Effect of fibrolytic enzymes and incubation pH on in vitro degradation of NDF extracts of alfalfa and orchardgrass

Alis Márquez¹,², Germán Mendoza³, Juan Manuel Pinos-Rodríguez⁴, Hilda Zavaleta⁵, Sergio González⁵, Silvia Buntinx¹, Octavio Loera⁶, Marcos Meneses⁵

¹Facultad de Medicina Veterinaria y Zootecnia. Universidad Nacional Autónoma de México, México City, México
²Decanato de Ciencias Veterinarias. Universidad Centroccidental Lisandro Alvarado, Barquisimeto, Venezuela
³Departamento de Producción Agrícola y Animal. Universidad Autónoma Metropolitana, Xochimilco, México
⁴Instituto de Investigación de Zonas Desérticas. Universidad Autónoma de San Luis Potosí, México
⁵Colegio de Postgraduados en Ganadería. Montecillo, México
⁶Departamento de Biotecnología. Universidad Autónoma Metropolitana, Iztapalapa, México

Corresponding author: Dr. Juan M. Pinos-Rodríguez. Department of Large Animal Clinical Science. College of Veterinary Medicine, Michigan State University. East Lansing, MI 48824, USA – Tel. +1 517 3559593 - Fax: +1 517 4321042. – Email: jpinos@uaslp.mx

Received August 27, 2008; accepted December 21, 2008

ABSTRACT

Two in vitro assays (one with alfalfa and the other with orchardgrass) were conducted to evaluate the effects of a fibrolytic enzyme preparation (enzyme) and initial incubation pH (5.6, 6.2 and 6.8) on the degradation of NDF extracts.

The enzyme increased (P≤0.05) degradation of alfalfa NDF: 1) at 6 h with pH 6.2 and 6.8; 2) at 9 and 12 h with pH 5.6 and 6.2; 3) at 24 h with all pH values; 4) at 48 h with pH 5.6 and 6.8; 5) at 72 h with pH 6.2 and 6.8. Alfalfa NDF degradation was changed (P≤0.05) by the enzyme with increasing pH, as follows: 1) a linear increase at 3 h; 2) a linear decrease at 6 and 12 h; 3) a quadratic effect at 24, 48, and 72 h, and the highest value was observed with pH 6.2. Further effects (P≤0.05) of the enzyme on the alfalfa cell wall were the following: 1) with pH 5.6, the undegradable NDF fraction was reduced and the degradation rate was increased; 2) with pH 6.2, the lag phase was decreased; 3) as pH increased there was a quadratic effect on the lag phase, and the lowest value was found at pH 6.2. For orchardgrass the effects (P≤0.05) of the enzyme were as follows: 1) an increased NDF degradation at 24 and 48 h; 2) a decreased linear degradation of NDF as pH increased, at 3, 6, and 9 h. Scanning electron micrographs of the alfalfa cell wall showed that parenchyma cells appeared broken and the vascular bundles exposed after incubation with the enzyme mixture at pH 6.2.

It may be concluded that a fibrolytic enzyme preparation at pH 6.2 enhanced in vitro degradation of alfalfa NDF.

Key words: Fibrolytic enzyme, Forages, NDF, Degradation.
RIASSUNTO

EFFETTI DI ENZIMI FIBROLITICI E DEL PH DI INCUBAZIONE SULLA DEGRADABILITÀ IN VITRO DELL’NDF DI ERBA MEDICA E DI ERBA MAZZOLINA

Sono state condotte due analisi in vitro (la prima con erba medica, la seconda con erba mazzolina) al fine di valutare gli effetti di una preparazione enzimatica fibrolitica sulla degradabilità dell’estratto NDF attraverso incubazione a diverso pH iniziale (5,6; 6,2 e 6,8).

La degradabilità della frazione NDF di erba medica è aumentata con la presenza della preparazione enzimatica (P≤0,05): 1) a 6h con pH 6,2 e 6,8; 2) a 9 e 12h con pH 5,6 e 6,2; 3) a 24h con tutti i valori di pH considerati; 4) a 48h con pH 5,6 e 6,8; 5) a 72h con pH, 2 e 6,8. La degradabilità della frazione NDF di erba medica è stata così modificata dal preparato enzimatico (P≤0,05) all’aumentare dei valori di pH: 1) incremento lineare a 3h; 2) diminuzione lineare a 6 e a 12h; 3) effetto quadratico a 24, 48 e 72h; il valore più alto si è rilevato a pH 6,2. Sono stati osservati ulteriori effetti dell’enzima (P≤0,05) sulla parete cellulare di erba medica: 1) a pH 5,6 la frazione non degradabile dell’NDF è diminuita e la velocità di degradazione è aumentata; 2) a pH 6,2 la lag-phase è diminuita; 3) all’aumentare del pH è stato osservato un aumento quadratico della lag-phase e il valore più basso è stato osservato a pH 6,2. Per quanto concerne l’erba mazzolina gli effetti della preparazione enzimatica (P≤0,05) sono stati i seguenti: 1) incremento della degradabilità della frazione NDF a 24 e 48h, 2) diminuzione lineare della degradabilità di NDF, con l’aumentare del pH, a 3, 6 e 9h. La scansione elettronica della parete cellulare di erba medica ha evidenziato la rottura del tessuto parenchimale e dei fasci vascolari dopo incubazione con il preparato enzimatico a pH 6,2.

Si può concludere che la preparazione enzimatica fibrolitica ha intensificato, a pH 6,2, la degradabilità in vitro della frazione NDF dell’erba medica.

Parole chiave: Enzimi fibrolitici, Foraggio, NDF, Degradabilità.

Introduction

Forage cell wall digestibility has been greatly improved through research, but ruminal fibre degradation continues to limit the nutritive value of forages (Buxton and Redfearn, 1997). Fibrolytic exogenous enzymes have improved forage cell wall digestibility (Krueger et al., 2008) and degradation of straws (Tang et al., 2008). However, kinetics of these enzymes is highly variable since rate, total digestion (Wang et al., 2004) and ruminal disappearance rates (Pinos-Rodriguez et al., 2008) may be enhanced or not changed at all. These variations are related to diverse factors, but pH is one of the most important (Colombatto et al., 2004; Colombatto et al., 2007). It is known that modern feeding practices often lead to ruminal pH being suboptimal to fibre degradation (Russell and Wilson, 1996). Given their optimal acidic pH, the addition of exogenous fibrolytic enzymes may alleviate the adverse effects on fibre degradation (Morgavi et al., 2000). Therefore, the objective of this experiment was to evaluate the effects of fibrolytic enzymes and incubation pH on in vitro ruminal degradation of NDF extracts of alfalfa and orchardgrass.

Material and methods

Alfalfa and orchardgrass (locally grown at Montecillo, State of México) were cut and sun dried for 24h; then both grasses were dried 48 h at 55°C and ground (2mm particle size; Thomas Willey, Philadelphia, USA). Samples of alfalfa or orchardgrass were boiled (95°C, 2h) using a neutral detergent solution with sodium sulfite (Van Soest et al., 1991). The insoluble fraction with the neutral detergent was recovered using a
Filter (Whatman 541). Insoluble NDF was relieved from the filter and then oven dried 24h at 85°C.

A fibrolytic enzyme preparation (enzyme; Fibrozyme, Alltech Inc., Nicholasville, KY, USA) with xylanase and cellulase activities was used. The enzyme was gently dissolved in distilled water (pH 6.5) and filtered (Whatman 541). One h before the incubation, 2mL of the enzymatic solution were applied directly onto the substrate (500mg NDF) contained in 120mL propylene tubes. This solution should provide an enzymatic activity equivalent to 300IU xylanases per g of NDF according to the enzyme characterization reported by Marquez et al. (2007). Substrate in the controls (non-enzyme treatment) was treated with 2mL of distilled water solution.

Ruminal fluid was obtained from a Holstein steer (400kg BW) fitted with a rumen cannula, fed a mixture of alfalfa hay:corn silage (500:500kg/kg, expressed as DM) and with free access to water and a mineral premix. Animal management and protocol were previously approved by the Graduate Committee from the Veterinary School of the Universidad Nacional Autónoma de México, according to laws enacted by the State of México. Ruminal fluid was collected before the morning feeding (0800h), strained through six layers of cheesecloth, and immediately mixed with McDougall’s artificial saliva (AS) (1mL ruminal fluid per 4mL AS). Artificial saliva, adjusted with glacial acetic acid to an initial pH of 5.6, 6.2 and 6.8, was added into propylene tubes (O2 free by flushing CO2) with substrate and enzyme solution or buffer without enzyme. Incubation periods were 3, 6, 9, 12, 24, 48 and 72h; fermentation was stopped by freezing (-20°C) for 12 h and residual material was collected by filtration (Whatman 541) and dried overnight at 100°C.

The DM (method 930.15), N (method 990.03), Ash (method 985.01), ADF (method 973.18), and lignin (method 973.18) of AOAC (1997), and NDF of Van Soest et al. (1991), were performed for the neutral detergent residues of alfalfa or orchardgrass. The NDF was determined without a heat stable amylase, and the ADF determination included residual ash. Lignin was determined by the sulphuric acid procedure. Chemical composition of NDF extracts of alfalfa and orchardgrass is shown in Table 1.

For NDF, 126 tubes for alfalfa and 108 for orchardgrass (i.e., three tubes for enzyme level 0 or 1; incubation pH 5.6, 6.2, 6.8; and incubation time 3, 6, 9, 12, 24, 48, 72h for alfalfa and 3, 6, 9, 12, 24, 48h for orchardgrass) were incubated; each combination was in triplicate. The in vitro incubation procedure was performed twice (runs).

Table 1. Chemical composition of NDF extracts of alfalfa and orchardgrass (values expressed as g/kg dry base except for DM values which are expressed as g/kg).

|                        | NDF Alfalfa | NDF orchardgrass |
|------------------------|-------------|------------------|
| Dry matter (65°C, 48h) | 99.8        | 98.4             |
| Crude protein          | 55          | 79               |
| Neutral detergent fibre | 933         | 944              |
| Acid detergent fibre   | 736         | 525              |
| Lignin                 | 201         | 104              |
| Ash                    | 18          | 59               |
The orchardgrass assay was run three days after that of alfalfa. At the end of each incubation time, tubes were cooled (4°C) and then were processed gradually.

The NDF degradation was calculated as:

\[ \text{(initial NDF)} - \text{(residual NDF)/initial NDF}. \]

These data were analysed using a first order model with lag phase (Mertens and Loften, 1980):

\[ R = D_0 \cdot e^{-kt} - L + U; \]

where: \( R \) is residue at time \( t \), \( D_0 \) is digestible fraction, \( k \) is digestible rate constant, \( L \) is discrete lag time, and \( U \) is indigestible fraction. The equation to calculate discrete lag time was:

\[ D_0 \cdot e^{-kt} + U \text{ when } t > L \text{ and } R = D_0 + U \text{ when } 0 < t < L, \]

where: \( R \) = cell wall residue (at time after inoculation = \( t \)), \( D_0 \) = digestible fraction (at time \( t < L \), \( D_0 = R-U \)), \( k \) = digestion rate constant; \( L \) = discrete lag time; \( U \) = indigestible fraction. The equation for the determinations of discrete lag time was:

\[ D_0 \cdot e^{-kt} = L_0 \text{ when } t > L; \]

\[ \ln D_0 = \ln D_0 - k(L); \]

\[ L_0 = (\ln D_0 - \ln D_0)/k \text{ where: } \]

\[ D_0 \text{ = intercept of the equation of } \ln(RU) \text{ over } \]

\[ \text{time at } t = 0. \]

Logarithmic transformation \((L-T)\) was used by determining the natural logarithm of potential digestible residue at the end of each fermentation period and regressing this over time. Potentially digestible residue was calculated assuming that digestion was completed at 72h of fermentation and subtracting this from the residue at each fermentation time, as described by Smith et al. (1972).

Data were analysed separately for each intact substrate using a generalized complete block design (run, n=2 was the blocking criteria) with a factorial arrangement 2x3, where factors were the enzyme addition (level 0, 1) and incubation pH (6.8, 6.2, 5.6). Orthogonal polynomials were used to test lineal and quadratic effects of pH levels in level 1 of enzyme. All the statistical analyses used the “GLM” option of SAS (2002). Significant differences were accepted when \( P \leq 0.05 \).

The effect of fibrolytic enzymes on microstructure and degradation of alfalfa cell wall was evaluated using scanning electron microscopy. Samples of substrates before and after in vitro incubation treatments were washed with hot (40°C) water and dried at 100°C for 12h. Tissue residues were attached to specimen holders with a double-stick carbon tape and coated with a 15nm thick gold layer using an ion sputter coater (Fine Coat JFC-1100, JEOL Ltd, Tokyo, Japan) and examined with a scanning electron microscope (JSM-6390, JEOL Ltd Tokyo, Japan) operating at 15 Kv. Micrographs were digitally captured and analysed.

**Results**

Degradation and kinetics of a NDF extract of alfalfa are shown in Table 2. From 6 to 72h alfalfa NDF degradation was affected by both enzyme and initial incubation pH. The interaction of enzyme with pH suggests that the enzyme activity depended on initial pH as follows: 1) at 6h the enzyme with pH 6.2 and 6.8, but not 5.6, increased NDF degradation; 2) at 9 and 12h the enzyme enhanced NDF degradation only with pH 5.6 and 6.2; 3) at 24h the enzyme increased NDF degradation at all pH values; 4) at 48h the enzyme increased NDF degradation only with pH 5.6 and 6.8; and 5) at 72h the enzyme increased NDF degradation with pH 6.2 and 6.8, but not 5.6. Increasing initial incubation pH and the enzyme had the following effects on NDF degradation: a linear increase at 3h, a linear decrease at 6 and 12h, and a quadratic response at 24, 48, and 72h, whereas the highest NDF degradation value was observed with pH 6.2. As for kinetics of alfalfa NDF degradation, there were enzyme with incubation pH interactions: 1) with pH 5.6 but not 6.2 or 6.8, the enzyme reduced the undegradable fraction of alfalfa NDF but increased the NDF degradation rate; 2) with pH 6.2 but not 5.6 or 6.8, the enzyme reduced the lag
Table 2. Degradation and kinetics of a NDF extract of alfalfa (values expressed as g/kg dry base).

|                      | Without enzyme | With enzyme | SEM | Significance |          |
|----------------------|----------------|-------------|-----|--------------|----------|
|                      | pH 5.6          | pH 6.2      | pH 6.8 | SEM          |          |
| pH                   | 21              | 30          | 38   | 18           | 30       | 32       | 2.1 ns | * ns | ns Linear |
|                      | 56abc           | 48bc        | 44c  | 48bc          | 65b      | 60ab     | 3.4 *** | ** *** | *** ns |
|                      | 90bc            | 74c         | 82bc | 141           | 125b     | 96b      | 4.1 *** | *** *** | *** Linear |
|                      | 128b            | 107b        | 113b | 163           | 157b     | 117b     | 5.0 *** | ** * | Linear |
|                      | 254c            | 269c,d      | 249d | 293c          | 353b     | 315b     | 7.1 *** | *** * | Quadratic |
|                      | 286c            | 405b        | 264c | 341           | 386b     | 329b     | 5.7 *** | *** *** | *** Quadratic |
|                      | 393b            | 421b        | 330c | 386b          | 456b     | 394b     | 10.7 *** | *** *** | *** Quadratic |
| Undegradable fraction| 271a            | 153b        | 181b | 117b          | 150b     | 169b     | 26.1 * | ns * | ns |
|                      | 0.4             |             | 3c   | 6a            | 4bc      | 5b       | 5b      | 5b | 0.03 |
|                      | ns              |             |       |               |          |          |         |     | 0.3 |

ns: not significant; *: P≤0.05; **: P≤0.01; ***: P≤0.001.

*Means in the same row with different superscripts differ (P≤0.05).
phase of NDF degradation. Finally, there was a quadratic effect on the lag phase of NDF degradation as pH increased; the lowest lag phase was with pH 6.2, and the highest with 5.6 and 6.8.

The enzyme increased NDF degradation at 24 and 48h incubation and, in addition, it caused a linear decrease of orchardgrass NDF at 3, 6, 9, and 12h, as initial incubation pH increased. However, neither the enzyme nor pH affected degradation kinetics of orchardgrass NDF, as well as undegradable fraction, degradation rate, or lag phase (Table 3).

Since the kinetics of orchard NDF were not affected by enzyme or pH, scanning electron microscopy results are presented only for alfalfa, where changes in microstructure were observed. The tissue organisation of the alfalfa cell wall was intact before incubation, but changes were detected in vascular bundles and parenchyma cells when alfalfa was incubated with ruminal fluid at pH 6.8 and further treated with the enzyme (Figure 1).

Discussion

The enzyme increased degradation of NDF extracts of alfalfa (~24%) from 6 to 72h, and NDF extracts of orchardgrass (~15%) at 24 and 48h. Indeed, Eun et al. (2007a) found that some fibrolytic enzymes preparations increased NDF degradation at 12h, 18h and 24h of incubation. However, Ranilla et al. (2008) observed that the effects of an exogenous fibrolytic enzyme on in vitro fermentation of the alfalfa cell wall were generally higher after 5h, intermediate after 10h and less marked, or even absent, after 24h.

Our results confirm that incubation pH is an important modulatory factor of the enzymatic response. The effect of the fibrolytic enzyme with the initial pH was variable: at 6, 24, 48 and 72h the effect was better with pH 6.2, but at 9 and 12h it was better with pH 5.6. The lowest undegradable fraction of alfalfa NDF induced by the enzyme was with pH 5.6, but the lowest lag phase was with pH 6.2. Contrarily to Colombatto et al. (2007), who suggested that some fibrolytic enzymes work better close to the neutral ruminal pH, the fibrolytic enzyme used in our study worked better at pH 5.6 and 6.2, as compared to 6.8. A large optimal pH range (4.0 to 6.0) was reported by Colombatto et al. (2004) for several fibrolytic enzyme preparations. According to Morgavi et al. (2000), commercial fibrolytic enzymes show their optimal activity in a smaller range of pH, which is lower than the optimal rumen pH for fibre degradation. Furthermore, Yang et al. (2002) found that NDF degradation was increased 8% by fibrolytic enzymes with pH 5.5, and 18% with pH 6.0, in diets for dairy cows.

The enzyme positively affected degradation rate and lag phase of NDF extracts of alfalfa. This indicates that those exogenous enzymes attached to the substrate before the native enzymes (Colombatto et al., 2003) to degrade a greater extent of NDF, and to increase the potentially digestible fraction (Nadeau et al., 1996). It is also possible that exogenous enzymes promote lignin solubility, reducing the indigestible fraction. Regarding the effects of enzymes on NDF kinetics, Varel et al. (1993) reported an increase in rate but not in extent of NDF degradation. The in situ lag phase of alfalfa NDF digestion was reduced when treated with fibrolytic enzymes, however, no effects were observed in the rate of degradation (Sievert and Shaver, 1993).

Stems and leaves of alfalfa are composed by parenchyma of mesophyll and cortex with primary cell walls (without lignin), and vascular tissues containing vessels and fibres with lignin. The preservation of cellular structure was not expected after treatment with exogenous fibrolytic enzymes, since
Table 3. Degradation kinetics of a NDF extract of orchardgrass (values expressed as g/kg dry base).

|                | Without enzyme | With enzyme | SEM | Significance |
|----------------|---------------|-------------|-----|--------------|
|                | pH 5.6   | pH 6.2  | pH 6.8 | pH 5.6  | pH 6.2  | pH 6.8 |
| pH 5.6         | 22       | 16       | 5      | 16       | 15       | 11      | 2.5     | ns         | *         | ns         | Linear    |
| pH 6.2         | 24       | 23       | 17     | 35       | 28       | 22      | 2.8     | ns         | ns         | ns         | Linear    |
| pH 6.8         | 52       | 43       | 34     | 54       | 38       | 23      | 3.9     | ns         | ns         | ns         | Linear    |
| 9 h            | 90       | 79       | 86     | 107      | 102      | 83      | 6.1     | ns         | ns         | ns         | ns        |
| 12 h           | 163      | 167      | 145    | 198      | 172      | 185     | 6.1     | *          | ns         | ns         | ns        |
| 24 h           | 177      | 180      | 194    | 220      | 196      | 218     | 11.7    | *          | ns         | ns         | ns        |
| 48 h           | 108      | 148      | 154    | 78       | 109      | 216     | 42.0    | ns         | ns         | ns         | ns        |
| Undegradable fraction |            |            |        |            |            |        |         |            |            |            |           |
| Degradation rate | g/h      |            |        |            |            |        |         |            |            |            |           |
| Lag phase      | h         |            |        |            |            |        |         |            |            |            |           |

*ns: not significant; *: P≤0.05.
they contain cellulose and xylanase which digest the main components of primary cell walls (Colombatto et al., 2007). Studies using scanning electron microscopy indicate that before incubation the alfalfa particles showed intact tissue organisation, where stems and leaves fragments could be recognised with the vascular tissues compactly surrounded by parenchyma cells (Figure 1A). The cells of vessels appeared intact, with primary cell wall covering the lignified wall thickenings, helicoidally arranged (Figure 1B). When alfalfa particles were incubated 72 h in ruminal fluid and pH 6.2, vascular bundles appeared exposed with few parenchyma cells covering the vessels (Figure 1C). The activity of bacteria on the primary cell wall could be detected at higher magnification (3700X). Bacteria appeared as granular deposits near the lignified cell wall thickening of the vessels, digesting the cellulosic primary wall (Figure 1D). After treating the alfalfa particles with ruminal fluid and the enzyme, the tissue was modified: parenchyma cells appeared broken and the vascular bundles exposed (Figure 1E). There was evidence of cell wall corrosion and complete degradation of the primary cell wall.
cell wall of the vessels (Figure 1F), resulting in smaller fragments composed mainly of lignified cells (vessels and botanical fibres) and broken residues of parenchyma cells.

Under these circumstances, exogenous enzymes may help fibre degradation when pH falls below 6.2 and ruminal fibrolytic activity is depressed (Russell and Wilson, 1996). Morgavi et al. (2000) reported that enzyme addition alleviated the drop in DM digestibility caused by low pH, but found no differences when pH was optimum for fibre degradation. If this is the case, it may imply that enzyme levels and type of activities not only have to be adjusted according to forage type (Colombatto et al., 2003) but also to expected rumen pH values (Colombatto et al., 2007).

Although our goal was not a comparison between alfalfa NDF and orchardgrass NDF, there were substantial differences in degradation caused by the action of fibrolytic enzymes on both NDF extracts. This may be partly explained by the fact that these forages have a very different structure and content of NDF, ADF, and lignin content forages, such as reported by Mertens (1993). Previous studies have shown that exogenous fibrolytic enzymes exert a direct catalytic action on cell wall components of alfalfa (Pinos-Rodriguez et al., 2002; Eun et al., 2007b).

Conclusions

The addition of fibrolytic enzymes with an initial incubation pH 6.2 enhanced in vitro degradation and decreased undegradable fractions, as well as lag phase of alfalfa NDF.

REFERENCES

AOAC, 1997. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Washington, DC, USA.

Buxton, R.D., Redfearm, D.D., 1997. Plant limitations to fiber digestion and utilization. J. Nutr. 127:814S-818S.

Colombatto, D., Hervás, G., Yang, W.Z., Beauchemin, K.A., 2003. Effects of enzyme supplementation of total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. J. Anim. Sci. 81:2617-2627.

Colombatto, D., Mould, F.L., Bhat, M.K., Owen, E., 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the in vitro ruminal fermentation of alfalfa stems. Anim. Feed Sci. Tech. 137:150-162.

Colombatto, D., Mould, F.L., Bhat, M.K., Phipps, R.H., Owen, E., 2004. In vitro evaluation of fibrolytic enzymes as additives for maize (Zea mays L.) silage. I. Effects of ensiling temperature, enzyme source and addition level. Anim. Feed Sci. Tech. 111:111-128.

Eun, J.S., Beauchemin, K.A., Schulze, H., 2007a. Use of an in vitro fermentation bioassay to evaluate improvements in degradation of alfalfa hay due to exogenous feed enzymes. Anim. Feed Sci. Tech. 135:315-328.

Eun, J.S., Beauchemin, K.A., Schulze, H., 2007b. Use of exogenous fibrolytic enzymes to enhance in vitro fermentation of alfalfa hay and corn silage. J. Dairy Sci. 90:1440-1451.

Krueger, N.A., Adesogan, A.T., Staples, C.R., Krueger, W.K., Kim, S.C., Littell, R.C., Sollenberger, L.E., 2008. Effect of method of applying fibrolytic enzymes or ammonia to bermudagrass hay on feed intake, digestion, and growth of beef steers. J. Anim. Sci. 86:882-889.

Márquez, A.A.T., Mendoza, M.G.D., Gonzalez, S.S., Buntinx, D.S.E., Loera, C.O., 2007. Actividad fibrolítica de enzimas producidas por Trametes sp. EuM1, Pleurotus ostreatus y Aspergillus niger AD96.4 en fermentación sólida. Interciencia 32:780-785.
Mertens, D.R., 1993. Kinetics of cell wall digestion and passage in ruminants. In: H.G. Hung, D.R. Buxton, R.D. Hatfield and J. Ralph (eds.) Forage Cell Wall Structure and Digestibility. ASA-CSSA-SSSA, Madison, WI, USA, pp 535-570.

Mertens, D.R., Loften, J.R., 1980. The effect of starch on forage fiber digestion kinetics in vitro. J. Dairy Sci. 63:1437-1446.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., Iwaasa, A.D., Yang, W.Z., McAllister, T.A., Wang, Y., 2000. Synergy between ruminal fibrolytic enzymes and enzymes from Trichoderma longibrachiatum. J. Dairy Sci. 83:1310-1321.

Nadeau, E.M., Buxton, D.R., Lindgren, E., Lingvall, P., 1996. Kinetics of cell-wall digestion of orchardgrass and alfalfa silages treated with cellulase and formic acid. J. Dairy Sci. 79:2207-2216.

Pinos-Rodríguez, J.M., González, S.S., Mendoza, G.D., Bárcena, R., Cobos, M., 2002. Efecto de las enzimas fibrolíticas exógenas en la digestibilidad in vitro de la pared celular de heno de alfalfa (Medicago sativa) o de ballico (Lolium perene). Interciencia 27:28-32.

Pinos-Rodríguez, J.M., Moreno, R., González, S.S., Robinson, P.H., Mendoza, G., Álvarez, G., 2008. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. Anim. Feed Sci. Tech. 142:210-219.

Ranilla, M.J., Tejido, M.L., Giraldo, L.A., Tricárico, J.M., Carro, M.D., 2008. Effects of an exogenous fibrolytic enzyme preparation on in vitro ruminal fermentation of three forages and their isolated cell walls. Anim. Feed Sci. Tech. 145:109-121.

Russell, J.B., Wilson, D.V., 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J. Dairy Sci. 79:1503-1509.

SAS, 2002. User’s Guide Statistics. Version 8.02. SAS Institute Inc., Cary, NC, USA.

Sievert, S.J., Shaver, R.D., 1993. Carbohydrate and Aspergillus oryzae effects on intake, digestion, and milk production by dairy cows. J. Dairy Sci. 76:245-254.

Smith, L.W., Goering, H.K., Gordon, C.H., 1972. Relationships of forage compositions with rate of cell wall digestion and indigestibility of cell walls. J. Dairy Sci. 55:1140-1147.

Tang, S.X., Tayo, G.O., Tan, Z.L., Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, H.F., Shen, S.B., 2008. Effects of yeast culture and fibrolytic enzymes supplementation on in vitro fermentation characteristics of low-quality cereal straws. J. Anim. Sci. 86:1164-1172.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Varel, V.H., Kreikemeier, K.K., Jung, H.-J.G., Hatfield, R.D., 1993. In vitro stimulation of forage fiber degradation by ruminal microorganisms with Aspergillus oryzae fermentation extract. Appl. Environ. Microb. 59:3171-3176.

Wang, Y., Spratling, B.M., ZoBell, D.R., Wiedmeier, R.D., McAllister, T.A., 2004. Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. J. Anim. Sci. 82:198-208.

Yang, W.Z., Beauchemin, K.A., Vedres, D.D., 2002. Effects of pH and fibrolytic enzymes on digestibility, bacterial synthesis, and fermentation in continuous culture. Anim. Feed Sci. Tech. 102:137-150.