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Abstract. Six rumen-fistulated Merino sheep were used in a crossover design experiment to evaluate the effects of a direct-fed exogenous fibrolytic enzyme (12 g/d; ENZ) on ruminal fermentation. Endoglucanase and xylanase activities were present in the enzyme preparation. Sheep were fed a mixed grass hay:concentrate (70:30; fresh matter basis) diet at a daily rate of 60 g/kg body weight$^{0.75}$. After 21 days of the ENZ supplementation, concentrations of NH_3-N and short chain fatty acids (SCFA) and enzymatic activities were determined in ruminal samples at 0, 4 and 8 h after feeding. Samples of the grass hay were incubated *in situ* in the rumen of each sheep to measure dry matter (DM) and neutral detergent fibre (NDF) degradation. ENZ treatment did not affect ($P > 0.05$) ruminal pH or concentrations of NH_3-N and total SCFA at any sampling time. In contrast, at 0 and 4 h after feeding, molar proportion of propionate tended ($P < 0.10$) to be greater and acetate:propionate ratio tended ($P < 0.10$) to be lower in ENZ-supplemented sheep. No effects of ENZ ($P > 0.10$) were detected 8 h after feeding. Both the ruminally insoluble potentially degradable fraction (*b*) of grass hay DM and its fractional rate of degradation (*c*) were increased ($P < 0.05$) by ENZ treatment. Supplementation with ENZ also increased ($P = 0.009$ to 0.023) effective and potential degradability of grass hay DM and NDF. Ruminal fluid endoglucanase and xylanase activities were greater ($P < 0.05$) at 4 h post-feeding in ENZ-supplemented sheep than in control animals. ENZ supplementation did not affect ($P = 0.151$ to 0.815) either exoglucanase or amylase activity at any sampling time.

Keywords. Fibrolytic enzyme – Rumen fermentation – *In situ* degradation.

Influence de l'administration directe d'enzymes fibrolytiques sur l'activité fibrolytique dans le rumen du mouton

Résumé. *Six moutons de race Mérinos, munis d'une fistule ruminale, ont été utilisés selon un dispositif en cross-over, pour évaluer les effets de l'administration directe d'une préparation enzymatique fibrolytique exogène (12 g/j ; ENZ) sur la fermentation ruminale. La préparation enzymatique a présenté des activités endoglucanase et xylanase. Les moutons ont reçu 60 g/kg poids vif^{0,75} par jour d'un régime composé d'herbe et de concentré (70:30 ; matière fraîche). Les concentrations d'azote ammoniacal et d'acides gras à courtes chaînes (SCFA) et les activités enzymatiques ont été déterminées dans des échantillons de jus de rumen prélevés à 0, 4 et 8 h après l'administration de l'aliment. Des échantillons du foin d'herbe ont été incubés in situ dans le rumen de chaque mouton pour mesurer la dégradation de la matière sèche (DM) et de la paroi totale (NDF). Le traitement ENZ n'a pas affecté ($P > 0,05$) le pH ou les concentrations ruminales d'azote ammoniacal et de SCFA total quel que soit le temps de prélèvement du jus de rumen. En revanche, à 0 et à 4 h après l'alimentation, la concentration de propionate a tendance ($P < 0,10$) à augmenter et le rapport acétate:priopionate est plus faible chez les moutons du groupe ENZ. Aucun effet n'a été détecté ($P > 0,05$) 8 h après l'alimentation. La fraction insoluble potentiellement dégradable dans le rumen (*b*) de la matière sèche du foin et la vitesse de dégradation (*c*) de cette dernière fraction ont été plus élevées ($P < 0,05$) avec le traitement ENZ. La supplémentation avec ENZ a également augmenté ($P = 0,009$ à 0,023) la dégradabilité théorique et potentielle de la matière sèche et de l'NDF de l'herbe. Les activités endoglucanase et xylanase du jus de rumen à 4 h ont été plus élevées ($P < 0,05$) chez les animaux supplémentés avec ENZ que chez ceux du groupe témoin. Le traitement ENZ n'a pas affecté ($P = 0,151$ to 0,815) les activités exoglucanase et amylase quel que soit le temps de prélèvement du jus de rumen.*

Mots-clés. *Enzyme fibrolytique – Fermentation ruminale – Dégradabilité* in situ.

I – Introduction

The use of exogenous fibrolytic enzymes as feed additives has been investigated in the last few years as a mean to enhance forage utilization by ruminants (Beauchemin *et al.*, 2003). Since it has been shown that a pre-feeding enzyme-feed interaction enhanced the beneficial effects of enzymes on ruminal fermentation (Wang *et al.*, 2001; Giraldo *et al.*, 2004), this method of enzyme application has been used in many studies. However, the addition of enzymes to diet immediately before feeding or direct addition of enzymes to ruminants have received considerably less attention. The objective of this study was to evaluate the effects of directly administering an exogenous fibrolytic enzyme into the rumen on ruminal parameters and fibrolytic activity in sheep fed a high-forage diet.

II – Materials and methods

Six rumen-fistulated Merino sheep (60.0 ± 4.36 kg live weight) were used in a crossover design experiment. Sheep were housed in individual pens and had continuous access to fresh water and vitamin-mineral block. Animals were fed a mixed diet of grass hay and concentrate (70:30; fresh matter basis). The concentrate was based on barley, gluten feed, wheat middlings, soybean meal, palmkern meal, wheat, corn and mineral-vitamin premix in the proportions of 21.5, 20.4, 20, 13.5, 11.5, 5, 5 and 3.1%, respectively (air-dry basis). The diet was offered to the animals twice daily (8:00 and 20:00 h) at a rate of 50 g fresh matter/kg $BW^{0.75}$. Individual feed intakes and refusals were recorded daily. Organic matter, crude protein, neutral detergent fibre (NDF) and acid detergent fibre contents of the mixed diet were 927, 124, 484 and 234 g/kg dry matter (DM), respectively.

The experiment consisted of two 29-day experimental periods, with a 20-day initial period for diet adaptation and a sampling period of 9 days. In each experimental period, three sheep were given 12 g/d of a commercial fibrolytic enzyme (ENZ; Fibrozyme™, Alltech Inc., Nicholasville, KY, USA), which was administered daily by direct introduction intro the rumen through the cannula immediately before the morning feeding. The other three sheep did not receive the enzyme (control; CON). The enzyme preparation was assayed for endoglucanase, exoglucanase, xylanase and amylase activities following the procedures described by Colombatto and Beauchemin (2003). All activities were measured at pH 6.5 and 39°C, conditions prevailing in the rumen. Solutions (10 g/l) of medium-viscosity carboxymethylcellulose, Avicel PH-101, oat spelt xylan and soluble starch were used as substrates for determination of endoglucanase, exoglucanase, xylanase and amylase activity, respectively. All substrates were commercialized by Fluka Chemicals (Seelze, Germany). One g of the enzyme liberated 148 µmol of glucose from carboxymethylcellulose and 791 µmol of xylose from oat spelt xylan per min at 39°C and pH 6.5, but no exoglucanase and amylase activities were detected.

On day 21 of each period, ruminal content was sampled through the cannula of each sheep at 0, 4 and 8 h after the morning feeding. Ruminal content was strained through four layers of cheesecloth, the pH of the fluid was immediately measured, and the following samples were taken: 5 ml were added to 5 ml of deproteinising solution [metaphosphoric acid (100 g/l) and crotonic acid (0.6 g/l)] for short chain fatty acids (SCFA) analyses, 2 ml were added to 2 ml 0.5 M HCl for NH_3-N determination, and 5 ml were immediately frozen at –80°C for determination of enzymatic activities. The nylon-bag technique (Mehrez and Ørskov, 1977) was used to measure the rumen degradation of grass hay over days 24-29 of each experimental period. Five-g samples of grass hay DM (3 mm screen ground) were incubated in polyester bags in the rumen of each sheep for 0, 3, 6, 9, 15, 24, 48 and 72 h (two replicates for each animal and time). The removed bags were washed thoroughly under running cold water for 1 min, and then washed in the cold rinse cycle (20 min) of a washing machine. Dry matter disappearance was measured from the loss in weight after oven drying at 60°C for 48 h, and the residues were analysed for NDF to estimate the loss of fibre.

The values for disappearance of DM from grass hay were fitted to the exponential model $y = a + b (1 – e^{-ct})$ (Ørskov and McDonald, 1979), where y represents the DM disappearance at each

incubation time (t) and *a*, *b* and *c* are constants that represent the soluble rapidly degradable fraction, the insoluble potentially degradable fraction and the fractional degradation rate for fraction *b*, respectively. The fitted equation was constrained so that *a* + *b* did not exceed 100%, and this value represented the potential degradability of grass hay (PD). The values for NDF disappearance were fitted to the model $y = b\ (1 - e^{-c(t - lag)})$, where *y* is the NDF disappearance at each incubation time (t), *b* is the potentially degradable fraction, *c* is the fractional degradation rate for fraction *b*, and *lag* is the lag time (h) before degradation commenced. Effective degradability (ED) was estimated in each sheep by using the parameters *a*, *b*, *c* and *lag*, and assuming a rumen particulate outflow rate (*kp*) of 3.5%/h according to the following equation: $ED = a + [b\ c / (c + k)] \times e^{(-\ kp \times lag)}$ (France *et al.*, 1990). A lag time value of 0 was assumed for calculation of DM effective degradability.

Procedures for analysis of DM, N, NDF, SCFA and NH_3-N have been described by Giraldo *et al.* (2007b). For determination of enzymatic activities in ruminal fluid samples, cells were lysed using a Mini-Beadbeater™ (BioSpec Products Inc., Bartlesville, OK, USA) to release intracellular enzymes. Cell material was removed by centrifugation (10,000 × *g*, 10 min, 4°C) and the supernatant was used to analyze enzymatic activities. Data were subject to ANOVA using the GLM procedure of SAS (2002). Effects included in the model were enzymatic treatment, sheep and period. Time-sequence data (pH, SCFA, NH_3-N and enzymatic activities in ruminal fluid) were analysed independently for each individual sampling time. Enzyme effects were declared significant at $P < 0.05$, and $P < 0.10$ values were considered as trends and discussed.

III – Results and discussion

As expected, ENZ supplementation did not affect daily feed intake (P = 0.334; 1093 g and 1075 g fresh matter for CON and ENZ, respectively). There were no effects (P = 0.106 to 0.666) of ENZ treatment on ruminal pH and concentrations of NH_3-N and total SCFA at any sampling time (Table 1).

Table 1. Influence of enzymatic treatment on pH, concentration of NH_3-N and total short chain fatty acids (SCFA), molar proportions of the main SCFA and acetate:propionate ratio in the rumen of sheep fed a 70:30 grass hay:concentrate diet†

Sampling time and treatment	pH	NH_3-N (mg/l)	Total SCFA (mmol/l)	Acetate (%)	Propionate (%)	Butyrate (%)	Others†† (%)	Ac/Pr (mol/mol)
0 h								
CON	6.54	99.0	84.1	70.2	14.8	12.0	3.06	4.86
ENZ	6.48	86.2	87.1	68.6	15.9	11.8	3.63	4.36
SED†††	0.066	6.50	8.85	0.82	0.50	0.40	0.36	0.219
P value	0.405	0.106	0.750	0.122	0.077	0.723	0.171	0.070
4 h								
CON	6.18	88.5	100.9	67.9	18.0	11.8	2.36	3.86
ENZ	6.07	99.0	98.2	66.2	19.6	11.8	2.69	3.40
SED†††	0.056	11.81	3.36	0.46	0.80	0.83	0.154	0.191
P value	0.276	0.417	0.891	0.015	0.092	0.999	0.084	0.065
8 h								
CON	6.33	72.5	97.2	69.4	16.6	11.5	2.45	16.6
ENZ	6.38	85.2	84.2	68.6	17.4	11.4	2.67	17.4
SED†††	0.116	13.4	3.08	0.88	0.50	0.64	1.12	0.149
P value	0.666	0.388	0.398	0.376	0.201	0.848	0.573	0.146

†Treatments, CON: control, ENZ: sheep received daily 12 g of exogenous fibrolytic enzyme.
††Calculated as the sum of isobutyrate, isovalerate and valerate.
†††Standard error of the difference.

Several *in vivo* (Hristov *et al.*, 2000; Pinos-Rodríguez *et al.*, 2002; Beauchemin *et al.*, 2003) and *in vitro* (Wang *et al.*, 2001; Giraldo *et al.*, 2007a) studies have shown that treating different feeds with fibrolytic enzymes produced a shift in the molar proportions of SCFA, but changes in rumen fermentation pattern seem to be affected by the characteristics of the diet fed to the animals and the type of supplemented enzyme. In the present experiment, molar proportion of propionate tended ($P < 0.10$) to be greater and acetate:propionate ratio tended ($P < 0.10$) to be lower at 0 and 4 h after the morning feeding in sheep supplemented with ENZ, compared to unsupplemented ones; in contrast, no effects ($P = 0.146$ to 0.848) of ENZ treatment on ruminal variables were observed 8 h after feeding. Several authors (Hristov *et al.*, 2000; Wang *et al.*, 2001) have suggested that changes in fermentation pattern may reflect concomitant changes in rumen bacterial populations.

As shown in Table 2, ENZ supplementation did not affect ($P = 0.895$) the soluble rapidly degradable fraction (*a*) of grass hay DM, but increased the insoluble potentially degradable fraction (*b*; $P = 0.002$) and its fractional rate of degradation (*c*; $P = 0.049$). As a consequence, both PD and ED of grass hay DM were greater ($P < 0.05$) in sheep supplemented with ENZ. Enzyme supplementation also increased ($P < 0.05$) *b* fraction and ED of grass hay NDF, and tended ($P = 0.099$) to increase the fractional rate of degradation of grass hay NDF. Similar results have been reported by Hristov *et al.* (2000) in heifers supplemented with a commercial enzyme containing mainly xylanase and β-glucanase activities, and by Pinos-Rodríguez *et al.* (2002) in lambs receiving a commercial enzyme with xylanase and endoglucanase activities.

Table 2. Influence of enzymatic treatment on *in situ* ruminal degradation parameters of dry matter (DM) and neutral detergent fibre (NDF) of grass hay in sheep fed a 70:30 grass hay:concentrate diet†

	DM††					NDF			
	***a* (%)**	***b* (%)**	***c* (%/h)**	**PD (%)**	**ED (%)**	***b***	***c* (%/h)**	***Lag***	**ED**
CON	26.9	47.0	7.50	73.9	58.8	58.7	6.53	0.61	37.2
ENZ	27.0	49.5	8.28	76.5	61.7	62.5	7.30	0.39	41.6
SED†††	0.43	0.34	0.280	0.54	0.80	0.95	0.358	0.129	1.10
P value	0.895	0.002	0.049	0.009	0.023	0.017	0.099	0.157	0.018

†Treatments, CON: control, ENZ: sheep received daily 12 g of exogenous fibrolytic enzyme.
††See text for a description of degradation parameters. PD: potential degradability; ED: effective degradability.
†††Standard error of the difference.

The greater *in situ* degradation of grass hay observed in ENZ-treated sheep is in accordance to the enhanced endoglucanase and xylanase activities observed in their ruminal fluid. The treatment with ENZ increased ($P = 0.011$ to 0.053) endoglucanase activity at all sampling times, and enhanced xylanase activity at 4 ($P = 0.046$) and 8 ($P = 0.100$) h after feeding (Table 3). In contrast, there was no effect of ENZ treatment ($P = 0.151$ to 0.815) on exoglucanase and amylase activities.

Enhanced fibrolytic activities in rumen fluid produced by the treatment of feed with exogenous fibrolytic enzymes have been reported in *in vitro* (Wang *et al.*, 2001; Giraldo *et al.*, 2007a) and *in vivo* studies (Hristov *et al.*, 2000; Morgavi *et al.*, 2000) demonstrated synergism between exogenous enzymes produced by *Trichoderma longibrachiatum* and those produced by rumen micro-organisms such that the net combined hydrolytic effect in the rumen was much greater than that estimated from the individual activities.

Table 3. Influence of enzymatic treatment on enzymatic activities in ruminal fluid from sheep fed a 70:30 grass hay:concentrate diet†

Sampling time and treatment	Enzymatic activities††			
	Endoglucanase	Exoglucanase	Xylanase	Amylase
0 h				
CON	691	26.6	9285	1652
ENZ	1071	34.6	10257	1998
SED†††	112.4	5.09	5236.4	256.0
P value	0.020	0.179	0.215	0.238
4 h				
CON	443	26.4	5874	1120
ENZ	751	28.1	8642	1037
SED†††	78.4	2.51	1045.3	334.2
P value	0.011	0.536	0.046	0.815
8 h				
CON	511	24.6	7916	1411
ENZ	766	32.9	10596	1253
SED†††	101.1	4.90	1303.0	412.7
P value	0.053	0.151	0.100	0.716

†Treatments, CON: control, ENZ: sheep received daily 12 g of exogenous fibrolytic enzyme.
††Endoglucanase, exoglucanase and amylase activities are expressed as nmol of glucose liberated from carboxymethylcellulose, Avicel PH-101 and soluble starch, respectively, per ml per minute at 39°C and pH = 6.5. Xylanase activity is expressed as nmol of xylose liberated from oat spelt xylan per ml per min at 39°C and pH = 6.5.
†††Standard error of the difference.

IV – Conclusions

The results of the present study showed that supplementing a fibrolytic enzyme directly into the rumen increased the fibrolytic activity in ruminal fluid, without a pre-feeding feed-enzyme interaction. Although enzyme supplementation did not affect significantly the concentration of total SCFA, molar proportions of propionate tended to increase during the initial hours postfeeding, which might indicate a change in rumen bacterial populations.

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