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Use of Exogenous Fibrolytic Enzymes to Enhance In Vitro Fermentation of Alfalfa Hay and Corn Silage

J.-S. Eun,* K. A. Beauchemin,*[1] and H. Schulze†
*Agriculture and Agri-Food Canada, Research Centre, Lethbridge, Alberta, T1J 4B1, Canada
†Danisco Animal Nutrition, PO Box 218, 2300 AE, Leiden, the Netherlands

ABSTRACT

Two in vitro experiments were performed to identify promising exogenous fibrolytic enzyme products (EFE) and optimum dose rates (DR) for improving the degradation of alfalfa hay and corn silage. The relationship between enzymatic activity and fermentation responses was examined to identify optimum formulations. In experiment 1, 5 EFE containing mainly endoglucanase and xylanase activities, with different ratios between the 2 activities, were assessed at a DR of 0.7, 1.4, and 2.1 mg/g of DM forage. Milled alfalfa hay or corn silage was incubated in an in vitro batch culture with buffer, ruminal fluid, and EFE. Gas production (GP) was measured during 24 h of incubation, and degradabilities of DM and fiber were measured after terminating the incubation at 24 h. Two (E1 and E3) EFE substantially improved GP and degradation of alfalfa hay and corn silage fiber. The optimum DR of these EFE was 1.4 mg/g of DM for both forages with improvements in NDF degradability up to 20.6% for alfalfa hay and up to 60.3% for corn silage. Whereas added activities of endoglucanase and exoglucanase were positively correlated with improvement in NDF degradability for alfalfa hay and corn silage, there was no relationship between added xylanase activity and NDF degradability. The 2 most promising EFE from experiment 1 were reevaluated in experiment 2, alone and in combination with a high xylanase EFE, to determine whether their effectiveness could be enhanced by decreasing the endoglucanase to xylanase ratio. The 2 EFE improved GP and fiber degradation in a manner similar to that observed in experiment 1, but the combination treatments resulted in no further beneficial effects. Exogenous fibrolytic enzyme products can greatly improve forage utilization, but DR and the activities supplied are critical for achieving this response. Products used with alfalfa hay and corn silage should contain high endoglucanase activity, with an ideal ratio of endoglucanse to xylanase.

Key words: alfalfa hay, corn silage, exogenous fibrolytic enzyme, forage digestibility

INTRODUCTION

Intake and digestibility of forages directly affect milk production, rumen function, and animal health. The cell wall components of forages represent a major source of energy for cattle, even though less than 50% of this fraction is readily digested and utilized (Hatfield et al., 1999). A 10% increase in cell wall digestion is projected to increase milk and meat sales in the United States by $380 million and also reduce manure solids by 2.3 million tonnes and grain usage by 3.0 million tonnes (Hatfield et al., 1999). In spite of significant improvements in forage cell wall digestibility achieved through forage breeding programs and agronomic advances, forage digestibility continues to limit the intake of digestible energy of dairy cows (Beauchemin et al., 2003).

The use of exogenous fibrolytic enzyme products (**EFE**) to improve feed utilization by ruminants has attracted growing attention. Some EFE increase cell wall digestibility in vitro (Colombatto et al., 2003) or in vivo (Schingoethe et al., 1999), but not all products are effective (Higginbotham et al., 1996; Vicini et al., 2003). Inconsistent responses are thought to be due in part to differences in product formulation and the key enzyme activities supplied (Beauchemin et al., 2003).

Despite the importance of enzyme formulation, the relationship between enzymatic activities and improvements in forage utilization is not well established. A high correlation between added endoglucanase activity and OM degradation improvement was reported for alfalfa hay (Eun and Beauchemin, 2007). Similarly, Wallace et al. (2001) reported that EFE with high endoglucanase activity increased the rate of gas production (**GP**) from corn silage compared with control (no added enzyme), but products with high xylanase activity did not increase GP. Most EFE contain endoglucanaces and xylanases, but whether there is an ideal combination of these 2 enzymes, or an optimum enzyme dose required for individual forages, is uncertain. In ruminant applications, EFE must act synergistically with the endogenous enzymatic activities of the rumen microbes

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[1]Corresponding author: beauchemink@agr.gc.ca

Table 1. Protein concentration and enzymatic activity of the exogenous fibrolytic enzyme products used in experiment 1

Enzyme product	Protein concentration[1]	Enzymatic activity[2]			
		Endoglucanase (E)	Xylanase (X)	Exoglucanase	E:X
E1	279	215 ± 8.8	495 ± 18.1	40 ± 2.1	0.42:1
E2	165	205 ± 9.1	334 ± 13.1	38 ± 1.5	0.61:1
E3	305	198 ± 11.2	406 ± 16.1	37 ± 2.2	0.49:1
E4	188	166 ± 4.9	1,165 ± 22.5	34 ± 2.4	0.14:1
E5	44	16 ± 2.5	1,685 ± 25.2	2.1 ± 0.71	0.01:1

[1]Protein concentration was expressed as milligrams per gram.

[2]Endoglucanase and exoglucanase activities were expressed as nanomoles of glucose released per minute per milligram. Xylanase activity was expressed as nanomoles of xylose released per minute per milligram.

(Morgavi et al., 2000), which complicates the situation further.

In addition to product formulation, it is important to establish optimum dose rate (**DR**) of individual EFE for various forages. Responses to enzyme addition can be nonlinear (Beauchemin et al., 1995; Kung et al., 2000); thus the most effective DR is not always evident. Furthermore, DR directly affects product cost. Thus, it is important to establish the optimum DR of EFE for different forages before enzyme technology can be used cost-effectively in dairy cattle diets for consistent positive improvements in forage utilization.

Our project focused on alfalfa hay and corn silage because they are the major forages fed to dairy cattle in North America. Our objectives were to 1) assess whether in vitro degradation of these forages could be enhanced using EFE, 2) determine the optimum DR of individual EFE, and 3) establish the relationship between the major enzymatic activities added and fiber degradation. Our hypothesis was that degradation of alfalfa hay and corn silage fiber could be improved using EFE products containing endoglucanases and xylanases, but that the response would be directly proportional to DR of these key activities.

MATERIALS AND METHODS

Experimental Layout

An in vitro batch culture system was used to evaluate 5 experimental EFE products for their potential to increase GP and degradation of alfalfa hay and corn silage. Experiment 1 was conducted as a completely randomized design with 4 replicates and a factorial arrangement of treatments: 5 EFE products × 4 DR of each EFE. Each forage was evaluated in a separate assay. Experiment 2 was performed as a completely randomized design with 5 treatments (4 enzyme treatments and control) using alfalfa hay and corn silage as substrates.

Forages and Enzyme Products

The alfalfa hay used contained 17.3% CP, 49.9% NDF, and 35.4% ADF (DM basis), whereas the corn silage contained 6.6% CP, 43.8% NDF, and 21.7% ADF. The same batches of alfalfa hay and corn silage were used throughout the series of experiments. Fresh alfalfa hay and freeze-dried corn silage were milled to pass a 1-mm screen using a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA) and stored for use in these experiments.

Five experimental EFE products (E1, E2, E3, E4, and E5) from Danisco (Stockport, UK) were used. These enzyme products are in liquid form and characterized with endoglucanase, exoglucanase, and xylanase activities (Table 1), compliant with the current specifications for food-grade enzymes.

Experiment 1

Experiment 1 sought to identify effective EFE products and the optimum DR needed to improve in vitro degradation of alfalfa hay and corn silage. The in vitro procedure used was based on that described by Colombatto et al. (2003). Approximately 0.9 g of DM of ground alfalfa hay or corn silage was weighed into acetone-washed and preweighed filter bags (F57, Ankom Technology, Macedon, NY). Exactly 0.5 g of the enzyme products was diluted with 25 mL of water, and 31.5, 63.0, or 94.5 μL of the diluted enzyme was added to the forage in the bags to achieve a DR of 0.7, 1.4, or 2.1 mg of concentrated enzyme product per gram of forage DM. In addition, forage substrate without enzyme was included (DR of 0) as a control. Four replications per treatment were prepared. The DR used were chosen based on rates used in previous in vitro studies (Colombatto et al., 2003; Eun and Beauchemin, 2005) and in vivo studies (reviewed by Beauchemin et al., 2003) conducted using other products.

Following enzyme addition, the bags were heat-sealed and gently placed in gas-tight culture vials (125 mL capacity, Wheaton Science Products, Millville, NJ) using tweezers. Because the EFE was applied to the feed within the bag using a pipette, uniform distribution of the EFE on the feed was not achieved. Thus, 3 h after adding the enzymes, 36 mL of anaerobic buffer medium, prepared as outlined by Hall et al. (1998) with pH 6.0, was added to each vial. The vials were gently shaken to disperse the EFE. The vials were then stored at 20°C for 17 h. A preincubation was used in this study to ensure that access of EFE to the substrate was not a limitation of the assay, although it is not known whether a preincubation period is required following EFE application to feed. In commercial applications of EFE to feed, a diluted solution of the product is usually sprayed or poured onto the feed to obtain uniform distribution. Furthermore, there is typically a delay between feed preparation and feed consumption.

Ruminal fluid was collected 4 h after the morning feeding (1100 h) from 2 ruminally cannulated, lactating Holstein cows fed a TMR formulated to meet the nutrient requirements of dairy cows in early lactation. Ruminal contents were obtained from various locations within the rumen, composited, and strained through polyester material (PeCAP, pore size 355 μm; B & S H Thompson, Ville Mont-Royal, Quebec, Canada) under a stream of oxygen-free CO_2. The strained ruminal fluid (pH of 6.14 and 6.15 for the assays with alfalfa hay and corn silage, respectively) was immediately transferred to the laboratory in a sealed flask and was kept at 39°C in a water bath. The inoculum was dispensed (9.0 mL per vial) into the culture vials that had been warmed to 39°C in an incubator and flushed with oxygen-free CO_2. Each vial was sealed with a 14-mm butyl rubber stopper plus aluminum crimp cap immediately after loading, and the vials were then stored at 39°C in an incubator. Negative controls (ruminal fluid plus buffer alone or ruminal fluid plus buffer and enzyme product without substrate) were also incubated in 4 replications. These controls were used to correct for gas release and fermentation residues resulting directly from the inoculum and the EFE. Headspace gas produced during substrate fermentation was measured at 12 and 24 h of incubation. The GP was measured by inserting a 23-gauge (0.6-mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering Inc., Laval, Quebec, Canada) connected to a visual display (Data Track, Christchurch, UK). The transducer was then removed leaving the needle in place to permit venting. The process was then repeated until a tray of culture vials (24) had been measured. All needles were then removed, and the flasks were swirled to mix and returned to the incubator (Mauricio et al., 1999). Pressure values, corrected for the amount of forage OM incubated and the gas released from negative controls, were used to generate volume estimates as described in detail by Eun and Beauchemin (2005).

After measuring GP at 24 h of incubation, the vials were placed in the refrigerator at 4°C for 2 h to stop fermentation. Then, the bags were gently folded and removed from the vials using tweezers. They were washed under cold tap water until excess water ran clear and dried at 55°C for 24 h. Degradability of DM was determined by the loss of DM. The bags and their contents were retained for subsequent analysis of fiber content.

Experiment 2

The aim of experiment 2 was to assess the relationship between enzyme activity and improvement in forage degradation. Specifically, the study addressed the effects of doubling the dose of xylanase activity without changing endoglucanase activity to examine the importance of the endoglucanase to xylanase ratio. Two EFE products (E1 and E3) were selected for further evaluation based on the results obtained in experiment 1. These EFE were evaluated alone or in combination with E5 (Table 2). The E1E5 treatment was prepared by adding 1.4 mg/g of DM of E1 and 0.30 mg/g of DM of E5, and E3E5 consisted of 1.4 mg/g of DM of E3 and 0.32 mg/g of DM of E5. Because E5 contained high xylanase activity and low endoglucanase activity, adding a small amount of E5 decreased the endoglucanase to xylanase activity ratio of E1 and E3 by about 50% without substantially changing endoglucanase activity. Thus, the combination treatments supplied similar activities of endoglucanase compared with those of E1 and E3.

Exactly 0.5 g of the enzyme products E1, E3, and E5 were diluted with 25 mL of water and 63.0 μL of the diluted enzymes E1 and E3 were added to the forage (0.9 g of DM) in the bags to achieve the DR of 1.4 mg concentrated enzyme product per gram of forage DM. For E1E5, 2.143 mL of diluted E5 was combined with 10 mL of diluted E1, and 76.5 μL of the combined preparation was added to the forage within the bags. For E3E5, 2.286 mL of diluted E5 was combined with 10 mL of the diluted E3, and 77.4 μL of the combined preparation was added to the forages. The remaining experimental procedures were similar to those described for experiment 1. The strained ruminal fluid used in experiment 2 had a pH of 6.12. After 24 h of incubation, 5 mL of the fermentation contents were added to 1 mL of 25% meta-phosphoric acid, and the samples were stored frozen at –40°C until analyzed for VFA.

Table 2. Enzyme treatments, dose rates, and enzymatic activities used in experiment 2

Enzyme treatment	Dose rate	Enzymatic activity added[1]		
		Endoglucanase (E)	Xylanase (X)	E:X
E1	1.4 mg/g of DM	301	693	0.43:1
E3	1.4 mg/g of DM	277	568	0.49:1
E1E5[2]	1.4 mg/g of DM of E1 + 0.3 mg/g of DM of E5	306	1,199	0.26:1
E3E5[2]	1.4 mg/g of DM of E3 + 0.32 mg/g of DM of E5	282	1,108	0.25:1

[1]Enzymatic activity added per gram of DM substrate. Endoglucanase and xylanase activity were expressed as nanomoles of glucose and xylose released per minute, respectively.

[2]Enzymatic activity of E1E5 and E3E5 was calculated based on their component enzymes.

Chemical Analyses

The amount of protein present in the enzyme products was determined using the BioRad protein determination kit (BioRad Laboratories, Hercules, CA), with bovine serum albumin as the standard according to the procedure described by Colombatto et al. (2003). The enzyme products were analyzed for their endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8) activity according to the procedures reported by Nelson (1944), Somogyi (1952), and Bailey et al. (1992) using medium-viscosity carboxymethylcellulose, Sigmacell 50, and birchwood xylan (1% in 0.1 *M* citrate phosphate buffer, pH 6.0), respectively as substrates (all obtained from Sigma Chemicals, St. Louis, MO). Birchwood rather than oat spelt xylan was used as the substrate for determining xylanase activity because of its low turbidity at 1% concentration and its extended range of linearity during the reaction (Bailey et al., 1992). The assay conditions were 39°C and pH 6.0 to reflect ruminal conditions. Suitably diluted enzyme (50 μL) and substrate solutions (450 μL) were incubated with the substrates for 5 min, and endoglucanase and exoglucanase or xylanase activity was expressed as nanomoles of glucose or xylose released per minute per milligram, respectively.

The VFA were quantified using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA) with a capillary column (30 m × 0.32 mm i.d., 1 μ phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detection. The oven temperature was 170°C held for 4 min, which was then increased by 5°C/min to 185°C, and then by 3°C/min to 220°C, and held at this temperature for 1 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium.

The alfalfa hay and corn silage substrates were analyzed for DM (method 930.15), OM (method 985.01), and CP (6.25 × N; method 990.03) according to AOAC (1995) methods. The NDF and ADF were determined using a digestion apparatus (Ankom^{200} Fiber Analyzer, Ankom Technology), following the procedures outlined by Van Soest et al. (1991) with heat-stable α-amylase and sodium sulfite in the NDF analysis.

Statistical Analyses

All the statistical analyses were conducted using the MIXED procedure of SAS (SAS Institute, 2001). Data from experiment 1 were analyzed as a completely randomized design by forage with EFE, DR, and the interaction between EFE and DR included in the model as fixed effects. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of DR. When there was an interaction ($P < 0.05$) between EFE and DR, as was the case for NDF and ADF degradability, an additional analysis by EFE was performed to identify the optimum DR. That model included DR as a fixed effect. When there was no interaction ($P > 0.05$) between EFE and DR, pooled means were compared among DR using an LSD test. In addition, individual EFE were compared across DR when their effect was $P < 0.05$. The relationship between added endoglucanase, exoglucanase, or xylanase activities and improvement of NDF degradability was determined by linear regression using the PROC REG procedure of SAS. A stepwise regression was used to examine the effects of the combined enzymatic activities on improving NDF degradability.

Exp. 2 was analyzed with a model that included the fixed effects of substrate (alfalfa hay and corn silage), EFE, and the interaction between substrate and EFE. Significant interactions were observed between substrate and EFE for GP ($P < 0.05$) and DM and fiber degradabilities ($P < 0.01$). Therefore, data were reanalyzed separately for each forage source as a completely randomized design using a model that included EFE as a fixed effect. Means were compared using a protected ($P < 0.05$) LSD test. Least square means are

Table 3. Effects of exogenous fibrolytic enzymes (EFE) on the cumulative gas production (GP; mL/g of OM) and 24-h DM degradability (DMD; %) of alfalfa hay in vitro (experiment 1, n = 4)

		Dose rate (DR)[1]						Significance of effects[3]		
Item	EFE	Mean	0	0.7	1.4	2.1	SEM[2]	EFE	DR[4]	EFE × DR
12-h GP	E1	71.8[c]	67.9	71.3	72.7	75.5	2.16	<0.01	L (<0.01)	NS
	E2	73.5[bc]	67.9	74.5	75.8	75.8				
	E3	77.9[a]	67.9	77.0	84.8	81.8				
	E4	74.8[b]	67.9	72.9	80.5	78.1				
	E5	73.2[bc]	67.9	72.4	74.9	77.6				
	SEM[5]	1.08								
24-h GP	E1	132.2[b]	126.9	131.5	135.8	134.7	2.91	<0.01	L (<0.01)	NS
	E2	133.5[b]	126.9	135.3	137.6	134.3				
	E3	138.6[a]	126.9	138.7	146.1	142.6				
	E4	133.3[b]	126.9	129.9	137.3	139.0				
	E5	130.1[b]	126.9	128.8	130.9	133.9				
	SEM[5]	1.45								
DMD	E1	42.4[b]	41.3	41.5	42.6	44.1	0.38	0.04	L (<0.01)	NS
	E2	42.6[ab]	41.3	43.2	42.8	43.0				
	E3	43.0[a]	41.3	42.5	44.1	44.1				
	E4	42.9[a]	41.3	42.6	43.7	44.2				
	E5	42.3[b]	41.3	42.3	42.8	43.0				
	SEM[5]	0.19								

[a–c]Means within an item that do not have a common superscript differ for the main effect of EFE at $P < 0.05$.

[1]Dose rate as milligrams per gram of DM forage substrate; mean = mean for individual EFE across dose rates; 0 = control without added EFE.

[2]SEM for EFE × DR.

[3]EFE × DR = interaction between EFE and DR. NS = EFE × DR was nonsignificant ($P > 0.05$).

[4]L = linear effect of dose rate. Gas production at all time points and DMD were increased ($P < 0.01$) at all DR compared with control.

[5]SEM for pooled mean of EFE.

reported throughout, and significance was declared at $P < 0.05$.

RESULTS

Experiment 1

The EFE products and doses used in experiment 1 provided a range of enzymatic activities (Table 1). Products E1, E2, and E3 supplied a range of endoglucanase and xylanase activities, but the endoglucanase to xylanase ratio was similar ranging from 0.43 to 0.61. In contrast, the EFE products E4 and E5 possessed mainly xylanase activity with lower endoglucanase to xylanase activity ratios (0.01 to 0.14). Exoglucanase activity was fairly low in all EFE products.

All EFE increased GP of alfalfa hay at 12 and 24 h in a linear manner with increasing DR, with no interaction ($P > 0.05$) between EFE and DR (Table 3). The most effective ($P < 0.05$) enzyme preparation for alfalfa hay, based on increased GP, was E3, with maximum effect at a DR of 1.4 mg/g of DM. The results for DM degradability were generally similar to the pattern of GP, but with E4 in addition to E3 more effective than the other EFE.

Degradability of NDF from alfalfa hay was increased by all EFE regardless of DR (Figure 1). The minimum DR required to maximize NDF degradability differed among EFE: 2.1 mg/g of DM for E1, 1.4 mg/g of DM for E3 and E4, and 0.7 mg/g of DM for E2 and E5. Although the minimum DR required to maximize NDF degradability was lower for E2 and E5 than for the other EFE, they were less effective. Degradability of ADF due to EFE addition followed a pattern similar to that of NDF degradability.

When corn silage was used as a substrate, the effects of DR on GP differed among EFE at 12 and 24 h of incubation (interactions, $P < 0.01$; Table 4), indicating that the optimum DR differed among EFE. At 12-h of incubation, E1, E2, and E3 increased GP with increasing DR, but for E4 and E5 maximum effects were observed at the lowest dose (0.7 mg/g of DM). At 24-h incubation, increasing the DR of most of the EFE (except E2) had quadratic effects on GP, with highest response at the DR of 0.7 or 1.4 mg/g of DM. In contrast, in the case of E2 GP continued to increase with increasing DR. Increasing the DR had quadratic effect on DM degradability, with highest response at the DR of 1.4 mg/g of DM.

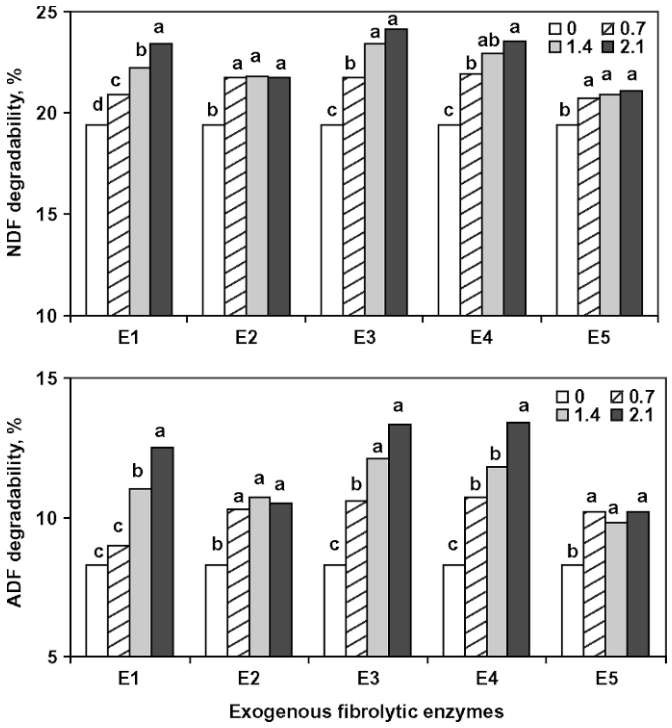

Figure 1. In vitro 24-h degradability of NDF and ADF from alfalfa hay in response to different dose rates (0, 0.7, 1.4, or 2.1 mg/g of DM alfalfa hay) of exogenous fibrolytic enzymes (experiment 1). [a–d]Bars within each enzyme treatment having a different letter differ ($P < 0.05$). Interactions between exogenous fibrolytic enzymes and dose rates were observed for NDF and ADF degradability ($P < 0.01$). The SEM for NDF and ADF degradability is 0.31 and 0.44 for E1, 0.27 and 0.39 for E2, 0.35 and 0.61 for E3, 0.41 and 0.52 for E4, 0.57 and 0.45 for E5, respectively.

Degradability of NDF and ADF from corn silage was considerably increased by E1, E2, and E3 (Figure 2), with maximum responses achieved at 1.4 mg/g of DM. Though E4 and E5 increased NDF and ADF degradability at 2.1 or 0.7 mg/g of DM, respectively, improvements due to these EFE were much smaller than for the other EFE.

Figures 3 and 4 depict the relationship between added activity of endoglucanase and exoglucanase and improvement in NDF degradability for both forages. Added activity of endoglucanase was positively correlated with improvement in NDF degradability from alfalfa hay (r = 0.77; $P < 0.001$) and corn silage (r = 0.77; $P < 0.001$; Figure 3). A similar relationship was observed for exoglucanase activity and improvement in NDF degradability for alfalfa hay (r = 0.79; $P = 0.008$) and corn silage (r = 0.75; $P < 0.001$; Figure 4). However, there was no relationship between added xylanase activity and improvement in NDF degradability for alfalfa hay ($P = 0.78$) or corn silage ($P = 0.29$). A step-wise multiple regression of added enzymatic activity and improvement in NDF degradability showed that endoglucanase activity alone explained 63 and 60% ($P < 0.001$) of the total variation for alfalfa hay and corn silage, respectively. Added exoglucanase tended ($P = 0.15$) to explain a further 6% of the variation in the improvement in NDF degradability in the case of alfalfa hay, and a further 11% ($P = 0.05$) of the variation in the case of corn silage. Xylanase activity explained none of the variation in NDF degradability after accounting for the effects of cellulases.

Experiment 2

Products E1 and E3 were selected for further investigation because these products showed the greatest improvements in NDF and ADF degradation of both forages tested in experiment 1. Product E5 was combined with E1 and E3 to determine if the effectiveness of these products could be further increased by increasing their xylanase activity (i.e., lowering endoglucanase to xylanase activity) without greatly changing endoglucanase activity. For the combination treatments, E5 was added in addition to E1 and E3 so that the basal DR of E1 and E3 was maintained at 1.4 mg/g of DM (Table 2).

The GP profiles of alfalfa hay and corn silage were similar between experiment 1 and 2 for control, E1, and E3 (Table 5). Products E1E5 and E3E5 or E3 increased GP of alfalfa hay compared with the control starting at 12 or 18 h of incubation, respectively, but E1 had no effect. At 12 h of incubation, decreasing endoglucanase to xylanase activity ratio by adding E5 to E1 and E3 further increased the GP beyond that attained by their component enzymes, but it failed to further increase the GP at 18 and 24 h of incubation. Products E1 and E3 increased the GP of corn silage compared with the control throughout the incubation. The combination enzyme treatments E1E5 and E3E5 increased the GP at 12 h of incubation, but they did not influence the GP at the later incubation times, resulting in lower GP compared with their component enzymes.

When alfalfa hay was used as a substrate, degradability of DM was increased by all enzyme treatments except for E1, whereas degradability of NDF and ADF was increased by all enzyme treatments (Table 6). Degradability of DM and fiber from corn silage was increased by only single enzyme treatments.

Total VFA concentration was increased by E1, E3, and E1E5 when added to alfalfa hay (Table 7). Molar proportion of acetate was decreased by adding E1, E3, and E3E5, whereas molar proportions of propionate and butyrate were not affected by EFE. All enzyme treatments decreased acetate to propionate ratio. Generally, VFA profiles were not affected by EFE when

Table 4. Effects of exogenous fibrolytic enzymes (EFE) on the cumulative gas production (GP; mL/g of OM) and 24-h DM degradability (DMD; %) of corn silage in vitro (experiment 1, n = 4)

		Dose rate (DR)[1]						Significance of effects[3]		
Item	EFE	Mean	0	0.7	1.4	2.1	SEM[2]	EFE	DR[4]	EFE × DR
12-h GP	E1	66.0[c]	62.4[e]	65.4[ed]	68.8[d]	67.3[d]	1.58	<0.01	L (<0.01)	<0.01
	E2	68.6[ab]	62.4[f]	69.0[e]	69.8[e]	73.4[d]			L (<0.01)	
	E3	70.6[a]	62.4[f]	69.2[e]	75.5[d]	75.1[d]			L (<0.01)	
	E4	70.6[a]	62.4[f]	78.0[d]	74.5[d]	67.5[e]			Q (<0.01)	
	E5	66.7[bc]	62.4[e]	69.4[d]	69.6[d]	65.2[e]			Q (<0.01)	
	SEM[5]	0.79								
24-h GP	E1	140.5[b]	132.5[e]	141.7[d]	146.0[d]	141.8[d]	2.84	0.02	Q (0.03)	<0.01
	E2	146.0[a]	132.5[f]	144.3[e]	151.8[d]	155.4[d]			L (<0.01)	
	E3	144.9[a]	132.5[e]	145.4[d]	151.9[d]	149.8[d]			Q (0.02)	
	E4	143.4[ab]	132.5[e]	154.5[d]	149.1[d]	137.4[e]			Q (<0.01)	
	E5	139.8[a]	132.5[e]	143.3[d]	143.8[d]	139.7[ed]			Q (<0.01)	
	SEM[5]	1.42								
DMD	E1	44.0	43.3	44.0	45.9	42.8	0.90	NS	Q (<0.01)	NS
	E2	44.4	43.3	43.2	45.5	45.5				
	E3	44.9	43.3	44.0	47.2	45.1				
	E4	44.5	43.3	45.3	44.6	44.9				
	E5	44.8	43.3	45.8	45.6	44.5				
	SEM[5]	0.45								

[a–c]Means within a column for EFE that do not have a common superscript differ at $P < 0.05$.

[d–f]Means within a row for dose rates of 0 to 2.1 mg/g of DM that do not have a common superscript differ at $P < 0.05$.

[1]Dose rate as milligrams per gram of DM forage substrate; mean = mean for individual EFE across dose rates; 0 = control without added EFE.

[2]SEM for EFE × DR.

[3]EFE × DL = interaction between EFE and DR. NS = EFE and EFE × DL were nonsignificant ($P > 0.05$). Degradability of DM was increased ($P < 0.05$) by DR of 1.4 and 2.1 mg/g of DM compared with control.

[4]L = linear; Q = quadratic effect of dose rate.

[5]SEM for pooled mean of EFE.

added to corn silage except that molar proportion of butyrate was increased by adding E3 and E3E5.

DISCUSSION

Effectiveness of Exogenous Enzymes and Optimum DR

Some of the EFE evaluated substantially improved the degradation of alfalfa hay and corn silage. For example, at the DR of 1.4 mg/g of DM, E1 increased NDF degradability of alfalfa hay by 14.4% (2.8 percentage unit increase) and corn silage by 54.5% (6.6 percentage unit increase), whereas E3 increased NDF degradation of alfalfa hay by 20.6% (4.0 percentage unit increase) and corn silage by 60.3% (7.3 percentage unit increase). These sizable increases in NDF degradation would be expected to increase DMI, milk production, and nutrient utilization by lactating dairy cows.

Improvements in vitro NDF degradability allows greater voluntary feed intake (Dado and Allen, 1995) by reducing physical fill in the rumen. Increased NDF degradability also increases the energy density of diets and stimulates microbial N production (Oba and Allen, 2000). A one percentage unit increase in forage NDF digestibility in vitro has been reported to elicit a 0.17 kg increase in DMI and a 0.25 kg increase in 4% FCM yield (Oba and Allen, 1999). For diets containing corn silage (>40% of the dietary DM), a one percentage unit increase in vitro NDF degradability of corn silage resulted in a 0.14 kg/d increase in 3.5% FCM yield and a 0.12 kg/d increase in DMI (Jung et al., 2004). Thus, the increases in NDF degradation observed in our study have the potential to substantially improve the productivity of dairy cows fed diets containing alfalfa hay or corn silage.

The economics of feeding these enzymes is currently unknown and will ultimately depend on enzyme cost. Because the products used in this study are not commercially available, it is not possible to calculate the cost:benefit ratio of adding them to the diet of commercial dairy cows. However, based on the retail cost of similar polysaccharidases, product costs would likely range between $5 and $10 per kilogram. Thus, for a lactating dairy cow consuming 23 kg of DM/d of TMR comprised of 50% forage, the cost of supplying the optimum dose (1.4 mg/g of forage DM) of enzyme would be about $0.08 to $0.16 per cow per day. This DR improved in vitro NDF degradability by about 3 to 7 percentage

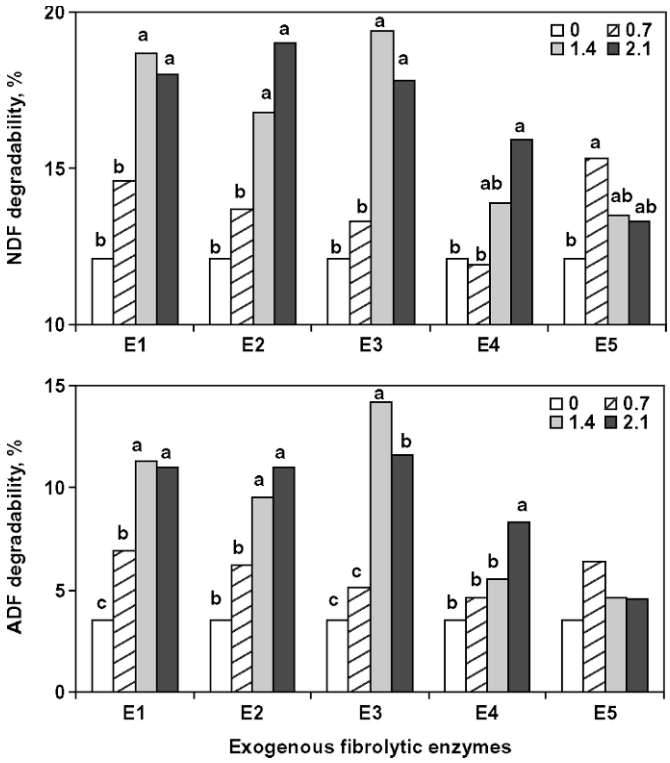

Figure 2. In vitro 24-h degradability of NDF and ADF from corn silage in response to different dose rates (0, 0.7, 1.4, or 2.1 mg/g of DM corn silage) of exogenous fibrolytic enzymes (experiment 1). [a–c]Bars within each enzyme treatment having a different letter differ ($P < 0.05$). Interactions between exogenous fibrolytic enzymes and dose rates were observed for NDF and ADF degradability ($P < 0.01$). The SEM for NDF and ADF degradability are 0.81 and 0.79 for E1, 1.09 and 1.21 for E2, 0.70 and 1.03 for E3, 1.59 and 1.14 for E4, and 0.89 and 0.87 for E5, respectively.

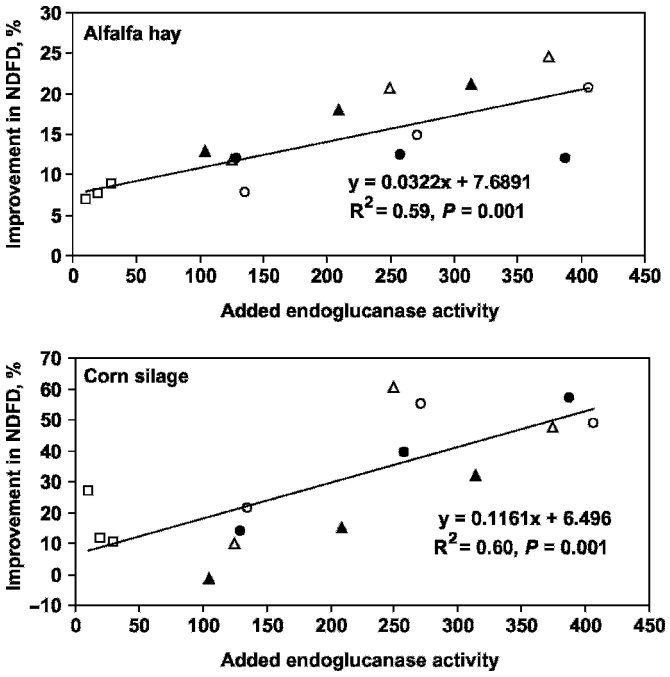

Figure 3. The relationship between added endoglucanase activity (nmol of glucose released/min) and improvement in NDF degradability (NDFD) for alfalfa hay and corn silage (experiment 1) due to the addition of exogenous fibrolytic enzymes: E1 (○), E2 (●), E3 (△), E4 (▲), and E5 (□). Improvement in NDFD (%) is calculated as [(Ye – Ycont)/Ycont] × 100, where Ye is the observed NDFD with enzyme addition and Ycont is the mean NDFD for control incubations.

units, which would correspond to an additional 1 to 2 kg of milk (Oba and Allen, 1999; Jung et al., 2004). However, it must be cautioned that research to determine the optimum in vivo DR and most effective method of adding the enzyme to the diet would need to be conducted before these products could be used in dairy cow diets.

The response to EFE was dose dependent for many of the products examined. Previously, several studies (Beauchemin et al., 1995; Kung et al., 2000; Nsereko et al., 2002) examined the ideal DR of EFE, but these studies were conducted using a single product. In contrast, we used a range of EFE, with DR (0.7, 1.4, and 2.1 mg/g of DM) chosen based on previous studies in which positive responses were observed for other EFE when applied within this range (Colombatto et al., 2003; Eun and Beauchemin, 2007).

The DR responses in GP and degradability were both linear and quadratic, depending upon the EFE. Quadratic responses to DR have been reported in several in vivo studies with other EFE products (Beauchemin et al., 1995; Kung et al., 2000; Nsereko et al., 2002). However, higher maximum doses were used in those animal feeding studies. It is possible that the relatively narrow range of DR used in our study may have favored linear responses, and it is possible that quadratic effects would have been observed more frequently had a higher maximum dose been used. Whereas higher DR could further increase forage utilization, these rates would likely not be cost effective.

The most promising EFE candidates, E1 and E3, both improved the NDF degradability of alfalfa hay and corn silage in experiment 1, with an optimum DR of 1.4 mg/g of DM. In addition, E4 was highly effective for alfalfa hay, but its effects on corn silage were only moderate, particularly at the DR of 1.4 mg/g of DM. The reverse occurred for E2. The cell wall compositions of alfalfa hay and corn silage are very different. In addition, anatomical structure plays an important role in limiting cell wall degradation (Wilson and Mertens, 1995). Therefore, it was expected that effectiveness of the products would differ for the 2 forages. Furthermore, the DR required to improve fiber degradation of these 2 forages was expected to differ because of differences in the chemical composition and structure of these forage

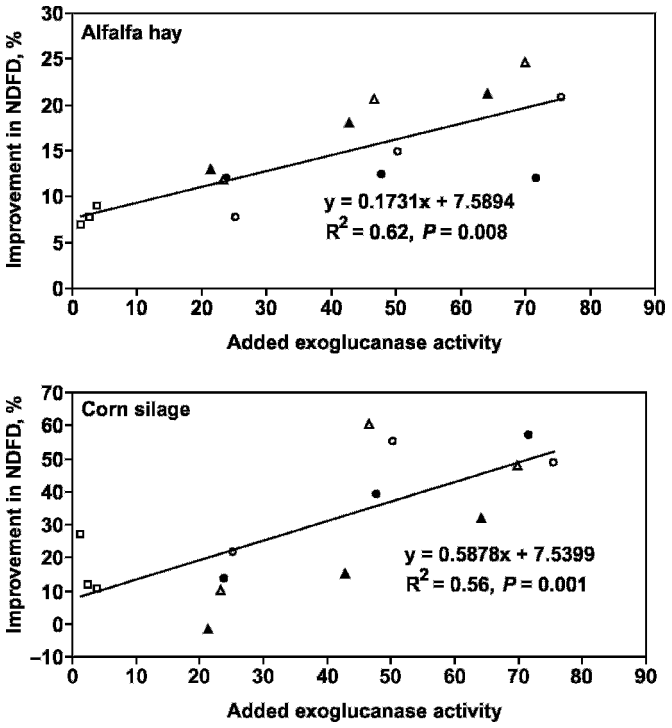

Figure 4. The relationship between added exoglucanase activity (nmol of glucose released/min) and improvement in NDF degradability (NDFD) for alfalfa hay and corn silage (experiment 1) due to the addition of exogenous fibrolytic enzymes: E1 (○), E2 (●), E3 (△), E4 (▲), and E5 (□). Improvement in NDFD (%) is calculated as [(Ye – Ycont)/Ycont] × 100, where Ye is the observed NDFD with enzyme addition and Ycont is the mean NDFD for control incubations.

(Beauchemin et al., 2003). Even though the optimum DR was the same for E1 and E3, the magnitude of the improvement was considerably greater for corn silage than for alfalfa hay.

Diets fed to lactating dairy cows usually contain several types of forages, including alfalfa and corn silage. To achieve maximum benefit under these circumstances, the ideal approach would be to use products such as E1 or E3 that are effective for both forages. Alternatively, E4 could also be used in alfalfa-based diets or E2 in corn silage-based diets. A targeted approach, in which EFE are formulated for specific types of feeds, is recommended to decrease the variability associated with using enzymes in ruminant diets (Beauchemin et al., 2004).

Relationship Between Fibrolytic Activities and Degradation of Forage

Identifying the ideal enzyme formulation needed for EFE products to be effective for ruminants is challenging because the mechanisms whereby EFE improve microbial digestion of feed are not well understood (Beauchemin et al., 2004). In vitro fiber digestion usually follows first-order kinetics of digestion, and it is generally thought that available surface area of the substrate, rather than the enzymatic activity of the microbial population itself, limits digestion (Weimer, 1998). At first, this principle may appear to be inconsistent with the observation that adding EFE increases forage degradation, at least when measured up to 24 h of incubation. Yet, the increase in fiber degradation observed in the present study confirms the previous study by Wallace et al. (2001) in which the rates of in vitro fermentation of corn and grass silage were increased when EFE containing endoglucanase activity was added immediately before the rumen fluid was added to the incubations. The authors concluded that enzyme activity may be in fact rate-limiting in the fermentation of some forages. Another possibility is that exogenous enzymes can access greater surface area compared with cell-bound microbial enzymes. In our study, the EFE were added to the forage 20 h before the ruminal fluid was added to the incubation, raising an additional possibility that the improvement in fiber degradation resulted from a pretreatment effect in which predigestion of forage substrate by exogenous EFE increased the accessibility of microbial enzymes

Table 5. Effects of exogenous fibrolytic enzymes on the cumulative gas production (mL/g of OM) from alfalfa hay or corn silage during in vitro fermentation (experiment 2, n = 4)

	Alfalfa hay (incubation time, h)			Corn silage (incubation time, h)		
Treatment	12	18	24	12	18	24
Control[1]	69.6^{bc}	105.0^{b}	127.8^{b}	55.6^{b}	98.7^{b}	130.4^{c}
E1	67.9^{c}	107.7^{ab}	135.6^{ab}	65.6^{a}	110.1^{a}	143.3^{ab}
E3	72.7^{b}	112.7^{a}	144.2^{a}	67.4^{a}	115.8^{a}	152.0^{a}
E1E5	77.4^{a}	117.1^{a}	145.5^{a}	65.6^{a}	104.7^{ab}	133.8^{c}
E3E5	76.5^{a}	116.1^{a}	143.8^{a}	65.6^{a}	104.4^{ab}	134.2^{bc}
SEM	1.62	2.41	4.05	2.15	3.41	5.12

$^{a–c}$Means within a column that do not have a common superscript differ at $P < 0.05$.

[1]Forage substrate without added enzymes.

Table 6. Effects of exogenous fibrolytic enzymes on the in vitro 24-h degradability (%) of alfalfa hay and corn silage (experiment 2, n = 4)

	Alfalfa hay			Corn silage		
Treatment	DM	NDF	ADF	DM	NDF	ADF
Control[1]	41.5[b]	18.4[b]	7.7[b]	43.8[c]	13.3[c]	4.4[b]
E1	42.7[ab]	20.8[a]	10.0[a]	49.0[a]	20.0[a]	11.4[a]
E3	43.9[a]	21.3[a]	10.5[a]	46.8[b]	17.3[b]	10.3[a]
E1E5	44.3[a]	21.9[a]	10.7[a]	44.7[bc]	11.4[cd]	3.9[b]
E3E5	44.3[a]	21.3[a]	9.5[a]	44.5[bc]	10.2[d]	4.0[b]
SEM	0.59	0.52	0.67	0.93	0.97	1.34

[a–c]Means within a column that do not have a common superscript differ at $P < 0.05$.

[1]Forage substrate without added enzymes.

to substrate once the ruminal fluid was added to the incubations.

The positive linear relationship between added endoglucanase activity and improvement in NDF degradability of forages indicates that the most effective EFE or DR were those with high endoglucanase activity (Figure 3). Furthermore, the effectiveness of EFE containing only moderate endoglucanase activity was improved by applying higher DR. Thus, for some enzyme products, such as E5, the lack of effect on fiber degradability can be explained by the low endoglucanase activity of this product.

The relationship between added endoglucanase activity and improvement in NDF degradability reported in the present study supports the findings of a previous study in which we used recombinant, single activity (endoglucanase or xylanase) experimental enzyme products (Eun and Beauchemin, 2007). In that study, the correlation between added endoglucanase activity (determined at ruminal conditions) and improvement in OM degradability of alfalfa hay was high (r = 0.71; $P < 0.01$). In contrast, added activity of xylanase was not associated with OM degradability improvement of alfalfa hay (Eun and Beauchemin, 2007), which supports the findings of the present study. Wallace et al. (2001) reported that several enzyme products were effective in stimulating fermentation of corn silage when matched for endoglucanase activity. In contrast, several preparations with high xylanase activity, but low endoglucanase activity, were considerably less effective. Overall, we conclude that endoglucanase activity is a good indicator of the ability of EFE to stimulate fermentation of alfalfa hay and corn silage.

The small improvement in NDF degradability that occurred due to exoglucanase activity after addition of endoglucanase activity indicates endoglucanases and exoglucanases act synergistically to hydrolyze the cellulose in forages. In general, endoglucanases hydrolyze cellulose at random to produce cellulose oligomers, whereas exoglucanases hydrolyze the cellulose chain from the nonreducing end, producing cellobiose. The crystalline regions of cellulose are not easily accessible to endocellulases, whereas the amorphous regions can be attacked by endocellulases and exocellulases (Bhat and Hazlewood, 2001). Therefore, the incremental effect of exoglucanase shows a small but important role of exoglucanase in addition to endoglucanase in EFE developed for alfalfa and corn silage.

The lack of further improvement in forage degradation with increased xylanase activity in experiment 2

Table 7. Effects of exogenous fibrolytic enzymes on the VFA profiles after 24-h in vitro fermentation of alfalfa hay and corn silage (experiment 2, n = 4)

	Alfalfa hay					Corn silage				
	Total VFA	Individual VFA[1]				Total VFA	Individual VFA[1]			
Treatment	(m*M*)	A	P	B	A:P	(m*M*)	A	P	B	A:P
Control[2]	90.4[b]	61.4[a]	18.2	12.2	3.38[b]	91.9	49.6	22.9	17.7[b]	2.17
E1	98.5[a]	59.1[b]	18.3	12.1	3.23[a]	101.1	48.8	23.3	18.3[ab]	2.11
E3	97.4[a]	58.0[b]	18.3	12.7	3.18[a]	102.6	48.0	23.1	19.0[a]	2.08
E1E5	97.1[a]	59.6[ab]	18.5	11.9	3.23[a]	92.7	49.3	22.8	17.8[b]	2.16
E3E5	93.0[ab]	58.9[b]	18.6	12.1	3.17[a]	96.0	47.6	23.2	18.7[a]	2.06
SEM	2.06	0.73	0.29	0.29	0.053	3.96	0.66	0.40	0.30	0.060

[a,b]Means within a column that do not have a common superscript differ at $P < 0.05$.

[1]Expressed as moles/100 moles; A = acetate; P = propionate; B = butyrate.

[2]Forage substrate without added enzymes.

further supports the limited role of xylanase in the fermentation of alfalfa hay and corn silage. The lack of effect of xylanase is somewhat surprising given that cellulases and xylanases usually act synergistically to hydrolyze forage cell wall (Bhat and Hazlewood, 2001). Thus, it is possible that an ideal ratio of endoglucanase and xylanase is needed to enhance the effectiveness of EFE. In experiment 1, products with an endoglucanase to xylanase ratio below 0.4:1 were not generally effective, particularly for corn silage. Likewise, in experiment 2, lowering the endoglucanase to xylanase ratio of the EFE below 0.4:1 without affecting the endoglucanase activity resulted in no further improvement in the degradation of alfalfa hay compared with the original EFE. Furthermore, none of the combination treatments improved the degradation of corn silage. In fact, the combination treatments generally decreased NDF degradabilities. The mechanisms for this apparent inhibition are not known. However, given the fact that added xylanase activity was not associated with NDF degradation, it is possible that further increases in added xylanase activity blocked enzyme binding sites, preventing exogenous and endogenous enzymes from digesting forage fiber.

For alfalfa hay, a minimum amount of endoglucanase and xylanase activity appears to be necessary for EFE to improve degradation, but a further increase in xylanase activity and decrease in the endoglucanase to xylanase ratio was neither beneficial nor detrimental. For corn silage, a further increase in xylanase activity was actually detrimental, indicating that the most effective enzyme preparations for corn silage may be those with a high endoglucanase to xylanase activity ratio. Hence, the endoglucanase to xylanase activity ratio needs to be considered when formulating exogenous feed enzymes for ruminants.

Because of the limited numbers of EFE used in this study, it is not possible to specify the ideal ratio of endoglucanase to xylanase for alfalfa hay and corn silage. However, based on the consistent effects of E1 and E3 observed in experiment 1 and 2, the ideal ratio appears to be >0.4:1 for both forages. In a previous study with alfalfa hay, there was no synergy when endoglucanase and xylanase from single activity enzyme products were combined in a 1:1 ratio (Eun and Beauchemin, 2007), thus the optimum ratio may be between 0.4:1 and 1:1. Evidently more research is required to identify the ideal endoglucanase to xylanase ratio for these forages and determine whether this ratio is affected by DR.

Impact of Exogenous Enzymes on Ruminal Fermentation

The effects of some EFE on forage degradation were substantial; therefore, it was considered important to examine changes in the formation of end products during fermentation. Increased GP and DM degradation led to a concomitant increase in VFA production, as expected. However, the changes in molar portion of VFA were inconsistent, which contrasts with our previous finding for alfalfa hay (Eun and Beauchemin, 2007), in which EFE addition resulted in a higher proportion of propionate and butyrate and a lower proportion of acetate. In the present study, EFE did not affect propionate proportion, but the decrease in acetate proportion decreased the acetate to propionate ratio. It is not uncommon to observe changes in VFA proportions as a direct effect of added enzymes, implying that added EFE affect microbial growth, shift the metabolic pathways by which specific microbes utilize substrates, or both. However, the changes may depend on the enzymatic activities supplied by the EFE and their impact on forage fiber degradation and ruminal microbial activities.

CONCLUSIONS

Several EFE substantially improved the fermentation of alfalfa hay and corn silage when assessed in vitro. Two of these products were effective for both forages at an optimum DR of 1.4 mg/g of DM, whereas some other products were moderately effective for either forage. The optimum doses of these products were within an economically feasible range, so further in vivo evaluation is warranted. Overall, the EFE were more effective when added to corn silage than alfalfa hay. Increased in vitro NDF degradability due to addition of the EFE would be expected to increase milk production and improve nutrient utilization of dairy cows. The high correlation between added activity of endoglucanase and the improvement of NDF degradability of alfalfa hay and corn silage signifies that endoglucanase, but not xylanase activity, is rate-limiting for microbial digestion of these forages by ruminal microorganisms. Decreasing the endoglucanase to xylanase ratio by increasing the xylanase activity resulted in no further beneficial effects on the degradation of either forage. Effective enzyme products for alfalfa hay and corn silage should supply high endoglucanase activity, with an optimum ratio of endoglucanase to xylanase activity.

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