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Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms in vitro[1,4]

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ABSTRACT: This study was done to determine the effectiveness of supplementary enzymes at increasing the fiber digestion by ruminal microorganisms and to assess whether enzyme activity limits the rate of fiber digestion in ruminal digesta. In vitro comparisons of enzyme activities in two feed enzyme preparations (A and B) with enzyme activities extracted from ruminal fluid indicated that the addition of fibrolytic enzymes at the application rates recommended by the manufacturers would not be expected to increase significantly glycanase and polysaccharidase activities in ruminal fluid. Preparations A and B both increased ($P < 0.001$) the rate of gas production from freeze-dried corn and grass silages in in vitro incubations with ruminal fluid, but only at concentrations much higher than recommended application rates. Autoclaved controls had little or no effect. Ultrafiltration of enzyme B indicated that most stimulation was due to components >100 kDa, which is consistent with the cause of the stimulation being enzyme activity. Fibrolytic enzymes from other sources were also able to stimulate gas production: increased rates of gas production were observed in seven out of eight combinations of "cellulase" and corn or grass silage ($P < 0.05$). The comparison of glycanase and polysaccharidase activities with gas-stimulatory activity in the different enzyme preparations indicated that the highest correlation was between increased gas production and enzyme activity against microgranular cellulose ($P < 0.05$). In a wider range of fibrolytic enzyme preparations, those with *endo*-(β-1,4)- or *exo*-(β-1,4)-xylanase activity equal to that of preparation A did not produce similar increased rates of fermentation of corn silage when glucanase activity was low ($P > 0.05$). In contrast, preparations with glucanase activity similar to enzyme A gave at least as great ($P < 0.05$) an improvement in gas production than enzyme A, irrespective of xylanase activity. It was concluded that enzyme activity, probably a type of *endo*-(β-1,4)-glucanase activity, limits the rate of fermentation of corn and grass silage in the rumen. Enzyme supplements of the type used in these experiments are unlikely to possess sufficient activity to overcome this limitation by direct application to ruminal digesta, implying that treatment of the ration prefeeding will be key to harnessing the potential of exogenous fibrolytic enzymes in ruminant nutrition.

Key Words: Corn Silage, Enzymes, Fiber, Grass Silage, Rumen, Ruminants

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Introduction

Fermentation extracts containing fibrolytic enzymes, which were previously used mainly for nonruminants (Graham and Balnave, 1995), are now being used in ruminant rations following feeding trials in which some benefit has been observed (Beauchemin et al., 1995; Feng et al., 1996; Yang et al., 1999). The nutritional benefit from the supplementation appears to depend on many factors, most of which have yet to be evaluated. The effectiveness of enzymes varies with diet (Beauchemin et al., 1995) and with the component of the diet to which the enzyme additive is added (Hristov et al., 1998a; Krause et al., 1998; McAllister et al., 1999). The prefeeding phase seems likely to be important, and increased postruminal digestion has been demonstrated (Yang et al., 1999). Some enzymes are sufficiently stable to pass from the rumen and be active in the abomasum and beyond (Hristov et al., 1998b; Morgavi et al., 2000b), suggesting that the enzymes may aid the digestion of feed materials that escape ruminal fermentation. However, one of the most interesting possibilities is that the enzymes stimulate fiber

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digestion in the rumen. Present information about this possibility is mixed. Carboxymethylcellulase (**CMCase**) and xylanase activities did not increase in response to enzyme addition to the feed (Hristov et al., 1998a), yet infusion of enzymes directly into the rumen increased enzyme activities in ruminal fluid (Hristov et al., 2000). Furthermore, a recent in vitro study (Morgavi et al., 2000a) indicated that there was some synergism between feed additive and ruminal enzymes.

The present study was undertaken to investigate the effects of adding enzymes directly to ruminal digesta in vitro; to determine whether enzyme supplements added without prior incubation with the feed, in order to eliminate prefeeding effects, could stimulate the rate of digestion of corn and grass silages; and to identify the likely nature of the key enzyme activities.

Materials and Methods

Animals and Dietary Components. Four rumen-cannulated sheep, 45 to 50 kg BW, received twice daily at 0800 and 1600 a maintenance ration of a total mixed ration (**TMR**), with no added enzyme. Sheep were penned individually, but they had contact with each other. The TMR contained, in grams per kilogram, the following: corn silage, 428; grass silage, 143; milled wheat, 201; rapeseed meal, 101; Hypro soybean meal, 58; fishmeal, 14; molasses, 40; vitamins, 6; and minerals, 10. The feedstuffs and samples of the individual silage ingredients were supplied by the Department of Agriculture, University of Reading, U.K. They were freeze-dried before being used in vitro.

Enzymes. We denoted the feed additive enzymes as **A**, an extract provided by Finnfeeds International, Marlborough, England, and enzyme **B** (Pro-Mote), from Biovance International, Omaha, Nebraska. Both enzymes A and B are extracts of *Trichoderma longibrachiatum*. Enzymes described as cellulases were obtained from Sigma (Poole, Dorset, U.K.) and stock solutions were prepared in 0.1 *M* sodium phosphate buffer, pH 6.5, as follows: *Penicillium funiculosum* (Sigma C0901), 1,000 U/mL; *Aspergillus niger* (Sigma C1184), 200 U/mL; *Trichoderma reesei* (Sigma C8546), 200 U/mL; *Trichoderma viride* (Sigma C9422), 200 U/mL. A further collection of fibrolytic enzymes, which included Celluclast, Depol, Liquicell, Sumizyme X, and XYL250, was supplied by Monsanto Company, St. Louis, MO. All dilutions of enzyme solutions were carried out in 0.1 *M* sodium phosphate buffer, pH 6.5.

Comparison of Enzyme Activity in Ruminal Fluid and Enzyme Additives. In order to measure enzyme activity of ruminal fluid, ruminal digesta were removed from the sheep 3 h after feeding, strained through four layers of muslin, and then centrifuged at $27{,}000 \times g$ for 20 min. The pellet was resuspended in ice-cold 0.1 *M* sodium phosphate, pH 6.5, then sonicated using 20 bursts, each of 30 s with intervening 30-s cooling intervals, in an MSE Soniprep 150 (MSE Instruments Ltd, Crawley, Sussex, U.K.) at an amplitude of 20 μm, maintained on ice throughout. This procedure exceeded the sonication time shown previously to be adequate to release maximal amounts of CMCase activity (Silva et al., 1987). The sonicated suspension was centrifuged once more ($27{,}000 \times g$ for 20 min), and CMCase and xylanase activities were measured using a tenfold dilution of the supernatant fluid in 0.1 *M* sodium phosphate buffer, pH 6.5. The enzyme activities of enzymes A and B were measured using 500-fold dilutions of the supplied liquid additive in the same buffer. Polysaccharidase activities against CMC and xylan were measured by measuring the reducing sugars released from the corresponding substrate, as described by Berger et al. (1989). The substrates were obtained from Sigma. Carboxymethylcellulose (**CMC**) was of medium viscosity; xylan was from oat spelts. They were suspended in 0.1 *M* sodium phosphate, pH 6.5, at concentrations of 2% (wt/vol) for CMC and 1% (wt/vol) for xylan. Two mL of diluted sonicated ruminal fluid was added to 2 mL of substrate solution/suspension, and the mixture was incubated at 39°C. Samples (0.2 mL) were removed immediately and after 1 h into 1.0 mL of an equal mixture of Somogyi-Nelson reagents A and B and 0.3 mL distilled water; the mixture was incubated at 100°C for 10 min, and then 0.5 mL of reagent C was added. Absorbance was measured at 600 nm. Standards were glucose for CMCase activity and xylose for xylanase activity.

Gas Production Measurements. Microbial fermentation in vitro was determined using the gas production technique described by Menke and Steingass (1988). Ruminal fluid was taken before feeding in the morning from each sheep in order to decrease endogenous substrates in the inoculum to a minimum. The ruminal fluid was kept at 39°C and strained through four layers of muslin before use. Equal volumes were pooled to provide the inoculum for in vitro incubations. The feedstuffs used were the component grass and corn silages of the TMR. Freeze-dried corn silage and freeze-dried grass silage were milled to pass a 1-mm screen and used as the fermentation substrate. Strained ruminal fluid (**SRF**; 1 vol) was added under CO_2 to 2 vol of buffer and minerals solution (Menke and Steingass, 1988) at 39°C. Thirty milliliters of diluted rumen fluid were added to 100-mL glass syringes containing 200 mg of freeze-dried substrate and fitted with glass pistons lubricated with petroleum jelly. Enzyme solution was added immediately before the diluted ruminal fluid, usually as a 1.0-mL volume in 0.1 *M* sodium phosphate buffer, pH 7.0. The syringes were then incubated at 39°C, and gas volume was measured every 2 h for the first 8 h of the incubation period and thereafter at 12 and 24 h. Between four and six replicates were carried out on different days for each treatment.

Autoclaving and Ultrafiltration. Enzymes A and B were autoclaved at 121°C for 15 min. Enzyme B was fractionated into different molecular mass fractions by ultrafiltration. The enzyme preparation was diluted, 10 mL to 50 mL, in 0.1 *M* sodium phosphate buffer, pH 6.5, and filtered by adding 4-mL aliquots to Vivaspin

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4 PES ultrafilters (Vivascience Ltd., Binbrook, Lincoln LN3 6BL, U.K.) of nominal molecular mass cut off 10, 50, and 100 kDa and centrifuging at 15,000 × *g* for 1 h. A 0.5-mL sample of filtrate was added to 0.5 mL of buffer and added to the gas syringes as described above.

Release of Reducing Sugars from Corn Silage by Enzyme B. Freeze-dried corn silage (60 mg) was suspended in 3 mL of clarified (27,000 × *g*, 30 min) ruminal fluid and 6 mL of 0.1 *M* sodium phosphate buffer, pH 6.5, and autoclaved at 121°C for 15 min. A 0.3-mL addition was made of a 10-fold dilution of enzyme B, the mixture was incubated at 39°C, and reducing sugars released were measured by the Somogyi-Nelson method as described above.

Measurement of Glycosidase and Polysaccharidase Activities in Enzymes A and B and in "Cellulases." β-Xylosidase, β-glucosidase, α-arabinosidase, and β-galactosidase were assayed using *p*-nitrophenyl substrates as described by Garcia-Campayo and Wood (1993). The assay mixture contained 50 μL of 1 mg/mL substrate dissolved in 0.1 *M* sodium phosphate, pH 6.5, and 50 μL of diluted enzyme solution. The mixtures were incubated in multititer plates at 39°C. The reaction was stopped at 10, 30, and 60 min by adding 100 μL of 0.4 *M* glycine-NaOH buffer, pH 10.8, and the absorbance was measured in a plate reader (Dynatech MR5000; Dynex Labsystems, Ashford, Middlesex, U.K.) at 420 nm. *p*-Nitrophenol released was calculated from the absorbance of a solution of *p*-nitrophenol in the same buffer. The concentrations used were 2 mL/L for enzymes A and B, and 20- or 100-fold dilutions of stock solutions for the other enzymes. Activities were calculated from readings corresponding to a release of less than 30 nmol *p*-nitrophenol in the assay, at which concentration the rate of release of *p*-nitrophenol was linear. Assays were performed in triplicate.

Polysaccharidase (exoglycanase) activities were assayed by reducing sugar release, as described above for CMCase and xylanase. The substrates were obtained from Sigma. Cellulose was microgranular. They were suspended in 0.1 *M* sodium phosphate, pH 6.5, at concentrations of 2% for CMC and 1% for other substrates. Solutions of 2 mL/L were used for enzymes A and B, and 50-fold dilutions of stock solutions were generally used for the other enzymes; exceptions were CMCase and xylanase activities of *Aspergillus niger* extract, which were carried out at 3.9 mL/L. Incubations were performed in duplicate.

Measurement of endo-*(β-1,4)-Glucanase and* endo-*(β-1,4)-Xylanase Activities.* Endoglycanase activities were measured by the release of dye from azo-substrates, based on methods described by McCleary (1988). Azo-Xylan (oat spelts) and azo-CMC were obtained from Megazyme International Ireland Ltd. (Bray, Co. Wicklow, Ireland). The substrates were suspended (20 g/L) in 0.1 *M* sodium phosphate buffer, pH 6.5. Enzymes, diluted in the same buffer, were mixed with substrate (0.1 mL each), vortexed, and incubated at 39°C. Reactions were terminated by adding 0.5 mL of 95% (vol/vol) ethanol (xylanase) or CMCase stopping solution, which contained sodium acetate trihydrate (40 g) and zinc acetate (4 g) dissolved in 150 mL of demineralized water, the pH adjusted to 5.0 with 5 *M* HCl and the volume made up to 200 mL with demineralized water and 800 mL of 95% (vol/vol) ethanol. The mixture was centrifuged at 12,000 × *g* for 5 min, and the absorbance of the supernate measured at 540 nm. All assays were carried out in duplicate. The activity of different enzyme preparations was equalized according to key enzyme activities by an iterative process, whereby dilutions of enzyme solutions were prepared in successive analyses until they matched the activity of enzyme A. The influence on gas production from corn silage was compared using preparations equalized for *endo*-(β-1,4)-glucanase, *exo*-(β-1,4)-glucanase, *endo*-(β-1,4)-xylanase, and *exo*-(β-1,4)-xylanase.

Data Analysis. The influence of different concentrations of enzymes A and B and autoclaved enzymes A and B on rates of gas production from either corn silage or grass silage was analyzed by a factorial analysis of variance. Concentration, autoclaving, and enzyme were treatment factors. Day of incubation was a blocking factor. The results are expressed as a single standard error of difference for all values. Rates of gas production were calculated by linear regression of net volumes of gas at 2-h intervals up to 8 h. The net volume of gas produced from the substrate was calculated at each time as follows:

(A) Gas production from silage =
(RF + S) − RF
(B) Gas production from silage in the
presence of enzyme =
(RF + S + E) − (RF + E)

where RF = ruminal fluid alone; RF + S = ruminal fluid + substrate; RF + E = ruminal fluid + enzyme; RF + S + E = ruminal fluid + substrate + enzyme. Thus, gas production from the enzyme itself was eliminated as a factor and the influence of enzymes on the digestion of the substrate could be determined from the difference between (A) and (B).

The rates of gas production from different molecular mass fractions of enzyme B were analyzed by a randomized block analysis of variance (ANOVA), with day of incubation as blocking factor and fraction as treatment. Xylanase and CMCase activities of the different molecular mass fractions were analyzed by one-way ANOVA.

The rates of gas production from different concentrations of commercial "cellulases" were analyzed by a randomized block ANOVA, with sheep ruminal fluid sample as blocking factor and concentration as treatment.

Correlation coefficients between the enzyme activity of different enzyme preparations and gas production rate were calculated. The *P*-value for significance was based on the number of enzyme preparations and obtained from statistical tables.

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Table 1. Comparison of CMCase and xylanase activities in sonicated ruminal microorganisms and enzyme additives A and B

	Enzyme activity, μmol/min per mL				Enzyme in the rumen, mmol/min[a]	
	CMCase		Xylanase			
Enzyme source	Mean[b]	SD	Mean[b]	SD	CMCase	Xylanase
Sonicated ruminal microorganisms	0.065	0.067	0.134	0.035	0.33	0.67
Enzyme A	48.8		30.7		0.05	0.03
Enzyme B	41.2		32.0		0.06	0.04

[a]Calculated on the basis of a 5-L ruminal volume, an addition rate of enzyme supplement of 1.5 and 2 L per megagram of feed for enzymes A and B, respectively, and a consumption of 0.7 kg of feed per meal.
[b]Mean of activities in sonicated ruminal fluid from four sheep and duplicate estimations with enzymes A and B.

Mean rates of gas production of enzyme preparations with equalized activities were calculated from measurements made using six different samples of ruminal fluid on different days. *P*-values were calculated from comparisons of the effect of enzyme A on the rate of gas production with the effects of other enzymes, calculated from a paired *t*-test of rates obtained on different days against enzyme A as control. Comparisons of gas production at different times in Figure 1 were carried out in a similar way.

Results and Discussion

Comparison of Enzyme Activity in Ruminal Fluid and Enzyme Additives

Xylanase and CMCase activities were measured in sonicated ruminal microorganisms and in the feed additive enzymes A and B in order to assess how great an impact the added enzyme activity might have on the total activity of these key enzymes in ruminal fluid. The activities associated with the feed additives were much higher than those present in sonicated ruminal microorganisms (Table 1). However, as only small quantities of the enzyme additives are applied to the feed, it emerged that the added activities were minor in comparison with those already present in ruminal microorganisms. Assuming that a sheep has a rumen volume of 5 L and that the highest concentration of enzyme would occur after the consumption of a 0.7-kg meal supplemented to an extent of 1.5 L/Mg—the recommended supplementation rate for enzyme A, these enzymes would lead to at most an increase of 5% in xylanase activity and 15% in CMCase activity (Table 1); both values are well within the variation of activities found in the ruminal fluid extracts. Moreover, the enzymes were extracted in this experiment using strained ruminal fluid; thus the solids-associated microbial population, which would be expected to have much more activity than the free-swimming population, was not measured. Therefore, the estimate of total fibrolytic enzymes measured in sonicated microorganisms made here is likely to be a significant underestimate. Other factors leading to the decline of enzyme activity in the rumen will be outflow from the rumen and the breakdown of the added enzyme (Hristov et al, 2000; Morgavi et al, 2000b). Thus, on the basis of the present results, supplementing feeds with enzymes A or B at the rates recommended by the manufacturers would not be expected to have a significant impact on total CMCase and especially xylanase activities of ruminal fluid. In contrast, Hristov et al. (2000) found that infusing a different enzyme additive directly into the rumen of heifers at a rate of 100 g/d increased ruminal xylanase activity by 56% and β-glucanase activity by 20%. It remains to be determined whether enzymes A and B would have the same effect in vivo. It is also relevant to note that many enzyme activities, particularly glycosidase activities, are present at such high concentrations in the feed that added enzymes have no detectable effect on feed-associated enzyme activities (data not shown).

There is a valid counterargument that the most beneficial enzyme activities that could be added to the feed would be synergistic with, rather than additive to, the main rumen microbial enzymes. The synergistic activity may not be detected by conventional enzyme assays. Researchers have identified such synergism in extracts from *Aspergillus oryzae* (Varel et al., 1994) and *Trichoderma longibrachiatum* (Morgavi et al. 2000a). Thus, simply determining the main enzyme activities in an extract may not indicate the true value of that extract as a feed additive.

Gas Production and Its Stimulation by Enzymes A and B

The in vitro gas production technique pioneered by Menke and Steingass (1988) was used in these experiments to give a measure of the rate of fermentation of corn or grass silage. Gas production in vitro is by its very nature an imperfect technique for measuring fermentation by rumen microorganisms. The ruminal fluid is dilute and heavily buffered. The substrate is milled to provide reproducible results. The rumen fluid is strained before being used, and ruminal fluid from sev-

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eral animals is pooled before use. But, most importantly, it is well known that the stoichiometry of fermentation changes according to various manipulations and diets, such that the volume of gas formed per unit of substrate fermented is bound to change. Nevertheless, the technique is convenient to use as a first approximation, and it is particularly useful for comparative purposes, where there is confidence that the manipulation does not affect fermentation stoichiometry. Measuring the impact of enzymes on rumen fermentation is one of those applications for which it would be expected to be most valid.

The results for enzyme A with corn silage (Figure 1) illustrate some of the most important features of the incubations; results for enzyme B were similar (not shown). Gas production was linear for the first 8 h, after which the rate decreased. Gas production was not measured after 48 h. We considered that increasing the initial rate of fermentation would be the most likely mode of action of enzyme additives. The pH was mea-

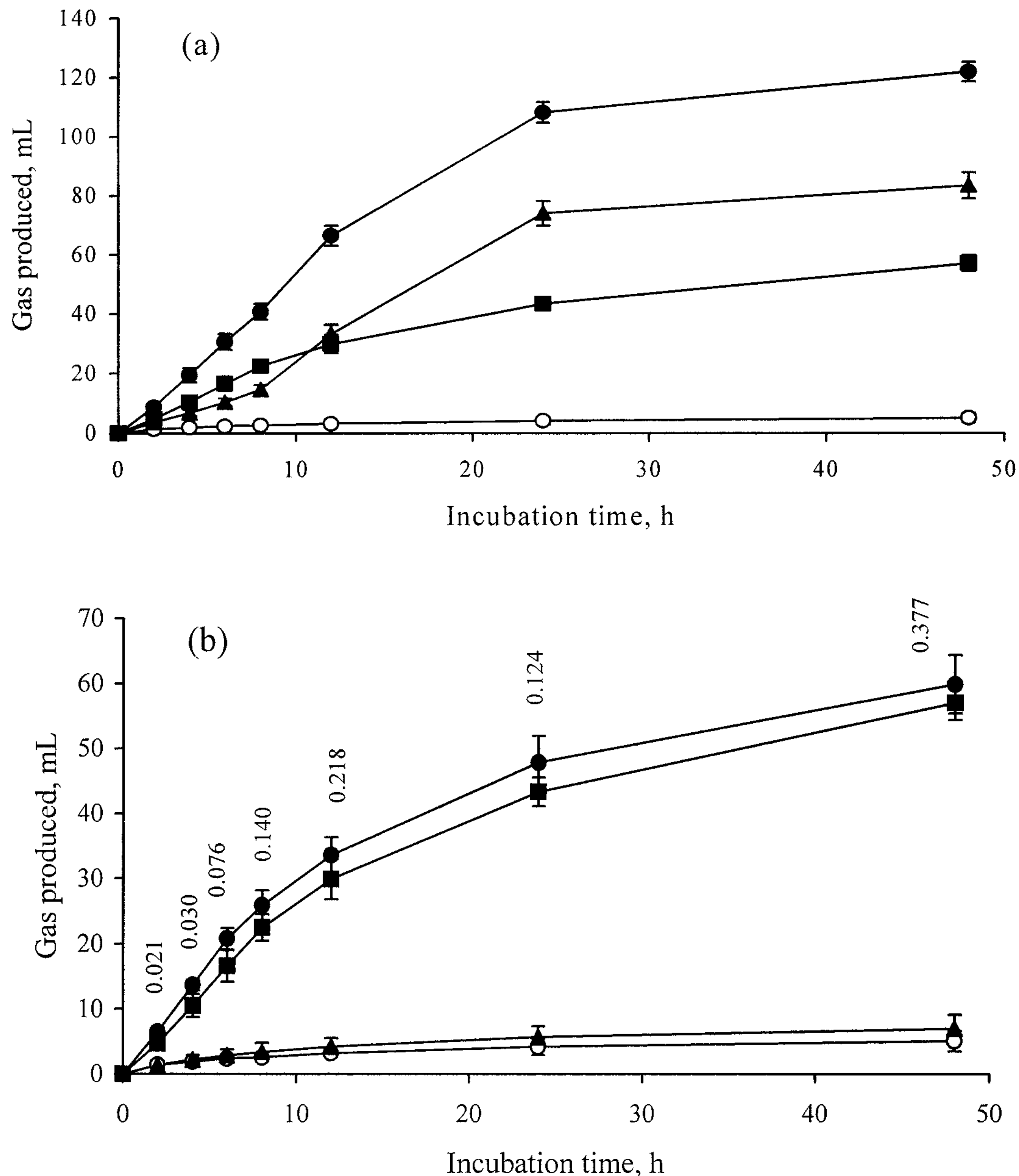

Figure 1. Influence of enzyme A on gas production from 0.2 g of corn silage by mixed ruminal microorganisms in vitro. (a) 1.0 mL of enzyme added to 30 mL of suspension; (b) 0.012 mL of enzyme added to 30 mL of suspension. ○, ruminal fluid blank; ■, ruminal fluid + corn silage; ▲, ruminal fluid + enzyme; ●, ruminal fluid + corn silage + enzyme. Results are means and SD from five incubations, each carried out on separate days with ruminal fluid pooled from four sheep. The numbers associated with each time point in Figure 1(b) are the probabilities that the volumes obtained with added enzyme in the presence of corn silage are significantly different to the volumes obtained without added enzyme.

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Table 2. Net gas production from corn and grass silages in response to the addition of different concentrations of untreated and autoclaved feed additive enzymes to ruminal fluid in vitro

Substrate	Concentration, mL/L:	Rate of gas production, mL/h								
		No addn.	1.11×10^{-2}	3.33×10^{-2}	0.111	0.333	1.11	3.33	11.1	33.3
Corn silage										
Enzyme A		3.34	3.25	3.33	3.46	3.58	3.79	3.87	4.36	4.43
Enzyme A, autoclaved		3.34	3.47	3.30	3.43	3.41	3.33	3.50	3.59	n.d.
Enzyme B		2.44	2.53	2.51	2.65	2.73	2.91	3.19	3.48	n.d.
Enzyme B, autoclaved		2.44	2.45	2.43	2.59	2.57	2.59	2.68	2.56	n.d.
SED = 0.209										
P-values		Concentration		<0.001		Concentration × autoclaving			<0.001	
		Autoclaving		<0.001		Concentration × enzyme			0.991	
		Enzyme		<0.001		Autoclaving × enzyme			0.502	
		Concentration × autoclaving × enzyme				0.984				
Grass silage										
Enzyme A		1.63	1.83	1.93	2.00	2.14	2.26	2.75	3.19	3.65
Enzyme A, autoclaved		1.63	1.79	1.84	1.73	1.83	1.87	1.78	1.94	n.d.
Enzyme B		2.03	2.03	2.02	2.13	2.28	2.53	2.73	3.10	n.d.
Enzyme B, autoclaved		2.03	1.96	2.00	2.21	2.23	2.26	2.41	2.56	n.d.
SED = 0.157										
P-values		Concentration		<0.001		Concentration × autoclaving			<0.001	
		Autoclaving		<0.001		Concentration × enzyme			0.746	
		Enzyme		<0.001		Autoclaving × enzyme			<0.001	
		Concentration × autoclaving × enzyme				0.135				

[a]Results are means of incubations done with ruminal fluid from four sheep. The dilution series of enzyme A and autoclaved enzyme A were done using the same samples of ruminal fluid for both corn silage and grass silage. Corresponding incubations with enzyme B and autoclaved enzyme B were done using samples from the same four sheep taken on different days.

sured in a few representative samples at 48 h. The pH of the medium varied between 6.5 and 6.7. Gas was produced from ruminal fluid in the absence of substrate or added enzyme, in spite of the sheep not having been fed for 16 h before sampling, but this (5 mL produced in 48 h) was a minor component of the total gas production from corn silage (57 mL). A greater problem was caused by endogenous gas-producing materials present in the enzyme solutions. With enzyme A and corn silage, when 1.0 mL of enzyme was added to 30 mL of microbial suspension [Figure 1(a)], more gas was produced from the enzyme solution (84 mL) than was produced from the corn silage (57 mL). As a consequence, the mixture had a much greater gas production than corn silage alone (122 mL). At 12 μL of enzyme solution, the effect was much less, but still not inconsequential [Figure 1(b)]. Analysis of reducing sugars in the enzyme solutions showed that some of the enzymes used in this study contained large amounts of sugars that would be expected to release gas during incubation with ruminal fluid (results not shown). Gas production from the enzyme extract was greatest for enzymes A and B and the *A. niger* extract (see below) when used at high concentrations. Endogenous gas production from ruminal fluid and from enzyme solutions was compensated for in the calculations, as described in Materials and Methods.

When data were subjected to linear regression analysis for the first 8 h of incubation and the gradients were compared, it became clear that significantly ($P < 0.001$) increased rates of fermentation of both types of silage resulted from the addition of both enzymes A and B (Table 2). The response declined ($P < 0.001$) as the concentration of the enzyme solution added to the fermentation liquid decreased.

In order to assess how relevant the in vitro data might be to ruminal fermentation in vivo, one must make some assumptions. If it is assumed that the key activity is the enzyme added per unit *volume* of ruminal digesta, as above, one would expect the volume of enzyme added to ruminal digesta to be $(1.5 \times 0.7) = 1.05$ mL in a sheep consuming 0.7 kg per meal of a ration treated with 1.5 L of enzyme A per megagram. With a rumen volume of 5 L, the concentration would be expected to be about 0.21 mL/L. This concentration is midway between the concentrations in Table 2 of 0.111 mL/L and 0.333 mL/L. The latter concentration gave a significant effect ($P < 0.05$) with enzyme A and corn silage, but the other enzyme/silage combinations gave nonsignificant increases in gas production. By contrast, if the appropriate addition of enzyme is considered to be measured per unit of *solid feed*, then the 200 mg of feed added to 30 mL of incubation would contain $200 \times 1.5 \times 10^{-6} = 0.3 \times 10^{-3}$ mL in the 30 mL, giving rise to a concentration of $(1{,}000/30) \times (0.3 \times 10^{-3}) = 0.01$ mL/L. At the closest concentration of 1.11×10^{-2} mL/L in Table 2, no effect was observed with any enzyme or silage ($P > 0.05$). We would therefore conclude that enzyme feed additives incorporated at the levels recommended by the manufacturers would be unlikely to stimulate the rate of fermentation directly.

Indirect or nonruminal effects, or adaptive effects on the fibrolytic rumen microbial population cannot be discounted, however. Adaptive effects in the microbial

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population might result from prolonged consumption of feed with added enzyme, such that a modified microbial population develops that is more potent in digesting fiber. Only longer-term experiments either in vivo or in vitro would be able to detect such an effect.

The experiments described here eliminated prefeeding effects because enzyme and feed were added to the incubations at the same time. A quadratic effect in vivo (Beauchemin et al., 1995) is not consistent with a ruminal mode of action; rather, efficacy tailing off at higher concentrations in vivo might result from a preruminal modification of the feedstuff that is too extensive at higher application rates. Furthermore, the small liquid volumes mixed with feeds would ensure a high enzyme concentration on the fiber surface prefeeding. We suggest that the most likely effect of fungal enzyme preparations is at prefeeding, and much more attention should be paid to this aspect of enzyme supplementation. Most articles do not specify for how long and under what conditions treated feeds are stored before being offered to the animals.

Postruminal effects cannot be ignored because increased xylanase activity in the duodenum has been detected (Hristov et al., 1998a, 2000). How this finding is consistent with the added enzymes being of such low activity compared with ruminal microorganisms is not clear; perhaps the supplementary enzymes are much less susceptible to inactivation in the abomasum and small intestine than are rumen microbial enzymes. The enzymes present in the fibrolytic enzyme preparations certainly seem to be unusually robust (Hristov et al., 1998a,b; Morgavi et al., 2000b).

The Influence of Autoclaving and Ultrafiltration on the Ability of Enzyme Additives to Stimulate Ruminal Fermentation

Parallel gas-production incubations were carried out with autoclaved enzymes A and B. No stimulation was observed with enzyme A, or with enzyme B with corn silage (Table 2), indicating that the stimulatory agent was heat-sensitive, possibly an enzyme activity or a heat-labile nutrient. A small increase ($P < 0.001$) was found with autoclaved enzyme B and grass silage, perhaps indicating incomplete destruction of enzyme activity, or some renaturation of the enzyme during incubation, or that enzyme B contained a stimulatory component that was not heat-labile.

When enzyme B was incubated with corn silage, reducing sugars were released rapidly (Figure 2). Autoclaving destroyed this activity (Figure 2). Thus, enzyme B had a high capability for digesting corn silage, and this was destroyed by autoclaving, suggesting that the effect of autoclaved enzyme A on gas production with grass silage was not due to the incomplete denaturation of enzyme activity.

Fractionation of enzyme B by ultrafiltration indicated that most of the response occurred with filtrates containing substances of molecular mass > 100 kDa

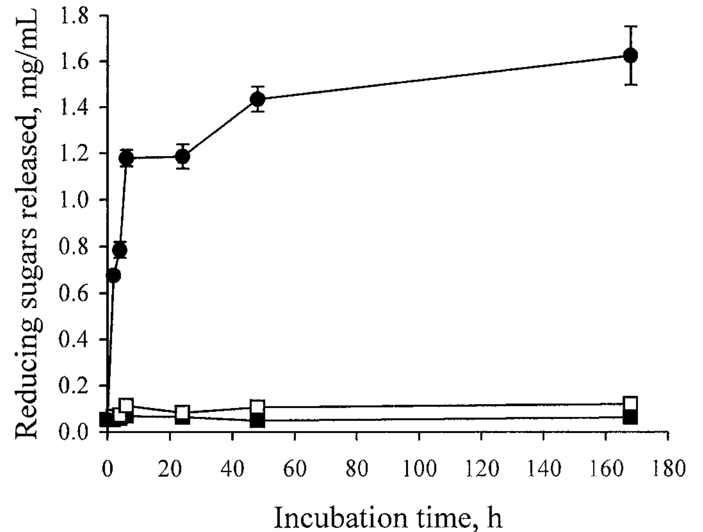

Figure 2. The release of reducing sugars from autoclaved corn silage by native (●) and autoclaved (□) enzyme B, compared with controls with no added enzyme (■). Freeze-dried corn silage (60 mg) was suspended in 3 mL of clarified ruminal fluid and 6 mL of 0.1 *M* sodium phosphate buffer, pH 6.5, and autoclaved at 121°C for 15 min. A 0.3-mL addition was made of a 10-fold dilution of enzyme B, the mixture was incubated at 39°C, and reducing sugars released were measured. Results are means and SD from three determinations.

(Table 3). As the nominal molecular mass cut-off decreased, the amount of stimulation of gas production decreased. Autoclaving destroyed the stimulatory activity of all of the fractions (not shown). The CMCase activity was mostly > 100 kDa, whereas most of the xylanase was < 100 kDa, suggesting a stronger relation between gas production and CMCase rather than xylanase activity.

Stimulation of Gas Production by Other Enzymes

Other commercial enzymes described as “cellulase,” consisting of preparations from *P. funiculosum*, *A. niger*, *T. reesei*, and *T. viride*, increased the rate of evolution of gas from both grass and corn silages in vitro (Table 4). As with enzymes A and B, the response increased as the concentration of enzyme increased ($P < 0.001$ in most cases). The response also tended to be greater with grass silage than with corn silage (Table 4; $P = 0.071$). For example, the responses to added cellulases at 11.1 mL/L of the various enzymes were 22.7 and 39.8% for corn silage and grass silage, respectively. As with enzymes A and B, significant responses were only seen at the highest concentrations of enzymes.

The finding that cellulases purchased commercially, which were not intended for use as feed additives, can function in the same way as feed additives A and B suggests that there is nothing particularly unusual about A and B, and that many other sources of fibrolytic enzymes might be expected to have a similar effect.

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Table 3. Net gas production from corn and grass silages by ruminal fluid in response to the addition of different molecular mass fractions of enzyme B prepared by ultrafiltration

Fraction	Rate of gas production, mL/h[a]		CMCase activity[b] % Total B	Xylanase activity[b] % Total B
	Corn silage	Grass silage		
Total B	3.75	3.03	100	100
>100 kDa	3.65	2.80	67.7	35.7
50–100 kDa	3.47	2.23	21.6	35.7
10–50 kDa	3.41	2.13	7.6	22.5
<10 kDa	—	—	3.12	6.02
No addition	3.02	1.83	—	—
SED	0.112***	0.054***	1.87	2.58

[a]Mean and SED of six incubations.
[b]Mean of duplicate measurements.
***$P < 0.001$.

The other conclusion that can be drawn for the data presented thus far is that ruminal fermentation of corn and grass silages is indeed enzyme-limited. Bhat et al. (1988) concluded that the rate of adhesion of cellulolytic microorganisms did not limit the rate of degradation of barley straw in the rumen. Dehority and Tirabasso (1998) manipulated the population size of cellulolytic bacteria in the rumen of sheep by changing the diet, but the rate of digestion of alfalfa cellulose was unaffected, indicating that “the concentration of cellulolytic bacteria is not the limiting factor in the digestion of cellulose in the rumen.” Other factors, such as the specificity of enzyme activity that might benefit ruminal fermentation most, have not been investigated. However, it is clear from the present experiments that increasing enzyme activity in ruminal fluid does increase the rate of fermentation, albeit at concentrations that cannot be provided by present feed supplements.

The next aim was to attempt to identify which, if any, of the enzyme activities that were present in the different preparations was rate-limiting in ruminal fermentation of the silages.

The Relationship Between Enzyme Activities of Different Preparations and Their Ability to Stimulate Gas Production

If it were possible to identify precisely which enzyme activity was causing the fermentation response, it might be possible to develop better feed additive enzymes in a rational way. Indirectly, it would also have implications for our understanding of the microbial enzyme requirements in order to improve fiber digestion in the rumen. The enzyme activities of enzymes A and B and the cellulases were therefore compared with the responses that had been observed in gas production.

Various glycosidase and polysaccharidase activities were measured in the enzyme extracts. β-Glucosidase activity was the highest glycosidase activity in all extracts, whereas α-arabinosidase showed minor activity

Table 4. Net gas production from corn and grass silages in response to the addition of different concentrations of commercial cellulases to ruminal fluid in vitro

Enzyme Concentration, mL/L:	Rate of gas production, mL/h									SED[a]
	No addn.	1.11×10^{-2}	3.33×10^{-2}	0.111	0.333	1.11	3.33	11.1	33.3	
P. funiculosum, 1000 U/mL										
Corn silage	2.57	2.52	2.59	2.64	2.76	2.66	2.91	3.19	n.d.	0.11***
Grass silage	1.94	1.96	1.98	1.94	2.14	2.11	2.36	2.89	n.d.	0.10***
A. niger, 200 U/mL										
Corn silage	2.57	2.67	2.63	2.60	2.63	2.69	2.89	3.59	6.04	0.29***
Grass silage	2.06	2.13	2.23	2.03	2.11	2.23	2.19	2.74	3.47	0.26***
T. reesei, 200 U/mL										
Corn silage	3.07	2.99	3.11	3.04	3.01	3.11	3.28	3.36	3.54	0.17
Grass silage	1.79	1.89	1.94	1.90	1.94	2.19	2.33	2.86	3.13	0.19***
T. viride, 200 U/mL										
Corn silage	2.78	3.04	2.93	3.14	3.22	3.24	3.28	3.35	3.41	0.19*
Grass silage	2.26	2.19	2.10	2.19	2.16	2.33	2.39	2.76	3.17	0.13***

[a]Results are means of incubations done with ruminal fluid from four sheep. Each dilution series was done using the same samples of ruminal fluid; different enzymes were done using samples from the same sheep taken on different days.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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Table 5. Relation between glycosidase and polysaccharidase activities of different enzyme extracts and the stimulation of gas production in vitro

Item	Enzyme A	Enzyme B	*A. niger*	*P. funiculosum*	*T. reesei*	*T. viride*	Correlation coefficient[a] Corn	Correlation coefficient[a] Grass
Gas responses[b]								
Corn silage	1.02	1.04	1.02	0.62	0.29	0.57	1.00	0.42
Grass silage	1.56	1.07	0.95	0.95	1.07	0.50	0.42	1.00
Glycosidase activities[c]								
β-Xylosidase	2.69	0.83	0.70	1.70	0.10	0.08	0.51	0.76
β-Glucosidase	4.17	3.45	1.17	2.74	0.76	2.66	0.54	0.38
α-Arabinosidase	0.17	1.48	0.05	0.12	0.02	0.01	0.49	0.16
β-Galactosidase	0.26	0.05	0.05	0.25	0.00	0.00	0.31	0.61
Polysaccharidase activities[d]								
Cellulose	5.74	3.72	n.d.	0.83	1.43	0.28	0.79	0.88
CMC	48.79	41.16	5.96	2.67	4.24	4.24	0.68	0.73
Lichenan	7.38	5.7	0.167	3.18	1.47	0.80	0.50	0.77
Laminarin	4.51	13.14	0.147	7.73	0.74	2.24	0.39	0.18
Arabinogalactan	0.47	0.26	0	0.03	0	0	0.62	0.80
β-Glucan	24.19	16.01	0.592	0.05	3.43	2.38	0.57	0.76
Starch	0.19	1.59	0.02	0.05	0	0.01	0.50	0.17
Xylan	30.71	31.98	1.66	1.67	1.48	2.73	0.67	0.65

[a]A correlation coefficient of 0.81 corresponds to a significance at $P < 0.05$.
[b]Difference in initial rate of gas production, mL/h, from 0.2 g of corn silage or grass silage in 30-mL incubations in gas syringes, in the presence of 1 mL of a threefold dilution of neat enzymes A and B, 1,000 U/mL *P. funiculosum* extract, 200 U/mL *A. niger* extract, 200 U/mL *T. reesei* extract, or 200 U/mL *T. viride* extract.
[c]Rate of release of *p*-nitrophenol, $\mu mol \cdot min^{-1} \cdot mL$ enzyme solution^{-1}, where the enzyme solution is neat enzymes A or B, 1,000 U/mL *P. funiculosum* extract, 200 U/mL *A. niger* extract, 200 U/mL *T. reesei* extract, or 200 U/mL *T. viride* extract.
[d]Rate of release of reducing sugars, $\mu mol \cdot min^{-1} \cdot mL$ enzyme solution^{-1}. n.d. = not determined due to high background sugar concentration.

except in enzyme B (Table 5). Enzyme activity was compared with the impact of the different preparations on rates of gas production at the 11.1 mL/L concentrations presented in Tables 2 and 4 (Table 5). None of the glycosidase activities correlated highly with gas production from either corn silage or grass silage.

The highest correlation between polysaccharidase activity and gas production was found between the activity of enzyme extracts toward microcrystalline cellulose and their ability to stimulate gas production from corn or grass silage (Table 5). It should be noted in considering the correlations that the response in terms of gas production was not proportional to enzyme concentration (Tables 2 and 4), nor was the estimation of polysaccharidase activities. The measurement of glycosidase activities by the release of chromophores was straightforward, in the sense that the rate of reaction was related linearly with enzyme concentration under the conditions of the assay. The measurement of polysaccharidase activities was not proportional to concentration, however, as illustrated in Figure 3. For example, the dye release from a 100-fold dilution was more than half that from a 10-fold dilution at 5 min, rather than one-tenth of the activity as would be expected if enzyme concentration were limiting in classical Michaelis-Menten kinetics. Also, although dye release was approximately linear with respect to time at the lowest concentrations of enzyme, the same was obviously not true at the highest concentrations (Figure 3). A similar lack of proportionality was found with CMCase and xylanase estimations by reducing sugar release (not shown). It was clear, therefore, that in order to assess properly the relationship between different enzyme activities and their ability to stimulate fermentation, the response would have to be measured in incubation to which exactly equal amounts of activity had been added.

The Comparison of Effects of Enzymes Equalized for endo- *and* exo-*(β-1,4)-Glucanase and* endo- *and* exo-*(β-1,4)-Xylanase Activities*

Four enzyme activities were selected for an analysis of gas production resulting from the addition of equal enzyme activities. These were *endo*- and *exo*-glycanase activities on two of the main polymers in plant fiber, cellulose and xylan. The polysaccharidase activities reported in Tables 1 and 4 were measured by the release of reducing sugars. This analysis detects free sugars and free reducing ends in oligosaccharides. The apparent enzyme activity would be dominated by free sugar release, resulting predominantly from exoglycanase activities. In contrast, the key activity in breaking down fiber might be expected to be endo in mechanism of action, breaking down the polymeric structure in the middle, rather than the ends, of chains. Such activity would be grossly underestimated from reducing sugar determinations. *endo*-(β-1,4)-Glucanase and *endo*-(β-

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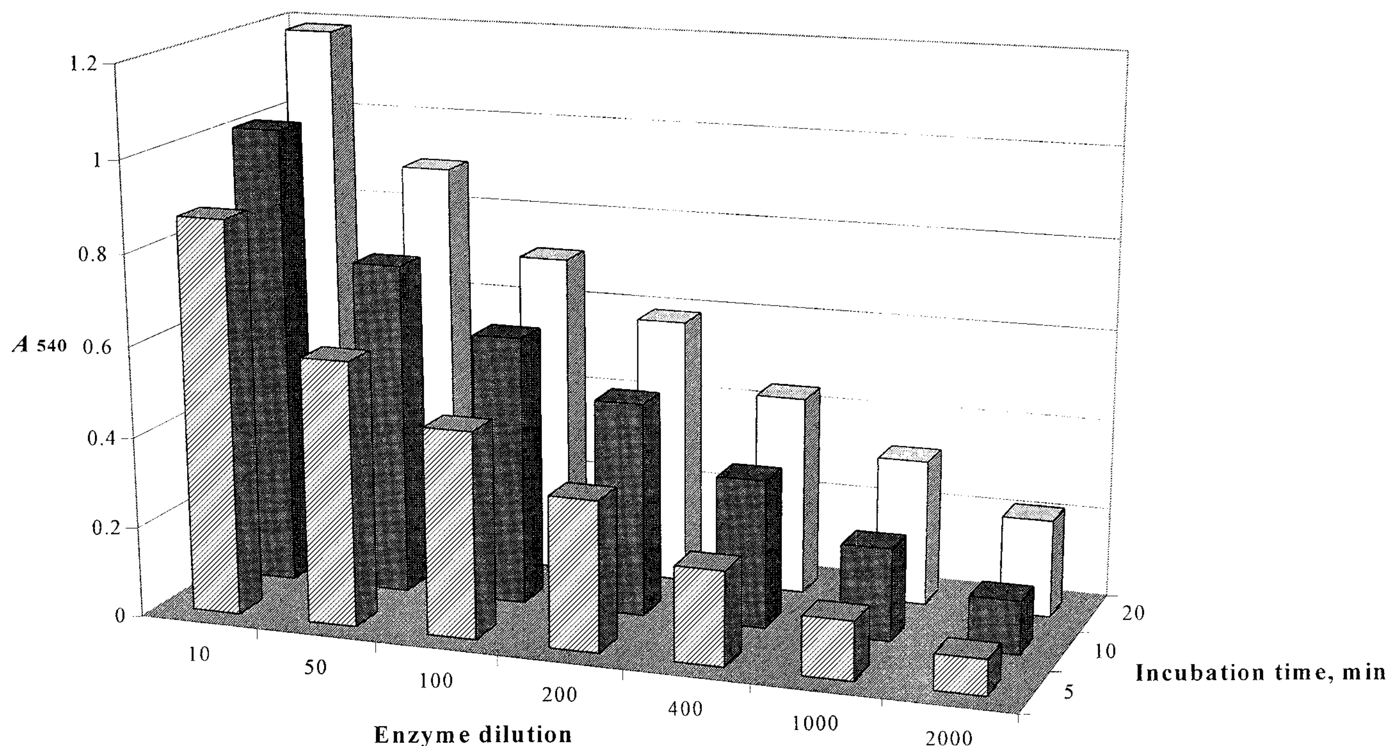

Figure 3. Influence of enzyme concentration on the hydrolysis of azo-xylan by enzyme B. Hydrolysis of the substrate was measured by the release of azo dye (A_{540}). Results are the means of duplicate incubations.

1,4)-xylanase assays were therefore undertaken using *azo*-CMC and *azo*-xylan, in which solubilized material, including solubilized oligosaccharides, is measured by dye release.

The standard addition in the gas incubations was designated to be the 11.1 mL/L concentration of enzyme A, in order to give a reasonable size of response. Other enzyme extracts were made up such that the addition of the same volume to the gas assay resulted in the addition of exactly the same amount of one of the enzyme activities (i.e., against *azo*-CMC, *azo*-xylan, CMC by reducing sugars, or xylan by reducing sugars). The process involved a process of iteration, whereby different dilutions were assayed successively until the activity in the dilution matched within ±10% of the corresponding activity of enzyme A.

Many different extracts, including those already examined and pure recombinant enzymes obtained from a variety of sources, were subjected to this laborious process. Many were eliminated, such as the *A. niger* extract, because it was not possible to dissolve the enzyme at a concentration high enough to attain the activity of enzyme A. None of the pure recombinant enzymes had activity sufficient to be included, even when a gas response test of a 10-fold lower scale was developed, because of either cost or availability. Only eight preparations, in addition to enzyme A, remained at the end of the process, and, of these, several were only included in one or two incubations because one or more of their activities were too low to match enzyme A.

The results of equalizing activities and the effects of equal enzyme activities on the rate of gas production are presented as four groups, equalized on activities against the four substrates (Table 6). By comparing the enzyme concentrations in the different groups, it is possible to obtain a semiquantitative impression of the relative enzyme activities of the different enzyme preparations. For example, *P. funiculosum* cellulase required a concentration of 750 U/mL to have the same *exo*-(β-1,4)-glucanase activity (by reducing sugars release; RS-CMC) as enzyme A against CMC, but only 113 U/mL to have the same *exo*-(β-1,4)-xylanase activity. It therefore had a much higher ratio of *exo*-(β-1,4)-xylanase to *exo*-(β-1,4)-glucanase than enzyme A. Where an enzyme appears in one group but not another, it means that it was not possible, in the conditions of the missing entry, to obtain a concentration of the enzyme high enough to match enzyme A. Thus, Sumizyme X had significant xylanase activities, but very low CMCase activities. Celluclast had the opposite properties.

When the enzymes were matched for reducing sugar release from CMC (RS-CMC), all were as effective as enzyme A in their ability to stimulate fermentation (Table 6). In contrast, several preparations with *exo*- and *endo*-xylanase activities matching enzyme A but much lower glucanase activities, including *P. funiculosum* extract, Xylanase 250 and Sumizyme X, had significantly lower stimulatory activity. Thus, the activity of enzymes in breaking down xylan is not a good indicator of their ability to stimulate fermentation, implying

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as well that xylan breakdown is not the rate-limiting fibrolytic activity in ruminal microorganisms. In contrast, the four preparations with matching activity against *azo*-CMC had higher stimulatory activity than enzymes A and B. The only preparation that was significantly different was Celluclast, which gave twice the stimulation of enzyme A, notably in the absence of significant xylanase activity.

The superior stimulation of corn silage digestion by Celluclast in comparison with the others suggests that evaluating cellulases as fermentation-stimulating additives using CMC as a substrate is inadequate; the ability of different enzymes to break down more ordered, crystalline cellulose may explain the difference. The finding that the *T. reesei* cellulase was much more effective with grass silage than corn silage suggests that such refinements should take into account the substrate feed materials as well.

Implications

Our main conclusion regarding enzyme feed additives is that a direct ruminal effect of the enzymes is improbable at the suggested application rates with these diets: gas responses were too low, and the enzyme activities added were insignificant in comparison with the enzymes already present in the feedstuffs or ruminal microorganisms. This does not eliminate adaptive, or pre- or postruminal effects. More experiments need to be carried out to focus on these aspects, particularly prefeeding storage times and conditions. Finally, the results demonstrate that microbial enzyme activity limits the rate of fiber digestion by ruminal microorganisms. The plant fiber itself does not limit the fermentation rate, at least when presented in the small particulate form used here, and direct stimulation of cellulolytic ruminal microbes and their enzyme activities would be

Table 6. Influence of equal activities of added enzymes on the rate of fermentation of corn silage by ruminal microorganisms in vitro

Enzyme assay[a]	Enzyme preparation	Concentration	Rate of gas production (No added enzyme = 100%)[b]	
			Mean	*P*-value[c]
Azo-CMC	A	100 mL/L	110.8	
	B	100 mL/L	110.6	0.922
	P. funiculosum	3,000 U/mL	117.7	0.115
	T. reesei	240 U/mL	113.8	0.404
	T. viride	380 U/mL	115.1	0.248
	Celluclast	200 mL/L	119.0	0.012
Azo-xylan	A	100 mL/L	110.8	
	B	105 mL/L	114.6	0.291
	P. funiculosum	1,200 U/mL	114.9	0.032
	T. viride	150 U/mL	110.0	0.471
	XYL 250	75 kU/mL	103.3	0.004
	LIQUICELL	160 mL/L	110.6	0.898
	Sumizyme X	150 kU/mL	104.5	0.001
RS-CMC	A	100 mL/L	110.8	
	B	105 mL/L	114.6	0.291
	P. funiculosum	750 U/mL	112.0	0.669
	T. reesei	80 U/mL	110.6	0.942
	T. viride	300 U/mL	113.4	0.207
	LIQUICELL	75 mL/L	110.8	0.998
	DEPOL	85 mL/L	109.6	0.523
	Celluclast	75 mL/L	113.3	0.274
RS-xylan	A	100 mL/L	110.8	
	B	105 mL/L	114.6	0.291
	P. funiculosum	113 U/mL	102.4	0.001
	T. reesei	500 U/mL	108.9	0.441
	T. viride	65 U/mL	105.7	0.096
	XYL 250	100 kU/mL	104.9	0.022
	LIQUICELL	50 mL/L	107.9	0.119
	Sumizyme X	200 kU/mL	102.6	0.006

[a]Azo-CMC means that all the concentrations of the enzymes added had the same activity as enzyme A against azo-carboxymethylcellulose under identical assay conditions. The RBB assays represent the release of dye from azo-substrate, a measure of *endo*-glycanase activity; RS is predominantly *exo*-glycanase activity, as measured by the release of reducing sugars.

[b]Results are means of six different incubations. The rate of gas production in each incubation was calculated by linear regression of gas released at 2-h intervals up to 6 h.

[c]*P*-values were calculated from comparisons of the effect of enzyme A on the rate of gas production with the effects of other enzymes, calculated from a paired *t*-test of rates obtained on different days against enzyme A as control. The stimulation of gas production from corn silage was corrected for gas production from the added enzyme in the absence of corn silage.

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expected to benefit the nutrition of ruminants receiving corn or grass silage-containing diets.

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