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in

Priolo A. (ed.), Biondi L. (ed.), Ben Salem H. (ed.), Morand-Fehr P. (ed.).
Advanced nutrition and feeding strategies to improve sheep and goat

Zaragoza : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 74

2007
pages 257-261

Influence of fibrolytic enzymes on *in vitro* methane production and rumen fermentation of a substrate containing 60% of grass hay

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SUMMARY – Batch cultures of mixed rumen microorganisms were used to study the effects of exogenous fibrolytic enzymes on the *in vitro* fermentation of a substrate composed by a 60:40 mixture of grass hay and concentrate. Three enzymatic preparations were tested: cellulase from *Aspergillus niger* (CEL), xylanase from *Trichoderma viride* (XYL), and a 1:1 mixture cellulase:xylanase (MIX). Samples of substrate (500 mg dry matter (DM)) were accurately weighed into 120-ml serum bottles and enzymes were added directly into the bottles at three levels: 0 (control; CON), 15 (-15) and 30 (-30) IU/g substrate DM. Bottles were kept at room temperature for 24 h before incubation with buffered rumen fluid. All enzymatic treatments, with the exception of CEL-15, increased ($P<0.05$) CH_4 production after 6 h of incubation, but after 24 h of incubation differences ($P<0.05$) with control were only detected for XYL-15, XYL-30 and MIX-30. Acetate, propionate and butyrate productions at both 6 and 24 h of incubation were increased ($P<0.05$) by all enzymatic treatments. NH_3-N concentrations, pH and gas production at 24 h of incubation were not affected ($P>0.05$) by the addition of enzymes. The treatment of substrate with XYL-15, MIX-15 and MIX-30 increased ($P<0.05$) neutral-detergent fibre degradability (61.3, 59.9 and 60.6%, respectively) compared to the control (56.5%). The results indicate that the xylanase was more effective than the cellulase in stimulating the *in vitro* fermentation of substrate, but for most of the measured parameters there were no differences ($P>0.05$) due to the level of enzymes (15 *vs* 30 IU/g DM).

Keywords: *In vitro* rumen fermentation, cellulase, xylanase, batch cultures, methane.

RESUME – *"Influence des enzymes fibrolytiques sur la production de méthane et la fermentation* in vitro *d'un substrat à 60% de foin de graminées". Les effets des enzymes fibrolytiques exogènes sur la fermentation d'une ration composée d'herbe et concentré (60:40) ont été étudiés en utilisant une technique* in vitro *de type «batch». Les trois préparations enzymatiques étaient une cellulase d'*Aspergillus niger *(CEL), une xylanase de* Trichoderma viride *(XYL) et un mélange 1:1: des deux (MIX). Des échantillons de la ration (500 mg matière sèche (MS)) ont été traités avec des préparations enzymatiques à trois doses : 0 (témoin ; CON), 15 (-15) et 30 (-30) UI/g MS, pendant 24 h à température ambiante, puis mis en suspension dans un milieu anaérobie, inoculé avec du contenu de rumen, et incubés à 39°C. La production de CH_4 après 6 h d'incubation a augmenté ($P<0,05$) avec tous les traitements enzymatiques, sauf avec CEL-15, tandis qu'après 24 h on n'a eu de différences qu'entre XYL-15, XYL-30, MIX-30 et le témoin. Les productions d'acétate, propionate et butyrate après 6 et 24 h d'incubation ont été plus importantes ($P<0,05$) avec les traitements enzymatiques qu'avec le témoin, mais aucune différence significative n'a été notée entre les traitements pour les concentrations en azote ammoniacal ou pour le gaz produit. Le traitement de la ration avec XYL-15, MIX-15 et MIX-30 a augmenté ($P<0,05$) la dégradabilité de la fibre (61,3, 59,9 et 60,6%, respectivement) par rapport au témoin (56,5%). Ces résultats montrent que la xylanase a été plus effective pour stimuler la fermentation* in vitro *de la ration, mais pour la plupart des paramètres étudiés aucune différence significative n'a été notée entre les doses d'enzymes (15* vs *30 UI/g MS).*

Mots-clés : *Fermentation ruminale* in vitro*, cellulase, xylanase, cultures en batch, méthane.*

Introduction

Cellulose and hemicellulose are quantitatively the most important structural carbohydrates present in forages. Rumen microorganisms produce enzymes that catalyse their hydrolysis, but the complex network formed by structural carbohydrates and lignin reduces their digestibility and restricts efficient utilization of forages by ruminants. Many attempts have been made to overcome this limitation, and in the last years the use of enzymes has received considerable attention. Results, however, have been highly variable. Exogenous fibrolytic enzymes might enhance attachment, and/or improve access to the cell wall matrix by rumen microorganisms and by doing so, accelerate the rate of digestion

(Nsereko *et al.*, 2000). The effects of enzymes are influenced by factors such as type and dose of enzyme, type of diets fed to the animals and enzymes application methods (Beauchemin *et al.*, 2003). The objective of this study was to evaluate the effects of two doses of three fibrolytic enzymes on the *in vitro* rumen fermentation of one substrate containing 60% of grass hay.

Material and methods

The substrate (dry matter (DM) basis) was composed of grass hay (60%) and a commercial concentrate (40%) based on barley, maize, soybean meal and a vitamin-mineral mixture. Crude protein, neutral-detergent fibre (NDF) and acid-detergent fibre content of substrate was 151, 470 and 213 g/kg DM, respectively. Samples of 500 mg of ground substrate (1-mm screen) were accurately weighed into 120-ml serum bottles. Three different enzymes preparations were tested: cellulase from *Aspergillus niger* (CEL; Cellulase 22178; Fluka Chemie GmbH), xylanase from *Trichoderma viride* (XYL; Xylanase 95595; Fluka Chemie GmbH), and a 1:1 mixture cellulase:xylanase (MIX). Enzyme preparations were added directly into the bottles at three levels: 0 (control; CON), 15 (-15) and 30 (-30) international units (IU)/g substrate DM. Solutions of each enzyme preparation containing either 3.75 or 7.50 units per ml were prepared in 0.1 M sodium phosphate buffer (pH 6.5). Two ml of the corresponding solution were added directly to each bottle 24 h before starting the incubation, and bottles were kept at room temperature (21-23ºC) until incubation. This pre-treatment of samples with enzymes was selected as previous *in vitro* studies (Giraldo *et al.*, 2004) have shown that this enzyme-feed interaction increased the efficacy of enzymes. Two ml of 0.1 M sodium phosphate buffer were added to the bottles corresponding to control treatment.

Rumen fluid was obtained from four rumen-cannulated Merino sheep fed medium-quality grass hay *ad libitum* and supplemented daily with 400 g of concentrate. Rumen contents of each sheep were obtained before the morning feeding, mixed and strained through four layers of cheese-cloth. Particle-free fluid was mixed with the buffer solution of Goering and Van Soest (1970) in a proportion 1:4 (vol:vol) at 39ºC under continuous flushing with CO_2. Fifty ml of buffered rumen fluid were added into each bottle, bottles were sealed with rubber stoppers and aluminium caps and incubated at 39ºC for 24 h. Four incubation runs were performed on different days and in each of them two bottles per treatment were included. Two blanks per treatment (bottles without substrate but with the corresponding enzymatic solution added) were included in each incubation run to investigate the effects of enzyme fermentation itself on gas and volatile fatty acids (VFA) production. After 6 hours of incubation, total gas production was measured in all bottles using a pressure transducer and a calibrated syringe, and a gas sample (about 15 ml) from each bottle was stored in an evacuated tube holder (Venoject®, Terumo Europe, Belgium) before analysis for CH_4 concentration. Two ml of bottle contents were sampled for VFA and NH_3-N analyses. Bottles were withdrawn from the incubator 24 h after inoculation, gas production was measured and a gas sample for CH_4 determination was taken as before described. Bottles were then uncapped, the pH was immediately measured and the fermentation was stopped by swirling the bottles in ice. Two ml of the bottle content were taken for VFA and NH_3-N analyses. Finally, the content of the bottles was transferred to previously weighed filter crucibles, the solid residue was washed with 50 ml of hot distilled water (50ºC) and the crucibles were dried at 50ºC for 48 h to estimate DM apparent degradability (DMAD). Residues were analysed for NDF and acid-detergent fibre (ADF) to estimate fibre degradability (NDFD and ADFD). Procedures for analysis of VFA, CH_4 and NH_3-N have been described by Carro *et al.* (1999).

The amounts of VFA produced were obtained by subtracting the amounts present initially in the incubation medium from those determined at the end of the incubation period. Data were analysed as an ANOVA with seven enzymatic treatments (CON, CEL-15, CEL-30, XYL-15, XYL-30, MIX-15 and MIX-30) and four rumen inocula as main effects. When a significant effect of the treatment ($P<0.05$) was detected, differences between means were assessed by the LSD test.

Results and discussion

The effects of the treatment with fibrolytic enzymes on *in vitro* production of CH_4 and VFA, and NH_3-N concentrations after 6 hours of incubation of substrate samples with rumen mixed microorganisms are shown in Table 1. There were no effects ($P>0.05$) of enzymes either on gas production or on NH_3-N concentration. All enzymatic treatments, with the exception of CEL-15,

increased (P<0.05) CH_4 production. Acetate, propionate and butyrate productions at 6 h of incubation were increased (P<0.05) by all enzymatic treatments and, as a consequence, total VFA production was increased (P<0.05) by 14, 14, 23, 18, 18 and 16% for CEL-15, CEL-30, XYL-15, XYL-30, MIX-15 and MIX-30 treatments, respectively. Compared to the buffer treated substrate, all enzymatic treatments increased (P<0.05) molar proportions of propionate without affecting those of acetate, and therefore decreased (P<0.05) the acetate/propionate ratio. Enzymes had no effect (P>0.05) on the CH_4/VFA ratio.

Table 1. Influence of different enzymatic treatments on production of gas (ml), CH_4 (µmol) and volatile fatty acids (VFA; µmol), acetate/propionate (Ac/Pr) and CH_4/VFA ratios and NH_3-N concentration (mg/l) after *in vitro* fermentation of a substrate (500 mg DM) containing 60% of grass hay in batch cultures of mixed rumen microorganisms for 6 h (n=8)

Item	Enzymatic treatment[†]							s.e.d. [††]
	CON	CEL-15	CEL-30	XYL-15	XYL-30	MIX-15	MIX-30	
Gas	44.6	45.9	44.9	47.0	46.6	47.3	45.7	1.20
CH_4	237 ^{a}	257 ^{ab}	269 ^{bc}	307 ^{d}	306 ^{cd}	283 ^{bcd}	298 ^{cd}	15.9
Total VFA	1031 ^{a}	1176 ^{b}	1177 ^{b}	1263 ^{c}	1220 ^{bc}	1219 ^{bc}	1198 ^{b}	27.7
Acetate	643 ^{a}	726 ^{b}	726 ^{b}	781 ^{c}	761 ^{bc}	755 ^{bc}	742 ^{b}	17.54
Propionate	267 ^{a}	316 ^{b}	315 ^{b}	339 ^{c}	326 ^{bc}	330 ^{bc}	318 ^{b}	7.63
Butyrate	91.6 ^{a}	99.9 ^{b}	100 ^{bc}	108 ^{c}	102 ^{bc}	101 ^{bc}	102 ^{bc}	4.01
Other VFA[†††]	30.2 ^{a}	33.7 ^{abc}	36.3 ^{c}	34.7 ^{bc}	31.4 ^{ab}	33.1 ^{abc}	35.4 ^{bc}	2.02
Ac/Pr	2.41 ^{b}	2.30 ^{a}	2.30 ^{a}	2.30 ^{a}	2.33 ^{a}	2.29 ^{a}	2.33 ^{a}	0.044
CH_4/VFA	0.230	0.218	0.228	0.243	0.251	0.233	0.249	0.0206
NH_3-N	179	184	179	175	178	179	177	4.6

[†]Treatments: CON: control; CEL-15 and CEL-30: cellulase at 15 and 30 IU/g substrate DM, respectively; XYL-15 and XYL-30: xylanase at 15 and 30 IU/g substrate DM, respectively; MIX-15 and MIX-30: cellulase:xylanase mixture (1:1) at 15 and 30 IU/g substrate DM, respectively.
[††]Standard error of the difference.
[†††]Calculated as the sum of isobutyrate, isovalerate and valerate acids.
[a, b, c, d]: Mean values within a row with unlike superscript letters differ (P<0.05).

No effects (P>0.05) of any enzymatic treatment were observed on final pH and gas production after 24 h of incubation. In agreement with the results obtained at 6 h of incubation (Table 2). There was no significant change (P>0.05) in the NH_3-N concentration with added enzymes, thus indicating no differences in protein degradability and/or NH_3-N incorporation by rumen microorganisms.

Although all enzymatic treatments (with the exception of CEL-15) increased CH_4 production after 6 h of incubation, most of the differences disappeared after 24 h and only XYL-15 and MIX-30 treatments increased (P<0.05) CH_4 production (by 7.9 and 10.5%, respectively). However, no effects (P>0.05) of enzymatic treatments on the CH_4/VFA ratio or molar proportions of acetate and propionate were detected. In agreement with the results observed at 6 h of incubation, all enzymatic treatments increased (P<0.05) the production of acetate, propionate and butyrate. Total VFA production was increased by 8.3, 7.9, 10.7, 8.1, 9.6 and 8.5% for CEL-15, CEL-30, XYL-15, XYL-30, MIX-15 and MIX-30 treatments, respectively. These results suggest that added enzymes stimulated the *in vitro* fermentation of substrate. The effects of enzymes were more marked at 6 than at 24 h of fermentation, thus indicating that enzymes produced their effects at early stages of fermentation. Dawson and Tricario (1999) also reported that fibrolytic enzyme effects *in vitro* were generally larger during the initial stages of degradation.

The treatment of substrate with CEL-15, CEL-30, XYL-15, XYL-30, MIX-15 and MIX-30 increased NDFD by 4.2, 4.8, 8.5, 6.7, 6.0 and 7.3%, respectively, when compared to CON, but the difference did not reach the significance level (P>0.05) for CEL-15 and CEL-30 treatments. Effects of enzymes on ADFD were only evident for XYL-15, which increased ADFD by 12.2% (P<0.05). For most of the

measured parameters, there were no effects (P>0.05) of level of enzyme (15 *vs* 30 IU/g DM) and no interaction (P>0.05) enzymatic treatment x level of enzyme was detected.

Table 2. Influence of different enzymatic treatments on final pH, production of gas (ml), CH_4 (µmol) and volatile fatty acids (VFA; µmol), acetate/propionate (Ac/Pr) and CH_4/VFA ratios, NH_3-N concentration (mg/l), dry-matter apparent degradability (DMAD; %) and neutral- and acid-detergent fibre degradability (NDFD and ADFD, respectively; %) of a substrate (500 mg DM) containing 60% of grass hay incubated in batch cultures of mixed rumen microorganisms for 24 h (n=8)

Item	Enzymatic treatment†							s.e.d. ††
	CON	CEL-15	CEL-30	XYL-15	XYL-30	MIX-15	MIX-30	
pH	6.58	6.59	6.57	6.56	6.58	6.56	6.57	0.016
Gas	113	116	114	116	113	116	111	2.8
CH_4	670 ^{a}	690 ^{ab}	717 ^{abc}	723 ^{bc}	731 ^{abc}	690 ^{abc}	740 ^{c}	7.7
Total AGV	2496 ^{a}	2703 ^{bc}	2692 ^{b}	2762 ^{c}	2697 ^{bc}	2735 ^{bc}	2708 ^{bc}	33.9
Acetate	1506 ^{a}	1631 ^{b}	1628 ^{b}	1645 ^{b}	1609 ^{b}	1642 ^{b}	1620 ^{b}	18.7
Propionate	591 ^{a}	654 ^{bc}	645 ^{b}	673 ^{c}	648 ^{b}	660 ^{bc}	648 ^{b}	11.6
Butyrate	295 ^{a}	309 ^{b}	312 ^{bc}	326 ^{c}	322 ^{bc}	318 ^{bc}	325 ^{c}	7.4
Other VFA†††	103 ^{a}	108 ^{ab}	106 ^{a}	116 ^{c}	117 ^{c}	113 ^{bc}	115 ^{c}	3.5
Ac/Pr	2.56 ^{b}	2.50 ^{ab}	2.55 ^{b}	2.46 ^{a}	2.49 ^{ab}	2.49 ^{ab}	2.51 ^{ab}	0.042
CH_4/VFA	0.268	0.255	0.267	0.252	0.273	0.262	0.271	0.0126
NH_3-N	258	262	259	257	266	255	259	11.1
DMAD	60.9 ^{a}	62.6 ^{ab}	63.5 ^{ab}	63.0 ^{ab}	63.8 ^{b}	63.8 ^{b}	63.8 ^{b}	1.32
NDFD	56.5 ^{a}	58.9 ^{ab}	59.2 ^{ab}	61.3 ^{b}	60.3 ^{b}	59.9 ^{b}	60.6 ^{b}	1.66
ADFD	50.1 ^{a}	53.3 ^{ab}	53.4 ^{ab}	56.2 ^{b}	53.5 ^{ab}	54.9 ^{ab}	54.7 ^{ab}	2.50

†Treatments: CON: control; CEL-15 and CEL-30: cellulase at 15 and 30 IU/g substrate DM, respectively; XYL-15 and XYL-30: xylanase at 15 and 30 IU/g substrate DM, respectively; MIX-15 and MIX-30: cellulase:xylanase mixture (1:1) at 15 and 30 IU/g substrate DM, respectively.
††Standard error of the difference.
†††Calculated as the sum of isobutyrate, isovalerate and valerate acids.
$^{a, b, c}$: Mean values within a row with unlike superscript letters differ (P<0.05).

Several authors (Nsereko *et al.*, 2000; Wallace *et al.*, 2001) have suggested that exogenous enzymes could increase fibre degradation through a hydrolytic action prior to feeding or *in vitro* incubation with rumen microorganisms. To test this hypothesis, we decided to analyse the effects of the 24 h pre-treatment with enzymes on the NDF and ADF content of substrate. Compared to the 24-h buffer treated substrate, all enzymatic treatments decreased (P<0.05) substrate NDF content (see Table 3), reductions ranging from 19 to 53 g NDF/kg DM. On the contrary, no effects (P>0.05) of enzymatic treatments on substrate ADF content were detected. These results would indicate that the 24 h pre-treatment with enzymes altered the fibre structure, as previously reported in other studies (Nsereko *et al.*, 2000; Colombatto *et al.*, 2003).

Table 3. Influence of 24 h pre-treatment of a substrate containing 60% of grass hay with different enzymatic treatments on its neutral- (NDF) and acid-detergent fibre (ADF) content (n=4)

Item	Enzymatic treatment†							s.e.d. ††
	CON	CEL-15	CEL-30	XYL-15	XYL-30	MIX-15	MIX-30	
NDF (g/kg DM)	499 ^{d}	461 ^{ab}	448 ^{ab}	480 ^{c}	466 ^{bc}	446 ^{a}	455 ^{ab}	0.89
ADF (g/kg DM)	271	270	263	273	271	263	269	0.63

†Treatments: CON: control; CEL-15 and CEL-30: cellulase at 15 and 30 IU/g substrate DM, respectively; XYL-15 and XYL-30: xylanase at 15 and 30 IU/g substrate DM, respectively; MIX-15 and MIX-30: cellulase:xylanase mixture (1:1) at 15 and 30 IU/g substrate DM, respectively.
††Standard error of the difference.
$^{a, b, c, d}$: Mean values within a row with unlike superscript letters differ (P<0.05).

Conclusions

The 24 h pre-treatment of a substrate that contained 60% of grass hay with fibrolytic enzymes increased *in vitro* NDF degradation and production of VFA by mixed rumen microorganisms. These effects were more marked at 6 than at 24 h of fermentation for all enzymes, which would indicate that enzymes enhanced the degradation at early stages of fermentation, probably by altering the fibre structure during the pre-incubation substrate/enzyme interaction and making it more amenable to rumen microorganisms. In general, there were no differences due to the dose of enzymes (15 *vs* 30 IU/g forage DM), and no additivity between the effects of enzymes was detected.

Acknowledgements

The authors wish to acknowledge the financial support received from the M.C.Y.T. of Spain (Project AGL2001-0130), Excma. Diputación Provincial de León and J.C.Y.L. (LE040A05).

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