 and 72 h of incubation. Before insertion into the rumen, all bags were washed with water (39°C) for 5 min.

Kinetics data of in vitro degradation and in sacco degradations were analyzed using a Gompertz model (Susmel et al., 1999) as follows:

\[
\text{dis}_t = (a+b) \exp\left(-c \exp\left(-Dt\right)\right)
\]

where: \( \text{dis}_t \) is the degradation of material (g/kg) from the bag or tube at time \( t \);
\( a \) is the ruminally soluble fraction (g/kg) at \( t = 0 \);
\( b \) is the insoluble, but potentially degradable fraction (g/kg);
\( c \) is the fractional degradation rate of \( a+b \);
\( D \) is a parameter to measure the rate of degradation.

According to Gompertz model, the fractional rate of degradation varies as a function of time, and the average value (i.e., a constant comparable to the exponential rate of degradation) is derived as: \( c = D/C \). The DM, NDF and ADF remaining at each incubation time were used to fit a nonlinear regression model using

Table 1. Chemical composition (g/kg DM, unless otherwise stated) of forages.

|                | Alfalfa hay | Corn silage | Elephant grass | Guinea grass | Corn stover | Oat straw |
|----------------|------------|-------------|----------------|--------------|-------------|-----------|
| Dry matter, g/kg | 893        | 895         | 920            | 915          | 906         | 925       |
| Crude protein   | 183        | 86          | 85             | 92           | 39          | 41        |
| Neutral detergent fibre | 363 | 509         | 624            | 676          | 656         | 757       |
| Acid detergent fibre | 239 | 304         | 373            | 410          | 425         | 553       |
| Ash             | 138        | 108         | 91             | 115          | 79          | 65        |

Table 2. Effects of fibrolytic enzymes on in vitro degradation of alfalfa hay, corn silage, corn stover, elephant grass, guinea grass and oat straw.

| Enzyme, g/kg DM | 0  | 1  | 2  | 3  | 4  | SEM |
|-----------------|----|----|----|----|----|-----|
| Alfalfa hay     |    |    |    |    |    |     |
| Dry matter      |    |    |    |    |    |     |
| \( a \)         | 16.5 | 16.3 | 16.2 | 16.9 | 17.4 | 0.79 |
| \( b \)         | 47.5 | 48.0 | 47.1 | 47.7 | 46.1 | 0.98 |
| \( a+b \)       | 64.0 | 64.3 | 63.3 | 64.6 | 63.5 | 1.47 |
| \( c \)         | 4.0  | 4.1  | 3.9  | 4.1  | 3.9  | 0.04 |
| Neutral detergent fibre |    |    |    |    |    |     |
| \( b \)         | 45.1 | 45.9 | 48.6 | 50.0 | 48.8 | 2.15 |
| \( c \)         | 3.0  | 3.1  | 3.3  | 3.7  | 3.3  | 0.02 |
| Acid detergent fibre |    |    |    |    |    |     |
| \( b \)         | 40.6 | 41.0 | 43.1 | 45.5 | 43.3 | 2.44 |
| \( c \)         | 2.5  | 2.6  | 2.7  | 2.9  | 2.7  | 0.02 |
| Corn silage     |    |    |    |    |    |     |
| Dry matter      |    |    |    |    |    |     |
| \( a \)         | 24.7 | 24.2 | 24.4 | 24.6 | 24.0 | 0.38 |
| \( b \)         | 41.9 | 43.3 | 42.9 | 42.7 | 44.2 | 0.39 |
| \( a+b \)       | 66.6 | 67.5 | 67.3 | 67.3 | 68.2 | 0.44 |
| \( c \)         | 4.2  | 4.3  | 4.3  | 4.3  | 4.4  | 0.03 |
| Neutral detergent fibre |    |    |    |    |    |     |
| \( b \)         | 51.3 | 53.0 | 54.0 | 54.1 | 54.1 | 0.82 |
| \( c \)         | 3.3  | 3.4  | 3.5  | 3.5  | 3.5  | 0.02 |
| Acid detergent fibre |    |    |    |    |    |     |
| \( b \)         | 39.1 | 41.0 | 41.9 | 40.7 | 41.7 | 1.08 |
| \( c \)         | 2.3  | 2.3  | 2.4  | 2.3  | 2.4  | 0.03 |
| Corn Stover     |    |    |    |    |    |     |
| Dry matter      |    |    |    |    |    |     |
| \( a \)         | 17.8 | 18.0 | 16.8 | 16.1 | 16.5 | 0.68 |
| \( b \)         | 37.9 | 35.3 | 37.5 | 39.0 | 39.5 | 1.23 |
| \( a+b \)       | 55.7 | 53.3 | 54.3 | 55.1 | 56.0 | 0.61 |
| \( c \)         | 3.4  | 3.2  | 3.3  | 3.4  | 3.4  | 0.03 |
| Neutral detergent fibre |    |    |    |    |    |     |
| \( b \)         | 53.8 | 50.8 | 52.8 | 53.8 | 54.2 | 0.78 |
| \( c \)         | 3.4  | 3.2  | 3.3  | 3.3  | 3.4  | 0.03 |
| Acid detergent fibre |    |    |    |    |    |     |
| \( b \)         | 49.7 | 49.1 | 51.2 | 52.7 | 52.8 | 1.31 |
| \( c \)         | 2.9  | 2.8  | 2.9  | 3.1  | 3.1  | 0.02 |
| Elephant grass  |    |    |    |    |    |     |
| Dry matter      |    |    |    |    |    |     |
| \( a \)         | 16.6 | 15.9 | 15.9 | 17.3 | 17.1 | 0.30 |
| \( b \)         | 46.5 | 46.7 | 47.0 | 47.3 | 47.7 | 0.36 |
| \( a+b \)       | 63.6 | 62.6 | 62.9 | 64.6 | 64.8 | 0.94 |
| \( c \)         | 4.0  | 4.0  | 4.0  | 4.1  | 4.2  | 0.03 |
| Neutral detergent fibre |    |    |    |    |    |     |
| \( b \)         | 61.8 | 61.8 | 61.3 | 61.6 | 61.6 | 0.41 |
| \( c \)         | 3.8  | 3.8  | 3.7  | 3.8  | 3.8  | 0.03 |
| Acid detergent fibre |    |    |    |    |    |     |
| \( b \)         | 44.6 | 44.5 | 44.1 | 46.5 | 46.5 | 2.64 |
| \( c \)         | 2.6  | 2.8  | 2.8  | 2.9  | 2.9  | 0.03 |

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the “NLIN” procedure of SAS (1999).

Kinetics data of in vitro assay were analyzed as a complete randomized design using the GLM procedure of SAS (1999). Statistical analysis included run as an experimental error in the model as described by Pinos-Rodriguez et al. (2002). Orthogonal polynomials were used to test linear and quadratic effects of enzymes levels on degradation of forages. Kinetic data of in sacco assay were analyzed as a cross-over design using the Mixed procedure of SAS (1999). Animal effect was included as a random component in the model. Significant differences were accepted when P<0.05.

This experiment was conducted under the supervision and approval of the Academic Committee of the Animal Science Department, Colegio de Postgraduados, Montecillo, México, according to regulations established by the Animal Protection Law enacted by the State of México.

Results and discussion

Corn stover, elephant grass, Guinea grass and oat straw exhibited the highest NDF contents and alfalfa hay and corn silage the lowest values. The same tendency was observed for ADF, although the elephant grass showed a lower ADF value than Guinea grass, corn stover and oat straw. The content of ashes was high in corn silage, probably due to soil contamination (Table 1).

Soluble fraction and kinetics of in vitro DM degradation for alfalfa hay was not affected by enzymes. The potentially degradable fraction as well as the degradation rate of NDF and ADF of alfalfa hay were increased quadratically (P<0.05) as the dose of enzyme treatments increased, and the highest values were obtained with 3 g of enzyme (50.0 and 45.5%; 3.7 and 2.9 %/h). The potentially degradable and soluble fractions and kinetics of in vitro degradation for DM, NDF and ADF of corn silage, corn stover, elephant grass, Guinea grass and oat straw were not affected by enzyme treatments (Table 2).

Kinetics results for in sacco degradation and in vitro assay for alfalfa hay were similar. Kinetics of DM degradation of alfalfa hay were not affected by enzymes; however, enzymes increased both potentially degradable fraction (65.7% vs 62.0%; 56.9% vs 52.8%) and degradation rate (4.2%/h vs 3.4%/h; 4.1%/h vs 3.3%/h) of NDF and ADF. The potentially degradable and soluble fractions and kinetics of in sacco degradation of DM, NDF and ADF of corn stover were not affected by enzymes. (Table 3)

| Table 2. Continued. |
|----------------------|
| Enzyme, g/kg DM      |
| 0       | 1       | 2       | 3       | 4       | SEM   |
| Guinea grass         |
| Dry matter           |
| a                   | 7.6     | 7.0     | 8.2     | 7.6     | 4.8    | 0.29   |
| b                   | 39.4    | 39.8    | 38.5    | 38.3    | 42.0   | 2.06   |
| a+b                 | 47.0    | 46.9    | 46.7    | 45.9    | 46.8   | 1.21   |
| c                   | 2.9     | 2.9     | 2.9     | 2.8     | 2.9    | 0.03   |
| Neutral detergent fibre |
| b                   | 45.9    | 44.0    | 44.3    | 44.1    | 44.1   | 1.16   |
| c                   | 2.6     | 2.5     | 2.5     | 2.5     | 2.5    | 0.03   |
| Acid detergent fibre |
| b                   | 38.2    | 35.5    | 38.3    | 37.9    | 39.9   | 1.09   |
| c                   | 2.2     | 2.2     | 2.2     | 2.2     | 2.3    | 0.03   |

Oat straw
Dry matter
| a + b                 | 65.7b   | 56.9b   | 3.4b    | 0.61    |
| c                   | 4.1b    | 4.2b    | 0.30    |

Neutral detergent fibre
| a                   | 55.1    | 59.6    | 0.57    |
| b                   | 62.0a   | 65.7b   | 0.59    |
| c                   | 3.4a    | 4.2b    | 0.29    |

Acid detergent fibre
| a                   | 52.8a   | 56.9b   | 0.61    |
| b                   | 3.3a    | 4.1b    | 0.30    |

Corn stover
Dry matter
| a                   | 16.4    | 15.8    | 0.89    |
| b                   | 36.8    | 39.1    | 0.98    |
| c                   | 53.2    | 54.9    | 0.88    |
| a+b                 | 3.0     | 3.1     | 0.51    |

Neutral detergent fibre
| b                   | 47.7    | 49.4    | 1.11    |
| c                   | 2.4     | 2.8     | 0.43    |

Acid detergent fibre
| b                   | 40.1    | 42.9    | 0.36    |
| c                   | 1.8     | 2.2     | 0.24    |

a, soluble fraction (%); b, potentially degradable fraction (%); a+b, total degradation (%); c, degradation rate (%/h). SEM, square error means. a, b Superscripts in row mean differences (P<0.05).

| Table 3. Influence of fibrolytic enzymes on in sacco degradation of alfalfa hay and corn stover. |
|---------------------------------------------------------|
| Control Enzyme SEM |
|------------------|------------------|
| Alfalfa hay:     |
| Dry matter       |
| a                | 25.2             | 22.9             | 0.42   |
| b                | 55.1             | 59.6             | 0.57   |
| a+b              | 80.3             | 82.5             | 0.67   |
| c                | 4.2              | 4.5              | 0.30   |
| Neutral detergent fibre |
| b                | 62.0a            | 65.7b            | 0.59   |
| c                | 3.4a             | 4.2b             | 0.29   |
| Acid detergent fibre |
| b                | 52.8a            | 56.9b            | 0.61   |
| c                | 3.3a             | 4.1b             | 0.30   |
| Corn stover:     |
| Dry matter       |
| a                | 16.4             | 15.8             | 0.89   |
| b                | 36.8             | 39.1             | 0.98   |
| a+b              | 53.2             | 54.9             | 0.88   |
| c                | 3.0              | 3.1              | 0.51   |
| Neutral detergent fibre |
| b                | 47.7             | 49.4             | 1.11   |
| c                | 2.4              | 2.8              | 0.43   |
| Acid detergent fibre |
| b                | 40.1             | 42.9             | 0.36   |
| c                | 1.8              | 2.2              | 0.24   |

a, soluble fraction (%); b, potentially degradable fraction (%); a+b, total degradation (%); c, degradation rate (%/h). SEM, square error means. a, b Superscripts in row mean differences (P<0.05).
With the exception of alfalfa hay, enzymes did not affect both in vitro degradation and in sacco degradation of forages under study; therefore, chemical composition of forages could influence enzyme efficiency. Indeed, differences in chemical composition of forages may be due to differences of forage species, stage of maturity at harvest, soil type, fertilization level, season, and weather conditions (Van Stralen and Tammenga, 1990). In addition, forage anatomical structure plays an important role in limiting cell wall degradation (Wilson and Mertens, 1995). According to Jalilvand et al. (2008), exogenous fibrolytic enzymes are more effective with higher fibre roughages such as wheat straw as compared to alfalfa hay and corn silage. Contrarily, our results show that the enzymes were only effective on alfalfa hay, which showed the lowest NDF content as compared to grasses and straws. In agreement with Mandevbu et al. (1999), Dean et al. (2008) and Avellaneda et al. (2009), who did not find positive effects of fibrolytic enzymes on degradation of high fibre grasses, in our study the fibrolytic enzyme did not impact the degradation of most of the forages evaluated.

The lack of effects of the fibrolytic enzyme on high fibre forages could also resulted to enzymatic activity, in our experiment. For alfalfa hay, a minimum amount of endoglucanase and xylanase activity would be needed for enzyme mixtures to improve degradation, but for corn silage a further increase in xylanase activity is detrimental (Eun et al., 2007). The enzyme product evaluated in our study showed higher xylanase than cellulase activity, which could have improved the degradation of alfalfa hay but not of high fibre forages. Cellulases and xylanases usually act synergistically to hydrolyze forage cell wall (Bhat and Hazelwood, 2001). Thus, it is possible that an ideal ratio of endoglucanase and xylanase is needed to enhance the effectiveness of exogenous fibrolytic enzymes (Eun et al., 2007). Indeed, exogenous fibrolytic enzymes can access greater surface area compared with cell-bound microbial enzymes. The crystalline regions of cellulose are not easily accessible to endocellulases, whereas the amorphous regions can be attacked by endocellulases and exocellulases (Bhat and Hazelwood, 2001). The quadratic effects of enzymes on in vitro degradation of alfalfa hay have also been found by other researchers. The response to enzyme addition could be not linear (Beauchemin et al., 1995; Kung et al., 2000); therefore, high levels of addition can be less effective than low levels (Jalilvand et al., 2008). It has been speculated that an excess of enzymes in the diet may bind to sites used by rumen bacteria and make them unavailable, creating a barrier against microbial colonization (Beauchemin et al., 2003).

The positive results of enzymes on in vitro degradation and in sacco degradation of alfalfa hay fibre are very consistent with those reported by Yang et al. (1999), Pinos-Rodriguez et al. (2002), Colombatto et al. (2007) and Elwakeel et al. (2007). The mode of action by which enzymes can improve degradation is still subject to speculation (Elwakeel et al., 2007), but it would be related to the fact that exogenous enzymes may enhance rumen enzyme activity (Hristov et al., 2000) due to increments of soluble carbohydrates released from undigested feed particles, which provides additional energy for microbial growth, shortening the lag time for microbial colonization (Yang et al., 1999; Wang et al., 2001). Thus, enzymes decrease the retention time of rumen digesta and the lag time for bacteria to attach to the feed (Sutton et al., 2002).

The exogenous fibrolytic enzymes increased 11% the potentially degradable fractions of NDF and ADF, and degradation rate of NDF (23%) and ADF (16%) in our in vitro assay. In sacco, the enzymes also increased the potentially degradation fraction of NDF (6%) and ADF (8%), as well as the degradation rate (24%) of NDF and ADF of alfalfa hay. These improvements on NDF degradation could induce greater dry matter intakes (Dado and Allen, 1995) by reducing physical rumen fill, increase the energy density of diets and stimulate microbial N production (Oha and Allen, 2000). A 1% increase in forage NDF in vitro degradation has elicited a 0.17 kg increase in DMI and a 0.25 kg increase in 4% FCM yield (Oba and Allen, 1999). Thus, the increases in NDF degradation observed in our in vitro and in sacco assays could potentially increase daily up to 1.0 kg of DMI, which might improve the productivity of cattle fed diets containing alfalfa hay.

Conclusions

It may be conclude that an exogenous fibrolytic enzymes powder preparation increased both in vitro and in sacco fibre degradation of alfalfa hay; however, corn silage, corn stover, elephant grass, Guinea grass and oat straw were not affected. Further studies with fibrolytic enzymes mixtures in order to determine the factors controlling degradation of high fibre forages are needed.

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