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Effects of Exogenous Enzymes on Ruminal Fermentation and Degradability of Alfalfa Hay and Rice Straw

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ABSTRACT : This study was conducted to evaluate the use of exogenous enzymes as a potential means of improving the ruminal digestion (i.e., degradability) of alfalfa hay and rice straw. Twenty six enzyme-additives were examined in terms of protein concentration and enzymic activities on model substrates. The exogenous enzymes contained ranges of endoglucanase, xylanase, β-glucanase, α-amylase, and protease activities. Six of the enzyme additives were chosen for further investigation. The enzyme additives and a control without enzyme were applied to mature quality alfalfa hay substrate and subsequently incubated in rumen batch cultures. Five of the enzyme additives (CE2, CE13, CE14, CE19, and CE24) increased total gas production (GP) at 48 h of incubation compared to the control ($p<0.05$). The two additives (CE14 and CE24) having the greatest positive effects on alfalfa hay dry matter, neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability were further characterized for their ability to enhance degradation of low quality forages. The treatments CE14, CE24, a 50:50 combination of CE14 and CE24 (CE14+24), and control (no enzyme) were applied to mature alfalfa hay and rice straw. For alfalfa hay, application of the two enzyme additives, alone and in combination, increased GP compared to the control at 48 h fermentation ($p<0.05$), whereas only CE14 and CE14+24 treatments improved GP from rice straw ($p<0.05$). Rumen fluid volatile fatty acid concentrations throughout the incubation of rice straw were analyzed. Acetate concentration was slightly lower ($p<0.05$) for CE14×CE24 compared to the control, although individually, CE14 and CE24 acetate concentrations were not different from the control. Increases ($p<0.05$) in alfalfa hay NDF degradability measured at 12 and 48 h of incubation occurred only for CE14 (at 12 h) and for CE14+24 (at 12 and 48 h). Similarly, ADF degradability increased ($p<0.05$) with CE14 and CE14+24. As for rice straw, increased DM degradability was observed at 12 and 48 h of incubation for all enzyme treatments with an exception for CE14 at 12 h. The degradability of NDF was improved by all the enzyme treatments at either incubation time, while ADF degradability was only enhanced at 48 h. Overall, the enzymes led to enhanced digestion of mature alfalfa and there was evidence of improved digestibility of rice straw, an even lower quality forage. (**Key Words :** Alfalfa, Rice Straw, Ruminal Digestion, Enzyme Additives)

INTRODUCTION

The use of exogenous enzymes as ruminant feed additives has shown variable effects on feed digestion and animal performance. In some studies, direct fed enzyme additives improved dry matter (DM) intake (Lewis et al., 1999; Beauchemin et al., 2000; Kung et al., 2000; Pinos-Rodríguez et al., 2002), *in vivo* fibre digestibility (Rode et al., 1999; Bowman et al., 2002; Pinos-Rodríguez et al., 2002), or animal productivity (Beauchemin et al., 1995; Lewis et al., 1999; Rode et al., 1999; Schingoethe et al., 1999; Yang et al., 2000). However, many other studies observed no beneficial effects of feeding enzyme additives (Knowlton et al., 2002; Vicini et al., 2003; Elwakeel et al., 2007; Miller et al., 2008). Inconsistencies in the responses to supplemental enzyme additives are thought to be due in part to the characteristics of the enzymes, including enzymic activities at ruminal conditions (temperature and pH) and composition of the target forage. Additionally, comparison of results between studies is confounded by a lack of standardization in testing of enzymes added to feed.

The biochemical activities of feed enzymes tested under controlled, optimal conditions does not always predict their ability to enhance ruminant feed digestion (McAllister et al., 2001). Mainly, this is because the enzymes must work synergistically with the ruminal microbial community to alter feed digestion (Morgavi et al., 2000). Therefore, the selection of enzymes for use as ruminant feed additives should be first tested in a ruminal environment (Colombatto

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and Beauchemin, 2003). It is also important to test enzymes on individual feeds, as their activity may be specific to the type of feed. For example, Beauchemin et al. (1995) demonstrated that a particular enzyme additive increased average daily gain of cattle when the additive was sprayed onto alfalfa hay, but the same additive had no effect when applied to whole crop barley silage.

There is interest in enhancing the nutritive value of poor quality forages. Alfalfa hay is a legume that is generally considered a high quality forage, and usually contains low concentrations of fibre (<30% acid detergent fibre; ADF, <40% neutral detergent fibre; NDF) when harvested at an early stage of maturity. However, its quality can decline dramatically when harvested at an advanced stage of maturity (>40% ADF, >50% NDF). Rice straw has a high lignocellulosic content, low crude protein content, poor palatability, and low organic matter (OM) degradation in the rumen (Jung et al., 1993). Increasing the digestibility of low quality feeds using enzymes could lead to significant improvements in animal performance in many parts of the world.

The purpose of this study was to analyze a range of commercially available enzyme additives that contained xylanase, endoglucanase, exoglucanase, glucanase, amylase, and protease activities. Based on these results, six enzyme additives were further characterized by *in vitro* ruminal incubations with alfalfa hay. Two enzyme additives showing high fibrolytic activity were then tested for their synergy for improving ruminal digestion of the low quality feeds, mature alfalfa hay and rice straw.

MATERIALS AND METHODS

Experiment 1: Biochemical enzymic activities

Protein concentration : The amount of protein present in 26 commercial enzyme products (denoted CE1 to CE26) was determined using the Bio-Rad DC protein determination kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the standard. Five microliters of diluted enzymes were added to microtiter plates, followed by 25 μl of Bio-Rad reagent A and 200 μl of reagent B. The reaction was allowed to proceed for 15 min at room temperature, and absorbance was read at 630 nm using a MRX-HD plate reader (Dynatech Laboratores, Inc., Chantilly, VA).

Enzymic activities : Polysaccharidase enzymic activities were determined using single polysaccharide substrates, in triplicate. Triplicate control samples were also prepared to account for background resulting from non-catalytic development and colour of the substrate in solution. The activities were determined as described by Bailey et al. (1992) using single polysaccharide substrates in solution or suspension (1% wt/vol) in 0.1 *M* citrate phosphate buffer (pH 6.0). Xylan (from birch wood or from oat spelts), carboxymethylcellulose, laminarin, and soluble starch (all obtained from Sigma Chemicals, St. Louis, MO) were used for determination of xylanase, endoglucanase, β-1,3-glucanase, and α-amylase, respectively. Assay conditions were 39°C and pH 6.0 to reflect ruminal conditions. Suitably diluted enzyme (50 μl) and substrate (450 μl) were incubated for 5 min. The enzymatic reaction was terminated by adding Nelson-Somogyi reagent (Nelson, 1944; Somogyi, 1952). The reaction contents were boiled for 15 min and cooled in a water bath. Reducing sugars were determined colorimetrically using a Dynatech MRX plate reader at 630 nm. One unit of activity was defined as the amount of enzyme required to release 1 nmol equivalent of glucose or xylose per minute per gram of enzyme additive, under the conditions of the assay. Protease activity was assayed using azocasein (Sigma Chemicals, St. Louis, MO) in 0.1 *M* citrate phosphate buffer (pH 6.0) in a similar manner as used by Brock et al. (1982) and Eun and Beauchemin (2005). Protease activity was expressed as mg of azocasein hydrolyzed/ml.

Experiment 2: Batch culture incubations of alfalfa hay treated with individual enzyme additives

Based on the enzyme activity data (Table 1), four enzyme additives showing high α-amylase and protease (CE14), β-1,3-glucanase (CE2), endoglucanase (CE19), or xylanase (CE8) activity were selected. Additionally, two enzyme additives (CE13 and CE24) that were newly marketed at the time of this study were investigated. The enzyme additives were applied individually to the alfalfa hay prior to incubation in ruminal batch culture.

A sample of mature alfalfa hay was obtained and ground to pass through a 1-mm screen using a Wiley mill. The alfalfa hay contained 94.8% DM, 52.8% NDF and 38.1% ADF. Alfalfa was weighed (1 g DM) into acetone-washed, preweighed filter bags (#57, ANKOM Corp., Fairport, NY). Enzyme additives dissolved in water (200 mg/L) or no additives (control) were applied (1.5 mg/g DM) to alfalfa substrates in the bags. Three replications were prepared for each treatment and each batch culture incubation time. The bags were then heat-sealed, placed into 100 ml vials and incubated at room temperature for 3 h, after which 40 ml of anaerobic buffer medium (Goering and Van Soest, 1970) were added. The vials were incubated at room temperature for an additional 17 h, prior to addition of rumen fluid for batch culture incubations.

Fresh rumen contents from two ruminally fistulated steers fed alfalfa hay were collected 3 h after the morning feeding for use in the batch cultures. Approximately 1.5 L rumen fluid was collected by straining digesta through four

Table 1. Activities of the 26 experimental enzyme additive (CE) products used in this study (Exp. 1)

Product	Protein	Enzymic activity[1]					
		BX	OSX	END	BG	AMYL	PROT
CE1	107	1,064	657	53	223	0.0	0.0
CE2	49	62	52	79	535	1.9	2.4
CE3	101	38	12	72	211	2.2	0.0
CE4	117	336	221	166	434	2.0	0.0
CE5	98	34	9.4	99	205	1.5	0.0
CE6	127	325	201	152	445	1.9	0.0
CE7	129	160	149	130	465	0.0	0.0
CE8	46	1,613	925	17	1.7	2.1	0.0
CE9	131	334	188	149	425	1.4	0.0
CE10	234	147	124	251	532	2.7	0.0
CE11	195	481	467	190	616	0.7	0.0
CE12	16	70	22	0.7	64	1.8	0.0
CE13	27	376	171	16	8.2	2.1	3.24
CE14	111	78	99	24	16.2	437	4.78
CE15	73	421	191	34	60	2.6	3.49
CE16	55	46	7.7	8.3	127	0.9	1.35
CE17	69	372	161	36	58	1.8	3.13
CE18	83	1,214	611	67	101	0.6	3.90
CE19	152	650	477	650	1.4	0.0	3.29
CE20	212	311	279	311	2.2	3.2	4.17
CE21	77	289	247	289	0.0	0.0	2.64
CE22	285	724	725	724	0.0	2.6	2.63
CE23	125	417	524	417	0.0	74.9	2.69
CE24	191	239	301	239	3.9	0.0	2.75
CE25	303	265	262	265	0.0	0.0	3.89
CE26	179	192	239	192	52.7	0.0	4.12

[1] Enzymic activities were expressed as nmol of sugar released min^{-1} mg^{-1} for the enzyme products except for protease activity which was expressed as mg of azocasein hydrolyzed per ml^{-1}.

BX = Xylanase (birchwood); OSX = Xylanase (oat spelt); END = Endoglucanase; BG = β-glucanase; AMYL = α-amylase; PROT = Protease.

layers of cheesecloth into a flask flushed previously with CO_2. Solid ruminal digesta was sealed in a bag and both fractions were taken back to the laboratory for processing under anaerobic conditions. Inoculum was prepared by blending 1.5 L rumen fluid with 375 g of solid digesta for 45 s three times. The homogenate thus obtained was passed through four layers of cheesecloth and the strained fluid (20 ml) was added to each of the vials containing substrate and enzyme treatments. The vials were sealed and incubated at 39°C on a rotary shaker for 48 h. Head space gas production (GP) resultant of substrate fermentation was measured at 2, 6, 12, 18, 24, 36, and 48 h post inoculation. Pressure values, corrected for the amount of substrate OM incubated and for the gas released from negative controls, were used to generate volume estimates using the quadratic equation reported by Mauricio et al. (1999). At 48 h, vials were removed from the shaker and placed on ice in preparation for DM, NDF, and ADF analyses.

Experiment 3: Batch culture incubations of alfalfa hay and rice straw treated with enzyme combinations

Two enzyme additives (CE14 and CE24) from Experiment 2 having the greatest positive effects on DM, NDF and ADF degradability of alfalfa hay were chosen for further evaluation, either as single enzyme treatments or as combinations. The alfalfa hay was the same as was used in Experiment 1. In addition, the enzyme additives were evaluated using rice straw containing 94.0% DM, 67.1% NDF and 41.0% ADF. Both substrates were ground to pass through a 1-mm screen and weighed (1 g DM) into filter bags (#57, ANKOM Corp., Fairport, NY). The treatments were: CE14, CE24, and a 50:50 combination of CE14 and CE24 (CE14+24). For the combination treatment, each component was applied at 100%, thus the response was expected to be equal to the sum of the response of CE14 and CE24. A control treatment with no added enzyme was also used. Four replications were prepared for each treatment and each batch culture incubation time. Enzyme

additives were applied (1.5 mg/g DM) to the substrates and processed as described above. The GP resultant of substrate fermentation was measured at 6, 12, 24, 48 h post-inoculation. Pressure values, corrected for the amount of substrate OM incubated and for gas released from negative controls, were used to generate volume estimates. At 12 and 48 h, vials were removed from the shaker and placed on ice in preparation for DM, NDF, and ADF analyses. Subsamples (5 ml) of the inoculant/buffer from rice straw incubations were collected for measurement of volatile fatty acid (VFA) concentrations.

Chemical analysis

The DM content of the forage residues after *in vitro* incubation was determined for 24 h at 105°C. Dry matter content of dried, ground forage samples was determined by oven drying at 100°C for 24 h. Organic matter was determined by placing samples in a muffle furnace at 550°C for 3 h, until all carbon was eliminated. Fibre (NDF and ADF) analyses were determined using the ANKON200 fibre analyzer, following the procedures outlined by the manufacturer (ANKOM Corp., Fairport, NY). Sodium sulfate (10 g/L NDF detergent) and heat-stable bacterial amylase (2 ml/L NDF detergent) were used in the analysis of NDF. Volatile fatty acids were determined by gas chromatography as described previously (Eun and Beauchemin, 2007).

Statistical analysis and calculations

Data for DM and fibre (NDF and ADF) degradation, GP, and VFA were analyzed separately for alfalfa hay and rice straw using the Proc Mixed procedure of SAS (SAS Institute, 2001). Multiple comparisons of the means at each time point were analyzed using the Tukey adjustment. Differences were considered significant if the p-values were less than or equal to 0.05.

To determine whether the effects of the combination treatment (CE14+24) were additive, an expected response was calculated from the sum of the response for the individual components. The calculated response was compared to the actual response using a t-test. When the response to CE14+24 was similar to the calculated response, the two enzymes were said to be additive. When the response to CE14+24 exceeded the calculated response, the response was said to be synergistic.

RESULTS AND DISCUSSION

Experiment 1: Biochemical enzyme activities

Each enzyme showed a unique array of enzymatic activities (Table 1). All of the enzymes had xylanase, endoglucanase, and β-glucanase activity, although the magnitude of activity varied greatly. Amylase and protease activity were present in some, but not all of the enzyme additives. Surprisingly, in some cases, xylanase activity differed remarkably for a single enzyme additive, depending on the substrate (birchwood vs. oat spelt). Generally, xylanase activity was greater when the substrate was birchwood. In contrast, three enzyme additives (CE23, CE24, and CE26) showed greater activity for oat spelt. These differences may account for variable results obtained for xylanases in other studies (Colombatto et al., 2003a). Additionally, these results emphasize the importance of testing enzymes on all substrates and feed samples to be supplemented, before they are used as feed additives.

Experiment 2: Batch culture incubations of alfalfa hay treated with individual enzyme additives

Gas production : Based on data from Experiment 1, enzyme additives that had high α-amylase and protease (CE14), β-1,3-glucanase (CE2), endoglucanase (CE19), or xylanase (CE8) activity, in addition to two newly marketed enzymes (CE13 and CE24) showing variable ranges of activity, were tested for their ability to improve digestion of alfalfa hay during *in vitro* batch culture fermentations. Gas production was significantly increased by enzyme treatment and incubation time (Figure 1, $p<0.05$ for both). Production increased throughout the fermentation, indicating continuous digestion. No interaction between time and enzyme treatment occurred ($p = 0.97$), implying that enzyme mixtures affected alfalfa digestion consistently regardless of the duration of the incubation. Thus, the enzymes improved digestion throughout the fermentation, thereby improving overall alfalfa degradation. Others have

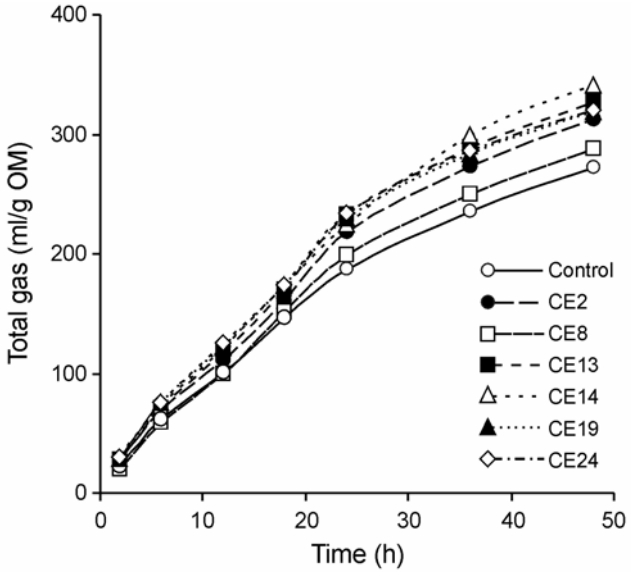

Figure 1. Mean (n = 3) cumulative gas production profiles for alfalfa hay treated with or without exogenous enzymes during an incubation in ruminal fluid. Enzyme additives (CE) activities are described in Table 1. Control, no enzyme treatment.

reported short-term effects of enzymes on digestion with limited effects later during the incubation (Tricarico, 2001; Colombatto et al., 2003a). Colombatto and Beauchemin (2003) suggested that enzymes enhance alfalfa digestion by removing structural barriers retarding microbial colonization, thus increasing the rate of degradation.

In the present study, five of the enzyme additives used (CE2, CE13, CE14, CE19, and CE24) had greater gas accumulation compared to the control. Enzyme CE14, which had high amylase and protease activity, improved GP the greatest compared to the control. In contrast, CE8, which had high xylanase activity, did not improve GP compared to the control throughout the incubation. Colombatto et al. (2003b) also reported greater increases in DM and NDF degradability of alfalfa hay and total mixed ration (TMR) as a result of supplementation with an exogenous proteolytic enzyme product.

In vitro degradability : After 48 h of incubation there was a trend ($p<0.2$) for enzyme additives that showed highest gas production (Figure 1) to also increase degradability of DM, NDF, and ADF, compared to the control (Table 2). Only CE14, which had high amylase and protease activity, and CE24, which had moderate polysaccharidase activity, significantly ($p<0.05$) improved alfalfa degradation. Based on the biochemical activities of the enzymes, it would be expected that CE8 (high xylanase activity) and both CE2 (high β-glucanase activity) and CE19 (high endoglucanase activity) would have the greatest effect on improvement of NDF and ADF degradation. These data would suggest that the biochemical activities of enzyme additives *in vitro* did not reflect activity on substrate incubated in a ruminal environment. Such findings have been reported before (Eun and Beauchemin, 2007) and highlight the importance of testing enzymes in an environment similar to the rumen.

Table 2. Effects of individual enzyme addition on apparent degradability (g/kg) of DM, NDF, and ADF from alfalfa hay after 48 h of incubation with ruminal fluid (Exp 2)

Treatment	Component[1]		
	DM	NDF	ADF
Control[2]	488	422	225
CE2	511	442	253
CE8	503	435	240
CE13	526	463	279
CE14	547[a]	479[a]	290[a]
CE19	530	464	281
CE24	536[a]	468	288[a]

[1] Data are the mean values (n = 3).
[2] Alfalfa hay without added enzymes.
[a] Different from the Control within a column ($P < 0.05$).

Experiment 3: Batch culture incubations of alfalfa hay and rice straw treated with enzyme combinations

Gas and VFA production : Enzyme additives CE14 and CE24 showed the greatest ability to improve digestion of alfalfa fibre in Experiment 2, and were therefore selected to test their ability alone, and in combination, to enhance digestion of alfalfa and rice straw. The GP from both substrates increased over time for all treatments, indicating active ruminal microbial fermentation (Tables 3 and 4). When compared to the control, enzyme additives CE14 and CE14+24 increased ($p<0.05$) total GP from alfalfa hay after 12 h of incubation with ruminal fluid, but none of the enzymes affected GP from rice straw ($p>0.05$) at this time point. Enzymes CE14 and CE14+24 increased GP from alfalfa hay by 15.8 and 25.2% at 12 h of incubation, respectively ($p<0.05$). All enzyme treatments resulted in greater GP by 48 h when applied to alfalfa. The relative increment in GP from alfalfa hay at 48 h of incubation was 12.9%, 9.9%, and 20.7% for CE14, CE24, and CE14+24, respectively. Only the CE14 and CE14+24 treatments improved GP from rice straw at 48 h of incubation ($p< 0.05$), suggesting that the recalcitrant fibre of rice straw took longer before the effects of the enzyme additives occurred. These results indicate that enzymes may have a greater potential to be used to improve the quality of higher quality forages than poorer quality forages.

Table 3. Cumulative gas production (ml/g OM) throughout ruminal batch cultures of alfalfa hay treated with feed enzymes individually or in combination (Exp. 3)[1]

Treatment	Incubation time (h)			
	6	12	24	48
Control[2]	70.5	114.5[a]	159.1[a]	229.9[a]
CE14	80.8	132.6[b]	182.8[b]	259.7[b]
CE24	78.5	129.0[ab]	177.4[ab]	252.8[b]
CE14+24	82.4	143.4[c]	195.1[c]	277.5[c]
CE14+24 (calculated)[3]	79.7	130.8*	180.1*	255.3*

[1] Data are the mean values (n = 4). [2] Alfalfa hay without added enzymes.
[3] Expected effect for CE14+24 treatment based on the responses for individual CE14 and CE24 treatments.
[a, b] Means in the same column with different superscripts differ ($p<0.05$), with the exception of CE14+24 (calculated), which was not included in the analysis.
* The expected effect was different ($p<0.05$) from the actual effect of CE14+24, within time point, indicating synergy between CE14 and CE24.

Table 4. Cumulative gas production (ml/g OM) during ruminal batch cultures of rice straw treated with feed enzymes individually or in combination (Exp. 3)[1]

Treatment	Incubation time (h)			
	6	12	24	48
Control[2]	33.3	62.3	102.3	142.5[a]
CE14	35.1	64.2	105.4	153.2[b]
CE24	31.1	60.6	102.6	150.9[a]
CE14+24	34.4	63.3	104.4	158.3[b]
CE14+24 (calculated)[3]	33.1	62.4	104.0	152.1

[1] Data are the mean values (n = 4). [2] Rice straw without added enzymes.
[3] Expected effect for CE14+24 treatment, based on the responses for individual CE14 and CE24 treatments. No differences were observed ($p>0.05$) between the expected effect and the actual effect of CE14+24, indicating additivity between CE14 and CE24.
[a, b] Means in the same column with different superscripts differ ($p<0.05$) with the exception of CE14+24 (calculated), which was not included in the analysis.

Gas production is an indirect measure of substrate degradation, particularly the carbohydrate fraction (Menke, 1979). However, gas production is not always positively related to microbial mass production. Because of the lack of effects of enzymes on GP from rice straw at the early incubation times, VFA profiles were measured to further explore any potential effects of enzymes on ruminal fermentation. After 12 h of incubation, total VFA and the proportion of propionate were similar for all treatments (Table 5). Acetate proportion was slightly lower ($p<0.05$) for CE14+24 compared to the control, although individually CE14 and CE24 showed an acetate proportion not different from the control. The difference observed for the individual enzymes and in combination suggests a potential synergistic effect between the two enzymes additives to lower acetate production. However, the decrease in acetate from CE14+24 was not enough to alter the acetate:propionate (A:P) ratio, which was similar across all treatments. There was some variation in the proportion of butyrate, where CE14 and CE14+24 resulted in higher amounts compared to the control at 12 h ($p<0.05$). By 48 h, total VFA concentrations tended to be greater than the control for CE24 ($p = 0.18$) and CE14+24 ($p = 0.07$), suggesting a slight increase in carbohydrate fermentation resulting from the addition of these enzyme additives (Table 5).

In some instances, enzyme treatments resulted in greater acetate proportions (CE14, CE24) and reduced propionate proportions (CE14, CE14+24) compared to the control after 48 h of incubation. This resulted in greater A:P ratios for treatments CE14 and CE14+24 ($p<0.05$). Propionate is the most important VFA precursor to glucose synthesis (Nagaraja et al., 1997) thus a lower A:P generally reflects improved nutritional value of a feed. Other studies have reported increased levels of propionate from fungal treatment (Karunanandaa and Varga, 1996) or reduced A:P from enzymatic treatment (Eun et al., 2006) of rice straw. However, differences in the substrate or length of fermentation may affect these results. Combined, the VFA

Table 5. The effect of enzyme addition on total and individual volatile fatty acids (VFA) 12 and 48 h following ruminal incubations of rice straw (Exp. 3)

Incubation time	Treatment[1]			
	Control	CE14	CE24	CE14+24
12 h				
Total VFA (m*M*)	95.1	97.1	94.1	96.3
Acetate (mol/100 mol)	63.2[a]	62.0[ab]	62.6[ab]	61.8[b]
Propionate (mol/100 mol)	18.0	18.0	18.2	18.2
Butyrate (mol/100 mol)	11.1[a]	11.9[b]	11.3[a]	11.7[b]
Acetate:propionate	3.5	3.4	3.4	3.4
48 h				
Total VFA (m*M*)	127.4	129.6	132.6	134.0
Acetate (mol/100 mol)	58.6[a]	62.0[b]	60.8[b]	60.3[ab]
Propionate (mol/100 mol)	21.6[a]	20.7[b]	21.1[ab]	20.7[b]
Butyrate (mol/100 mol)	12.2[a]	11.0[b]	11.3[ab]	12.0[a]
Acetate:propionate	2.7[a]	3.0[b]	2.9[b]	2.9[b]

[1] Data are the mean values (n = 4).
[a, b] Means in the same row with different superscripts differ ($p<0.05$).

Table 6. Effects of enzyme addition on apparent degradability (g/kg) of DM, NDF, and ADF from alfalfa hay after 12 and 48 h of incubation with ruminal fluid (Exp. 3)

Incubation time and treatment	Component[1]		
	DM	NDF	ADF
12 h			
Control[2]	390.2[a]	190.8[a]	108.4[a]
CE14	413.2[b]	212.8[b]	126.3[b]
CE24	408.7[b]	194.1[a]	111.6[a]
CE14+24	423.7[c]	213.7[b]	135.2[b]
CE14+24 (calculated)[3]	410.9	203.4	118.9
48 h			
Control	556.4[a]	400.7[a]	248.8[a]
CE14	584.9[b]	422.2[a]	287.0[b]
CE24	572.3[b]	410.0[a]	270.5[a]
CE14+24	589.8[b]	440.7[b]	296.6[b]
CE14+24 (calculated)[3]	578.6	416.1*	278.7

[1] Data are the mean values (n = 4). [2] Alfalfa hay without added enzymes.
[3] Expected effect for CE14+24 treatment, based on the responses for individual CE14 and CE24 treatments.
[a, b] Means in the same column with different superscripts differ ($p<0.05$) with the exception of CE14+24 (calculated), which was not included in the analysis.
* The expected effect was different ($p<0.05$) from the actual effect of CE14+24 for this time period, indicating synergy between CE14 and CE24.

profiles indicated that enzymatic treatment altered the degradability of rice straw in a way that shifted VFA end-product synthesis towards higher A:P ratio. This may have resulted from altered growth rates of bacteria (Russell and Wallace, 1997) or perhaps a shift in bacteria populations. Overall, fermentation after enzyme supplementation appeared to be at least equal to the control or slightly improved, based upon VFA end-product synthesis.

In vitro degradability : Effects of enzyme addition on the degradability of DM, NDF and ADF are shown in Table 6 (alfalfa hay) and Table 7 (rice straw). Similar to GP, DM degradability of alfalfa hay was higher ($p<0.05$) for all enzyme treatments after 12 and 48 h of incubation. Significant increases ($p<0.05$) in alfalfa hay NDF degradability occurred only for CE14 (at 12 h) and CE14+24 (12 and 48 h). Similarly, ADF degradability increased ($p<0.05$) with CE14 and CE14+24 only, but at both time points. Overall increases in 48-h degradabilities due to added enzymes ranged from 2.8 to 6.0% for DM, 2.3 to 9.9% for NDF, and 8.7 to 19.2% for ADF. Furthermore,

Table 7. Effects of enzyme addition on apparent degradability (g/kg) of DM, NDF, and ADF from rice straw after 12 and 48 h of incubation with ruminal fluid (Exp. 3)

Incubation time and treatment	Component[1]		
	DM	NDF	ADF
12 h			
Control[2]	330.4[a]	201.9[a]	241.9
CE14	344.4[ab]	241.6[b]	264.7
CE24	351.8[b]	229.3[b]	242.6
CE14+24	353.9[b]	235.9[b]	260.0
CE14+24 (calculated)[3]	347.7	235.4	253.7
48 h			
Control[2]	524.0[a]	434.4[a]	464.6[a]
CE14	539.7[b]	463.3[b]	485.8[b]
CE24	548.5[b]	470.6[b]	506.7[c]
CE14+24	538.5[b]	459.8[b]	490.4[b]
CE14+24 (calculated)[3]	544.1	448.9	496.3

[1] Data are the mean values (n = 4). [2] Rice straw without added enzymes.
[3] Expected effect for CE14+24 treatment, based on the responses for individual CE14 and CE24 treatments. No differences were observed ($p>0.05$) between the expected effect and the actual effect of CE14+24 ($p<0.05$), within each time point, indicating additivity between CE14 and CE24.
[a, b] Means in the same column with different superscripts differ ($p<0.05$) with the exception of CE14+24 (calculated), which was not included in the analysis.

the response to CE14+24 for NDF at 48 h exceeded the calculated response, indicating synergistic effects resulted from combining enzyme additives.

For rice straw (Table 7), DM degradability was increased at 12 and 24 h of incubation for all enzyme treatments (except CE14 at 12 h). Therefore, the results for DM do not reflect the results for GP, which was unexpected. It is difficult to explain these discrepancies. However, in a study by Elwakeel et al. (2007), total VFA were inversely proportional to DM disappearance data when alfalfa was treated with commercial enzymes before incubation in rumen fluid. The results in that study and ours warrant further investigation into how some exogenous enzymes affect degradation of substrates in the rumen.

The degradability of NDF was improved by all enzyme treatments at both incubation times, whereas ADF degradability was only enhanced at 48 h. It has previously been suggested that the application of enzyme directly to feed results in the slow release of enzyme into ruminal fluid as the feed is digested (Beauchemin et al., 1999). Degradability of ADF for treatment CE24 was greater than both CE14 and CE14+24. Morgavi et al. (2000) found that enzyme supplementation worked in synergy with ruminal enzymes to enhance xylan and cellulose digestion. It is possible that the ADF fraction required a prolonged action of supplemental enzyme in addition to ruminal enzymatic activity to improve digestion of this fibrous rice straw fraction after 48 h of incubation. Unlike the positive synergistic effect from CE14+24 during alfalfa digestion, there was no noticeable synergy between CE14 and CE24 for any of the parameters measured during rice straw.

Generally, enzyme additives have a multitude of types of enzyme activities. The primary enzyme activities of CE14 and CE24 were previously determined to be xylanase and endoglucanase. The enzyme activities of CE14 and CE24 significantly improved degradability parameters for alfalfa digestion when applied separately and showed positive synergy in combination. In contrast, the effects of CE14 and CE24 on rice straw were less pronounced compared to that of alfalfa, and increased degradability was limited to the actions of single enzyme additives. The differences in digestion between alfalfa and rice straw likely reflects differences in their composition, with rice straw containing greater amounts of lignin. Additionally, the structure of rice straw may have impeded the full effects of the enzymes. High silica content in the cuticle of rice straw has been shown to act as a barrier and inhibit microbial degradation (Van Soest, 2006). It appeared that the enzymes in CE14 and CE24 were less able to penetrate structural barriers in rice straw, compared to alfalfa hay. Ammonia treatment of rice straw improves digestion by damaging the cuticle, allowing microbial penetration. Eun et al. (2006) showed that ammonia treatment in combination with certain exogenous enzymes increased digestion of rice straw more than enzyme or ammonia treatment alone. Thus, the full potential of CE14 and CE24 may have been limited by access to substrate within the cell wall contents, whereas for alfalfa, the substrate was more readily available.

In conclusion, the degradability of mature quality alfalfa hay was improved by the enzymes studied and synergism between the enzyme additives was apparent. While improvement was seen after enzyme application to rice straw, the effects were less pronounced. It appeared that the same physical barriers to energy utilization of rice straw in ruminants limited the improvement in digestion by application of exogenous enzymes. However, the results suggest that enzyme treatment of rice straw may increase its degradability thus improving digestion. Further studies investigating novel enzymes and the mechanism by which they alter feed digestibility are warranted.

REFERENCES

Bailey, M. J., P. Biely and K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23:257-270.

Beauchemin, K. A., L. M. Rode and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641-644.

Beauchemin, K. A., W. Z. Yang and L. M. Rode. 1999. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. J. Dairy Sci. 82:378-390.

Beauchemin, K. A., L. M. Rode, M. Maekawa, D. P. Morgavi and R. Kampen. 2000. Evaluation of a nonstarch polysaccharidase feed enzyme in dairy cow diets. J. Dairy Sci. 83:543-553.

Bowman, G. R., K. A. Beauchemin and J. A. Shelford. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. J. Dairy Sci. 85:3420-3429.

Brock, F. M., C. W. Forsberg and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl. Environ. Microbiol. 44:561-569.

Colombatto, D. and K. A. Beauchemin. 2003. A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. Can. J. Anim. Sci. 83:559-568.

Colombatto, D., D. P. Morgavi, A. F. Furtado and K. A. Beauchemin. 2003a. Screening of exogenous enzymes for ruminant diets: relationship between biochemical characteristics and *in vitro* ruminal degradation. J. Anim. Sci. 81:2628-2638.

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

Elwakeel, E. A., E. C. Titgemeyer, B. J. Johnson, C. K. Armendariz and J. E. Shirley. 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. J. Dairy Sci. 90:5226-5236.

Eun, J. -S. and K. A. Beauchemin. 2005. Effects of a proteolytic

feed enzyme on intake, digestion, ruminal fermentation, and milk production. J. Dairy Sci. 88:2140-2153.

Eun, J. -S. and K. A. Beauchemin. 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. Anim. Feed Sci. Technol. 132:298-315.

Eun, J. -S., K. A. Beauchemin, S.-H. Hong and M. W. Bauer. 2006. Exogenous enzymes added to untreated or ammoniated rice straw: Effects on *in vitro* fermentation characteristics and degradability. Anim. Feed Sci. Technol. 131:86-101.

Goering, H. K. and P. J. Van Soest. 1970. Forage fibre analysis. Agricultural Handbook no. 379. Agricultural Research Service, US Department of Agriculture, Washington, USA.

Jung, H. G., D. R. Buxton, R. D. Hatfield and J. Ralph. 1993. Forages cell wall structure and digestibility. American Society for Agronnomics, Wisconsin, USA.

Karunanandaa, K. and G. A. Varga. 1996. Colonization of rice straw by white-rot fungi (*Cyathus stercoreus*): Effect on ruminal fermentation pattern, nitrogen metabolism, and fiber utilization during continuous culture. Anim. Feed Sci. Technol. 61:1-16.

Knowlton, K. F., J. M. McKinney and C. Cobb. 2002. Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. J. Dairy Sci. 85:3328-3335.

Kung, L. Jr., R. J. Treacher, G. A. Nauman, A. M. Smagala, K. M. Endres and M. A. Cohen. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. J. Dairy Sci. 83:115-122.

Lewis, G. E., W. K. Sanchez, C. W. Hunt, M. A. Guy, G. T. Pritchard, B. I. Swanson and R. J. Treacher. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. J. Dairy Sci. 82:611-617.

Mauricio, R. M., F. L. Mould, M. S. Dhanoa, E. Owen, K. S. Channa and M. K. Theodorou. 1999. A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. Anim. Feed Sci. Technol. 79:321-330.

McAllister, T. A., A. N. Hristov, K. A. Beauchemin, L. M. Rode and K-J. Cheng. 2001. Enzymes in ruminant diets. Pages 273-298 in Recent Advances in Enzymes in Farm Animal Nutrition (Ed. M. R. Bedford and G. G. Partridge). CABI Publishing, Wallingford, Oxon, UK.

Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz and W. Schneider. 1979. The estimation of digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they incubated with rumen liquor *in vitro*. J. Agric. Sci. (Cambridge). 92:217-222.

Miller, D. R., B. C. Granzin, R. Elliott and B. W. Norton. 2008. Effects of an exogenous enzyme, Roxazyme® G2 Liquid, on milk production in pasture fed dairy cows. Anim. Feed Sci. Technol. 145:194-208.

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

Nagaraja, T. G., C. J. Newbold., C. J. Van Nevel and D. I. Demeyer. 1997. Manipulation of ruminal fermentation. In: The Rumen Microbial Ecosystem, 2nd edn (Ed P. N. Hobson), pp. 523-632. New York: Blackie.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

Pinos-Rodríguez, J. M., S. S. González, G. D. Mendoza, R. Bárcena, M. A. Cobos, A. Hernández and M. E. Ortega. 2002. Effect of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. J. Anim. Sci. 80:3016-3020.

Rode, L. M., W. Z. Yang and K. A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82:2121-2126.

Russell, J. B. and R. J. Wallace. 1997. Energy-yielding and energy-consuming reaction. In: The rumen Microbial Ecosystem (Ed. P. N. Hobson and C. S. Stewar). Blackie and Professional, New York, NY, USA, pp. 246-882.

SAS. 2001. SAS User's Guide: Statistics Release 8.01. SAS Inst. Inc., Cary, NC, USA.

Schingoethe, D. J., G. A. Stegeman and R. J. Treacher. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. J. Dairy Sci. 82:996-1003.

Somogyi, M. 1952. Determination of blood sugar. J. Biol. Chem. 195:19-23.

Tricarico, J. M. and K. A. Dawson. 2001. Contribution of an acetyl esterase containing enzyme preparation to the action of exogenous enzyme supplements for ruminants. J. Anim. Sci. (Abstract only).

Vicini, J. L., H. G. Bateman, M. K. Bhat, J. H. Clark, R. A. Erdman, R. H. Phipps, M. E. Van Amburgh, G. F. Hartnell, R. L. Hintz and D. L. Hard. 2003. Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. J. Dairy Sci. 86:576-585.

Van Soest, P. J. 2006. Rice straw, the role of silica and treatments to improve quality. Anim. Feed Sci. Technol. 130:137-171.

Yang, W. Z., K. A. Beauchemin and L. M. Rode. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. J. Dairy Sci. 83:2512-2520.