Rumen Microbes, Enzymes and Feed Digestion-A Review**

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ABSTRACT : Ruminant animals develop a diverse and sophisticated microbial ecosystem for digesting fibrous feedstuffs. Plant cell walls are complex and their structures are not fully understood, but it is generally believed that the chemical properties of some plant cell wall compounds and the cross-linked three-dimensional matrix of polysaccharides, lignin and phenolic compounds limit digestion of cell wall polysaccharides by ruminal microbes. Three adaptive strategies have been identified in the ruminal ecosystem for degrading plant cell walls: production of the full slate of enzymes required to cleave the numerous bonds within cell walls; attachment and colonization of feed particles; and synergetic interactions among ruminal species. Nonetheless, digestion of fibrous feeds remains incomplete, and numerous research attempts have been made to increase this extent of digestion. Exogenous fibrolytic enzymes (EFE) have been used successfully in monogastric animal production for some time. The possibility of adapting EFE as feed additives for ruminants is under intensive study. To date, animal responses to EFE supplements have varied greatly due to differences in enzyme source, application method, and types of diets and livestock. Currently available information suggests delivery of EFE by applying them to feed offers the best chance to increase ruminal digestion. The general tendency of EFE to increase rate, but not extent, of fibre digestion indicates that the products currently on the market for ruminants may not be introducing novel enzyme activities into the rumen. Recent research suggests that cleavage of esterified linkages (e.g., acetylesterase, ferulic acid esterase) within the plant cell wall matrix may be the key to increasing the extent of cell wall digestion in the rumen. Thus, a crucial ingredient in an effective enzyme additive for ruminants may be an as yet undetermined esterase that may not be included, quantified or listed in the majority of available enzyme preparations. Identifying these pivotal enzyme(s) and using biotechnology to enhance their production is necessary for long term improvements in feed digestion using EFE. Pretreating fibrous feeds with alkali in addition to EFE also shows promise for improving the efficacy of enzyme supplements. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No.11 : 1659-1676)***

Key Words : Exogenous Fibrolytic Enzymes, Ruminant, Feed Digestion, Alkali

INTRODUCTION

Upon ingestion by ruminants, feedstuffs enter the rumen and are degraded to various extents by rumen microbial populations. The ruminal ecosystem comprises a diverse, symbiotic population of obligately anaerobic bacteria, fungi and protozoa (Forsberg and Cheng, 1992) that have adapted for survival in the face of high dilution rates, high cell densities and protozoal predation. Moreover, they have evolved the capacity for efficient utilization of complex and recalcitrant plant polymers such as cellulose and hemicellulose. Carbohydrates, mainly polysaccharides and structural (cell wall) polysaccharides such as cellulose, hemicellulose and pectin, are the major component of ruminant diets, and are the primary source of energy in forage-based diets. Degradation and metabolism of structural carbohydrates is accomplished through synchronous activities of the multitude of microbial enzymes present in the rumen. The insolubility, structural complexity and initial inaccessibility of cell wall components, however, often limits the extent to which they are fermented in the rumen (Nagaraja et al., 1997). Manipulation of ruminal metabolism to maximize the efficiency with which plant cell wall materials are degraded has become an important goal in modern livestock production.

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Composition and organization of plant cell wall

Plant cell walls are composed primarily of sugars (e.g., rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, galacturonic acid and glucuronic acid) arranged as polysaccharides with varying compositions and structures and complexed with hydroxycinnamic acids, lignin, protein, ions and water. Analytically, these polysaccharides can be grouped into cellulose, hemicellulose and pectin fractions.

Cellulose is formed from linear chains of β-1,4-linked glucose units. It is the most abundant polysaccharide in the cell wall, accounting for 20 to 30% of the dry weight of most plant primary cell walls (Chafe, 1970; McNeil et al., 1984). Hemicellulose is composed mainly of xylans with a backbone structure of β-1,4-linked xylose residues and attachment of various side chains (e.g., acetic acid, arabinose, coumaric acid, ferulic acid, glucuronic acid, 4-*O*-methylglucuronic acid) to the xylose residues (Waite and Gorrod, 1959; Wilkie, 1979; Chesson et al., 1983; McNeil et al., 1984). Xylan polymers may be cross-linked to other hemicellulose backbones or to lignin through ferulic acid or 4-*O*-methyl-α-D-glucuronic acid residues (Hartley and Ford, 1989; Lam et al., 1990, 1992a,b). One surface of the linear

xylan backbone binds strongly also to cellulose and interlocks with other xylan polymers, forming an extensive network of cross-links between cellulose microfibrils (Carpita and Gibeaut, 1993). In the cell wall, the ratio of cellulose to hemicellulose ranges from 0.8:1 to 1.6:1 (Wilkie, 1979). Pectin exists in the primary cell wall and its primary structure is a backbone of α-1,4-linked residues of D-galacturonate. The rhamnogalacturonan backbone may be interspersed with either rhamnose or galacturonic acid residues substituted with methyl ester groups or sugar side chains (Jarvis, 1984; McNeil et al., 1984; Rombouts and Pilnik, 1986). Structural proteins called extensins are also commonly found in association with the polysaccharide in dicotyledonous cell walls (Fry, 1986). They frequently form intermolecular cross-links (Fry, 1986) and as a consequence entrap other polymers within the wall.

Although the composition of the cell wall is known, the model of how these individual compounds are organized is still not clear. It is known, however, that the polysaccharides, hydroxycinnamic acids, lignin, protein and ions are intermolecularly cross-linked through various ionic-, hydrogen- and covalent (glycosidic, ester and ether) bonds to form a three-dimensional matrix that entraps polysaccharide within the cell wall. Hydrolysis of the cell wall polysaccharides, therefore, requires not only hydrolytic enzymes but also those capable of cleaving the bonds within the cross-linked matrix; the latter are perhaps the key steps that limit the degradation of cell wall in the rumen.

The outer layers of epicuticular waxes, cuticle and pectin represent the plant's first line of defense against dehydration and penetration by phytopathogens. The cuticular layers of grasses, legumes and cereal grains also represent a potent barrier to penetration by ruminal microbes (Forsberg and Cheng, 1992). Although the cuticle is resistant to microbial and digestive enzymes in the rumen, mastication of forages and pretreatment of cereal grain disrupts the cuticular layer minimizing its deleterious effect on digestion (Akin, 1989).

Microbial strategies for plant cell wall digestion

Rumen microbes involved in degradation of plant cell wall : Plant cell walls are degraded by a combination of bacteria, fungi and protozoa (Table 1), with bacteria and fungi contributing approximately 80% of the degradative activity, and protozoa 20% (Dijkstra and Tamminga, 1995). The fibrolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are generally considered as the primary organisms responsible for degradation of plant cell walls in the rumen (Cheng et al., 1991; Forsberg and Cheng, 1992).

Compared to bacteria, the role of the fungi is not as well understood. Ruminal fungi produce a broad array of enzymes and generally degrade a wider range of substrates than do the ruminal bacteria (Wubah et al., 1993; Trinci et al., 1994). Furthermore, ruminal fungi are able to degrade the most resistant plant cell wall polymers (Forsberg and Cheng, 1992; Wubah et al., 1993; Trinci et al., 1994) and the cellulases and xylanases they produce are among the most active fibrolytic enzymes described to date (Gilbert et al., 1992; Trinci et al., 1994). Growth of the fungi, however, is apparently restricted to the recalcitrant sclerenchymal fraction of plant cell walls; this may be due to their much slower growth rate relative to bacteria (Forsberg and Cheng, 1992). Fungi also possess the unique capacity to penetrate the cuticle at the plant surface and the cell walls of lignified tissues (Akin, 1989).

The activities of ruminal protozoa contribute significantly to the digestion of plant cell wall polymers and their absence from the rumen may have a negative effect on the extent of fibre digestion (Coleman, 1986; Bonhomme, 1990; Williams and Coleman, 1991). All of the major fibrolytic enzyme activities can be detected in the rumen protozoal population, but study of these enzyme systems has been hampered by difficulties in culturing the protozoa.

Production of a full slate of enzymes : The major bonds in plant cell wall polymers have been defined and extensively mapped. Clearly, degradation of plant cell walls occurs as a result of concerted and complex interactions. Hydrolysis of the recalcitrant substrates encountered by ruminal microbes to their constituent monomers requires numerous enzyme types and specificities (Table 2).

The enzyme activities confirmed to exist in the rumen are diverse, including those that degrade plant cell wall polymers (e.g., cellulases, xylanases, β-glucanases, pectinases), amylases, proteases, phytases and those that degrade specific plant toxins (e.g., tannases). The variety of enzymes present in the rumen arises not only from the diversity of the microbial community but also from the multiplicity of fibrolytic enzymes produced by individual microorganisms (Doerner and White, 1990; Malburg and Forsberg, 1993; Flint et al., 1994; Ali et al., 1995; Yanke et al., 1995).

Efficient digestion of complex substrates in the rumen requires the coordinated activities of these enzymes. Two models for individual cells have been proposed to describe the organization of fibrolytic enzyme systems following synthesis and secretion from the cell. In the first model, enzymes act individually and synergistically to effect hydrolysis of cellulose. This model, reviewed by Wood (1992) and Béguin and Aubert (1994), originated largely from research on aerobic fungi representing several genera, including *Trichoderma* and *Phanerochaete*. In the second model, individual enzymes are assembled into multi-enzyme complexes (e.g., cellulosomes). The cellulosomal multi-enzyme complex of the thermophilic bacterium *Clostridium thermocellum* is the most extensively studied

Table 1. Identity and enzyme activities of ruminal microbes involved with degradation of plant cell walls in the rumen

Organism	Degradative activity		
	Cellulolytic	Hemicellulolytic	Pectinolytic
Bacteria			
Fibrobacter succinogenes	+	+	+
Ruminococcus albus	+	+	+
Ruminococcus flavefaciens	+	+	+
Butyrivibrio fibrisolvens	+	+	+
Eubacterium cellulosolvens	+		+
Clostridium longisporum	+		
Clostridium locheadii		+	+
Prevotella ruminantium		+	+
Eubacterium xylanophilum		+	
Ruminobacter amylophilus		+	
Succinimonas amylolytica		+	
Succinivibrio dextrinosolvens		+	
Selenomonas ruminantium		+	
Selenomonas lactilytica		+	
Lachnospira multiparus		+	+
Streptococcus bovis		+	+
Megasphaera elsdenii		+	
Protozoa			
Eudiplodinium maggii	+	+	+
Ostracodinium dilobum	+	+	+
Epidinium caudatum	+	+	
Metadinium affine	+	+	+
Eudiplodinium bovis	+	+	+
Orphryoscolex caudatus	+	+	+
Polyplastron multivesiculatum	+	+	+
Diplodinium pentacanthum	+		
Endoploplastron triloricatum	+		
Orphyroscolex tricoronatus	+		
Ostracodinium gracile	+		
Entodinium caudatum	+	+	
Isotricha intestinalis	+	+	+
Isotricha prostoma	+	+	+
Fungi			
Neocallimastix frontalis	+	+	+
Neocallimastix patriciarum	+	+	+
Neocallimastix joyonii	+	+	
Caecomyces communis	+	+	+
Piromyces communis	+	+	+
Orpinomyces bovis	+	+	
Ruminomyces elegans	+	+	

(Adapted from Dehority 1993).

example of this model (Bayer et al., 1994). High molecular mass complexes containing numerous cellulases have been identified in a number of rumen bacteria, including *Butyrivibrio fibrisolvens, R. albus* and *F. succinogenes*, and fungi, including *Neocallimastix frontalis* and *Piromyces sp.* (Forsberg et al., 1993; Bayer et al., 1994; Ali et al., 1995; Fanutti et al., 1995).

Pectin, a minor component of grass cell walls (Jarvis, 1984), is digested in the rumen either by strictly pectinolytic species or by those species possessing a combination of pectinases (e.g., pectin lyase, polygalacturonase, pectin methylesterase) and xylanases (Orpin, 1984; Cheng et al., 1991; Gordon and Phillips, 1992). One of the major pectinolytic bacterial species inhabiting the rumen, *Lachnospira multiparus*, produces a pectin lyase and a pectin methylesterase (Silley, 1985).

Table 2. Major enzyme activities required for hydrolysis of plant cell wall polymers and present in the rumen

Substrate polymer	Target bond for hydrolysis	Enzyme effecting hydrolysis
Cellulose	β-1,4-glucose linkage	Endo-β-1,4-glucanase
Cellulose (non-reducing end)	β-1,4-glucose linkage	Exo-β-1,4-glucanase
Cellobiose	β-1,4-glucose linkage	β-1,4-Glucosidase
Soluble cellooligomers	β-1,4-glucose linkage	Cellulodextrinase
Cellulose or xylan	β-1,4-glucose linkage or xylose linkage	Xylocellulase
Xylan	β-1,4-xylose linkages	Endo-β-1,4-xylanase
Xylobiose	β-1,4-xylose linkage	β-1,4-Xylosidase
Arabinoxylan	α-1,3-linkage	α-L-Arabinofuranosidase
Glucuronoxylan	α-1,3 or α-1,2 linkage	α-Glucuronidase
Acetylxylan	Acetylester bond	*O*-Acetyl xylan esterase
Ferulic acid cross bridge or linkage	Feruloylester bond	Ferulic acid esterase
p-Coumaric acid cross bridge	*p*-Coumaryl ester bond or linkage	*p*-Coumaric acid esterase
Laminarin	β-1,3-glucanase	β-1,3-hexose linkage
Lichenin	β-1,3- and β-1,4-hexose linkages	Mixed linkage β-1,3-β-1,4-glucanase
Polygalacturan	α-1,4-Galacturonide linkages	Pectate lyase
Pectin	α-1,4-Galacturonide linkages	Pectin lyase
Pectin	Methylester bond	Pectin methylesterase

Ruminal fungi and protozoa also express one or more of these enzymes (Orpin, 1984; Bonhomme, 1990; Gordon and Phillips, 1992; Chesson and Forsberg, 1997).

Adhesion and colonization of feed particles by ruminal microorganisms : Rumen microbes digest feed through the action of enzymes they produce. Contact between these enzymes and their feed substrates is necessary for hydrolysis to occur. Rumen contents comprise a heterogeneous mixture of liquid and solid. Polysaccharide-degrading enzymes secreted into the liquid fraction are at risk of inactivation by proteolysis or of being washed out of rumen before they contact their substrate(s). Clearly, attachment to feed particles is the most efficient way for microbes to prolong their residence in the rumen and to bring their enzymes into contact with substrates. The process of adhesion is absolutely essential for efficient digestion of forages and cereal grains in the rumen (McAllister et al., 1994; McAllister and Cheng, 1996). Bacterial strains that can not adhere carry out only limited cellulolysis (Morris and Cole, 1987).

Ruminal microorganisms that interact with feed particles can be functionally described as three distinct subpopulations: 1) those associated with the ruminal fluid, 2) those loosely attached to feed particles and 3) those firmly attached to feed particles (Cheng and McAllister, 1997). Microorganisms associated with the ruminal fluid include those newly detached from feed particles, as well as those that survive on soluble feed components within ruminal fluid and have little direct involvement in the digestion of insoluble feed particles (Latham, 1980). This subpopulation is an integral part of the rumen ecosystem, as these microbes colonize and initiate digestion of newly ingested feed particles. Association with and attachment to feed particles by ruminal microorganisms is rapid (Cheng et al., 1983/84; Craig et al., 1987), occurring within five minutes of feed entering rumen (Bonhomme, 1990).

Bacteria loosely associated with feed particles can be removed by gentle washing, whereas bacteria in the tightly adherent population remain attached. These two subpopulations are numerically predominant and account for 70-80% of the microbial matter in the rumen (Forsberg and Lam, 1977; Craig et al., 1987). It has been estimated that these two populations are responsible for 80% of the endoglucanase activity, 70% of the amylase activity and 75% of the protease activity in the rumen (Minato et al., 1966; Brock et al., 1982). Hemicellulase and cellulase activities are also notably higher in the particulate fraction of ruminal contents than in the fluid (Williams and Strachan, 1984), leaving no doubt that particle-associated microbial populations are responsible for the majority of ruminal feed digestion.

Ruminal microorganisms that attach to feed particles have a competitive advantage over their non-attaching counterparts. The digestive enzymes of bacteria are often stabilized and protected within a fibrous polysaccharide glycocalyx on the cell surface (Cheng et al., 1981; Lappin-Scott et al., 1992). Presumably, because these microorganisms are close to the digestion site, they receive a large proportion of the nutrients released during digestion of feed particles. Furthermore, depending on size, density, and susceptibility to digestion, feed particles are generally retained in the rumen two to three times longer than the

fluid (Owens and Goetsch, 1986). Microorganisms that attach to feed particles with a slow rate of passage remain in the rumen longer and therefore increase the reaction time between enzymes and substrates. This prolonged residency is particularly important for slow-growing organisms such as ruminal fungi and protozoa. With generation times of 5 to 14 h for ruminal protozoa (Williams and Coleman, 1988) and 24 to 30 h for ruminal fungi (Bauchop, 1981; Joblin, 1981), populations of these microorganisms are rapidly depleted if they are unable to attach to feed particles to delay their passage from the rumen.

Synergetic interaction among rumen microbes : Although some individual rumen microbes produce enzymes with multiple activities which can hydrolyze certain compounds of the cell wall, complete degradation of polymers within the intact cell wall requires a wide range of hydrolytic enzymes that are able to act both simultaneously and systematically. These multiple hydrolytic enzyme activities required to degrade the cell wall in the rumen are attained by a niche of diversified ruminal microbes that produce enzymes capable of cleaving certain linkages within the cell wall. This fibrolytic activity, however, is not just a simple quantitative accumulation. Rather, it is a strategic organization and interaction of ruminal microbes to facilitate cell wall digestion.

Synergism between microorganisms has been defined as the increase in activity that exceeds the additive effects of each individual organism when two or more function in the same fermentation (Dehority, 1993). Synergetic action is evident in the organization of ruminal microbial activity that has evolved for efficient digestion of plant cell wall. Examples include the increased xylanase and cellulase synthesis (Joblin et al., 1990; Teunissen et al., 1992) and increased rate and extent of cellulose digestion (Bauchop and Mountfort, 1981; Mountfort et al., 1982) that result from co-culturing rumen methanogens with anaerobic fungi and by combination of *R. albus* or *F. succinogenes* with *P. ruminicola* (Gradel and Dehority, 1972). Dehority (1993) proposed that there are likely two types of synergism with regard to the digestion of cellulose from intact forages: "unmasking", in which a microbial species is enzymatically capable of removing a component that limits a second species' access to the substrate; and end product utilization. In addition, the rumen fungi act synergistically in the digestion of forages by physically disrupting the lignified stem tissue and allowing entrance of the rumen microbes into plant stems, thereby accessing the digestible portions of the plant.

In contrast to the synergism observed in structural polysaccharide digestion when certain microbial species are combined, some combinations can result in less activity. Some examples of this are the inhibitory effects of *R. albus* and *R. flavefaciens* on the cellulolytic activity of ruminal fungi (Fonty and Joblin, 1991; Stewart et al., 1992; Bernalier et al., 1993) and the inhibitory effect of *R. albus* 8 on growth of *R. flavefaciens* FD1 (Odenyo et al., 1994). This negative interaction among ruminal microbes may be due to the different organisms producing different depolymerases which act on alternative sites of the polysaccharide, producing oligosaccharides that are not further degradable by the available glycosidases (Dehority, 1993). Competition for the adhesion site and production of bacteriocins may be also responsible for negative interactions.

It is important to note, however, that these positive or negative effects of microbial interaction on cell wall degradation were obtained by *in vitro* culturing of pure species or their combinations, and may not represent the real situation in the rumen in which hundreds of microbial species are supported by the different compounds arising from the same feedstuff. Furthermore, some ruminal microbes express multiple activities simultaneously (e.g., fibrolytic and proteolytic activities) and their activity against certain compounds can vary depending on the feed source. Thus, interactions among ruminal microbes *in vivo* may be much more complicated than those elucidated through *in vitro* culturing. Similarly, a negative microbial interaction attributed *in vitro* to end product inhibition may not occur in the ruminal environment as other microbes not present in the *in vitro* system may serve to utilize these end products. Probably, the only safe conclusion that can be drawn regarding the complex ruminal ecosystem is that the digestion of the feed materials is the net result of the interactions among ruminal microbes. Evolutionary pressures would suggest that the synergetic effects of the microbial interaction in feed digestion are greater than existing antagonistic effects.

Limitations of cell wall digestion by ruminal microbes

It has been reported that the maximum rate constant for digestion of crystalline cellulose by rumen cellulolytic bacteria under optimal growth condition is 0.08 h^{-1} (Pavlostathis et al., 1988; Weimer, 1993, 1996). However, the rates of digestion of cellulose in forages by mixed ruminal microflora rarely approach the rates for crystalline cellulose (Weimer, 1996). Dehority (1993) compared digestion of cellulose in 11 forages between *in vitro* and *in vivo* experiments and determined average cellulose digestibilities of 64.6% (*in vitro*) and 61.7% (*in vivo*). Thus, although ruminants have evolved a powerful and sophisticated microbial ecosystem to digest fibrous feedstuffs, ingested cell wall polysaccharides are rarely completely degraded by the ruminal microflora. Reasons for this incomplete digestion include the combination of the biochemical and physical barriers present in the ingested substrates and limits on retention time of the ingested

substances in the rumen.

Feed consumed by ruminants comprises not only the nutrients required by the animal, but also naturally occurring plant secondary compounds such as polyphenolics and saponins (Bae et al., 1993; McAllister et al., 1993; Wang et al., 2000a,b) and cell wall compounds such as phenolic acids and silica (Chesson et al., 1982; Bae et al., 1997) that usually have negative effects on cellulolytic activity. The greatest obstacle to cell wall degradation in the rumen, however, is likely the cross-linkages among cellulose, hemicellulose, lignin and other compounds that limit the access of enzymes to the substrate trapped inside. Free phenolic acids and soluble phenolic-carbohydrate complexes have both been shown to inhibit rumen microbial activity (Jung and Sahlu, 1986; Hartley and Akin, 1989; Hartley and Ford, 1989) and additional evidence suggests that concentrations of these materials on the surface of feed particles may prevent microbial attachment (Chesson et al., 1982). Accumulation of lignin-carbohydrate complexes mediated by phenolic compounds (PC-LCC) at the surface of the feed particles, therefore, constitutes both a physical and a biochemical protective layer that limits the availability of surface area for microbial colonization and protects the underlying cell wall from further attack by rumen microbes.

Limited retention time in the rumen represents a second impediment to complete digestion of plant cell materials. This factor is usually determined by the particle size - the larger the particle size, the longer the feed particles are likely to be retained in the rumen and the greater the resulting degradation. Retention time, therefore, affects the extent rather than the rate of digestion. In modern livestock feeding operations, feed processing is often not closely defined; feedstuffs are commonly processed "to an appropriate extent". Under these conditions, the efficiency of cell wall degradation in the rumen will depend largely on the rate of microbial colonization and the efficacy with which microbial enzymes remove the physical-biochemical barrier posed by PC-LCC from the particle surfaces to expose the contained polysaccharides. Hence, any means that would render the feed particles more colonizable will increase the initial rate of digestion and thereby enhance overall digestion of cell wall in the rumen.

Use of exogenous fibrolytic enzymes to enhance microbial digestion of cell wall

Exogenous fibrolytic enzymes (EFE) are used extensively to improve feed value for nonruminant animals, particularly for broilers fed diets containing barley, oats or wheat. The use of EFE first attracted the attention of ruminant nutritionists over 40 years ago (Burroughs et al., 1960; Rovics and Ely, 1962; Rust et al., 1965). More recent developments in the enzyme production industry (mainly reductions in production costs) have prompted researchers to re-examine the role of EFE in ruminant production (Judkins and Stobart, 1988; Chen et al., 1995; Feng et al., 1996; Beauchemin et al., 1997; Hristov et al., 1998; McAllister et al., 1999; Wang et al., 1999, 2001a, 2002c; Yang et al., 1999). Most of these reports, however, focus on animal responses to particular products; only a few of the studies were designed to study the mechanisms of the effect of the enzymatic action. Comprehensive reviews of the use of EFE as feed additives for ruminant animals are available (McAllister et al., 2001; Rode et al., 2001), therefore this paper will summarize newer developments and discuss the efficacy and restrictions in effects of EFE on enhancing rumen microbial activity.

Effect of EFE on rumen microbial activity : Until recently it was assumed that upon introduction into the rumen, EFE would be rapidly degraded by the array of proteases produced by ruminal microorganisms (Kung, 1996). Indeed, fungal cellulases incubated with ruminal fluid were rapidly degraded to the extent that after 6 h of incubation, less than 25% of their original activity remained (Kopecny et al., 1987; Vandevoorde and Verstraete, 1987). However, experimentation with other enzyme products showed that cellulase activity and xylanase activity remained constant after 6 h of incubation with ruminal fluid (Hristov et al., 1998; Morgavi et al., 2001). There is evidence that declining EFE activity in ruminal fluid is associated both with inactivation of the enzymes and with their outflow with the fluid phase of ruminal contents (Hristov et al., 1996b; Morgavi et al., 2001).

The fact that EFE remain active in the rumen raises the possibility that they may play a role in manipulating ruminal digestion. Great variation in ruminal responses to supplementary EFE has been reported (McAllister et al., 2001), but one commonly observed phenomenon is increased cellulolytic activity (Figures 1 and 2) in association with EFE (Wiedmeier et al., 1987; Newbold et al., 1992a,b; Feng et al., 1996; Yang et al., 1999; Wang et al., 2001a,b). Increased numbers of total culturable and cellulolytic bacteria is also one of the most consistently reported responses to direct-fed microbes for ruminants (Najaraja et al., 1997). Proposed modes of action for these products include the presence of as yet unidentified heat-labile and heat-stable components (Nisbet and Martin, 1993; Girard and Dawson, 1995), products of microbial metabolism, such as vitamins, in the culture medium (Martin and Nisbet, 1992) and scavenging of oxygen by the live microorganism (Rose, 1987). However, published reports suggest that the mode of action for EFE is likely different than that of direct-fed microbial products.

Applying autoclaved (inactivated) enzymes to feed did not affect any of the ruminal parameters measured and actually reduced initial microbial colonization (Wang et al.,

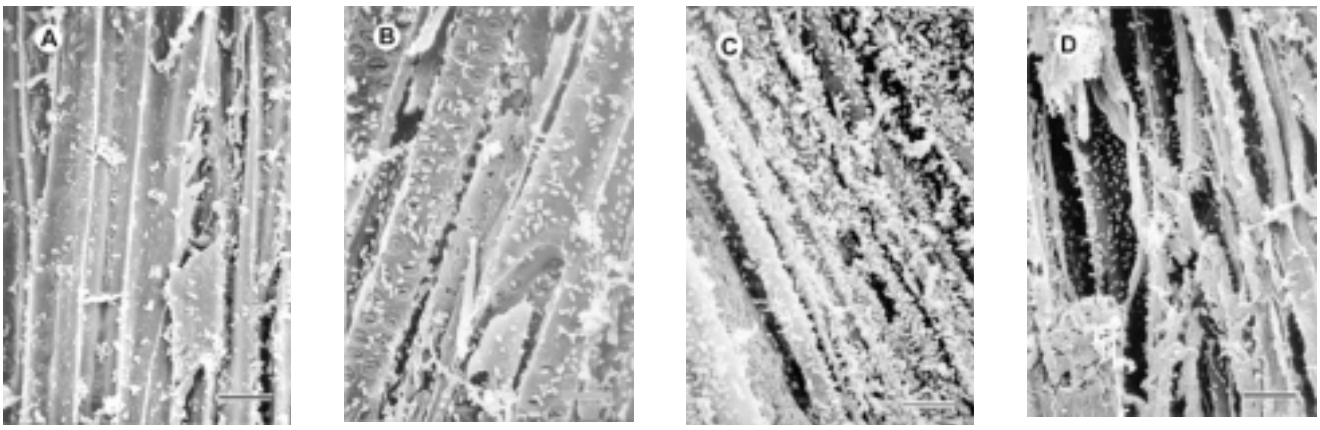

Figure 1. Effect of exogenous fibrolytic enzymes (EFE) on colonization of alfalfa hay by *Ruminococcus flavefaciens* during 48 h of *in vitro* incubation. A) No EFE; B) 28 μg/mL β-glucanase; C) 280 μg/mL β-glucanase; D) 280 μg/mL xylanase. Bars = 10 μm (Wang et al. 2001b).

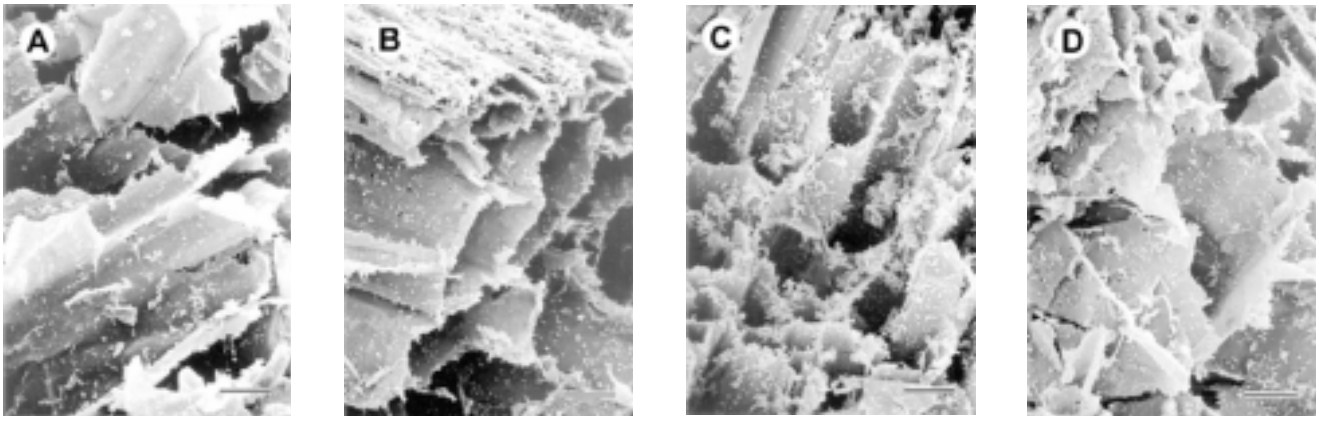

Figure 2. Effect of exogenous fibrolytic enzymes (EFE) on colonization of barley straw by *Ruminococcus flavefaciens* during 48 h of *in vitro* incubation. A) No EFE; B) 28 μg/mL β-glucanase; C) 280 μg/mL β-glucanase; D) 280 μg/mL xylanase. : Bars = 20 μm (Wang et al. 2001b).

2001a), indicating that non-enzyme components of EFE preparations had no role in promoting microbial colonization. It was proposed that the positive effect of EFE on enhancing microbial colonization is likely due to enzymatic hydrolysis of substrate, which produces reducing sugars that attract secondary colonization, or to removal of barriers to microbial attachment to feed particles by cleaving the linkage between phenolic compounds and polysaccharide (Wang et al., 2001a). Further studies, however, showed that the ability of EFE to cleave phenolic compounds from feed particles is limited (Wang et al., 2002a). Moreover, although the reducing sugars produced from the hydrolysis of straw increased microbial adhesion to the feed particles, extensive enzymatic hydrolysis of barley straw prior to exposure to ruminal microorganisms actually reduced colonization compared to that of minimally hydrolyzed straw (Wang et al., 2002b).

These observations indicate that EFE activity may not create new attachment sites for ruminal microbes by cleaving phenolic compounds within feed particles as originally proposed. Rather, they may be hydrolyzing the polysaccharides of the cell wall and leaving the PC-LCC on the surface of the feed particles to block the ruminal microbial colonization. This is supported by our recent research with alkali-treated barley straw (Wang et al., unpublished data). Disappearance of DM during *in situ* incubation increased with higher rates of EFE application, but the percentage of phenolic compounds in the residue also increased (Figure 3), indicating that the DM disappearance resulted mainly from the hydrolysis of polysaccharides by the EFE; this is consistent with our previous work with wheat straw (Wang et al., 2002a). Given the chemical and physical effects of the PC-LCC matrix on microbial digestion of cell wall, it follows that accumulation of PC-LCC on the surface of feed particles would hamper microbial colonization. This may partially explain why residues from EFE hydrolysis had lower fibre digestibility than non-hydryolyzed substrates (Morrison,

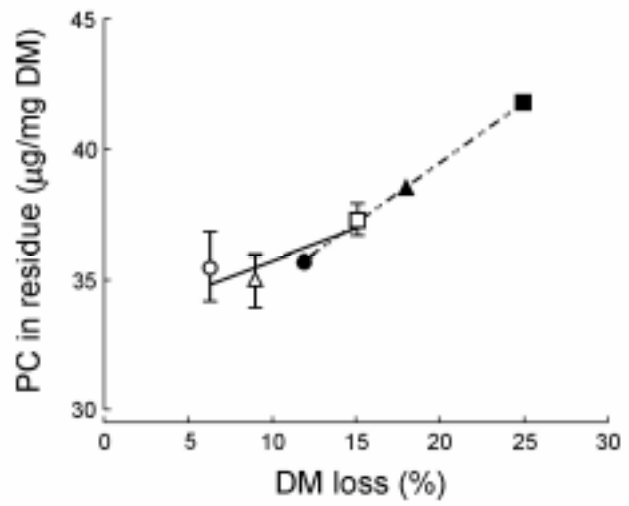

Figure 3. Relationship between loss of DM and concentration of phenolic compounds (PC) in residues from ruminal incubation of native (open symbols, ——) and alkali-treated (closed symbols, - · · - ·) wheat straw sprayed with no (○, ●), low (△,▲) or high (□, ■) levels of exogenous fibrolytic enzymes prior to *in vitro* incubation.

1988, 1991; Wang et al., 2002b).

At least 21 different enzymatic activities have been identified as being involved in the hydrolysis of the structural polysaccharides of the plant cell wall, and all of them are produced by a normally functioning ruminal microflora (White et al., 1993). Although researchers have shown that extracts of *Aspergillus oryzae* and of *Trichoderma longibrachiatum* can work synergistically with ruminal microorganisms to enhance release of soluble sugars from hay or silage (Newbold, 1995; Morgavi et al., 2000; Wang et al., 2001b), the significance of this synergistic effect on the extent of the fibre digestion *in vivo* is questionable, as discussed above. In summarizing recent reports on the topic, McAllister et al. (2001) noted that the activities supplemented to the rumen by EFE are not novel to the ruminal environment, i.e., they would act upon the same sites of the feed particles as endogenous enzymes. Hence, within the rumen, the most significant effects of EFE are probably occurring in the interval between arrival of the feed and its colonization by ruminal microorganisms. This concept is consistent not only with reported quadratic effects of increasing EFE application rates on digestibility (Deniels and Hashim, 1977; Morgavi et al., 2001; Wang et al., 2001c) but also with the general observation that EFE usually only increase the rate and not the extent of digestion (Varel et al., 1993; Feng et al., 1996; Hristov et al., 1996a; Wang et al., 2002c).

Efficacies of EFE from different sources : Exogenous fibrolytic enzyme products marketed for livestock number in the hundreds, but they are derived primarily from only four bacterial *(Bacillus subtilis, Lactobacillus acidophilus*, *L. plantarum*, and *Streptococcus faecium*) and three fungal (*A. oryzae*, *Trichoderma reesei,* and *Saccharomyces cerevisiae*) species (Muirhead, 1996). Other fungal species including *Humicola insolvens* and *Thermomyces* spp. are being marketed to a lesser extent. It is unlikely that this list of source organisms will expand substantially, given that no recent petition to the United States Food and Drug Administration to add a new organism has been successful (Pendleton, 1996).

Enzyme preparations for ruminants are marketed primarily on the basis of their capacity to degrade plant cell walls and as such, are often referred to as cellulases or xylanases. However, none of these commercial products comprise single enzymes; secondary enzyme activities such as amylases, proteases, or pectinases are invariably present. Degradation of cellulose and hemicellulose alone requires multiple enzymes, and differences in the relative proportions and activities of these individual enzymes may impact the efficacy of cell wall degradation by the marketed products. Even within a single microbial species, the types and activity of enzymes produced can vary widely depending on the strain selected and the growth substrate and culture conditions employed (Considine and Coughlan, 1989; Gashe, 1992).

The diversity of enzyme activities present in commercially available EFE preparations is advantageous, in that a wide variety of substrates can be targeted by a single product, but it presents problems in terms of quality control and extrapolation of research findings between studies. For ruminants, enzyme products are usually standardized by blending crude enzyme extracts to obtain specified levels of one or two defined enzyme activities, such as xylanase and/or cellulase. However, because numbers of cell wall-degrading organisms are not limiting under normal conditions (Chesson and Forsberg, 1997), xylanase or cellulase activity of the preparations is probably of little consequence in their efficacy in enhancing cell wall digestion. Rather, it seems that some unknown secondary activit(ies) exert the greatest impact on the efficacy of a given EFE product. It is of value, therefore, to identify these influential activities. One proposed strategy is to shift from a "whole product testing" model, which is currently being used extensively, to an "evaluation of individual effective compounds" model. Information obtained from this model would be useful not only for elucidating the modes of action of current products, but also for designing new products.

Most of the EFE products on the market are generated from the fermentation industry, in which conditions typically are optimized for maximal production, and as such, the substrates employed could be quite distinct from ruminant feeds. Thus, the enzyme products currently

available and in use may not be optimal for digesting ruminant feed, although they may have been tested and shown to hydrolyze some pure substances under laboratory conditions. Overcoming this discrepancy will require closer coordination and transfer of information between researchers in animal production and those in fermentation industries.

Efficacy of EFE applied in different methods : A number of research teams have shown that EFE can enhance fibre degradation by ruminal microorganisms *in vitro* (Forwood et al., 1990; Varel et al., 1993; Hristov et al., 1996a; Feng et al., 1996) and *in situ* (Lewis et al., 1996) and this effect has been confirmed in some (Beauchemin et al., 1999; Yang et al., 1999) but not in all (Firkins et al., 1990; Varel and Kreikemeier, 1994) studies conducted using ruminally and duodenally cannulated cattle. In each of these studies, EFE were administered by applying them directly onto the feed. Administering an aqueous solution of mixed EFE directly into the rumen of cannulated sheep, however, lowered DM digestion (McAllister et al., 1999), and similar results were reported when EFE were infused into beef steers (Lewis et al., 1996). These studies suggest that EFE are not as efficacious when ruminally infused as when applied directly to the feed, and imply that differences in EFE application methods may be contributing to the observed differences in their effectiveness.

Methods of EFE application reported in studies to date have included spraying onto concentrate, forage or total mixed rations, top-dressing onto total mixed rations, and direct introduction into the rumen. Current information suggests that spraying EFE onto dry feed components (either forage or concentrate) prior to feeding is most effective for eliciting positive response (Table 3). This observation has led researchers to speculate that this method of EFE application may have increased the stability of the enzymes through binding with substrate and/or provided opportunity for EFE to effect pre-ruminal hydrolysis.

Wang et al. (2001a) found that spraying EFE onto feed prior to incubation increased the xylanase activity in both the solid and the liquid fractions of ruminal incubation fluid (Table 4). It is generally recognized that the most active xylanolytic and pectinolytic microbes do not show strong adhesion to their respective substrates, and in many cases their hydrolases are secreted into the *in vitro* incubation medium (Chesson and Forsberg, 1997). Fluid-associated enzyme activity usually represents less than 30% of the total enzyme activity in the rumen (Minato et al., 1966; Brock et al., 1982); the greatest influence on feed digestion is therefore most probably exerted by those EFE that are attached to feed particles during ruminal processes. The increased particle-associated xylanase activity afforded by spraying enzymes onto feeds prior to consumption may enhance this aspect of rumen ecosystem function.

A solution of EFE applied onto both barley grain and alfalfa hay (10 mL/100 g DM) caused pre-ruminal hydrolysis (as evidenced by increased reducing sugars and reduced NDF content in the substrate) of barley grain, but not alfalfa hay (Table 5; Wang et al., 2001a). Other researchers (Beauchemin and Rode, 1996; Hristov et al., 1996a,b) have also reported pre-ruminal hydrolysis. These studies demonstrate that the degree of sugar release is dependent on both the type of feed and the type of EFE.

Release of sugars from feeds arises at least partially from the solubilization of NDF and ADF (Hristov et al., 1996a; Gwayumba and Christensen, 1997; Wang et al., 2001a). The observation that pre-ruminal enzymatic hydrolysis occurred even when enzymes were applied onto dry feed at first seemed improbable, given that the role of water in the hydrolysis of soluble sugars from complex polymers is a fundamental biochemical principle (Lehninger, 1982). However, feed offered to ruminants is seldom absolutely dry; even feeds that nutritionists would describe as "dry" (e.g., grain, hay) contain 6 to 10% moisture; as well, EFE are usually dissolved in water for spraying onto feeds. Moreover, although the percentage of the water applied is low (usually 1%), the aqueous solution is only spread on the surface of the feed particle, thus water content in this film may be sufficient to support enzymatic activity prior to evaporation or penetration into the feed particles. These points suggest that administering EFE by spraying onto feeds is advantageous for pre-ruminal hydrolysis compared to top-dressing EFE onto the diet in a supplement. However, the significance of this hydrolysis on ruminal digestion of the feeds is unclear.

Wang et al. (2002b) conducted research to assess the pre-ruminal hydrolysis of exogenous fibrolytic enzymes on the ruminal fermentation of barley straw. Native and ammoniated ground barley straw (S) were prepared six ways for use in batch culture incubation: 1) control (treated with water), 2) sprayed with EFE (and used directly), 3) sprayed with EFE, held at 39ºC for 24 h and freeze-dried prior to incubation (i.e., prehydrolyzed), 4) prehydrolyzed and washed (PW) to remove EFE and hydrolysis products, 5) PW followed by reapplication of autoclaved (inactivated) EFE, and 6) PW followed by reapplication of hydrolysis product (reducing sugars). Applying enzymes onto straw prior to incubation increased DM loss and microbial colonization as measured by ^{15}N incorporation (Table 6). Extensive pre-ruminal hydrolysis reduced rumen microbial colonization, however, even though it increased DM loss compared to that of less extensively hydrolyzed straw. The reduced microbial colonization was not due to the greater soluble sugars released by the enzymatic action, since addition of the reducing sugars up to the level same as that of extensively pre-hydrolyzed substrate actually increased DM loss and microbial colonization. This suggested that

Table 3. Summary of effects of exogenous fibrolytic enzymes (EFE) on *in vitro* and *in vivo* digestion of feedstuffs, and animal performance

Enzyme	Application method	Main diet ingredients	Apparatus or animal(s)	Main responses	Reference
***In vitro* fermentation responses**					
Xylanase from *Trichoderma longibrachiatum*	Applied onto feed or incorporated into medium	Alfalfa hay, barley grain	Rusitec	On feed, EFE increased cellulolytic bacteria and colonization of feeds; not so when incorporated into medium	Wang et al. (2001a)
Xylanase from *T. longibrachiatum*	Applied onto wet silage or onto dried silage	Barley silage or corn silage	Batch culture	On dried silage, EFE increased initial bacterial colonization; on wet silage, reduced it	Wang et al. (2002c)
Extract of *Aspergillus oryzae* with multiple enzyme activities	Top dressed onto total mixed ration (3 g/d)	Alfalfa hay, bromegrass, supplement	*In situ*	No effects on rate or extent of fibre degradation	Varel and Kreikemeier (1994)
Fungal cellulase and xylanase	Applied onto dried, fresh or re-hydrated grass (2.1 or 5.26 mL/kg DM)	Grass	*In vitro* and *in situ*	On dried grass, EFE increased degradability of DM and NDF; on wet grass, reduced degradability of DM or NDF; applied onto dry grass, increased colonization compared to application onto wet grass	Feng et al. (1996)
Ruminal digestion and metabolism responses					
Cellulase and xylanase	Applied onto silage, or introduced into rumen (1.25, 3.5 or 5 L/t TMR)	Barley silage and barley	Sheep	No effects on digestion by either method as compared to control	McAllister et al. (1999)
Extract of *A. oryzae* with mutiple enzyme activities	Mixed with supplement and fed (3 g/d)	Alfalfa hay, bromegrass, supplement	Nonlactating cows	With bromegrass, but not alfalfa, EFE increased total bacteria; no effect on rate or extent of fibre digestion	Varel and Kreikemeier (1994)
Extract of *A. oryzae* with multiple enzyme activities	Mixed with supplement and topdressed onto TMR (90 g/d)	Alfalfa hay, barley, barley straw and molasses	Nonlactating cows	EFE increased digestibility of DM, protein and hemicellulose; rumen cellulolytic bacteria also increased	Weidmeier et al. (1987)
Cellulase and xylanase	Sprayed onto TMR (2.5 g/kg TMR)	Barley silage, barley grain, alfalfa hay	Lactating cows	No effect on feed intake, but increased microbial N in post-ruminal non-ammonia N fraction	Beauchemin et al. (1999)
Cellulase and xylanase	Applied onto forage 0 or 24 h prior to feeding; onto barley 0 h prior, or infused into rumen (1.65 mL/kg DM)	Grass hay, concentrate	Beef steers	No effect on intake of feed or NDF; applying onto forage increased digestibility of DM and NDF compared to control or infused enzymes	Lewis et al. (1996)
Extract of *A. oryzae* with multiple enzyme activities	Mixed with groundsorghum and topdressedonto TMR (3 g/d)	Alfalfa and milo	Nonlactating cows	With alfalfa hay, EFE increasedfibre digestion and rate of rumenfermentation; effects not observedwith milo or wheat straw	Gomez-Alarcon et al. (1990)
Cellulase and xylanase from *T. longibrachiatum*	Applied onto TMR or onto Concentrate (50 mg/kg TMR)	Corn silage; alfalfa hay; barley concentrate	Lactating cows; sheep	In cows EFE increased DMD when applied to concentrate but not to TMR; no effects observed in sheep	Yang et al. (2000)
Animal production responses					
Mixture of xylanase and β-glucanaseoriginating from *T. longibrachiatum*	Sprayed onto concentrate (0, 37.5 or 75 g/t DM)	Barley silage; barley grain	Feedlot cattle	EFE increased growth rate, but not feed intake or feed efficiency	Wang et al. (1999)
Xylanase and cellulase	Sprayed onto alfalfa hay during cubing (1 or 2 g/kg DM)	Alfalfa cubes; barley-based concentrate	Lactating cows	Applied at 2 g/kg DM, EFE increased milk yield; no effects on DMI or milk composition	Yang et al. (1999)
Mixture of fungal cellulase and xylanase	Applied onto silage during backgrounding; applied onto TMR during finishing (1.25, 3.5 or 5 L/t DM)	Barley grain; barley silage	Feedlot cattle	Quadratic increase in ADG during initial 56 d of backgrounding, but not overall (0 to 120 d); during finishing, EFE at 3.5 L/t DM only increased ADG	McAllister et al. (1999)
Enzymes (mainly xylanase) from *T. longibrachiatum*	Applied onto forages (2 or 5 L/t forage)	Corn silage; alfalfa hay; concentrate	Lactating cows	Applied at 2 L/t, EFE increased milk yield, but not milk composition	Kung, Jr. et al. (2000)
Cellulase and xylanase	Applied onto forage (0.7, 1.0 or 1.5 L/t)	Alfalfa hay; corn silage; concentrate	Lactating cows	Enzymes increased milk yield, milk fat and protein yield	Schingoethe et al. (1999)
Cellulase and xylanase from *T. longibrachiatum*	Applied onto TMR or onto concentrate (50 mg/kg TMR)	Corn silage; alfalfa hay and barley concentrate	Lactating cows	Milk yield increased by EFE applied onto concentrate, but not onto TMR; no effects on DMI or milk composition.	Yang et al. (2000)
Cellulase and xylanase	Applied to concentrate (1.4 L/t concentrate)	Barley and barley silage	Feedlot cattle	Average daily gain and feed efficiency were increased by EFE; no effect on DMI	Beauchemin et al. (1999)
Extract of *A. oryzae* with multiple enzyme activities	Mixed with supplement and topdressed onto TMR (1.5, 3 or 6 g/d)	Alfalfa hay; concentrate	Lactating cows	No effect on milk yield, milk composition or body weight change	Denigan et al. (1992)
Extract of *A. oryzae* with multiple enzyme activities	Mixed with supplement and topdressed onto TMR (3 g/d)	Alfalfa hay; steam flaked or rolled corn	Lactating cows	No effect on DMI, milk yield, or milk composition	Yu et al. (1997)

Table 4. Effect of exogenous fibrolytic enzyme on xylanase activity in fermenter effluent and in feed particle-associated (FPA) fractions in the Rusitec

Item	Treatment[1]				
	C	EF	AEF	EI	SEM
Xylanase activity [μg RS/(ml/min)][2]					
Effluent	0.95[b]	1.91[a]	0.79[b]	1.91[a]	0.159
FPA fraction [μg RS/(g DM/min)]					
24 h	79.7[b]	133.3[a]	73.9[b]	73.1[b]	7.25
48 h	134.2[b]	237.5[a]	144.1[b]	123.1[b]	7.60

[1]Xylanase concentrate applied at 1 mg/g DM. C: control (no enzyme); EF: enzyme applied to feed; AEF: autoclaved enzyme applied to feed; EI: enzyme infused with buffer.

[2]Expressed as release of reducing sugars (RS). In effluent, activity is expressed as [μg RS/(ml/min)]. On feed particles, activity is expressed as [μg RS/(g DM/min)].

[a,b] Within a row, means bearing different superscripts differ ($p<0.05$). (From Wang et al. 2001a)

EFE and ruminal microbes may compete for reaction sites on the substrate, and/or that some products of enzymatic action may have been inhibitory to colonization. Given that the EFE had limited capacity to cleave the esterified and etherified linkages (Wang et al., 2002a), PC-LCC matrix would also have accumulated on the surface of the feed particles during EFE hydrolysis, slowing down and eventually preventing the attachment of ruminal microbes. Limited hydrolysis prior to ruminal incubation, however, may promote microbial growth by increasing the availability of reducing sugars without the accumulation of substantial amounts of PC-LCC at the feed surface. It is unknown, however, what extent of pre-ruminal hydrolysis would be optimal for maximizing the efficacy of EFE in rumen microbial digestion.

Another speculation presented in the literature on EFE is that their efficacy when applied onto feed with low moisture content (dry feed) would be greater than when applied onto feed with high moisture (wet feed). The argument made is that high moisture feeds would have relatively lower binding capacity, so that EFE would be dissolved into ruminal fluid upon ingestion of the feedstuff.

Table 5. Pre-ruminal hydrolytic effects of spraying alfalfa hay and rolled barley grain with exogenous fibrolytic enzymes

	Treatment[1]			
	Control	EF	AEF	SEM
Rolled barley				
Organic matter (%)	97.28	97.26	97.10	0.939
Neutral detergent fibre (%)	29.13[a]	26.18[b]	29.06[a]	0.572
Reducing sugars (mg/g DM)	1.68[d]	5.58[c]	1.99[d]	0.352
Chopped alfalfa hay				
Organic matter (%)	92.15	91.39	91.42	0.817
Neutral detergent fibre (%)	54.53	52.67	54.07	0.785
Reducing sugars (mg/g DM)	33.96	37.91	36.19	2.683

[1]Treatments comprised spraying feedstuffs (100 mL/kg DM) with water (control), a 1% solution of crude xylanase preparation (EF), or the same solution after autoclaving (AEF).

[a,b] Within a row, values followed by different superscripts differ ($p<0.05$).

[c,d] Within a row, values followed by different superscripts differ ($p<0.01$).

This hypothesis has arisen mainly from studies involving application of EFE onto silages, mixed rations or concentrate. While silages certainly contain more moisture than concentrate, they also bear high numbers of aerobic microbes, which may play a significant role in determining the efficacy of enzymatic action as shown by Wang et al. (2002a).

To date, no studies have examined the effect of feed moisture per se on the efficacy of EFE. Feng et al. (1996) reported that EFE applied to dried grass exerted a greater effect than when applied to fresh or re-hydrated grass. However, all of the substrates in that study were dried prior to evaluation, thus the effects of water content were not assessed. The authors proposed that the lower efficacy of enzyme applied onto wet feed may have been due to reduced enzymatic activity caused by the drying process. It is clear that more studies are required to determine the significance of the moisture content of the diet on the efficacy of EFE.

Table 6. Effects of applying exogenous fibrolytic enzyme[1] to barley straw on dry matter loss and incorporation of ^{15}N into particle-associated microbial N (^{15}N-PAMN) during batch culture incubation

Substrate[2]	DM loss (%)			^{15}N-PAMN (μg/g DM)		
	4 h	12 h	48 h	4 h	12 h	48 h
Straw (S)	7.26	12.73	43.92	32.75	111.1	419.1
Straw+Enzyme (SE)	9.8	16.31	45.76	59.27	132.2	437.5
Prehydrolyzed SE (SE+P)	16.59	20.46	47.85	45.89	109.7	459.6
Washed SE+P	2.52	2.52	35.41	24.64	113.7	510.2
Washed SE+P plus AE	-0.08	4.5	36.32	28.79	125.6	504.5
Washed SE+P plus RS	2.25	7.93	38.12	47.36	150.0	498.8
SEM	0.2	0.233	0.88	0.88	3.91	3.9

[1]The exogenous fibrolytic enzyme (EFE) was a 1% (w/v) solution of a powdered preparation from *Trichoderma longibrachiatum*.

[2]Treatment SE was used in batch culture directly after enzyme treatment. Prehydrolysis comprised incubating the enzyme-treated straw at 39ºC for 24 h prior to batch culture. Washing was done to remove EFE and prehydrolysis products. Autoclaved (inactivated) enzyme (AE) was added back at the concentration applied to SE and SE+P. Reducing sugars (RS) were added back in amounts equivalent to those accumulating during the prehydrolysis period. (From Wang et al. 2002b).

Table 7. Summary of animal responses to exogenous fibrolytic enzyme added to different diets

Enzyme	Animals	Diets	Responses	Reference
Mixture of xylanase and β-glucanase from *Trichoderma longibrachiatum*	Feedlot cattle	High barley silage; low barley grain	Increased growth rate	Wang et al. (1999)
		Low barley silage; high barley grain	No effect on growth rate	
Mixture of xylanase and cellulase from *Trichoderma* spp.	Feedlot cattle	Alfalfa diet	Low to moderate levels of application increased growth rate	Beauchemin et al. (1995)
		Timothy diet	High level of application increased growth rate	
Extract of *Aspergillus oryzae*	Non-lactating beef cows	Bromegrass diet	Increased ruminal bacterial population; increased proportion of *Ruminococcus albus* in the cellulolytic bacteria	Varel and Kreikemeier (1994)
		Alfalfa hay diet	Above effects not observed	

Efficacy of EFE on different diets : There is ample evidence that EFE preparations exert dissimilar effects on different feed types, and that each preparation has specific activities towards different substrates (Hristov et al., 1996a; Wang et al., 2001a,b). This is understandable considering that these preparations are produced from monocultures on specific substrates. Logically, optimal activities would be expected to be expressed on substrates identical or chemically similar to those upon which the products were developed. These EFE-feed specificities have been observed not only with single substrates *in vitro* but also in total mixed rations, as shown in Table 7. The net effects of EFE on different feeds are influenced not only by the fitness of enzyme spectrum with the chemical structure of the substrate, but also by the reaction environment (e.g., ruminal pH) that arises from the diets themselves. Conditions of use are not typically specified for enzyme products marketed for ruminants. This may lead to misconceptions that an enzyme supplement might be equally effective with all ruminant diets, which is certainly not the case.

Considering the low fibre content of high concentrate diets, it is surprising that EFE have improved feed digestion (Krause et al., 1998) and performance of cattle fed high cereal grain diets (Beauchemin et al., 1997; Iwassa et al., 1997). An explanation of this phenomenon may come from comparing the pH optima of the fibrolytic enzymes produced by ruminal microorganisms with the pH optima of EFE produced by aerobic fungi. It is well documented that growth of fibrolytic bacteria is inhibited and fibre digestion is severely compromised when pH falls below 6.2 (Russell and Dombrowski, 1980; Hoover et al., 1984). Most of the fibrolytic enzymes produced by ruminal microorganisms function optimally at pH above 6.2 (Greve et al., 1984; Matte and Forsberg, 1992). In contrast, the pH optima of fibrolytic enzymes produced by aerobic fungi typically range from 4.0 to 6.0 (Gashe, 1992; Muzakhar et al., 1998). This point was illustrated by observations that the extent to which *T. longibrachiatum* enzymes enhanced gas production increased as the pH declined from 6.5 to 5.5 (Morgavi et al., unpublished data). Further, although reducing the pH from 6.5 to 5.5 decreased DM disappearance from corn silage in mixed ruminal cultures supplemented with *T. longibrachiatum* enzymes, the negative effect of low pH on DM disappearance was more pronounced in the absence of added enzyme. Ruminal pH can remain below 6.0 for a significant portion of the day in dairy cattle (Nocek, 1998; Yang et al., 1999) and in feedlot cattle (Krause et al., 1998). Under these conditions, EFE could make a meaningful contribution to ruminal fibre digestion. The higher fibre content of barley, as compared to corn, may explain why EFE improved feed conversion in finishing cattle fed barley grain but did not affect feed conversion by finishing cattle fed corn (Beauchemin et al., 1997).

The foregoing observations indicate that the mechanism of EFE in regulating fibre digestion in forage diets differs to that in concentrate diets. When forage diets are fed, ruminal pH is not normally low enough to limit cellulolytic activity, thus the fitness of enzyme-substrate specificities and the degree of enhancement of microbial digestion are the predominant indicators of enzyme effectiveness. With concentrate-based diets, however, cellulolytic activity is typically inhibited by low ruminal pH, thus pre-ruminal and ruminal hydrolytic effects of the EFE play a greater role in fibre digestion than for forage diets.

Improving efficacy of EFE in enhancing rumen microbial digestion: Although some EFE preparations have been observed to increase ruminal fermentation, the general trend is that only the rate, and not the extent, of the plant cell wall digestion is enhanced. The positive effects of EFE on animal productivity that have been reported are probably due more to the particular ruminal conditions arising from a certain diet (e.g., high grain diet/low ruminal pH) than to a generalized increase in cell wall digestion by EFE.

The ultimate goal of using EFE in ruminants is to increase cell wall digestion so that the animals can utilize low quality fibrous feedstuffs (e.g., straw). The literature suggests, however, that commercially available enzyme preparations still lack the novel activities that can overcome

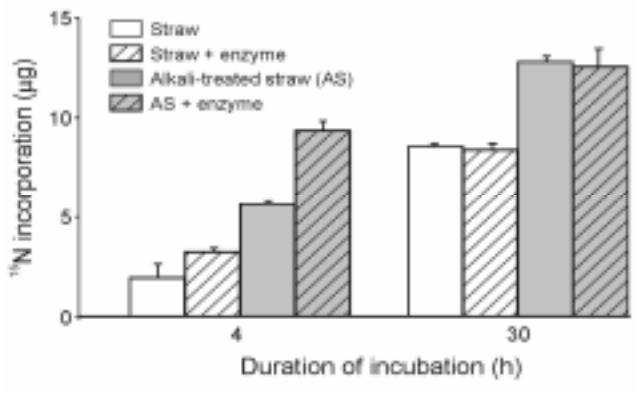

Figure 4. Incorporation of ^{15}N into microbial protein during *in vitro* incubation of native- or alkali-treated straw that had been sprayed (10 mL/100 g DM) with water (control) or with a 1.5% (w/v) solution of exogenous fibrolytic enzyme. Bars indicate standard error.

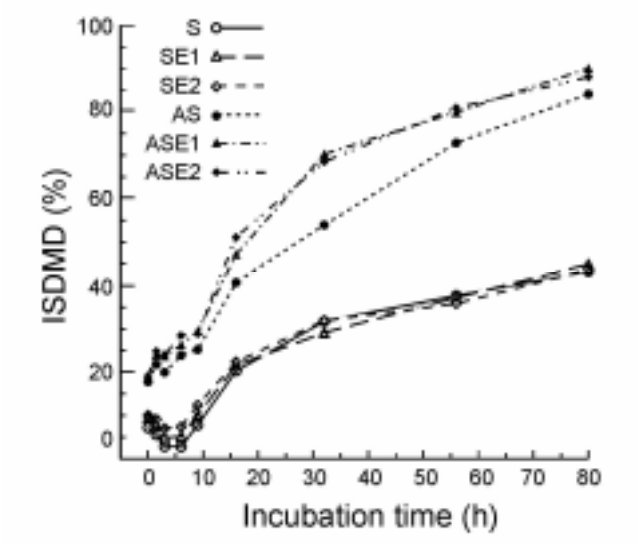

Figure 5. *In sacco* dry matter disappearance (ISDMD) from untreated (S; ○, △, ◊) or alkali-treated (AS; ●, ▲, ◆) wheat straw sprayed with water (control) or exogenous fibrolytic enzyme at 0.15 or 1.5 mg/g DM (E1 and E2, respectively) prior to 80 h of incubation in the rumen of a heifer.

the factors limiting ruminal digestion of plant cell walls. In the long run, identifying and producing such activities is vital to the success of the concept of using EFE to increase feed utilization for ruminants. In the meantime, however, certain measures can be taken to improve the efficacy of EFE currently marketed for ruminants.

Gould (1984) reported that hydrolysis of the insoluble fraction of wheat straw with cellulase from *T. reesei* subsequent to having treated the straw with alkaline peroxide yielded glucose with almost 100% efficiency, demonstrating the effectiveness of alkaline peroxide pre-treatment for enhancing enzymatic degradation of lignocellulosic crop residues. Similar increases in ruminal digestibility following alkaline treatment of straw were observed *in vitro* with some but not all EFE investigated by Ben-Ghedalia and Miron (1981). Applying enzymes directly onto alkaline pre-treated straw prior to consumption increased the efficacy of the EFE for enhancing ruminal digestion (Wang et al., 2002b).

The synergetic effects of alkali pretreatment and EFE are clearly related to increasing the availability of substrates for microbial protein synthesis (Figure 4). With the alkali pre-treatment, not only the rate but also the extent of the degradation of the straw was increased (Figure 5).

Research involving alkaline pretreatment and EFE has demonstrated that esterified bonds within the PC-LCC matrix represent a barrier common to both EFE and endogenous enzymes in the digestion of straw. Alkali treatment cleaves esterified bonds in the PC-LCC matrix which reduces the entrapment of cellulose and removes inhibitory compounds (PC) from feed particles. This improves access by microbial enzymes (Fahey et al., 1993) and increases adhesion and colonization by ruminal bacteria (Kerley et al., 1985). The synergetic effects of alkaline pretreatment and EFE are dependent on both the extent of pretreatment and the source of the EFE (Ben-Ghedalia and Miron, 1981; Wang et al., 2002b). Current findings suggest that future research into developing EFE as feed additives for ruminants ought to include screening enzyme preparations for high activity against esterified bonds rather than high activity against cellulose or hemicellulose. It should be noted, however, that this strategy (i.e., cleaving esterified bonds either by chemical or enzymatic action) may be less effective for increasing digestibility of cell wall fractions from dicotyledonous plants than from monocotyledonous plants, because ester bonds are less important in the LCC of dicots (Ben-Ghedalia et al., 1982).

Steam pre-treatment has also been investigated as a means of improving the hydrolytic efficacy of EFE (Grous et al., 1986; Poutanen et al., 1986; Liu and Ørskov, 2000). During a 24-h *in vitro* incubation, EFE increased gas production from steam-treated rice straw but not from untreated rice straw (Liu and Ørskov, 2000). Similar to Feng et al. (1996), however, all substrates in the Liu and Ørskov (2000) study were dried prior to incubation, so only the pre-ruminal hydrolytic effects, and not the effects of moisture from the steam, could be assessed. Cereal straw represents a huge potential feed source in Asian countries, and alkali treatments (e.g., ammoniation) have been adopted in many areas of this region. In those situations, EFE applied by techniques such as described by Wang et al. (2002b) would undoubtedly increase the feeding value of

these products. Further research is necessary to determine the most effective enzyme(s) and the optimal conditions of application for maximizing the efficacy of this treatment procedure.

CONCLUSION

Even though the ruminal ecosystem represents a sophisticated microbial community for attacking fibrous substrates, digestion of these feeds in the rumen is still less than desirable. Attempts to provide supplementary EFE to increase ruminal fibre digestion has produced mixed results. The variation of responses both *in vitro* and *in vivo* is due mainly to widely ranging enzyme sources, differences in application methods, and poorly defined enzyme-feed specificities. The general trend that EFE increase rate but not extent of fibre digestion suggests that currently available products are not introducing novel enzyme activity into the rumen. Recent research indicates that cleavage of the esterified bonds within the phenolic compound-mediated LCC matrix is key to effecting more complete digestion of cell wall polysaccharides. Thus, provision of some kind of esterase activity not currently included, measured or listed in most commercial enzyme preparations may be crucial to developing an effective enzyme additive for ruminants. In the long term, identifying such enzyme(s) and using bio-techniques to produce them are necessary. In the meantime, alkali pretreatment combined with EFE application could produce similar results.

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