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Enhancing In Vitro Degradation of Alfalfa Hay and Corn Silage[1] Using Feed Enzymes

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ABSTRACT

A series of in vitro fermentation experiments was performed to assess the effects of 4 feed enzyme products (FE) that varied in enzymatic activities on the degradation of alfalfa hay and corn silage. The FE contained a range of endoglucanase, exoglucanase, xylanase, and protease activities, and a range of dose rates (DR) was used. The objective of the study was to identify effective formulations and optimum DR, and to establish if combining FE would further improve fiber degradation. For alfalfa hay, quadratic increases in gas production and degradation of dry matter (DM) and fiber were observed for all FE, with maximum responses at low to medium DR. For corn silage, none of the FE increased gas production or DM degradation, but all FE increased NDF degradation, with optimum DR in the low to medium range. The proteolytic enzyme papain improved fiber degradation of alfalfa hay and corn silage in a manner similar to that observed for polysaccharidase FE. Among the polysaccharidase FE, added activities of endoglucanase and exoglucanase were positively correlated with improvement in neutral detergent fiber (NDF) degradability of corn silage, whereas only added endoglucanase activity tended to be correlated with improvement in NDF degradability of alfalfa hay. Combining effective polysaccharidase FE further improved fiber degradation of both forages, with greater improvements for corn silage. Combining polysaccharidase and proteolytic FE further improved NDF degradation of corn silage, but not alfalfa hay. Combination treatments generally resulted in additive effects with increases in fiber degradation equal to the sum of the improvements for the individual enzyme components. Improved fiber degradation of corn silage was associated with decreased acetate to propionate ratios. Enzyme products that improve in vitro degradation of forages may have the potential to improve lactational performance of dairy cows.

Key words: alfalfa hay, corn silage, feed enzymes, fiber degradation

INTRODUCTION

Supplementing ruminant diets with feed enzymes (**FE**) to improve forage utilization has attracted growing attention (Beauchemin et al., 2003). Products that contain polysaccharidases have been shown to increase fiber digestion in some (Rode et al., 1999; Bowman et al., 2002), but not all (Knowlton et al., 2002; Sutton et al., 2003), feeding studies. Increased ruminal fiber digestion is expected to increase DMI and milk production of dairy cows. Using numerous forage species ranging in NDF digestibility (24 to 87%), Oba and Allen (1999) reported that a 1-percentage unit increase in NDF digestibility (measured in vitro or in situ) was associated with a 0.25-kg/d increase in 4% FCM yield and a 0.17-kg/d increase in DMI.

Ideal enzyme formulations and effective dose rates (**DR**) need to be identified using in vitro methods before FE products can be used cost effectively in commercial dairy production. The structure of plant cell walls is complex (Wilson and Mertens, 1995) and ruminal microorganisms produce numerous enzymatic activities that hydrolyze the plant cell wall to its constituent monomeric components. Exogenous FE are thought to improve fiber degradation in the rumen by acting synergistically with the rumen microflora (Morgavi et al., 2000), thereby increasing the hydrolytic capacity within the rumen environment (Beauchemin et al., 2004). The major activities involved are cellulases and xylanases, which degrade cellulose and hemicellulose, respectively, with synergy occurring between these 2 activities (Bhat and Hazlewood, 2001). A previous study showed a positive relationship between added endoglucanase activity and improvement in in vitro NDF degradability from alfalfa hay and corn silage (Eun et al., 2007). In addition to these key enzymes, Colombatto et al. (2003a) indicated that proteases improved the in vitro degradation of alfalfa hay.

The objective of this study was to evaluate the potential of various FE products differing in enzymatic activities to improve in vitro degradation of alfalfa hay or

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Table 1. Protein concentration and enzymatic activities of the feed enzyme products used in experiments 1 and 2[1]

Feed enzyme[2]	Protein concentration, mg/g	Enzymatic activity[3]			
		Endoglucanase	Xylanase	Exoglucanase	Protease
FF	625	1,405 ± 69.5	12,990 ± 1,611.7	38 ± 5.0	0.1 ± 0.05
FS	1,212	1,099 ± 51.7	5,785 ± 174.8	40 ± 4.5	0.2 ± 0.10
FT	875	1,613 ± 139.5	955 ± 108.0	79 ± 3.0	0
P	522	0	0	9.1 ± 2.48	63 ± 0.9

[1]Data are for the concentrated enzyme solutions.

[2]Four developmental feed enzyme products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2).

[3]Substrates were (1% in 0.1 *M* citrate phosphate buffer, pH 6.0) medium-viscosity carboxymethylcellulose for endoglucanse, birchwood xylan for xylanase, and Sigmacell 50 for exoglucanase activities. Endoglucanase and exoglucanase or xylanase activity was expressed as nanomoles of glucose or xylose released per minute per milligram; protease activity was expressed as milligrams of azocasein hydrolyzed per minute per gram.

corn silage. A range of doses was used for each product to determine optimum DR. We hypothesized that improvements in fiber degradability would be proportional to endoglucanase or protease activity (or both) supplemented. Feed enzyme products that improved forage degradation in the first experiment were combined to determine whether combination products, particularly those containing proteases, further improved in vitro fiber degradation.

MATERIALS AND METHODS

Forages and Enzyme Products

Two forage substrates were used in the study. The alfalfa hay was of moderate quality and had the following chemical composition (DM basis): 17.3% CP, 49.9% NDF, and 35.4% ADF. The corn silage contained (DM basis): 6.6% CP, 43.8% NDF, and 21.7% ADF. The same batches of alfalfa hay and corn silage were used throughout the study. 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 the in vitro incubations.

Four developmental FE products (**FF**, **FS**, **FT**, and **P**) from Dyadic International Inc. (Jupiter, FL) were used (Table 1). Enzyme products FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2). These source organisms are acceptable for use in animal feeds in North America (AAFCO, 2002; CFIA, 2005). All of the FE products were in powder form.

Experiment 1: Identifying Potential Candidate FE and Their Optimum DR for Alfalfa Hay and Corn Silage

Experiment 1 was undertaken to identify the promising enzyme candidates and their optimum DR. The in vitro procedures used in this series of incubations were the same as those described by Eun et al. (2006) for screening exogenous enzymes for their effectiveness in increasing forage degradability. The incubations were performed in separate runs for alfalfa hay and corn silage. Each run consisted of a 24-h in vitro batch culture fermentation with treatments applied in a 4 (FE) × 5 (DR) factorial design.

For the incubations, approximately 0.7 g (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 each enzyme powder was solubilized using 25 mL of water, and 8.75, 17.5, 26.25, or 35.0 μL of the diluted enzyme was added to the forage in the bags to achieve a DR of 0.25, 0.5, 0.75, or 1.0 mg of concentrated enzyme product per g of forage DM. In addition, substrate without enzyme was included (DR of 0) as a control. The bags were heat-sealed and placed in gas-tight culture vials (125-mL capacity, Wheaton Science Products, Millville, NJ) with 4 replications. Because the enzymes were applied to the feed within the bag using a pipette, uniform distribution of the enzymes on the feed may not have been 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 enzymes. The vials were then stored at 20°C for 17 h. The incubation with buffer, but without ruminal fluid, was used

to ensure adequate interaction time between the forage substrate and the exogenous enzymes.

Ruminal fluid was collected 4 h after the morning feeding (1100 h) from 2 ruminally cannulated, lactating Holstein cows fed a TMR composed of barley silage (46.6%), chopped alfalfa hay (4.5%), rolled corn grain (6.8%), and concentrate (42.1%) on a DM basis. The diet consumed was formulated to meet the nutrient requirements of a dairy cow in early lactation. To prepare the ruminal fluid, 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 6.2 and 6.0 in alfalfa hay and corn silage incubations, respectively) was immediately transferred to the laboratory in a sealed flask and kept at 39°C in a water bath. The inoculum was dispensed (7.0 mL per vial) into the culture vials, which 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 using 4 replications. These controls were used to correct for gas release and fermentation residues resulting directly from the inoculum or the enzyme product itself. The incubation was terminated at 24 h. Then, the vials were placed in a refrigerator at 4°C for 2 h to stop fermentation. Headspace gas production (**GP**) produced during substrate fermentation was measured at 2, 6, 12, 18, and 24 h of incubation using the procedure reported by Mauricio et al. (1999). The vials were handled in the same order during the entire process to ensure that the time interval (6 h) between procedures was the same for each vial.

At the end of the incubation, the bags were removed from the vials and washed under cold tap water until excess water ran clear. The bags were dried at 55°C for 24 h, and degradability of DM was determined by the loss of DM. The contents of the bags were retained for subsequent analysis of fiber content. Profiles of VFA were measured using 5 mL of the fermentation contents added to 1 mL of 25% meta-phosphoric acid. The fermentation samples were stored frozen at –40°C until analyzed.

Experiment 2: Effects of Combining Enzyme Treatments on In Vitro Fermentation of Alfalfa Hay and Corn Silage

The aim of experiment 2 was to confirm the efficacy of selected enzymes from experiment 1 and to determine whether combining these treatments further improved their effectiveness. A completely randomized design was conducted in a single run. From experiment 1 with alfalfa hay, FF, FT, and P at 0.25, 0.75, and 0.25 mg/g of DM, respectively, were selected as single enzyme treatments. Two combination treatments were produced by combining each of the polysaccharidase treatments with the proteolytic enzyme treatment (i.e., FF + P and FT + P). A third combination treatment was produced by combining the 2 polysaccharidases (i.e., FF + FT). Enzyme product FS was not used in experiment 2 with alfalfa hay due to its lack of effect when used with alfalfa hay in experiment 1. From experiment 1 with corn silage, FF, FS, FT, and P at 0.25, 0.75, 0.75, and 0.5 mg/g of DM, respectively, were chosen as single enzyme treatments. Three combination treatments were produced by combining each polysaccharidase with the protease (i.e., FF + P, FS + P, and FT + P). A fourth combination of FF and FT (FF + FT) was made, similar to the combination used for alfalfa hay. A summary of the enzyme treatments and DR used in experiment 2 is given in Table 2.

The incubations were conducted using alfalfa hay and corn silage as previously described for experiment 1. For combination treatments, component enzyme treatments were added separately to the forage substrates at the respective DR chosen. The strained ruminal fluid used in experiment 2 had a pH of 6.3.

Chemical Analyses

The amount of protein present in the enzyme products was determined using the Bio-Rad DC protein determination kit (Bio-Rad Laboratories, Hercules, CA), with BSA as the standard according to the procedure described by Colombatto et al. (2003a). 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) activities 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

Table 2. Enzyme treatments and dose rates used in experiment 2

Substrate	Enzyme treatment[1]	Dose rate
Alfalfa hay	FF	FF at 0.25 mg/g of DM
	FT	FT at 0.75 mg/g of DM
	FF + FT	FF at 0.25 mg/g of DM + FT at 0.75 mg/g of DM
	P	P at 0.25 mg/g of DM
	FF + P	FF at 0.25 mg/g of DM + P at 0.25 mg/g of DM
	FT + P	FT at 0.75 mg/g of DM + P at 0.25 mg/g of DM
Corn silage	FF	FF at 0.25 mg/g of DM
	FT	FT at 0.75 mg/g of DM
	FF + FT	FF at 0.25 mg/g of DM + FT at 0.75 mg/g of DM
	FS	FS at 0.75 mg/g of DM
	P	P at 0.5 mg/g of DM
	FF + P	FF at 0.25 mg/g of DM + P at 0.5 mg/g of DM
	FT + P	FT at 0.75 mg/g of DM + P at 0.5 mg/g of DM
	FS + P	FS at 0.75 mg/g of DM + P at 0.5 mg/g of DM

[1]Four developmental feed enzyme products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2).

milligram, respectively. Protease activity was assayed using azocasein (lot 25H7125, Sigma Chemical) in 0.1 *M* citrate phosphate buffer (pH 6.8) as a substrate in a similar manner as used by Brock et al. (1982) and Eun and Beauchemin (2005a). Protease activity was expressed as milligrams of azocasein hydrolyzed per minute per gram.

The alfalfa hay and corn silage were analyzed for DM (method 930.15) and N (method 990.03) according to AOAC (1995). The NDF and ADF, both inclusive of residual ash, were determined according to Hall et al. (1998) with the method modified for use with an Ankom^{200} Fiber Analyzer (Ankom Technology). Heat stable α-amylase and sodium sulfite were used in the NDF analysis.

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 μm 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.

Statistical Analyses

All the statistical analyses were conducted using the MIXED procedures (SAS Institute, 2001). Data from experiment 1 were analyzed separately by forage substrate as a completely randomized design with FE, DR, and the FE × DR interaction included in the model as fixed effects. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of DR. Cubic and quartic effects were not examined, because they could not be interpreted biologically. The relationship between added endoglucanase, exoglucanse, or xylanase activities and improvement of NDF degradability was determined among polysaccharidase FE by linear regression using the PROC REG procedure of SAS. Data for experiment 2 were also analyzed as a completely randomized design according to substrate with treatment as a fixed effect in the model. Differences between control (no added enzyme) and enzyme treatments were detected using the Dunnett adjustment option. To evaluate the benefits of the combination treatments in experiment 2, the actual response was compared with the individual responses obtained for the control and each of the single component enzymes, as well as to the overall calculated response for the combination. The calculated response was determined by summing the response for the control and the incremental response to each of the component enzymes. These treatment means were compared using a protected ($P < 0.05$) LSD test from a model that included treatment as a fixed effect. The response to the combination treatment was said to be additive when the observed response was similar to the calculated response, and synergistic when the observed response exceeded the calculated response. Least squares means are reported throughout, and significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

The DR used in this study were half of those used in a previous study that examined formulation of ruminant FE (Eun et al., 2007). The lower DR range was used because these FE had higher enzymatic activities

Table 3. Influence of feed enzymes (FE) on the cumulative gas production (mL/g of OM) and the degradability (%) of DM and NDF from alfalfa hay during in vitro fermentation (experiment 1, n = 4)

		Dose rate[3]							Significance of effect[5]		
Item[1]	FE[2]	Mean	0	0.25	0.5	0.75	1.0	SE[4]	FE	DR	FE × DR
GP	FF	102[b]	100[ef]	105[e]	103[e]	96[f]	105[e]	2.9	$P < 0.01$	Q ($P = 0.03$)	$P < 0.01$
	FS	93[c]	100[e]	88[f]	93[ef]	99[e]	93[ef]			Q ($P < 0.01$)	
	FT	100[b]	100[ef]	93[f]	95[f]	107[e]	107[e]			Q ($P = 0.02$)	
	P	119[a]	100[g]	118[ef]	119[ef]	126[e]	114[f]			Q ($P = 0.05$)	
	SE[6]	1.7									
DMD	FF	44.9[b]	43.0[f]	46.0[e]	46.2[e]	43.2[f]	44.3[ef]	0.69	$P < 0.01$	Q ($P < 0.01$)	$P < 0.01$
	FS	41.6[d]	43.0[e]	41.2[ef]	40.5[f]	43.2[e]	41.5[ef]			Q ($P < 0.01$)	
	FT	43.7[bc]	43.0[f]	41.5[f]	41.2[f]	46.0[e]	46.2[e]			Q ($P < 0.01$)	
	P	46.3[a]	43.0[f]	47.6[e]	46.6[e]	46.4[e]	44.7[f]			Q ($P = 0.05$)	
	SE[6]	0.51									
NDFD	FF	22.0[a]	19.7[fg]	22.5[e]	22.7[e]	20.8[ef]	22.0[e]	0.58	$P < 0.01$	Q ($P < 0.01$)	$P < 0.01$
	FS	19.1[c]	19.7[ef]	18.2[f]	18.5[f]	20.3[e]	19.5[ef]			Q ($P < 0.01$)	
	FT	20.7[b]	19.7[f]	19.2[fg]	18.0[f]	22.3[e]	23.2[e]			Q ($P = 0.02$)	
	P	21.8[a]	19.7[g]	23.5[e]	22.1[ef]	21.6[f]	20.1[g]			Q ($P = 0.05$)	
	SE[6]	0.44									

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

[e–g]Means within a row for dose rates of 0 to 1.0 mg/g of DM that do not have a common superscript differ at $P < 0.05$.

[1]All items were measured at 24 h of incubation. GP = gas production; DMD = DM degradability; NDFD = NDF degradability.

[2]Four developmental FE products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2).

[3]Dose rate as mg/g of DM forage substrate; Mean = mean for individual FE across dose rates except dose rate of 0; 0 = control without added FE.

[4]SE for FE × DR.

[5]DR = dose rate; Q = quadratic effect of DR; FE × DR = interaction between FE and DR.

[6]SE for pooled mean of FE excluding the dose rate of 0.

per unit of product than those used previously (Table 1). Enzymes FF, FS, and FT had high endoglucanase and xylanase activities, moderate exoglucanase activity, and negligible protease activity. Enzyme P contained mainly protease activity with no endoglucanase or xylanase and little exoglucanse activity.

Experiment 1

For alfalfa hay, the effect of each of the 4 FE depended upon the DR used, as evidenced by significant FE × DR interactions (Table 3). A quadratic response to DR was observed for all FE, but the optimum DR varied among FE. Some FE affected both GP and degradability of DM and fiber, whereas others only affected fiber degradability. In this study, optimum DR was considered to be the minimum dose required to elicit the greatest significant increase in degradability of fiber (NDF or ADF) compared with the control. Fiber degradability, rather than GP and DM degradability, was selected to ensure that improvements due to FE supplementation lead to an increase in fiber utilization.

None of the polysaccharidases (FF, FS, or FT) increased GP from alfalfa hay compared with the control, whereas the lowest DR of P (0.25 mg/g of DM) increased GP by 18% compared with control. The optimum DR of FE for improving degradability of NDF was 0.25 mg/g of DM for FF and P, and 0.75 mg/g of DM for FT. Relative improvements in the degradability of NDF were 14% for FF, 19% for P, and 13% for FT. Enzyme FS did not increase GP or degradability at any DR.

For corn silage, none of FE evaluated affected GP or DM degradation regardless of DR used (Table 4). However, the FE produced variable effects on NDF degradability across DR, indicated by the FE × DR interaction. Optimum DR for increasing NDF degradability was 0.25 mg/g of DM for FF, 0.75 mg/g of DM for FS and FT, and 0.5 mg/g of DM for P. Relative improvements in NDF degradability at these DR were 14, 26, 54, and 17% for FF, FS, FT, and P, respectively.

Summarizing the data for all polysaccharidase FE and DR, improvement in NDF degradability of alfalfa hay was positively correlated with added endoglucanase activity ($r = 0.51$, $P = 0.09$; Figure 1), but not with added xylanase activity ($P = 0.37$). A similar relationship was observed for corn silage; improvement in NDF degradability was positively correlated with endoglucanase activity ($r = 0.66$, $P = 0.02$), but not with xylanase

Table 4. Influence of feed enzymes (FE) on the cumulative gas production (mL/g of OM) and the degradability (%) of DM and NDF from corn silage during in vitro fermentation (experiment 1, n = 4)

		Dose rate[3]							Significance of effect[5]		
Item[1]	FE[2]	Mean	0	0.25	0.5	0.75	1.0	SE[4]	FE	DR	FE × DR
GP	FF	114	112	119	112	113	113	3.4	NS	NS	NS
	FS	120	112	118	119	117	126				
	FT	116	112	114	115	116	117				
	P	117	112	115	121	115	117				
	SE[6]	1.8									
DMD	FF	42.2^{b}	42.8	42.8	42.2	41.1	42.9	0.85	$P < 0.01$	NS	NS
	FS	43.4^{ab}	42.8	42.3	42.1	44.1	45.0				
	FT	44.6^{a}	42.8	43.8	44.4	45.2	44.9				
	P	42.2^{b}	42.8	41.3	44.0	42.4	41.2				
	SE[6]	0.48									
NDFD	FF	13.3^{b}	11.5^{f}	13.1^{e}	13.8^{e}	11.8^{f}	14.5^{e}	0.73	$P < 0.01$	L ($P < 0.01$)	$P < 0.01$
	FS	13.9^{b}	11.5^{f}	13.6^{f}	12.4^{f}	14.5^{e}	15.3^{e}			Q ($P < 0.01$)	
	FT	15.7^{a}	11.5^{g}	12.8^{g}	14.5^{f}	17.7^{e}	17.4^{e}			L ($P = 0.04$)	
	P	11.6^{c}	11.5^{f}	10.8^{gf}	13.5^{e}	12.6^{ef}	9.4^{g}			Q ($P = 0.05$)	
	SE[6]	0.52									

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

$^{e–g}$Means within a row for dose rates of 0 to 1.0 mg/g of DM that do not have a common superscript differ at $P < 0.05$.

[1]All items were measured at 24 h of incubation. GP = gas production; DMD = DM degradability; NDFD = NDF degradability.

[2]Four developmental FE products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2).

[3]Dose rate as mg/g of DM forage substrate; Mean = mean for individual FE across dose rates except dose rate of 0; 0 = control without added FE.

[4]SE for FE × DR.

[5]DR = dose rate; NS = nonsignificant ($P > 0.05$); L = linear effect of DR; Q = quadratic effect of DR; FE × DR = interaction between FE and DR.

[6]SE for pooled mean of FE excluding the dose rate of 0.

activity ($P = 0.36$). In addition, added exoglucanase activity was positively associated with improvement in NDF degradability for corn silage (r = 0.85, $P < 0.01$; Figure 1), but not alfalfa hay. Combining endoglucanase and exoglucanase in the model explained 87% of the variation ($P < 0.01$) in the improvement in NDF degradability of corn silage due to the addition of the enzymatic activities.

Cellulose is hydrolyzed through a complex process involving cellulases. In general, endoglucanases hydrolyze cellulose chains at random to produce cellulose oligomers of varying degree of polymerization, whereas exoglucanases hydrolyze the cellulose chain from the nonreducing end, producing cellobiose (Bhat and Hazlewood, 2001). Thus, it is not surprising that endoglucanase was shown in this study to be linked to NDF degradability of both forages. The incremental effect of exoglucanase on NDF degradability of corn silage indicates that the ideal enzyme formulations for these 2 forages differ as a result of their differences in chemical composition. One of the main characteristics of exoglucanases is that they act on cellulose chains in a progressive manner. They progress along the polymer chain while releasing cellobiose in a recurrent fashion (Tomme et al., 1996; Reverbel-Leroy et al., 1997), resulting in thinning of crystalline cellulose (Boisset et al., 2000). The versatility of exoglucanases may be important for the degradation of more recalcitrant fiber, such as the corn silage used in this study.

The relationship between added endoglucanase activity and improvement in NDF degradability from alfalfa hay and corn silage observed in this study supports the findings of previous studies with other FE products. Wallace et al. (2001) suggested that endoglucanase activity was rate limiting in the fermentation of corn silage. Similarly, we previously reported a strong relationship (r = 0.77, $P < 0.001$) between added endoglucanase activity and improvement in NDF degradability from alfalfa hay and corn silage (Eun et al., 2007). In that study, the added activity of endoglucanase averaged 224 ± 144.3 (n = 15) nmol of glucose released/min, whereas in the present study the activity was 858 ± 428.1 (n = 12) nmol of glucose released/min. The much higher endoglucanase levels used in this study may have contributed to the weaker relationship between its activity level and improvement in NDF degradability.

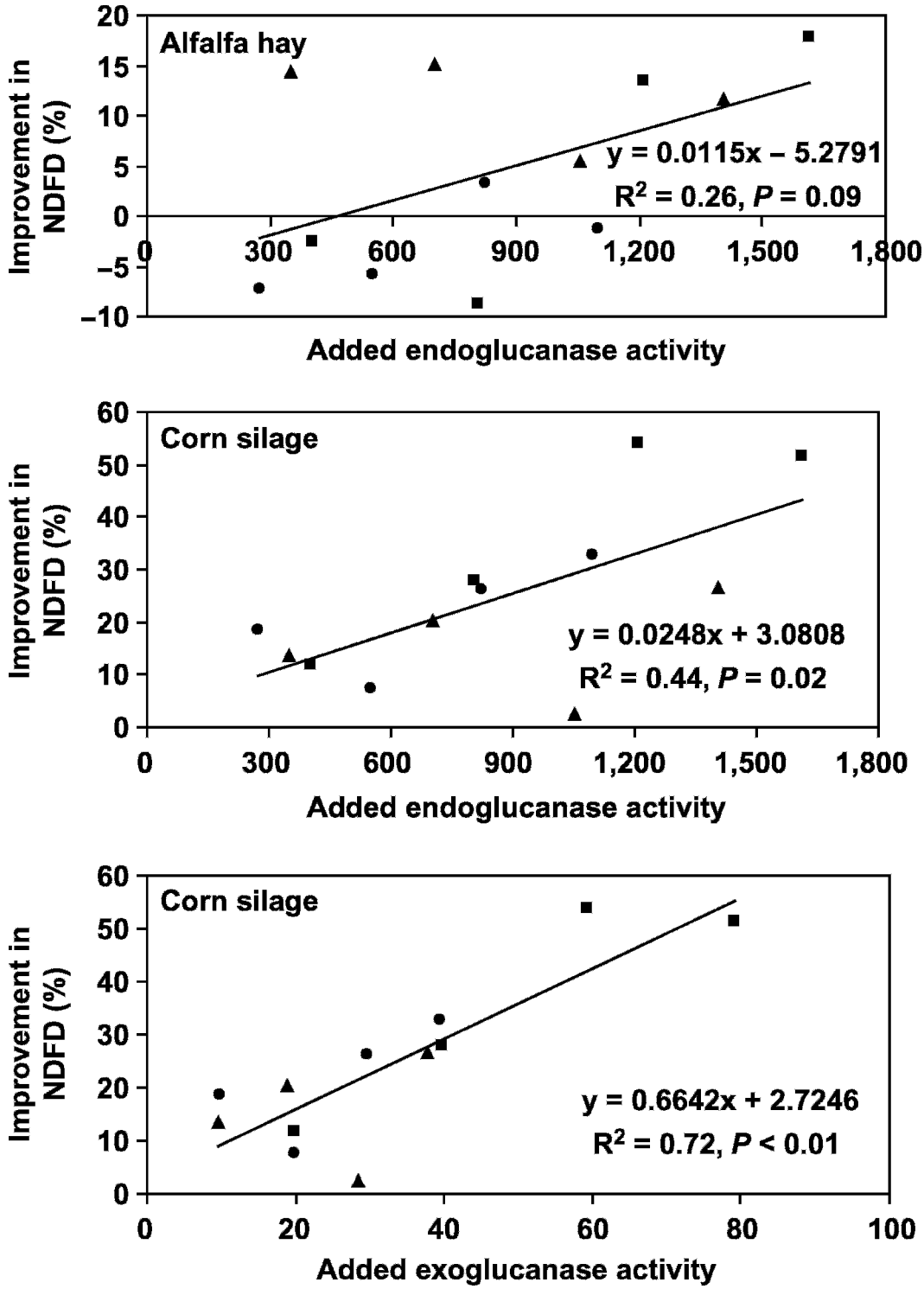

Figure 1. The relationship between added endoglucanase or exoglucanase activity (nmol of glucose released/min) and improvement in NDF degradability (NDFD) for alfalfa hay and corn silage in experiment 1 due to the addition of exogenous feed enzymes (n = 12): FF (▲), FS (●), and FT (■). Improvement in 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. The root mean square error (RMSE) for the improvement of NDFD with added endoglucanase activity for alfalfa hay is 8.71. The RMSE for the improvement in NDFD with added endoglucanase and exoglucanase activities for corn silage are 12.49 and 8.80, respectively.

Table 5. Influence of adding single or combination feed enzymes (FE) on cumulative gas production (GP) and 24-h degradability of alfalfa hay measured in vitro (experiment 2, n = 4)

Treatment[1]	Dose rate (mg/g of DM)	GP (mL/g of OM)		Degradability (%)		
		12 h	24 h	DM	NDF	ADF
Control	0	58.8	99	45.5	22.5	13.6
FF	0.25	61.0	102	46.9	24.4	13.9
FT	0.75	64.3[a]	107	48.4[b]	27.7[a]	19.6[a]
FF + FT	0.25 + 0.75	62.7[b]	109[b]	50.0[a]	28.8[a]	20.7[a]
P	0.25	59.4	103	46.0	25.0[b]	15.3
FF + P	0.25 + 0.25	60.2	114[b]	47.9[b]	25.7[b]	15.6
FT + P	0.75 + 0.25	60.0	108	49.0[a]	27.5[a]	18.5[a]
SE		1.01	3.4	0.69	0.62	0.86

[a,b]Different from the control within columns at $P < 0.01$ and $P < 0.05$, respectively.

[1]Developmental FE products (FF, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum*, whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2). Control was alfalfa hay without added enzymes.

The linear relationship between individual enzymatic activities (i.e., endoglucanase or exoglucanase activities) and NDF degradability presented in Figure 1 differs from the quadratic responses in NDF degradability observed with increasing DR of FE presented in Tables 3 and 4. This apparent discrepancy is likely the result of the interrelationships among the various enzymatic activities within FE, which cause quadratic rather than linear responses.

The substantial increase in NDF degradability with the use of a proteolytic FE supports previous studies that used a proteolytic FE derived from *Bacillus licheniformis* (Protex 6L, Genencor International, Rochester, NY) that did not contain cellulolytic or xylanolytic activities (Colombatto et al., 2003a,b). These studies reported large increases in DM and NDF degradability of alfalfa hay and TMR with use of a proteolytic FE. When that same product was fed to dairy cows, total tract digestibilities of DM, OM, N, NDF, and ADF were increased (Eun et al., 2005a). It is unclear how proteases positively affect forage degradation; however, Colombatto et al. (2003a) speculated that proteolytic enzymes remove some of the cell wall components that are physical barriers to degradation.

To our knowledge, ours is the first study to examine the effects of papain on forage fiber degradation. Papain is a proteolytic enzyme produced by the tropical fruit papaya and is used widely as a meat tenderizer. In ruminant nutrition research, papain has been used to predict ruminal degradation of feed protein (Tománková and Kopečný, 1995; Mirza and Miller, 2005). Proteolytic activity from papain in this study was apparently greater than that from *Bacillus* used previously (63 vs. 36 mg of azocasein/min per g; Eun et al., 2005a, 2006). Low levels of proteolytic enzymes from *B. licheniformis* increased DM degradation of alfalfa hay (Colombatto et al., 2003a), but not corn silage (Colombatto et al., 2003a), alfalfa haylage (Eun and Beauchemin,

Table 6. Influence of adding single or combination feed enzymes (FE) on cumulative gas production (GP) and 24-h degradability of corn silage measured in vitro (experiment 2, n = 4)

Treatment[1]	Dose rate (mg/g of DM)	GP (mL/g of OM)		Degradability (%)		
		12 h	24 h	DM	NDF	ADF
Control	0	54.4	99	54.2	19.8	13.9
FF	0.25	55.5	104	52.8	19.4	11.5
FT	0.75	53.1	106	55.8	22.3[b]	16.5
FF + FT	0.25 + 0.75	59.8[b]	111	57.8[b]	26.0[a]	20.4[a]
FS	0.75	53.6	102	56.0	20.9	14.5
P	0.5	52.6	109	54.4	22.8[b]	13.3
FF + P	0.25 + 0.5	54.8	100	52.8	18.8	14.1
FT + P	0.75 + 0.5	53.3	98	57.7[b]	24.4[a]	17.7[b]
FS + P	0.75 + 0.5	54.2	100	56.4	19.9	14.1
SE		1.42	5.3	0.93	0.77	1.15

[a,b]Different from the control within columns at $P < 0.01$ and $P < 0.05$, respectively.

[1]Four developmental FE products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2). Control was corn silage without added enzymes.

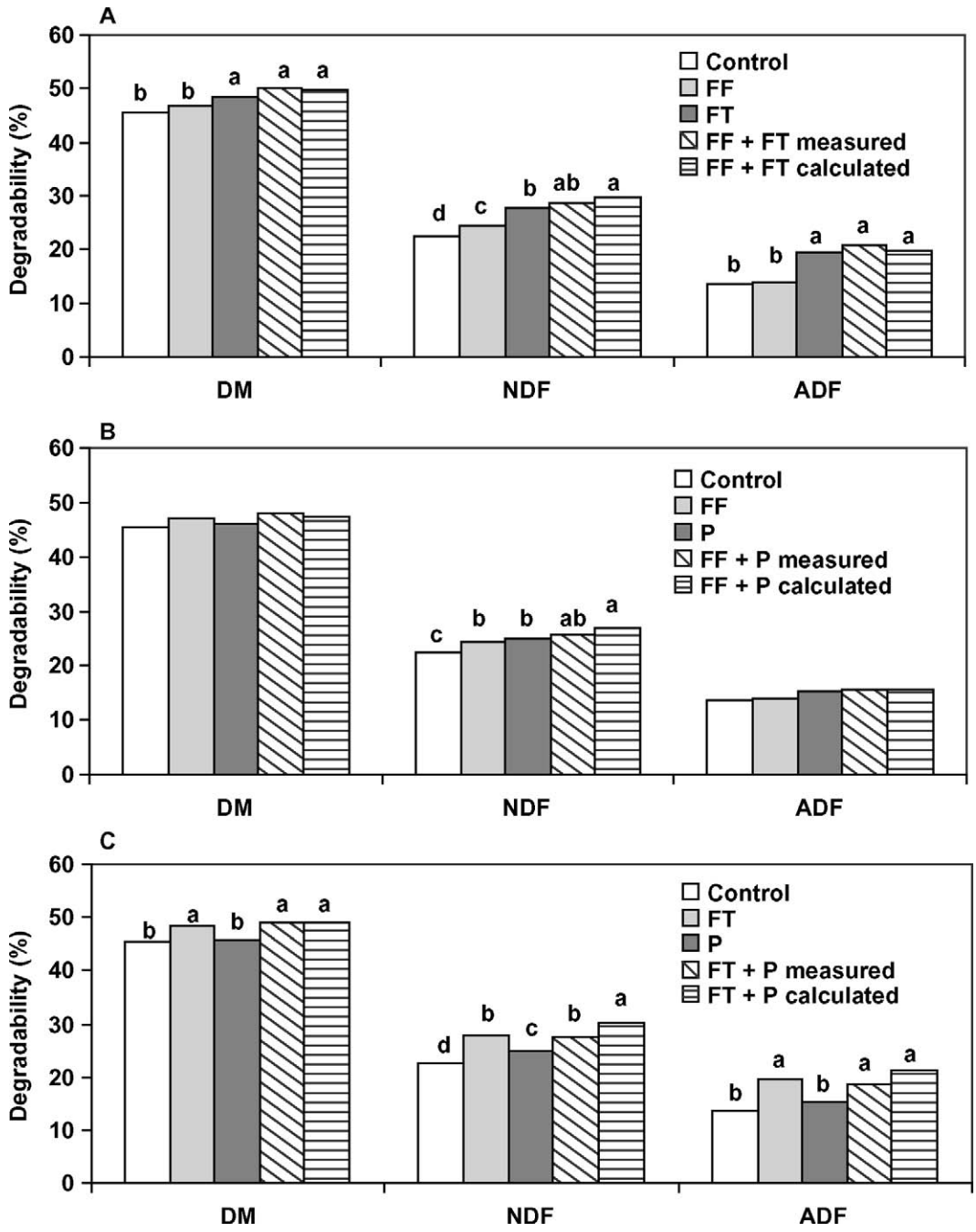

Figure 2. Comparison of single vs. combination enzyme treatments for the degradability of DM, NDF, and ADF of alfalfa hay after 24 h of in vitro fermentation in experiment 2 (n = 4 for each mean). A) Effect of the combination of FF and FT. Calculated values for FF + FT are the sum of control and increments due to FF and FT enzyme treatments. The SE for DM, NDF, and ADF degradability are 0.70, 0.52, and 0.88, respectively. B) Effect of the combination of FF and P. Calculated values for FF + P are the sum of control and increments due to FF and P enzyme treatments. The SE for DM, NDF, and ADF degradability are 0.65, 0.67, and 0.87, respectively. C) Effect of the combination of FT and P. Calculated values for FT + P are the sum of control and increments due to FT and P enzyme treatments. The SE for DM, NDF, and ADF degradability are 0.69, 0.61, and 1.06, respectively. Bars within each fraction having a different letter differ ($P < 0.05$). Control = alfalfa hay without enzyme treatment.

2005b), or barley silage (McGinn et al., 2004; Eun and Beauchemin, 2005a). In the present study, papain improved both alfalfa hay and corn silage NDF degradability, indicating that papain may have a wide range of forage specificity or that higher supplementation rates are needed for some forages. Although papain was effec-

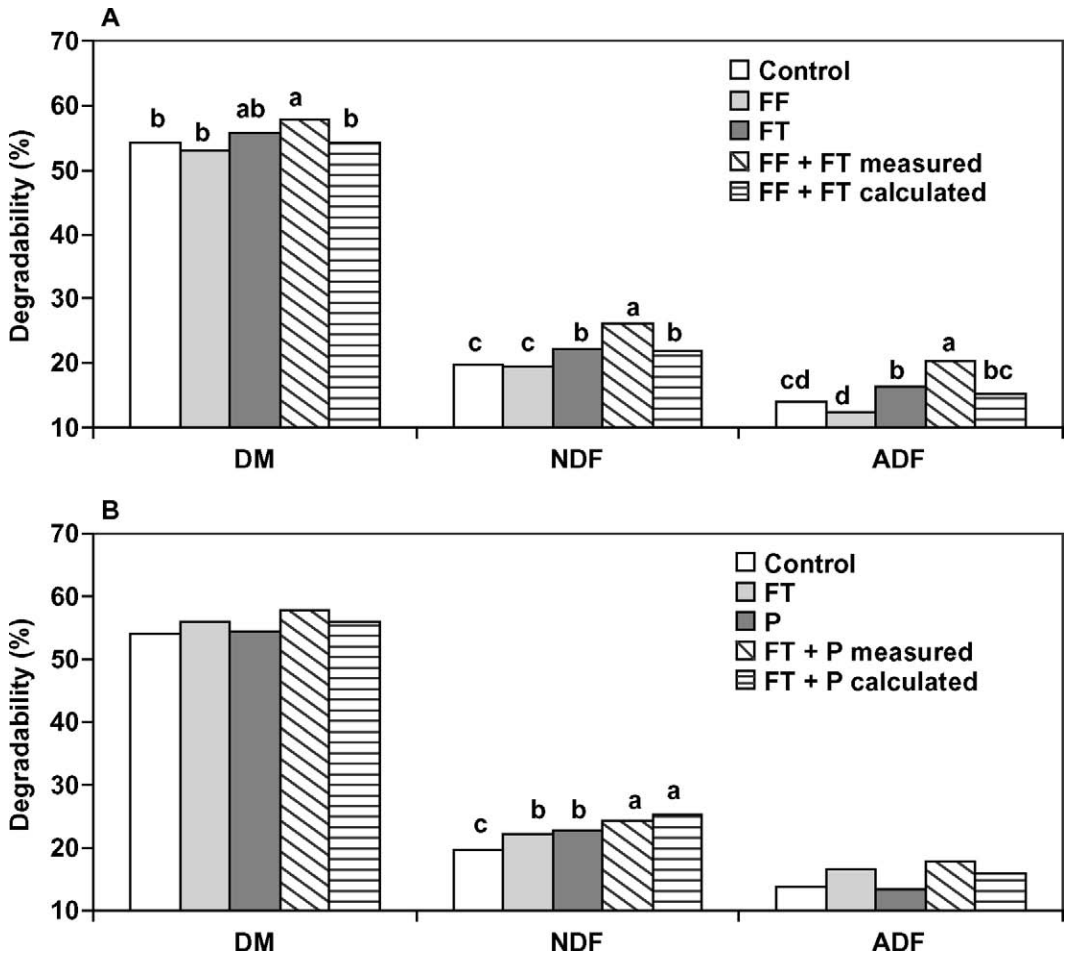

Figure 3. Comparison of single vs. combination enzyme treatments on the degradability of DM, NDF, and ADF of corn silage after 24 h of in vitro fermentation in experiment 2 (n = 4 for each mean). A) Effect of the combination of FF and FT. Calculated values for FF + FT are the sum of control and increments due to FF and FT enzyme treatments. The SE for DM, NDF, and ADF degradability are 1.08, 0.77, and 0.98, respectively. B) Effect of the combination of FT and P. Calculated values for FT + P are the sum of control and increments due to FT and P enzyme treatments. The SE for DM, NDF, and ADF degradability are 1.43, 0.81, and 1.23, respectively. Bars within each fraction having a different letter differ ($P < 0.05$). Control = corn silage without enzyme treatment.

tive for both alfalfa hay and corn silage, its optimum DR differed for each forage: 0.25 and 0.5 mg/g of DM, respectively. At these DR, papain improved NDF degradability to a similar extent for each forage.

Experiment 2

For alfalfa hay, the single enzyme treatment FT increased GP at 12 h and FT and P improved degradability of NDF, whereas unlike in experiment 1, there was no effect of FF (Table 5). Relative to the control, all combination treatments increased GP or DM degradability, with substantial improvements in NDF and ADF degradability: 28 and 52% for FF + FT, 14 and 15% for FF + P, and 22 and 36% for FT + P, respectively. These improvements generally reflected the additivity of the responses to the component enzymes (Figure 2A and B), with the exception of FT + P, for which responses were similar to responses obtained using FT alone (Figure 2C). Thus, in most cases, P and FF were both improved by combining them with FT, but FT was not improved by combining it with other enzymes (Figure 2A). The difference in the response may be due to the higher exoglucanase activity of FT compared with the other products. In our previous study with alfalfa hay (Eun and Beauchemin, 2007), we observed that combination treatments did not increase degradation of alfalfa hay beyond that of the component enzymes when endoglucanase and xylanase from single-activity enzyme products were combined in a 1:1 ratio. From this finding, we suggested that there might be an ideal ratio between the major enzymatic activities to achieve further improvement of degradation with combination treatments. However, the information from the present

Table 7. Influence of adding single or combination feed enzymes (FE) on the VFA profiles after 24 h of in vitro fermentation with alfalfa hay (experiment 2, n = 4)

Treatment[1]	Dose rate (mg/g of DM)	Total VFA (m*M*)	Individual VFA (mol/100 mol)			
			Acetate (A)	Propionate (P)	Butyrate	A:P
Control	0	71.4	57.1	22.4	8.60	2.55
FF	0.25	73.3	56.3	22.7	8.92	2.48
FT	0.75	77.6	57.1	22.9	8.79	2.50
FF + FT	0.25 + 0.75	83.7[a]	57.6	23.0[a]	8.69	2.50
P	0.25	77.6	57.5	22.7	8.37	2.54
FF + P	0.25 + 0.25	76.0	56.8	22.9	8.65	2.48
FT + P	0.75 + 0.25	81.2[a]	56.9	23.1[a]	8.70	2.47
SE		3.34	0.61	0.17	0.202	0.042

[a]Different from the control within columns at $P < 0.05$.

[1]Developmental FE products (FF, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum*, whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2). Control was alfalfa hay without added enzymes.

study suggests that in addition to high endoglucanse and low xylanase activities, high exoglucanse activity (as in the case of FT) may be beneficial in an FE formulation for alfalfa forage.

For corn silage, most FE failed to increase GP but the single enzyme treatments (FT and P) increased NDF degradability (Table 6). Only the combination treatments that contained FT increased degradabilities of fiber, with improvements in NDF and ADF of 31 and 47% for FF + FT, and 23 and 27% for FT + P, respectively. In the case of FF + FT, the substantial increase in fiber degradability exceeded that obtained by the component enzymes (Figure 3A). Therefore, for this particular combination of FF + FT, the response was synergistic. The FF + FT combination was also the only treatment that increased GP throughout fermentation. In contrast to the results with alfalfa hay for which adding P to FT did not improve the response, the effects of FT + P were additive for corn silage (Figure 3B).

The additivity of FF + FT for alfalfa hay can be explained based on the results of experiment 1, in which improvements in NFD degradation were correlated positively to added endoglucanase activity. However, of particular interest is the synergy between these FE for corn silage fiber degradation. This synergy could have been due to the specific cellulases and xylanases within these 2 products or other secondary enzymes that were not measured, such as esterases. Although the primary enzymes involved in xylan degradation are xylanases, the side-chain components of xylans are removed by several enzymes that include acetyl esterase, arabinosidase, and glucuronidase (Hespell and Whitehead, 1990). When arabinosidase or xylanase were incubated individually with alfalfa cell walls, only small amounts of sugars were released; however, sugar release increased 5- to 10-fold when the enzymes were used together (Hespell and Whitehead, 1990). Therefore, enzymatic synergism for the degradation of the side-chain

Table 8. Influence of adding single or combination feed enzymes (FE) on the VFA profiles after 24 h of in vitro fermentation with corn silage (experiment 2, n = 4)

Treatment[1]	Dose rate (mg/g of DM)	Total VFA (m*M*)	Individual VFA (mol/100 mol)			
			Acetate (A)	Propionate (P)	Butyrate	A:P
Control	0	84.0	45.3	29.9	13.4	1.52
FF	0.25	87.7	43.3	31.4[a]	13.6	1.38[b]
FT	0.75	76.4	42.2[b]	31.2[a]	14.3	1.35[b]
FF + FT	0.25 + 0.75	74.9	42.2[b]	31.3[a]	14.4	1.35[b]
FS	0.75	75.4	41.5[b]	31.5[a]	14.4	1.32[a]
P	0.5	76.7	41.6[b]	31.0[b]	14.6	1.34[a]
FF + P	0.25 + 0.5	81.7	42.8	31.4[a]	13.9	1.37[b]
FT + P	0.75 + 0.5	77.0	43.6	30.8[b]	13.7	1.42
FS + P	0.75 + 0.5	79.5	42.2[b]	31.5[a]	14.1	1.34[a]
SE		4.49	0.83	0.26	0.45	0.035

[a,b]Different from the control within columns at $P < 0.01$ and $P < 0.05$, respectively.

[1]Four developmental FE products (FF, FS, FT, and P) from Dyadic International Inc. (Jupiter, FL) were used. FF, FS, and FT were polysaccharidases from a strain of *Trichoderma longibrachiatum* (for FF and FT) or *Penicillum funiculosum* (for FS), whereas P was a proteolytic enzyme originating from papaya (papain; EC 3.4.22.2). Control was corn silage without added enzymes.

components of xylans may account for the increased fiber degradation observed when FF + FT was added at an optimum DR. Further work on enzyme formulation needs to address the possible role of the secondary enzymes.

Colombatto et al. (2003a) reported that the increase in DM degradability of alfalfa hay using FE was directly related to xylanase and protease activities. Therefore, we expected additive effects on fiber degradation of alfalfa hay by combining fibrolytic and proteolytic enzymes. Grabber et al. (2002) reported that the degradation of xylans from alfalfa cell walls was severely restricted when compared with that of other polysaccharides, probably as a result of cross-linkages involving lignin. Therefore, xylanase alone might be ineffective if not accompanied by other enzymes capable of cleaving the cross-linkages. Ferulic acid does not appear to be involved in the interactions between xylans and lignin in alfalfa (Grabber et al., 2002). Although the specific mechanisms are not known, it has been suggested (Jung, 1997) that tyrosine residues could play a role in the cross-linking of dicotyledonous plants, which supports the potential role of protease activity to enhance alfalfa cell wall degradation. However, in our study, combining polysaccharidase and protease treatments had neither additive nor synergistic effects on the degradation of alfalfa hay. In contrast, combinations of polysaccharidase and proteolytic enzymes acted synergistically in improving corn silage fiber degradability when an effective polysaccharidase (i.e., FT) was used (Figure 2B). One possible explanation for the lack of synergy between the key enzymatic activities for alfalfa hay fiber degradation may be that a certain combination of endoglucanase, xylanase, and protease is required for different forage substrates.

Total VFA production from alfalfa hay was increased by FF + FT and FT + P, and these combination treatments increased molar proportions of propionate but did not affect the acetate to propionate ratio (Table 7). Total VFA production from corn silage was not influenced by enzyme treatment (Table 8). However, enzyme treatment generally decreased the molar proportion of acetate and increased the molar proportion of propionate, resulting in decreased acetate to propionate ratio. The molar proportion of butyrate was not affected by enzyme treatments.

Changes in VFA proportions corresponded to increased fiber degradation of corn silage. However, the changes in VFA proportions due to enzyme addition were relatively small for alfalfa hay. In our previous experiment (Eun and Beauchemin, 2007), adding exogenous fibrolytic enzymes to alfalfa hay resulted in more propionate and butyrate and less acetate and corresponded to a considerable increase in fiber degradation. The inconsistent effects of enzyme addition on ruminal fermentation may indicate that changes in VFA composition depend on the enzyme activities added and the forage substrates used. The most pronounced changes in VFA composition in response to enzyme addition in the current experiment were decreased acetate to propionate ratio from corn silage fermentation. Decreased acetate to propionate ratio is another potential benefit of supplementing diets with FE. Increasing availability of glucogenic precursors to cows could improve nutrient utilization, particularly for dairy cows in early lactation when nutrient intake lags behind nutrient requirement.

CONCLUSIONS

Exogenous enzymes containing endoglucanase, exoglucanase, and xylanase or protease activities improved in vitro degradability of alfalfa hay and corn silage fiber, and their optimum DR varied depending upon the forage. In general, low to medium DR of some individual polysaccharidase products resulted in substantial increases in NDF degradability (13 to 19% for alfalfa hay, 14 to 54% for corn silage). The proteolytic enzyme product papain also improved NDF degradability of both forages (19 and 17%, respectively). In most cases combining polysaccharidases with polysaccharidases or proteolytic products had additive, and sometimes synergistic, effects on fiber degradation of forages. It is recommended that the combination treatments FF + FT and FT + P be further evaluated in a dairy cow feeding study using diets based on corn silage or alfalfa forage.

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