EXOGENOUS FIBROLYTIC ENZYME OR ANHYDROUS AMMONIA EFFECTS ON THE NUTRITIVE VALUE, INTAKE, AND DIGESTION KINETICS OF BERMUDAGRASS AND THE GROWTH OF BEEF CATTLE

By

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To my dear parents, Luz and Marino

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADG	Average daily gain
ADICP	Acid detergent insoluble crude protein
ADL	Acid detergent lignin
AMN	Ammonia-treated bermudagrass hay
BW	Body weight
CBM	Carbohydrate binding molecule
CON	Untreated bermudagrass hay
CP	Crude protein
CSA	Cross sectional area
DM	Dry matter
DMI	Dry matter intake
EFE	Exogenous fibrolytic enzymes
ENZ	Enzyme-treated bermudagrass hay
EPI	Epidermis
IVDMD	In vitro dry matter digestibility
MES	Mesophyll
NAD-ME	Nicotinamide adenine dinucleotide – malic enzyme
NADP-ME	Nicotinamide adenine dinucleotide phosphate – malic enzyme
NCPAR	Non-chlorenchymatous parenchyma
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber digestibility
NDS	Neutral detergent solubles
NMR	Nuclear magnetic resonance

OM	Organic matter
PBS	Parenchyma bundle sheath
PEP-CK	Phosphoenolpyruvate carboxykinase
RI	Regrowth interval
RUBISCO	Ribulose biphosphate carboxylase/oxygenase
SCL	Sclerenchyma
SE	Standard error
VFA	Volatile fatty acid
VT	Vascular tissue
Wt	weight

Abstract of Thesis Presented to the Graduate School
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EXOGENOUS FIBROLYTIC ENZYME OR ANHYDROUS AMMONIA EFFECTS ON THE NUTRITIVE VALUE, INTAKE, AND DIGESTION KINETICS OF BERMUDAGRASS AND THE GROWTH OF BEEF CATTLE

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The objectives were to compare the effect of exogenous fibrolytic enzyme (Biocellulase A20) or anhydrous ammonia (4% DM) treatment on the nutritive value, voluntary intake, and digestion kinetics of Coastal bermudagrass [*Cynodon dactylon* (L.) Pers.] hay harvested at two regrowth intervals and to determine if feeding the treated hays improves the growth of beef cattle. In Experiment 1, 6 individually housed, ruminally-cannulated Brangus steers (BW 216 ± 6 kg) were used in an experiment with a 6 x 6 Latin square design with a 3 (additives) x 2 (regrowth intervals) factorial arrangement of treatments. Each period consisted of 14 days of adaptation, 7 days of digestibility measurements, 4 days of in situ degradability, 1 day of rumen rest and 1 day of rumen fluid fermentation measurements. Steers were fed hay ad libitum supplemented with sugar cane molasses and distillers grain at a rate that met maintenance energy requirements. Differences in prevailing weather conditions (e.g. temperature) during growth of the 5- and 13-wk hays were associated with greater fiber concentrations in the 5-wk hay. Ammonia treatment decreased most fiber fractions and increased the crude protein (CP) concentration particularly for the mature lignified 13-wk

hay. Enzyme treatment did not affect most nutritional components but slightly increased CP concentration. Enzyme application did not affect intake measures but ammoniation decreased intake. Ammoniation increased digestibility of DM, OM, NDF, hemicellulose ADF and cellulose across regrowth intervals, but reduced CP digestibility across regrowth intervals. Enzyme application increased NDF and hemicellulose digestibility of the 5-wk hay. Ammoniation increased the ruminal in situ DM degradation of the hay and ruminal ammonia concentration but enzyme treatment did not. In Experiment 2, 90 Angus and Brangus steers (308 ± 37 kg) were stratified by weight and randomly allocated to 18 1.01-ha pens containing bahiagrass pasture (*Paspalum notatum* Flügge). Three pens were assigned randomly to each treatment. The same hays and supplement fed in Experiment 1 were fed in Experiment 2. The treated hays from both regrowth intervals were fed in round-bale feeders to cattle in respective pens in quantities sufficient to ensure *ad libitum* access for 56 d. Steers were adapted to diets for 6 d, and full body weights were obtained on two consecutive days at the beginning (d 7 and 8) and end (d 50 and 51) of the measurement period. Weekly supplement allocations were fed in open troughs in equal amounts three times per week. Refused hay was weighed on d 30 and 49. Ammoniation increased hay DMI and tended to increase final BW and ADG. Enzyme treatment increased DMI of the 5-wk hay but had no effect on growth performance. In conclusion, ammoniation improved the nutritional composition and digestibility of the hays and resulted in a trend for increased growth. Enzyme treatment improved hay CP concentration, and improved the intake and NDF and hemicellulosedigestibility of the 5-wk hay but did not improve growth.

CHAPTER 1
INTRODUCTION

Forages are the major feed source for ruminant animals and they represent approximately 61% and 83% of the ration of dairy and beef cattle in the U.S., respectively (Barnes and Nelson, 2003). In tropical areas of the world, warm-season grasses make up around 85% of the feed supply for meat, milk, and fiber production (Coleman et al., 2004). In the southeastern of the U.S., warm-season grasses are the basis of livestock production (Pitman, 2007) and all beef cattle operations in Florida rely on forages as the primary source of nutrients (FASS, 2000).

During the growing season, plant maturity and environmental conditions such as elevated temperatures and prolonged drought stress, reduce the quality of warm-season grasses (Pitman and Holt, 1982). For instance, the higher temperatures at which warm-season grasses grow are associated with increased lignification and reduced tissue and cell wall degradability (Coleman et al., 2004). Because of these and other complex factors, warm-season grasses often have lower quality than cool-season grasses (Minson, 1980; Pitman, 2007).

Several methods have been developed to improve forage quality (Fahey et al., 1993; Berger et al., 1995) and anhydrous ammonia application is one of the most effective methods (Berger et al., 1995). Ammoniation has improved the forage quality of cool- (Wanapat et al., 1985; Flachowsky et al., 1996; Wang et al., 2004) and warm-season forages (Brown, 1988; Brown and Kunkle, 2003; Krueger et al., 2008) but it is not used widely because it is costly, potentially toxic and caustic (Rotz and Shinners, 2007). Recent research has focused on using exogenous fibrolytic enzymes (EFE) as a potential alternative to improve forage quality and animal performance (Beauchemin et

al., 2004; Adesogan, 2005). Supplementing dairy cow and feedlot cattle diets with EFE has improved cell wall digestion and animal performance (McAllister et al., 1999; Beauchemin et al., 2003; Arriola et al., 2007) and a few EFE have been used successfully to enhance the digestibility of warm-season grasses (Dean, 2005; Krueger et al., 2008). However, the results of EFE application on forage quality and animal performance have been equivocal (McAllister et al., 2001; Wang and McAllister, 2002; Adesogan, 2005). Thus, more research is needed to develop consistently effective enzyme products and improved application strategies. This research is needed particularly for intrinsically recalcitrant warm-season forages, since more attention has been focused on using EFE on cool-season forages. Krueger et al. (2008) reported that an EFE was more effective at improving the intake and digestibility of a 5-wk regrowth of bermudagrass hay when applied at cutting instead of at hay baling or feeding,. Limited information is available on whether EFE-induced increases in forage intake and digestion depend on forage regrowth interval. If EFE efficacy is independent of regrowth interval, application to higher-yielding mature forages may be advisable.

The objectives of this study were to evaluate the effect of applying an EFE enzyme or anhydrous ammonia to bermudagrass hay [*Cynodon dactylon* (L.) Pers.] on its nutritive value, voluntary intake, digestion kinetics and the growth of beef cattle.

CHAPTER 2
LITERATURE REVIEW

Warm-Season Grasses

Definition and Importance

Warm-season grasses are the backbone of livestock systems in much of the world and make up perhaps 85% of the feed supply for meat, milk and fiber production in warm-climate areas (Coleman et al., 2004). In the southeast of the United States and in the tropics, they are the basis of most livestock systems (Pitman, 2007). The optimum growth temperature of warm-season grasses is 35 to 38°C (Long, 1999) and they are characterized by the C_4 photosynthetic system (Moser et al., 2004).

Characteristics of C_4 Photosynthesis

In order to comprehend C_4 photosynthesis, it is necessary to understand the C_3 pathway, because the former is an adaptation of the latter (Moser et al., 2004). In the C_3 pathway, ribulose biphosphate carboxylase/oxygenase (rubisco) in mesophyll cells (MES) fixes CO_2 to the 5-carbon sugar, ribulose-1,5-biphosphate generating an unstable six-carbon compound that immediately forms two 3-carbon molecules of 3-phosphoglycerate, which are subsequently converted to glucose (Nelson and Cox, 2008) (Figure 2-1). However, rubisco can also fix O_2 and cause photorespiration because of its oxygenase activity (McAdam and Nelson, 2003). Photorespiration reduces the efficiency of C fixation during photosynthesis by 10 to 50% or more, depending on the prevailing temperature (McAdam and Nelson, 2003). Warm-season grasses possess unique anatomical features that reduce photorespiration and make the C_4 photosynthetic pathway more efficient. The most notable feature is the “Kranz anatomy” (Rudall, 2007), which has many vascular bundles surrounded by a

specialized, large, chloroplast-containing parenchyma bundle sheath (PBS; Rudall, 2007), which in turn is surrounded by a concentric layer of MES cells (Moore et al., 2004). The Kranz structure acts to inhibit photorespiratory CO_2 loss by maintaining high CO_2 concentrations in the bundle sheath cells, or by promoting refixation of evolved CO_2 as it diffuses outwardly through the MES cells (Kennedy, 1976).

In the C_4 pathway, CO_2 is fixed initially in the MES as four-carbon intermediates by PEP carboxylase, which has a greater affinity for CO_2 than rubisco. The four-carbon intermediates are then transported to the PBS where they are decarboxylated, releasing CO_2. This process prevents photorespiration and increases the concentration of CO_2 in the PBS, where the rest of the photosynthetic reactions occur (Moore et al., 2004). Consequently, photosynthesis is more efficient in C_4 grasses.

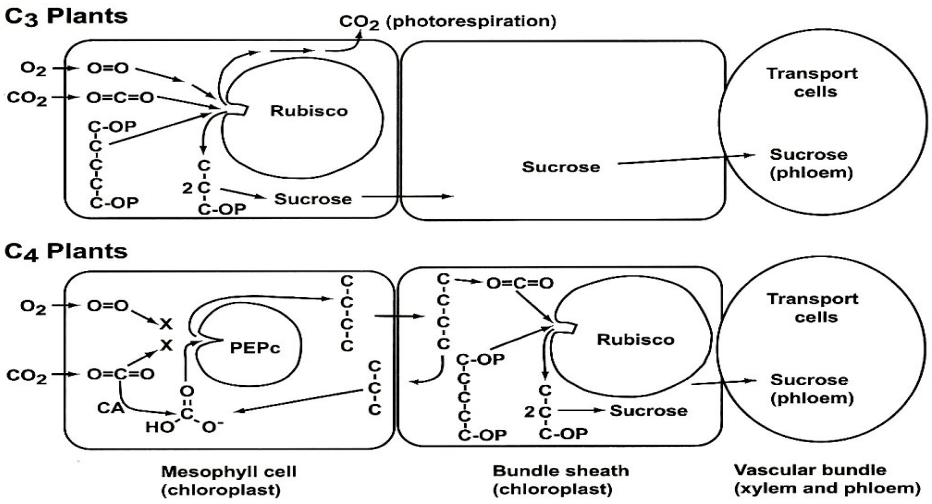

Figure 2-1. The C_3 and C_4 anatomy and photosynthetic pathway. (Volenec and Nelson, 2007) CA= Carbonic anhydrase, PEPc= Phosphoenolpyruvate carboxylase.

Subtypes of C_4 Photosynthesis

There are three subtypes of C_4 photosynthesis depending on the decarboxylating enzyme in the PBS and chloroplast structure and arrangement (Moser et al., 2004). The enzymes and associated subtypes include phosphoenolpyruvate carboxykinase

(PEP-CK), nicotinamide adenine dinucleotide – malic enzyme (NAD-ME) and nicotinamide adenine dinucleotide phosphate – malic enzyme (NADP-ME). Decarboxylation occurs in the cytoplasm in PEP-CK types and in the mitochondria in NAD-ME and NADP-ME types (Moore et al., 2004). Subtypes of C_4 grasses also vary in PBS anatomy and function, which influences their photosynthetic efficiency (Moore et al., 2004). From a photosynthetic and cell wall anatomy perspective, NADP-ME types seem to be more efficient, because their bundle sheath cells have a suberized lamella between the border of their secondary and primary wall (Hattersley and Browning, 1981). This suberized layer keeps the CO_2 inside the cell reducing its leakage, thus greatly reducing the risk of photorespiration, and consequently increasing efficiency (Moore et al., 2004). This group includes some of the most productive C_4 grasses such as corn (*Zea mays*), sorghum (*Sorghum bicolor*) and bahiagrass (*Paspalum notatum*). In contrast, NAD-ME types are more sensitive to O_2 and may be less efficient because their bundle sheath cells lack the suberized layer and leak more O_2 (Ehleringer and Pearcy, 1983). Examples of NAD-ME type are broomcorn (*Panicum miliaceum*) and bermudagrass (*Cynodon dactylon*). The PEP-CK subtype will not be discussed further because very few species in this group are used in agriculture.

Practical Implications of C_4 Photosynthesis

Water- and N-use efficiency. Warm-season grasses use water efficiently, requiring about one-third to one-half as much water to produce a unit of dry matter (DM) compared to C_3 grasses (Moser et al., 2004). This is because C_4 plants maintain high photosynthetic rates at lower rates of stomatal conductance than most C_3 plants, resulting in higher rates of carbon fixation per unit of water transpired (Ehleringer and Monson, 1993). Furthermore, C_4 grasses have a higher N-use efficiency than C_3

grasses (Wedin, 2004). High rates of leaf photosynthesis can occur in C_4 grasses with one-half or less of the leaf N required for the same photosynthetic rate by C_3 species (Brown, 1978). This is because C_4 plants use less rubisco than C_3 plants for the same photosynthetic rate due to their "Kranz Anatomy" and their special pathway (Wedin, 2004). Long (1991) estimated that at 30°C with normal atmospheric CO_2 concentrations, a C_4 leaf will need 13 - 20% of the rubisco found in C_3 leaves to maintain the same photosynthetic rate.

Nutritional quality. Warm-season grasses vary tremendously in forage quality depending on maturity, leaf:stem ratio, inherent nutritive value of leaves and stems and environmental factors during their growth (Moser et al., 2004). Nevertheless, they generally are of poorer quality than C_3 grasses. The C_4 grasses have lower protein concentrations compared to C_3 grasses because they have less rubisco and associated photosynthetic enzymes that can account for 50% of leaf N in C_3 grasses (Wedin, 2004). Minson (1980) also noted that CP concentrations of a large number of C_4 grasses averaged 4 - 6% less than that of C_3 species and concluded that occurrence of CP deficiency among livestock fed C_4 grasses is much greater. Moreover, C_4 grasses possess special anatomical features and cell wall components that limit their intake and digestibility.

Factors Affecting Digestibility of Forages

Impact of C_4 Grass Anatomy on Forage Digestibility

The anatomy of plant organs and their constituent tissues is important in ruminant nutrition because they affect critically the intake and digestibility of forages (Coleman et al., 2004). Firstly, they affect intake by affecting chewing and rumination time (Coleman et al., 2004), particle size reduction (Wilson and Kennedy, 1996), and passage rate

(Kennedy and Doyle, 1993). Secondly, the extent of lignification of plants largely determines their digestibility (Akin, 1989).

In most of the ruminant nutrition literature, nutritional value of forages is estimated from chemical composition. However, this review will focus on chemical and anatomical features because both influence forage quality. Anatomical features are particularly critical in C_4 grasses because the Kranz structure of their leaves has a detrimental impact on plant degradation (Akin et al., 2007).

Influence of organs and their tissue proportions

The digestibility of blades, sheaths and stems is associated with the relative proportion of tissue types in each organ with digestible or indigestible cell walls (Wilson, 1993; Wilson and Hatfield, 1997). However, focusing on tissue proportions alone ignores the impact on digestibility of intercellular air spaces or the thickness or degree of lignification of cell walls in the various tissues; consequently, tissue proportions may not explain smaller differences in vitro DM digestibility (IVDMD) between species or cultivars (Wilson, 1993).

Leaf Blade. Due to the anatomical structures associated with the C_4 photosynthetic pathway, C_4 grasses generally have significantly more vascular bundles in the leaf than C_3 grasses (Dengler et al., 1994). Consequently, there is a higher proportion of the less digestible, thick-walled, lignified tissues (PBS, sclerenchyma, and vascular tissue) in C_4 than C_3 grasses (Table 2-1). The NADP-ME type of C_4 grasses have lower proportions of PBS than PEP-CK and NAD-ME types (Dengler et al., 1994). Studies conducted to evaluate the impact of these anatomical differences between C_4 grass subtypes on digestibility have not been conclusive (Akin et al., 1983a; Wilson and Hattersley, 1989).

Table 2-1. Tissue proportions in organs of different forage types (From Wilson, 1993).

Cell Type	Proportion of tissue in cross sectional area (%)					
	C_4 grass (*Panicum maximum*)			C_3 grass (*Lolium multiflorum*)		
	Blade	Sheath	Stem	Blade	Sheath	Stem
Epidermis	22	4	2	23	NM	2
Mesophyll	31	7	2	66	86	2
PBS[1]	24	7	0	5	0	0
Sclerenchyma	2	6	8	1	10	12
NCPAR[2]	14	66	75	2	NM	75
Vascular tissue (without phloem)	6	9	12	3	4	9
Phloem	<1	1	1	<1	<1	<1

[1]PBS= parenchyma bundle sheath, [2]NCPAR= non-chlorenchymatous parenchyma. NM= not mentioned

Leaf Sheath. The anatomy of leaf sheaths is intermediate between those of the blade and stem, but more like that of the stem. Sheaths also have lignin concentrations between those of blades and stems and consequently their IVDMD falls between those of blades and stems (Wilman and Altimimi, 1982). Sheath tissue proportions do not appear to change with maturity (Cherney and Marten, 1982), but there are marked increases in wall thickness of lignified cells (Wilson, 1976) and a marked decrease in sheath IVDMD with maturity (37.2% for young vs. 55.8% for mature bermudagrass tissue, Akin et al., 1977).

Stem. Stems differ from leaf blades in that their tissue characteristics change greatly with maturity (Cherney and Marten, 1982) such that stem IVDMD can be similar to or greater than that of leaves when young, but lower than that of leaves when mature due to a faster rate of decline in IVDMD (Hacker and Minson, 1981). When the stem is young (Fig. 2-2) the vascular tissue (VT) is in isolated bundles but as it matures, the bundles link together through lignification of the interfascicular nonchlorenchymatous parenchyma cells (NCPAR), which form a strong, indigestible tissue (Wilson, 1993) .

Eventually, this tissue forms a ring that embraces the entire cortical region of sclerenchyma (SCL) and epidermis in most grasses and constitutes a powerful barrier to digestion (Wilson, 1993). For instance, the neutral detergent fiber (NDF) digestibility (NDFD) of *Sorghum bicolor* stem was 55% at the 12-leaf stage but it deceased to 30% at the mature grain maturity stage (Wilson and Hatfield, 1997).

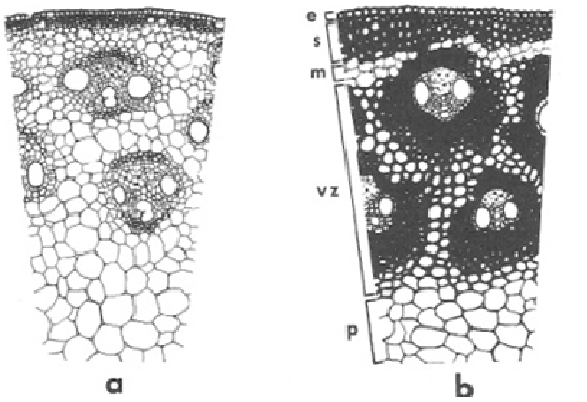

Figure 2-2. Cross sections of young (a, head emergence) and mature (b, grain maturity) stems of *Sorghum bicolor*. Solid black areas indicate lignification. e=epidermis, s=sclerenchyma, m=mesophyll, vz= vascular lignified zone. p= pith parenchyma. Modified from Wilson (1993).

Influence of tissue type on digestion

Epidermis (EPI). The epidermis is the outermost (dermal) cell layer in plants. It covers the entire plant surface (Rudall, 2007) and constitutes approximately 26% of the cross sectional area (CSA) of bermudagrass leaf blades (Table 2-2). The outer tangential walls of the EPI become thickened, lignified, and completely covered with cuticle with increased maturity (Akin, 1989; Wilson, 1993). The cuticle consists of complex waxes, cutin and phenolic compounds (Gevens and Nicholson, 2000); therefore, it is not usually degraded by ruminal microbes (Akin, 1989). Thus, the outer surface of the EPI is impervious to microbial digestion and penetration (Monson et al. 1972) except through stomata or breaks caused by prehension or chewing (Wilson,

1993). After 48 h of digestion in rumen fluid, the cuticle of bermudagrass and orchardgrass leaves were intact (Fig. 2-3). Other tissues were lost from orchardgrass leaves except SCL and lignified VT; whereas changes in bermudagrass leaves only included loss of MES and partial degradation of EPI and PBS.

Table 2-2. Percentage of tissue types in cross-sections (CS) of warm-and cool-season grass leaf blades from 4 to 8-wk-old plants. (Modified from Akin, 1989). Number of samples not stated.

	Tissue type						
Grass	Total vascular tissue	Lignified vascular tissue	PBS[1]	Phloem	EPI[2]	SCL[3]	MES[4]
Cynodon dactylon	37	5	28	4	26	10	27
C_4 grass mean[+]	22±8	4±2	15±7	2±1	35±10	6±3	38±9
C_3 grass Mean[+]	15±5	7±2	6±2	2±1	23±6	6±2	57±5

[1]PBS= parenchyma bundle sheath, [2]EPI= epidermis, [3]SCL= sclerenchyma, [4]MES= mesophyll
[+]Average value for several species.

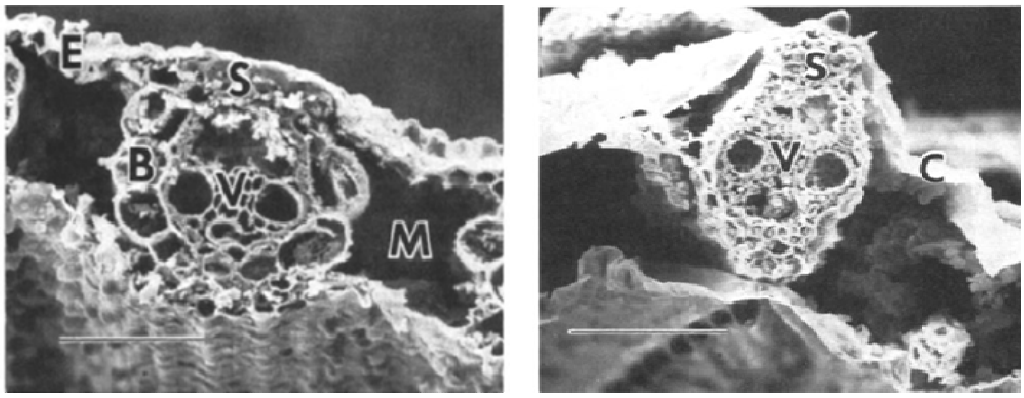

Figure 2-3. Scanning electron micrograph of bermudagrass (left) and orchardgrass (right) leaf blades incubated for 48 h in rumen fluid. M= mesophyll, E= epidermis, B= parenchyma bundle sheath, C= cuticle, S= sclerenchyma and V= vascular tissue. Modified from Akin (1989).

In most C_3 grasses, the EPI is lost from the leaf quickly during chewing and digestion because it is attached to MES cells, whereas in C_4 grasses the EPI is not lost readily (Akin et al., 1983a), because it is attached to the major and intermediate-sized vascular bundles through thick-walled SCL girder cells (Wilson, 1993). The epidermis is

not easily broken down in C_4 grasses because the walls of the adjacent long cells in paradermal view are linked together with strong “dove tailed” joints (Coleman et al., 2004). Consequently breakages must occur by splitting across walls rather than separation at the middle lamella (Wilson et al., 1989). The comparable joints in most C_3 grasses are straight sided, which appears to facilitate easy splitting along the middle lamella (Wilson et al., 1989).

The EPI in C_4 grasses can be a strong barrier to digestion depending on maturity and environment (Akin, 1989). Nevertheless, chemical treatments like sodium hydroxide and anhydrous ammonia can dissolve or crack the cuticle, respectively and potentially increase microbial colonization of cell walls and the rate and extent of digestion. Certain plant aerobic pathogenic fungi produce cutinases (Kolattukudy, 1985), which may degrade the cuticle and EPI, but this hypothesis is yet to be validated by research.

Mesophyll (MES). The MES comprises the chlorenchymatous tissue internal to the EPI in leaves (Rudall, 2007) and represents around 27% of leaf blade CSA tissue in bermudagrass (Akin, 1989). It is composed of thin-walled cells that form the main volume of tissue in all leaves but only a small part of the volume of stems (Wilson, 1993). In C_3 leaves, MES cells are arranged more loosely than in the C_4 grasses because significantly more intercellular spaces exist in C_3 (30%) than C_4 grasses (22%) (Dengler et al., 1994). This creates a larger surface for bacteria to attach and helps detachment of individual cells, which can aid rate of passage (Wilson, 1993). Mesophyll cell walls never lignify; therefore, they are one of the most rapidly digested cell types (Akin, 1989). For such cells, digestion is essentially completed in less than 12 h (Chesson et al., 1986). However, MES cells of C_4 grasses like bermudagrass are

digested more slowly than those of C_3 grasses partly because of the reasons mentioned previously but also because phenolic compounds are more abundant in C_4 grass MES cells of C_4 grasses (Akin, 1989).

Parenchyma Bundle Sheath (PBS). The PBS is a highly specialized group of chlorenchymatous cells surrounding the VT in leaves (PBS in C_3 grasses do not have chloroplasts). It is commonly called the “Kranz” sheath in C_4 grasses, along with the radial distribution of MES cells around the PBS (Moore et al., 2004). The PBS cells contain a high proportion of protein, because of their large chloroplasts, and starch and hence are a significant source of easily digestible substrates in C_4 grasses (Wilson, 1993). They represent around 28% of the leaf blade CSA tissue in bermudagrass (Akin, 1989). Secondary thickening occurs in PBS cell walls with increasing maturity, resulting in about five times the thickness of MES walls (Wilson, 1990). The degree of lignification of the PBS can vary with many factors (Akin, 1989) including stressful growth conditions. Akin et al. (1983b) reported that irrigated C_4 grasses had significantly lower digestibility than their non-irrigated counterparts, partially because of increased PBS lignification. They also reported that PBS digestion in *Panicum spp*. can be incomplete and is always slow, taking 48 to 72 h or longer. The NADP-ME sub-types of C_4 grasses have a suberized lamella in their PBS but NAD-ME types do not. It was hypothesized that this suberized lamella acts as a barrier to the digestible nutrient-rich cytosol contents of PBS since it cannot be digested by rumen microbes (Wilson and Hattersley, 1983). Akin et al. (1983a) evaluated the impact of the suberized layer on digestibility of the PBS among different C_4 grasses by visual estimation of tissue loss in cross-sections after incubation in rumen fluid. Interestingly, they reported that for a

group of *Panicum spp.*, the PBS walls of the NAD-ME grasses were more slowly digested than those of NADP-ME. However, subsequent comparison with a wider range of genera did not substantiate this conclusion (Wilson and Hattersley, 1989). Akin and Burdick (1977) highlighted the potential benefits of improving the rate of digestion of the PBS of bermudagrass. They stated that in order for bacteria to access the PBS cytosol, they would first have to degrade the PBS cell wall fast enough before it leaves the rumen. It takes 48 -72 h to digest the PBS (Akin et al., 1983a), which implies that they could be usually incompletely digested in high-producing cattle that have high passage rates.

Chemical treatments have been explored to improve digestion of the PBS in bermudagrass. After treatment with NaOH and incubation in rumen fluid, almost complete degradation of PBS was reported (Akin and Hartley, 1992). The main component of the suberized layer is suberin, which is similar to cutin in composition (Kolattukudy, 1985); therefore, cutinases (and suberinases) may also aid PBS degradation by degrading suberin.

Nonchlorenchymatous Parenchyma (NCPAR). Nonchlorenchymatous parenchyma cells are typically thin-walled and often polyhedral or variously shaped (Rudall, 2007). They can be quite large in size (40-140 μm) and variable in function (Rudall, 2007). Leaf NCPAR represents about 14% of the leaf blade tissue CSA in C_4 grasses but only 2% in C_3 grasses (Wilson, 1993). Nevertheless, they are thin-walled in leaves and usually are rapidly and almost entirely digested (Wilson et al., 1991).

In contrast, leaf sheath and stem NCPAR can be large contributors to the low digestibility of leaf sheath and stem, respectively. This problem is exacerbated by their

extensive presence in such tissues (66 and 75% of CSA tissue, respectively; Wilson, 1993), which reflects their large volume and their capacity to develop a thick secondary wall that can undergo lignification. The NCPAR cells form a solid tissue joined by a middle lamella with little intercellular space (Wilson, 1993). When stems are young, the NCPAR cells are easily digested and lost from stem sections; however, as stems mature, the NCPAR tissue becomes more lignified from the outer stem inwards, particularly for the cells between the vascular bundles in the main vascular ring (Akin, 1989; Wilson, 1993). Clearly, NCPAR have a pivotal role in limiting sheath and stem digestion. The stem is usually a key fraction that influences overall plant digestibility, because it comprises much of the weight and decreases rapidly in quality with maturity (Cherney and Marten, 1982). Since NCPAR comprises 75% of the tissue CSA in the stem, improving its digestion should be central in any attempt to improve forage digestibility. Therefore, more research should be conducted on improving the accessibility of enzymes and microbes to NCPAR.

Sclerenchyma (SCL). The sclerenchyma is the supporting or protective tissue categorized as fibers (long and narrow) and sclereids (variously shaped), which develop a thickened lignified wall with maturity (Rudall, 2007). The SCL represents10, 6 and 8% of blade, sheath and stem CSA tissue respectively, in C_4 grasses (Akin 1989). In grass blades and sheaths, SCL are more or less universally present as discrete patches above and below the vascular bundles, and at the leaf margins (Wilson, 1993). In the grass stem, they are similarly associated with vascular bundles as SCL caps, and may form a complete ring of tissue around the outside of the stem between the EPI and vascular tissue (Wilson, 1993). This ring could act as another protective “sheath” that

hinders access of enzymes and microbes to the inner NCPAR in the stem. Therefore, enhancing the degradation of the SCL would likely improve the intake and digestibility of the stem. The SCL also can have a critical impact on intake by slowing down particle size reduction because of their structural role in the plant (Rudall, 2007).

Vascular Tissue (VT). In this document, xylem and phloem tissue will be jointly referred as the VT. Phloem tissue consists of thin-walled cells and because they do not lignify, they are quickly digested (Akin, 1989). However, they form a small fraction of the tissue volume in leaves, sheaths, and stems (approximately 4% of leaf blade CSA tissue in bermudagrass; Akin, 1989). Xylem tissue consists of thick-walled cells that are heavily lignified whether in blades, sheaths, or stems. In these plant organs, xylem accounts for 6, 9 and 12% of CSA tissue of C_4 grasses, respectively and it is considered indigestible (Wilson, 1993). Most rumination activity is directed towards destruction of the VT structure and most of the undigested fiber particles appearing in feces are parts of the VT with attached or isolated SCL strands (Wilson, 1993). Thus, degradation of this structure is crucial to improve intake, digestion and passage of C_4 grasses.

Middle lamella: Intercellular component. The middle lamella is the layer between walls of neighboring cells (Rudall, 2007). The middle lamella-primary wall region is where lignification starts and is more concentrated (Jung and Allen, 1995); therefore, the middle lamella is the most powerful barrier to microbial degradation in recalcitrant tissues (Coleman et al., 2004). It cements cells together reducing exposure of the cell outer surface (Wilson, 1993). Consequently, cell wall digestion can only start from the less lignified lumen (Jung and Allen, 1995) after cells have been physically ruptured (Wilson, 1993).

Impact of the Forage Cell Walls on Digestibility

Chemical composition of forage cell walls

The major chemical components of cell walls are various structural polysaccharides and lignin (Theander and Westerlund, 1993). Protein, minerals, and lipids are minor components. Grasses also contain small but important amounts of hydroxycinnamic acids (ferulates and p-coumarates) (Hatfield et al., 2007). Structural polysaccharides can have complex molecular structures, yet if removed from the cell wall they are degraded readily to their component monosaccharides by microbial enzymes (Hatfield et al., 1999a). This section briefly describes such structural polysaccharides and then details their interaction in the cell wall matrix.

Cellulose. Cellulose is the most abundant structural polysaccharide in forage cell walls. It is a linear polymer of glucose linked by β 1,4 glycosidic bonds. It has a simple primary structure and a complex tertiary structure and the repeating unit is cellobiose (Bhat and Hazlewood, 2001). Individual cellulose molecules are extremely large and are arranged into bundles known as microfibrils (Nelson and Cox, 2008). Hydrogen bonds between cellulose molecules hold the microfibrils together (Nelson and Cox, 2008). In some regions the cellulose chains are highly ordered and strong hydrogen bonds hold them together in structures called crystallites, whereas loosely-arranged cellulose molecules form the amorphous regions (Bhat and Hazlewood, 2001). Pure cellulose is quickly and completely degraded ruminally as is cellulose cross linked to hemicelluloses alone (Hatfield et al., 1999a).

Hemicellulose. Hemicellulose is the second most abundant plant structural polysaccharide. It is present in association with cellulose in the walls of most plant species and can be extracted with alkalis from delignified walls (Bhat and Hazlewood,

2001). It is composed of a range of cell wall heteropolysaccharides different in component sugars and linkages (Hatfield et al., 2007). The most abundant of the heteropolysaccharides are the xylans, which are composed of a β-1,4-linked xylose backbone with branch substitutions (Hatfield et al., 2007). The type and frequency of branch substitutions varies with species and stage of development (Hatfield et al., 2007). Grass xylans have complex structures and contain substitutions of arabinose or glucuronic acid or both (Hatfield, 2007). In addition, some of the arabinose residues contain ferulic acid and to a much lesser extent p-coumaric acid ester linked to the C-5 hydroxyl group (Hartley, 1972). The ferulates can form linkages to one another, creating diferulate structures that crosslink arabinoxylan chains in grass cell walls (Ralph et al., 1994a). Several complementary enzymes are needed for hemicellulose degradation (Bhat and Hazlewood, 2001) and these are discussed later in the enzyme section. In the absence of lignin, intimate association of xylan and cellulose does not inhibit the rate of digestion of either polysaccharide in rumen fluid (Weimer et al., 2000).

Pectins. Pectins account for a very small fraction of grasses (10 μg/g or less of DM; Hatfield et al., 1999a) and they are rapidly and extensively degraded from cell wall matrices (Hatfield et al., 1999a). Due to their minor role in grass cell walls, they will not be discussed further.

Proteins. Proteins generally make up less than 5 μg/g of the grass cell wall depending on tissue type and maturity (Hatfield et al., 1999a). As with polysaccharides, proteins outside the wall matrix are susceptible to degradation, but those within the wall may be completely resistant to degradation and pass intact through the digestive tract.

Structural proteins like extensin, appear to play critical roles in cross-linking wall components, particularly in primary walls (Hatfield et al., 1999a).

Lignin. Lignin is defined as a group of polymeric natural products arising from an enzyme-initiated dehydrogenative polymerization of its primary precursors, coniferyl, sinapyl, and p-coumaryl alcohol (Sarkanen and Ludwig, 1971). Coniferyl and sinapyl alcohols are the most important in grasses (Hatfield et al., 2007). Peroxidases and/or oxidases react with lignin precursor alcohols to form lignin (Fig. 2-4). These reactions result in one-electron oxidized products that undergo radical coupling reactions to produce a growing polymer of lignin (Hatfield et al., 1999a). However, the strict chemical definition of lignin may not be compatible with the idea of lignin from a nutritional perspective, because other components can become incorporated into the "lignin" polymer (Hatfield et al. 1999a). Consequently, Hatfield et al. (1999a) proposed that lignin should be defined as a phenolic-derived macromolecule that interacts with other wall polymers to provide structural integrity, resistance to degradation, and water impermeability. Deleterious effects of lignin on digestibility result from its interaction with cell wall polymers (Hatfield et al., 1999a).

Hydroxycinnamic acids. The difunctional hydroxycinnamates (ferulic acid, *p*-coumaric acid and sinapic acid) are structurally related to lignin precursors and they may attach to lignin, playing critical roles in regulating wall matrix organization (Hatfield et al. 1999a). Hydroxycinnamic acids can cross-link polysaccharides with other polysaccharides or lignin (Fig 2-5; Ralph, 1996), and both of these result in decreased digestibility (Hatfield, 1993).

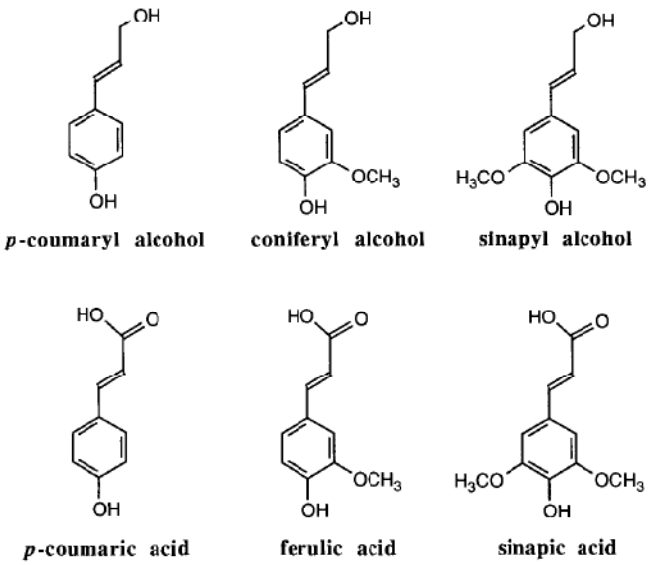

Figure 2-4. Chemical structural characteristics of typical lignin precursors (coniferyl, sinapyl and *p*-coumaryl alcohols) and hydroxycinnamic acids (ferulate, *p*-coumarate and sinapate) found in forage cell walls (Hatfield, 1999).

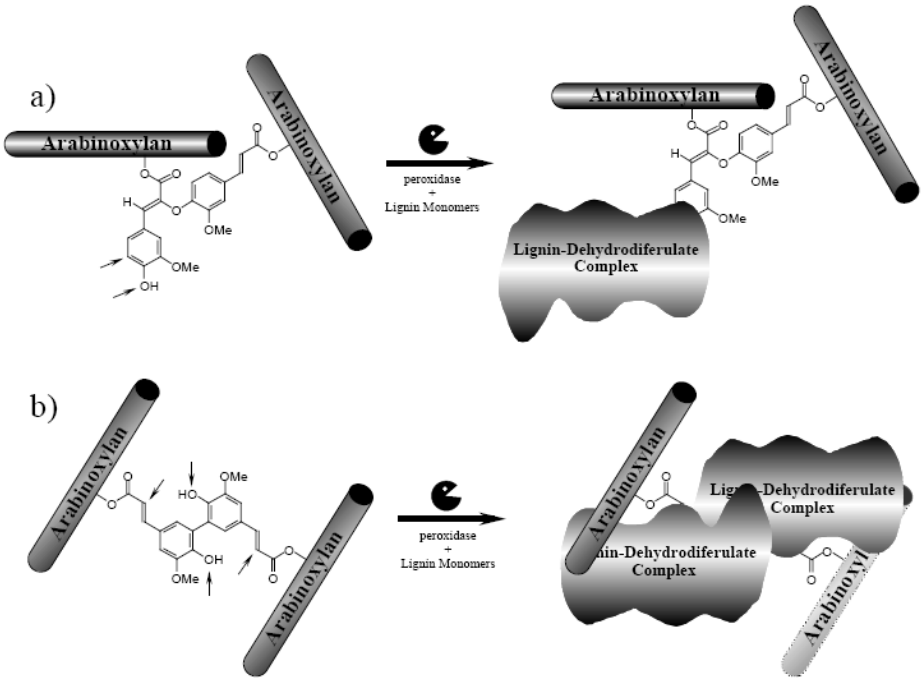

Figure 2-5. Diferulate cross-linking of arabinoxylan chains and incorporation into lignin via active mechanisms. Schematically shown are incorporation of a)the 8-O-4 diferulate and b) the 5-5 diferulate, which can produce a very highly cross-linked matrix. Lignin potential attachment points are signaled by arrows. Me= methyl group (Modified from Ralph, 1996)

Cell wall development and its impact on digestion

According to Terashima et al. (1993), the growth and development of the cell wall in plants can be divided into two phases:

Primary Wall Phase of Development. Primary wall growth is the phase when the plant cell is increasing in size through wall elongation (Jung and Allen, 1995). After plant cells have reached mature size, additional development of the primary wall or deposition of a secondary wall structure can occur (Hatfield et al., 2007). However, any additional material is deposited on the cell lumen side of the wall; thus, decreasing cytoplasmic space (Terashima, 1993) (Figure 2-6). Primary cell walls are composed of cellulose, hemicellulose (primarily xylans), pectin and small amounts of proteins at this stage (Hatfield, 1993). In grasses, hydroxycinnnamic acids are also present particularly ferulates and small amounts of p-coumaric acid; both are esterified to arabinoxylan polymers (He and Terashima, 1989; 1990). There is no deposition of lignin during this phase (Jung and Allen, 1995). For the few tissues that develop thickened primary cell walls, the additional wall material appears to be similar in composition to previously deposited primary cell wall (Jung and Engels, 2002). However, during the secondary stage of growth, the primary wall becomes the most lignified portion of the cell wall (Jung and Allen, 1995).

Secondary Wall Phase of Development. The process of secondary wall thickening starts after mature cell size has been attained. Polysaccharides added are richer in cellulose than xylans and pectin is no longer aggregated (Jung and Allen, 1995). Also, ferulates (Jung, 2003) and p-coumaric acids (Lam et al., 1992) are being continuously added. Lignin deposition starts at this phase in the middle lamella - primary wall region

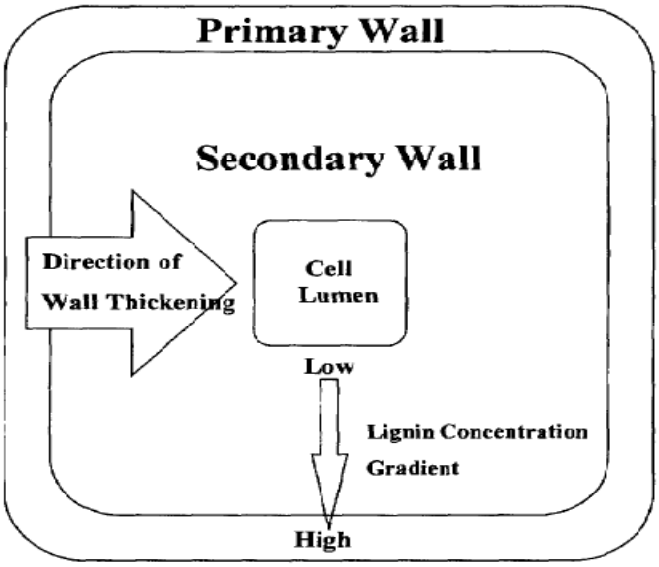

Figure 2-6. Schematic representation of a plant cell and wall development (Jung and Allen, 1995).

(Jung and Allen, 1995) and continues on the lumen side of the wall (Terashima et al., 1993). Because lignin deposition lags behind polysaccharide aggregation, the most recently deposited polysaccharides are not lignified; therefore, they are more digestible (Jung and Allen, 1995). In contrast, the middle lamella - primary wall region is the most intensely lignified and least digestible (Jung and Allen, 1995) region. This partly explains why microbes digest recalcitrant cells from the inside out (Grant, 2009).

Cross-linking mediated by hydroxycinnamic acids and its impact on digestion

Conclusive evidence of the cross-linking function of hydroxycinnamic acids was initially provided by Lam et al. (1991; 1992), who developed a technique to quantitatively distinguish hydroxycinammic acids that are ester linked, ether linked and both ester and ether linked. Hydroxycinnamic acids can form ester or ether bonds with lignin but only form ester bonds with polysaccharides (Ralph and Helm, 1993). Lam et al. (1991; 1992) reported that *p*-coumaric acid often is esterified to lignin, but also quite frequently involved in ether bonding; however, it did not appear to form cross-linked

structures with both ester and ether linkages. Whereas, ferulic acid was mostly both esterified and etherified and thus it was involved in cross-linkages between lignin and arabinose, though it occurred also in the ‘esterified only’ form. The fact that no ferulic acid was found in the ‘etherified only’ form suggests that only the pre-esterified form is incorporated to lignin via ether bonds to form the cross-link (Lam et al., 1992). In another study, Ralph et al. (1994b) showed that ferulate and *p*-coumarate molecules are esterified to arabinoxylan in grasses; however, the majority of *p*-coumarates are ester linked to lignin. In grasses, the mode of attachment seems to be similar across species, with the acid group esterified to the primary hydroxyl at the C-5 position when arabinose is in the furanose form. Mueller-Harvey et al. (1986) estimated that ferulic acid was substituted on 1 in 15 arabinose units in barley *(Hordeum vulgare* L.) straw. However, Ralph et al. (1995) mentioned that this level of substitution is based on extractable ferulates and it probably underestimates total feruloylation within the grass. Cross-linking of cell wall components has a marked influence on numerous wall properties such as accessibility, extensibility, plasticity, digestibility, and adherence (Hatfield et al., 1999b).

The formation of diferulates from ferulic acid monomers ester linked to arabinose in a polysaccharide chain is all that is required to covalently couple two polysaccharides, and this can occur in two ways (Hatfield et al., 1999b). The first one is a photochemical process, which produces cyclodimers and the second is by radical mediated dimerisation, which produces a range of dehydrodimers (Hatfield et al., 1999b). In the photochemical process, the plant has no control over the process but the radical-mediated dimerisation process involves sufficient control by the plant to optimize

wall cross linking (Hatfield et al., 1999b). Diferulate concentrations have been underestimated for many years due to limitations of analytical techniques and they could account for up to 70% of total ferulates present in grasses (Ralph, 1996). Cross-links mediated by diferulates between polysaccharides have a structural role in plants and depress the rate and possibly the extent of polysaccharide degradation (Grabber 1998b) (Figure 2-7).

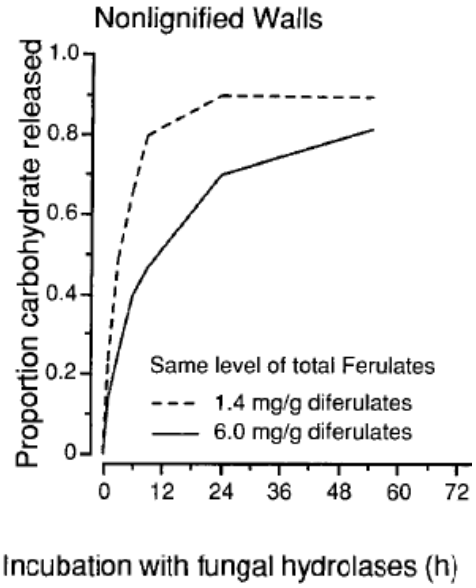

Figure 2-7. Impact of diferulate formation in nonlignified walls upon wall structural polysaccharide degradation by fungal hydrolases (Grabber 1998b)

Using nuclear magnetic resonance (NMR) spectrometry on ryegrass, Ralph et al. (1995) demonstrated that ferulates attached to C-5 of arabinose units do form covalent linkages to coniferyl and sinapyl alcohol residues in lignin. Moreover, they confirmed that ferulates bound to arabinoxylans could become covalently linked to monolignols by radical coupling reactions (active mechanism) besides the “commonly” accepted lignin-ferulate α-ethers (passive mechanism; Figure 2-8). This means that ferulates act as nucleation or initiation sites for the lignification process (Ralph et al., 1995) because

radical coupling to lignin can only occur if ferulates react with monolignols. Hatfield et al. (1999) suggested that the positioning of ferulates within the wall might regulate lignin formation patterns and control cross-linking within wall matrices. Consequently, controlling the level of total feruloylation should affect directly extent of cross-linking.

Except for β-ether, linkages arising from radical coupling reactions cannot be released by common solvents used for ferulate quantification (Ralph et al., 1995). This is because high-temperature alkaline hydrolysis only works for α- and β-ether structures, but many other structures are involved when radical coupling occurs (Ralph et al., 1995). Consequently, about 50% of total ferulates are not released from lignified walls during quantification, resulting in underestimation of cross linking (Grabber et al., 1995). Ralph et al. (1995) suggested that NMR is the only accurate method for their total quantification.

Grabber (2005) mentioned that each unit of lignin depressed cell wall degradability by two units in his model based on maize (*Zea mays* L.) cell walls. Lignin appears to act as a physical barrier to the microbial enzymes that degrade cell-wall polysaccharides (Jung and Deetz, 1993; Hatfield et al., 2007). Therefore, lignified forage tissues are only partially digestible, whereas non-lignified tissues of mature forages remain completely digestible (Jung and Engels, 2002). Rumen microbes cannot overcome this barrier to cell wall polysaccharide digestion because lignin cannot be degraded in the anaerobic rumen environment (Hatfield et al., 2007).

In the past, the composition of lignin was hypothesized to impact the degree to which cell wall digestion is limited by lignin (Jung and Deetz, 1993). However, recent reports indicate that lignin composition does not play a direct role in cell wall digestibility

(Grabber et al., 1998a; Jung et al., 1999). Apparently, the decrease in cell wall digestion associated with increased syringyl- to guaiacyl-type lignin observed previously (Jung and Deetz, 1993) reflects accumulation of poorly digested, lignified, secondary cell walls that are intrinsically higher in syringyl lignin concentration (Jung and Engels, 2002; Grabber, 2005).

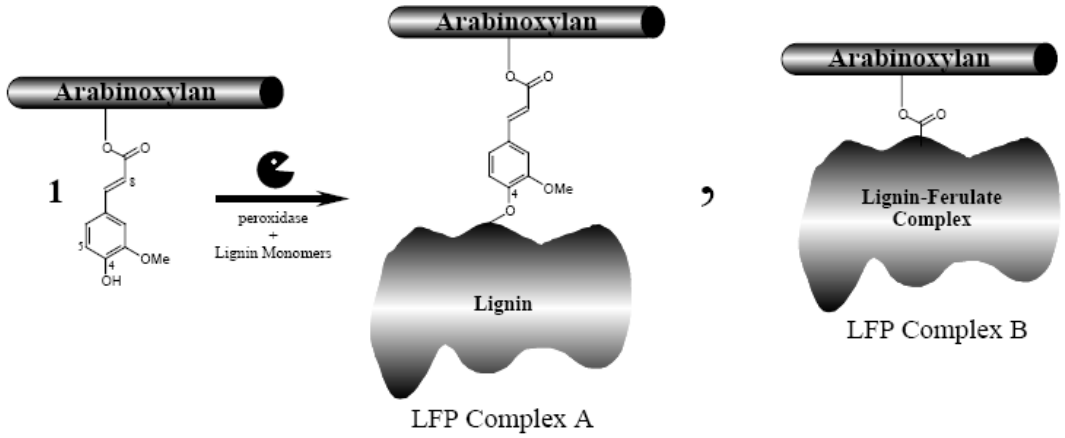

Figure 2-8. Attachment of ferulates to lignin by two distinct mechanisms: 'Passive' mechanism (LFP Complex A) and 'Active' radical-coupling mechanism (LFP Complex B; Ralph, 1996).

Jung and Allen (1995) suggested that ferulic acid esters of arabinoxylan alone (excluding the ether cross-linkage with lignin) could interfere with digestion of the polysaccharides they are esterified to by hindering the alignment of xylanase with its substrate, which is necessary for hydrolysis (Gorbacheva and Rodionova, 1977; Mitsuishi et al., 1988). Furthermore, Jung et al. (1991) demonstrated that the presence of hydroxycinnamic esters reduced polysaccharide digestion. When ferulates cross-link arabinoxylans and lignin via ester and ether linkages, respectively, the extent of digestion is reduced dramatically (Jung and Deetz, 1993; Grabber et al., 1998 a,b). Jung and Allen (1995) hypothesized that this happens because the ester portion of the

ferulate bridge is no longer available to be cleaved by enzymes since the lignin polymer is in such close proximity that ferulate esterase can no longer attach appropriately to its substrate. In addition, the authors mentioned that anaerobic cleavage of ether linkages is not known to occur. Hence, ferulate ethers accumulate in the residues remaining after forage digestion (D.R. Mertens and H.G. Jung, unpublished data cited by Jung and Allen, 1995).

Grabber et al. (1998 a,b) demonstrated that when lignin was added and cross-linked to a cell wall model based on maize cells, the rate and extent of cell wall degradation was depressed with a more pronounced impact on the arabinoxylans than on any other wall polysaccharide. This was clear proof of the impact of ferulate cross-linking of hemicelluloses to lignin on digestion. Casler and Jung (1999) reported that higher levels of ferulate cross-linking at the same lignin concentration reduced NDF digestibility of smooth bromegrass.

In summary, forages are very complex substrates for ruminal digestion. So far, this review has described impacts of different anatomical and chemical features on forage digestion. It is clear that lignification of the EPI of the whole plant, the PBS of the leaf, and the SCL ring of the stem impedes microbial degradation of the plant cell wall. In addition, though to a lesser extent, diferulate crosslinking of polysaccharides hinders cell wall degradation (Hatfield et al., 1999a). The rest of the review focuses on methods that have been used to attempt to improve cell wall digestion in order to enhance animal performance.

Methods to Improve Forage Nutritive Value and Intake

Many treatments have been applied to low quality forages to improve their nutritive value, voluntary intake and rate and extent of digestion in order to improve nutrient

availability to the ruminant animal (Berger et al., 1995). Detailed descriptions of such physical, chemical, and biological treatments were outlined by Fahey et al. (1993) and Berger et al. (1995). Among the existing methods, ammonia application is a proven technology to improve forage quality and is the most widely used chemical method in the US (Berger et al., 1995). Application of EFE is a more recent technology that holds promise for improving forage quality but avoids some problems associated with ammoniation. The rest of this review will summarize key effects of ammoniation on forage nutritive value and animal performance and discuss the underlying principles and efficacy of EFE application to forages.

Anhydrous Ammonia

Several studies have compared the efficacy of improving forage digestibility with ammonia and other alkaline compounds like NaOH, CaOH and urea. In a classical study, Wanapat et al. (1985) demonstrated that NaOH was the most effective treatment at improving digestibility of barley straw (*Hordeum vulgare*), followed by anhydrous ammonia. Similar results were obtained by Haddad et al. (1995) and Flachowsky et al. (1996) with wheat straw (*Triticum spp.*). Nevertheless, NaOH is less widely used than ammonia (Berger et al., 1995) because it exacerbates the N deficiency of low quality forages (Moss, 1990), places a high sodium load on the animal (Haddad et al., 1995), and is very dangerous to handle due to its caustic nature.

Anhydrous ammonia acts as an alkali after it dissolves in and deprotonates water and is transformed into ammonium hydroxide (Solomons and Fryhle, 2004). The hydroxide functional group saponifies ester bonds (Solomons and Fryhle, 2004) that bind lignin-crosslinked hydroxycinnamic acids to sugars on a hemicellulose chain. Consequently, ammoniation increases hemicellulose solubilization in forages (Berger et

al., 1995), leading to increases in the rate and extent of forage digestion (Brown, 1988; Wang et al., 2004). These factors culminate in increased intake, energy supply to and performance of animals fed ammoniated forages (Brown, 1988; Fahey et al. 1993; Flachowsky et al., 1996), particularly when such forages are mature and highly lignified (Fahey et al., 1993; Brown and Kunkle, 2003).

An additional benefit of ammoniation that is lacking with other forage treatment methods is the increased N concentration of the ammoniated forage (Berger et al., 1995; Sollenberger et al., 2004). This attribute is particularly important for low quality forages like warm-season grasses that have characteristically low protein concentrations (Brown and Kunkle, 2003). However, applying excessive levels of ammonia can depress intake due to the strong odor, and more ammonia than desired may be trapped in forages with moisture concentrations exceeding 25-30%, even at the 4% (DM basis) recommended rate (Brown and Kunkle, 2003). Consequently, ammonia should be applied only at 4% to forages with moisture concentrations at or below 25 - 30%.

Ammoniation improves the quality of lignified forages, but animal and human safety concerns have limited its use (Rotz and Shinners, 2007). It can be toxic to cattle when applied to forages with high soluble sugar concentrations, due to formation of 4-methyl imidazole. In addition, direct exposure of ammonia to humans can cause severe burns, blindness, and even death (Rotz and Shinners, 2007). For these reasons and others like the cost and availability of ammonia, other forage treatment methods are required (Adesogan, 2003).

Exogenous Fibrolytic Enzymes

Recent reductions in enzyme manufacturing costs along with more active and better defined enzyme preparations have stimulated researchers to re-examine the role of EFE in ruminant diets (McAllister et al., 2001). Some studies reported that adding EFE to diets of dairy and feedlot cattle has considerable potential to improve milk production and weight gain (Sanchez et al., 1996; Yang et al. 1999; McAllister et al., 1999; Krueger et al. 2008; Arriola et al., 2007); however, experimental results have been inconsistent (Luchini et al., 1997; Dhiman et al. 2002; Beauchemin et al. 2003; Yescas-Yescas et al., 2004). Wang and McAllister (2002) argued that much of the EFE research has been focused on animal responses to different commercial enzyme products, yet little attention has been paid to how they differ and the mechanisms of enzyme action. An additional, related problem is that commercial EFE products are marketed based on specified levels of activity of one or two key enzymes (typically xylanase and cellulase) without reference to others that could be present (McAllister et al., 2001). This implies that enzyme products with similar activities of main enzymes may have other enzyme activities that complement or hinder activity of the main enzymes. This presents a serious problem for interpreting and comparing results from enzyme studies in the literature (Wang and McAllister, 2002).

In most cases, enzyme activities supplied by commercial EFE products are not novel to the rumen. Therefore, EFE act on the same cell wall targets as endogenous enzymes (Wang and McAllister, 2002). This might explain why many EFE treatments have improved the rate but not the extent of plant cell wall digestion (Feng et al., 1996; Wang et al., 2004) and had equivocal effects on forage nutritive value and animal performance. The remainder of this review focuses on the science underlying fibrolytic

enzyme action and effects of EFE application on cell wall concentration, digestibility and animal performance.

Types of enzymatic activities

Cellulases. The term “cellulase” refers to a broad group consisting of many fibrolytic enzymes. Fig. 2-9 gives an overview of the successive action of some of such enzymes.

a) **Endoglucanases** (1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4))

Endoglucanases specifically cleave the internal β-1,4-glycosidic bonds of amorphous and swollen celluloses as well as cello-oligosaccharides but they are generally inactive towards crystalline cellulose and cellobiose (Bhat and Hazlewood, 2001). Microorganisms secrete multiple endoglucanases (I, II, III, IV, V) with a wide range of substrate specificities and thereby cause efficient hydrolysis of complex substrates (Bhat et al., 1990). End products of endoglucanase hydrolysis include oligosaccharides of various lengths (Lynd et al., 2002).

b) **Exoglucanases** (4-β-D-glucan glucanohydrolases (cellodextrinases, EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases, EC 3.2.1.91)

Exoglucanases act on the reducing or non-reducing ends of cellulose fibrils, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products (Lynd et al., 2002). Cellobiohydrolases are highly active on amorphous and swollen cellulose, but degrade poorly crystalline cellulose and cello-oligosaccharides (Wood and Bhat, 1988). These enzymes are specific for β-1,4-glycosidic bonds, but are inactive on cellobiose. There are two classes of cellobiohydrolases. Cellobiohydrolase I hydrolyses the cellulose chain preferentially from the reducing end whereas Cellobiohydrolase II attacks the chain from the non-reducing end (Bhat and Hazlewood,

2002). Glucanhydrolases only release glucose from the non-reducing end (Wood and McRae, 1982).

c) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21).

β-glucosidases can be classified as either aryl β-D-glucosidases (hydrolyzing aryl-β-D-glycosidases exclusively), cellobiases (hydrolyzing diglucosides and cellooligosaccharides) or β-glucosidases with broad substrate specificities (Bhat and Hazlewood, 2001). β-glucosidase sequentially removes one glucose unit from either the reducing end, the non-reducing end or both ends (Bhat and Hazlewood, 2001).

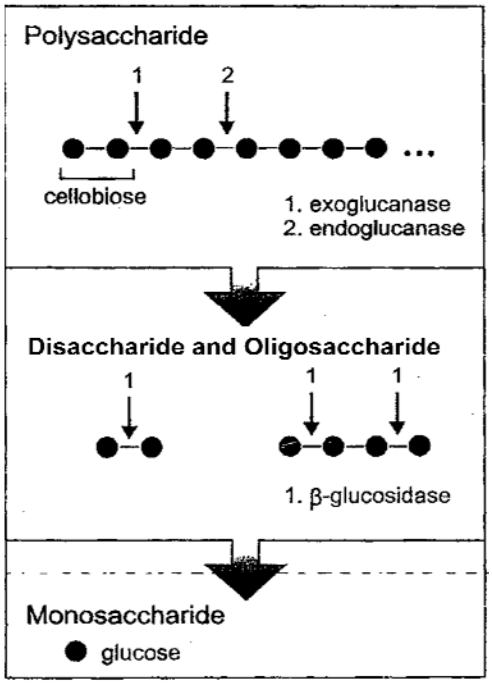

Figure 2-9. Schematic representation of the major enzymes involved in cellulose hydrolysis (Beauchemin et al., 2004). Black circles indicate glucose units.

Hemicellulases. "Hemicellulases" are composed of a myriad of enzymatic activities. A broad schematic representation of their action is shown in Fig. 2-10. Xylanases are specific for the internal β-1,4 linkages of polymeric xylan and are designated as endoxylanases (EC 3.2.1.8), which yield xylooligomers, and β-1,4

xylosidase (EC 3.2.1.37), which yields xylose (Bhat and Hazlewood, 2001). Most endoxylanases are specific for unsubstituted (not branched with acetic acid, glucuronic acid or arabinose) xylosidic linkages of xylans and release both substituted and unsubstituted xylo-oligosaccharides. In contrast, some endoxylanases are specific for xylosidic linkages adjacent to substituted groups in the main xylan chain (Bhat and Hazlewood, 2001). Other hemicellulase enzymes which degrade the side chains are β-mannosidase (E.C. 3.2.1.25), α-L-arabinofuranosidase (E.C. 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), α-D-galactosidase (EC 3.2.1.22), acetyl-xylan esterases (3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Beauchemin et al., 2004).

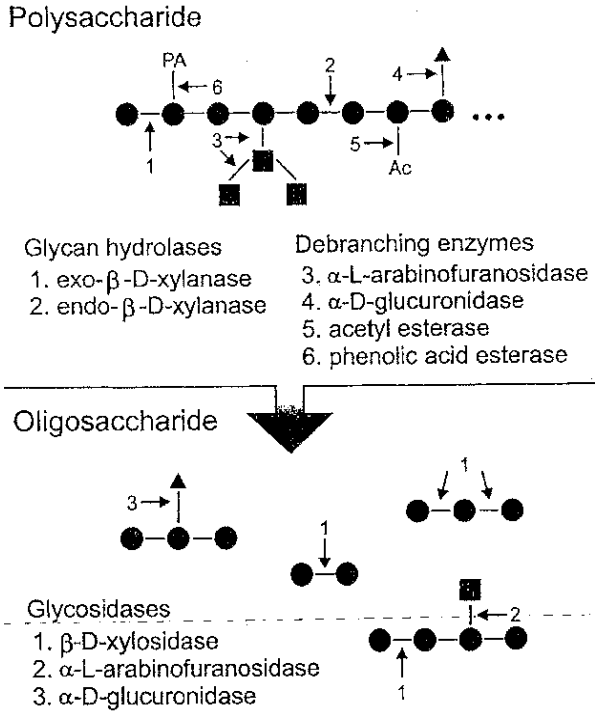

Figure 2-10. Schematic representation of the hemicellulose enzymes involved in the degradation of arabinoxylan (Beauchemin et al., 2004). PA= phenolic acid, Ac= acetic acid, triangle= glucoronic acid, square= arabinose.

Synergy between enzymes

Synergism between cellulases. Cellulase enzyme systems exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism. Five forms of synergism have been reported (Lynd et al., 2002):

- Endo-exo synergy between endoglucanases and exoglucanases,
- Exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains,
- Synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of exoglucanases, and
- Endo-endo synergy between different types of endoglucanases (Klyosov, 1990)
- Intramolecular synergy between catalytic domains and carbohydrate -binding molecules

Bhat and Hazlewood (2001) speculated that the exo-exo synergism was due to their ability to expose new hydrolysis sites to each other, as well as their ability to act from reducing and non-reducing ends (Barr et al., 1996). The presence of endoglucanases with different substrate specificities (I, II, III, IV, V) would increase the synergistic efficiency further, as shown in Fig. 2-11. Wood (1992) suggested that the five endoglucanases of *T. reesei* are primarily responsible for decreasing the degree of polymerization by internally cleaving cellulose chains at relatively amorphous regions, thereby generating new cellulose chain ends susceptible to the action of cellobiohydrolases (Teeri et al., 1998).

Synergy between hemicellulases. Efficient and complete hydrolysis of xylan requires the synergistic action of main and side-chain enzymes with different

specificities (Coughlan et al., 1993). Two types of synergy between such enzymes have been described in vivo (Coughlan et al., 1993):

- Homeosynergy: This occurs between two or more different types of side-chain-cleaving enzymes or between two or more types of main-chain cleaving enzymes. (e.g., ferulic acid esterase and α-L-arabinofuranosidase; endoxylanases and β-xylosidases)

- Heterosynergy: This occurs between side-chain and main chain cleaving enzymes (e.g., ferulic acid esterases and endoxylanases).

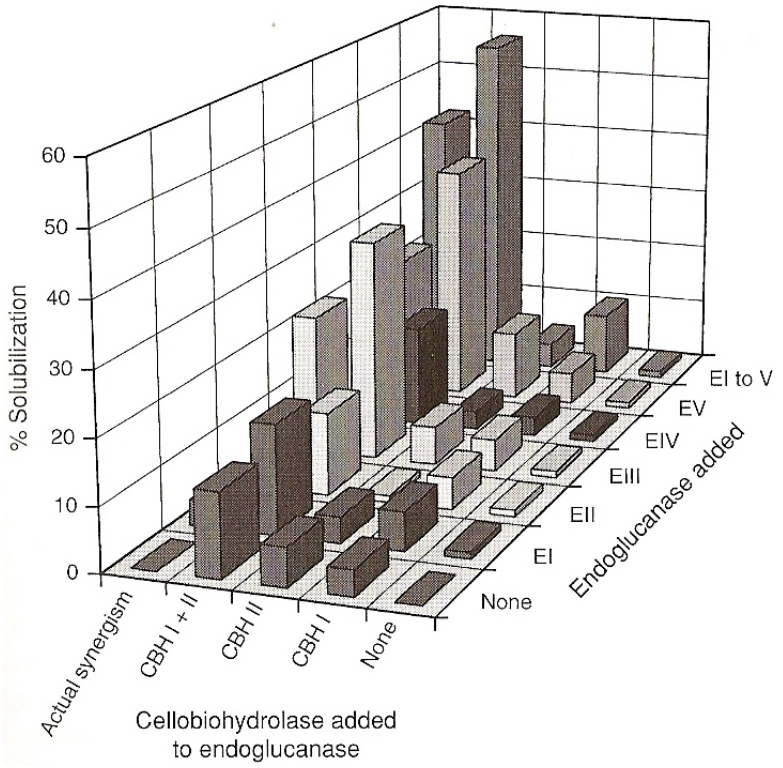

Figure 2-11. Synergism between *Penicillium pinophilum* cellobiohydrolase (I and II) and endoglucanases (EI to EV) in solubilizing cotton fiber (Bhat and Hazlewood, 2001).

Attachment of enzymes

Carbohydrate-binding molecules (CBMs) are non-catalytic structures that have been found in most cellulases. The CBM affects binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate (Lynd et al., 2002). The presence of CBMs is particularly

important for the initiation and activity of exoglucanases (Teeri et al., 1998). Cellulases that adsorbed to a greater extent increased the rate and extent of crystalline cellulose hydrolysis when compared with cellulases that adsorbed less strongly (Klyosov, 1990).

Product inhibition

As with most enzymes, high concentrations of the hydrolysis products of cellulases and xylanases often inhibit their action. Endoglucanases (Bhat et al., 1989) and most cellobiohydrolases are inhibited by cellobiose (Wood and McCrae, 1986). However, concentrations of glucose up to 100 mM had little effect on many cellobiohydrolases and endoglucanases (Bhat et al., 1989). Likewise, xylanases and endoxylanases are believed to be inhibited by high concentrations of xylobiose, but not by xylose (Bhat and Hazleewood, 2001). In contrast, β-glucosidases are inhibited by glucose and other mono- and disaccharides (Bhat et al., 1993). These inhibitions suggest strongly that different enzymes, each with sufficiently high activity levels are needed to ensure unimpeded and thorough degradation of plant cell walls. The challenge for researchers is to formulate products with ideal proportions of each type of enzyme to overcome product inhibition and ensure enzyme efficacy.

Cellulose degradation in nature

An understanding of mechanisms of cellulose degradation in nature is essential to appreciate differences between degradation capabilities of EFE and ruminal fibrolytic enzymes. There is a distinct difference in the strategy for degrading cellulose among cellulolytic microorganisms, depending on whether they are aerobes or anaerobes (Lynd et al., 2002). Most EFE are produced by aerobes whereas, ruminally - produced fibrolytic enzymes are from anaerobes (Lynd et al., 2002).

Complexed cellulase systems (Cellulose degradation by anaerobes). In this system, the majority of cellulolytic anaerobes (bacteria and fungi) do not release extracellular cellulase, instead their complexed cellulases are localized directly on the surface of the cell or the cell-glycocalyx matrix, which is called the cellulosome (McAllister et al., 1994; Fig. 2-12). Most cellulolytic anaerobes including those in the rumen grow optimally on cellulose when attached to the substrate, and this contact appears to be necessary for catalysis (Wang and McAllister, 2002). Due to their anaerobic metabolism, such microbes have low yields of enzymes compared to aerobes; consequently, production of EFE from aerobes is preferred in the industry (Bhat and Hazlewood, 2001). In addition, such low yields partly explain why supplemental EFE complement the activity of ruminal fibrolytic enzymes despite similarities in the types of enzymes involved.

Lynd et al. (2002) hypothesized that anaerobic bacteria develop cellulosomes to degrade cellulosic material because they cannot effectively penetrate cellulosic material and also because their anaerobic metabolism requires them to be as efficient as possible in enzyme synthesis. The cellulosome seems to allow cooperative enzyme activity in close proximity to the bacterial cell, enabling optimum synergism between the cellulases presented on the cellulosome (Lynd et al., 2002). At the same time, it minimizes the distance over which hydrolysis products must diffuse, allowing efficient uptake of oligosaccharide products by the microbes (Bayer et al., 1994). According to Lynn et al. (2002), cellulosomes are efficient at hydrolyzing microcrystalline cellulose because they have adequate ratios of enzymatic activities that optimize synergism and their structure spaces enzymes and CBMs in a manner that favors synergism.

Many rumen bacteria including *Ruminococcus* species (Ding et al., 2001) and *Fibrobacter succinogenes* (Miron and Forsberg, 1999) seem to produce cellulosomes. There is evidence indicating that anaerobic fungi also utilize cellulosomes for hydrolysis of crystalline cellulose (Hazlewood and Gilbert, 1998). Inhibition of adhesion of microbes to cellulose or detachment of microbes already adherent can reduce or completely prevent cellulose utilization by ruminal microbes (Weimer et al., 1993).

Non-complexed cellulase systems (cellulose degradation by aerobes). Aerobic cellulolytic bacteria and fungi produce high cell yields characteristic of aerobic respiratory growth (Bhat and Hazlewood, 2001). Aerobic cellulose degraders utilize cellulose through production of substantial amounts of extracellular cellulase enzymes that can be recovered from culture supernatants (Schwarz, 2001).

While many aerobic bacteria adhere to cellulose, physical contact between microbes and cellulose does not appear to be necessary for their cellulolytic action (Lynd et al., 2002). Cellulolytic filamentous fungi and actinomycete bacteria can penetrate plant cell walls through hyphal extensions, thus presenting their cellulase systems in close proximity to the substrate in confined cavities, even if they are not strictly adhering (Eriksson et al., 1990). The production of “free standing” cellulases may therefore suffice for efficient hydrolysis of cellulose under these conditions because loss of enzymes and hydrolytic products due to diffusion is likely to be limited (Lynd et al., 2002). In contrast, when enzymes from aerobic organisms are sprayed on forages or added to diets, they may be less effective because they cannot cross anatomical barriers to the digestible cell wall components. More research is needed in this area to understand how to optimize the interaction of EFE with dietary substrates.

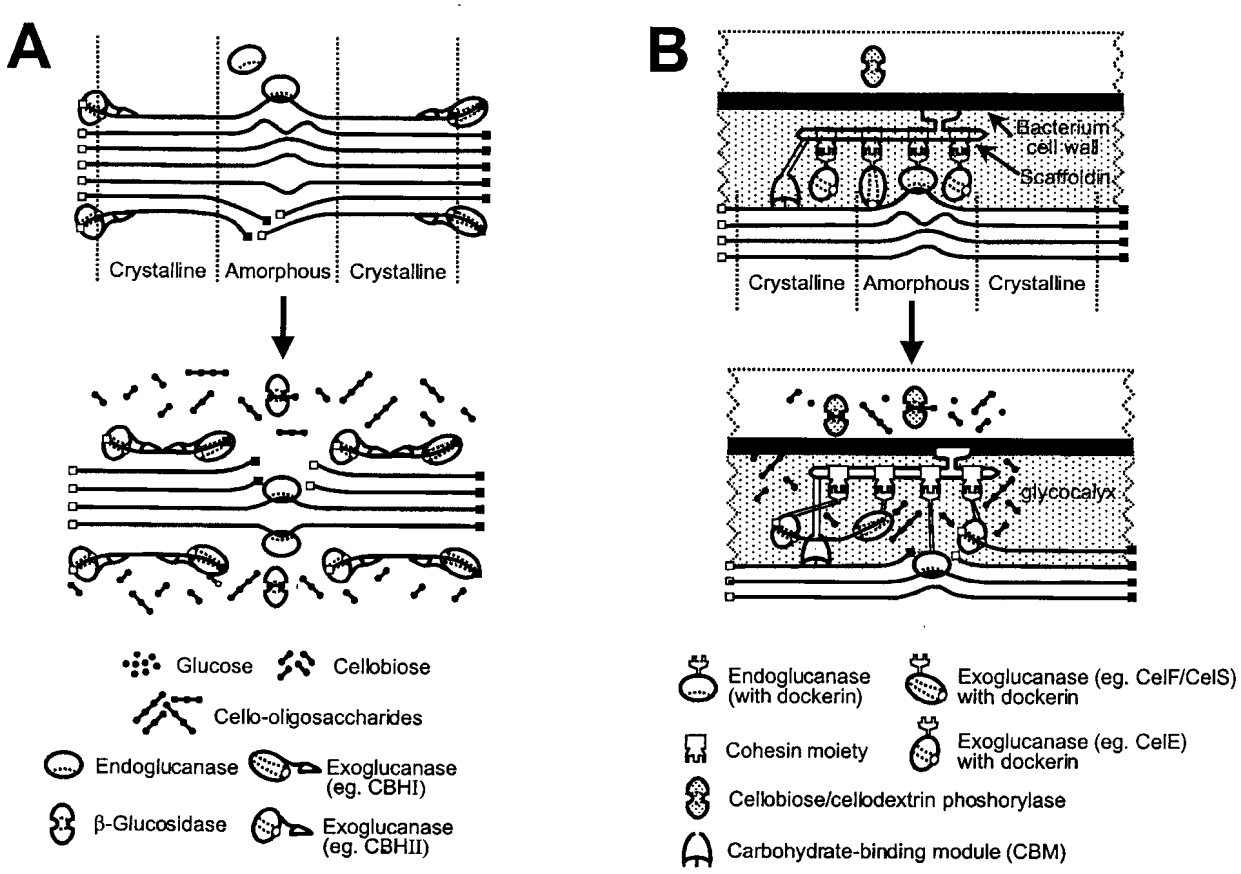

Figure 2 -12. Hydrolysis of amorphous and microcrystalline cellulose by non-complexed (A) and complexed (B) cellulase systems. The solid squares on the right extremes of cellulose chains represent reducing ends, and the open squares on the left extremes represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale. From Lynd et al. (2002)

Exogenous fibrolytic enzymes in ruminant diets

This section initially describes the mode of action of EFE in ruminant diets, then discusses the non-enzymatic factors affecting the response to EFE addition to forages and diets fed to ruminants.

Mode of action of EFE in ruminant diets. McAllister et al. (2001) divides the mode of action of EFE in ruminant diets as follows:

a) Pre-consumption effects

Application of EFE to ruminant diets has caused pre-consumptive effects, particularly the release of soluble carbohydrates (Hristov et al., 1996; Nsereko et al., 2000) due to partial solubilization of NDF and ADF (Gwayumba and Christensen, 1997; Krause et al., 1998; Nsereko et al., 2000; Krueger, 2007) but this effect is not always observed (Krueger et al., 2008). This response most likely depends on the degree of lignification of the forage and the type of enzyme used. Nsereko et al. (2000) demonstrated compelling evidence that applying enzymes to feed causes structural changes that make feed more amenable to further degradation. In their trial, EFE application (Multifect Xylanase, experimental preparation and Sumizyme X, Monsanto Co., St. Louis, MO) improved alfalfa hay (*Medicago sativa*) digestibility in rumen fluid even when substrates were autoclaved and washed to remove enzyme residues and hydrolysis products, leaving only structural changes as the possible explanation for the digestibility improvement. McAllister et al. (2001) reported that EFE application caused appearance of “digestive pits” in cell walls of barley straw though the application rates tested were greater than those recommended by the manufacturer. Collectively, these studies demonstrate that EFE exert pre-ingestive effects that enhance cell wall utilization.

b) Ruminal effects

Most of the improvements in forage quality resulting from EFE application are attributable to ruminal effects (Beauchemin et al., 2003). In the rumen, EFE are generally more stable than previously thought (Hristov et al., 1998; Morgavi et al., 2000a, 2001). Application of enzymes to feeds prior to ingestion enhances the

adhesion of the enzyme to the substrate, which increases the resistance of the enzymes to proteolysis and prolongs their residence time within the rumen (McAllister et al., 2001).There are two mechanisms by which EFE influence ruminal fiber utilization:

a.1) Direct hydrolysis

The fact that EFE remain active in the rumen raises the possibility that they may improve digestion through direct hydrolysis of ingested feed within the rumen (Rode et al., 2001). Dong (1998 cited by McAllister et al., 2001) noted that applying an EFE to a grass hay diet for sheep increased endoglucanase activity in ruminal fluid, but this activity accounted for 0.5% of the total endoglucanase activity in the rumen. This suggests that addition of EFE hardly affected the hydrolytic capacity conferred by endoglucanase in the rumen but the author acknowledged problems with his sampling procedure for particle-associated activity. More work is needed to substantiate the latter report and to quantify improvements in digestion that are entirely due to direct cell wall hydrolysis in the rumen by added EFE.

a.2) Synergism with rumen microbes

Synergism between EFE and rumen microbes has enhanced ruminal fiber digestion (Morgavi et al., 2000c). Adding exogenous enzymes to the diet increases the hydrolytic capacity within the rumen mainly due to increased bacterial attachment (Yang et al., 1999; Morgavi et al., 2000b; Wang et al., 2001) and stimulation of rumen microbial populations (Wang et al., 2001; Nsereko et al., 2002). These responses may have occurred because nutrients released by initial enzyme action attracted bacteria to the digestion site, stimulating further microbial degradation (McAllister et al., 1994).

The model described above is widely accepted. However, Krueger et al. (2008) reported that EFE improved forage digestion even when applied to the harvested hay several months before it was fed. In that study, the activity of EFE on the stored hay was not evaluated. Therefore, it is not clear if the improvement was caused before or during ingestion and ruminal digestion of the hay. Perplexingly, applying the same EFE to the hay at feeding did not improve digestibility. Nevertheless, the success of the harvest time application holds promise for EFE use in production systems where adding EFE at feeding is impractical.

Non-enzymatic factors affecting efficiency of EFE. Many factors influence EFE effects on ruminant diets, and the most important of these will be discussed in this section.

a) Manufacturing process

Exogenous fibrolytic enzymes are produced by a batch fermentation process, mostly with aerobic fungi or bacteria (Cowan, 1994). Once the fermentation is over, EFE are separated from fermentation residues and the source organism (Beauchemin et al., 2004) by chemical and physical processes (Gashe, 1992). The types and activity of EFE produced depend on the strain selected, the growth substrate and culture conditions (Considine and Coughlan, 1989). Batch to batch variations in enzyme activity may arise from changes in factors that influence the fermentation process including substrate moisture content, culture depth, O_2 and CO_2 concentrations, and prevailing temperature and pH (Considine and Coughlan, 1989).

Morgavi et al. (2000a; 2001) reported that in some cases carriers and stabilizers added during manufacturing increased the survival of EFE in the rumen. van de Vyver

et al. (2004) and Adesogan (2005) also mentioned that co-factors and natural or artificially-induced enzyme glycolysation are important for ensuring ruminal stability and function of EFE. These factors also may be important determinants of how long EFE can survive outdoors, especially when applied to feeds stored for long periods before they are fed.

b) Influence of pH and temperature

Most commercial EFE products that have been evaluated in ruminant diets were originally designed for non-feed applications (Bhat and Hazlewood, 2001). This partially explains why the pH and temperature optima of most such enzyme products are 4 - 5 and 60°C, respectively (Beauchemin et al., 2004). In general, fungal cellulases are optimally active between pH 4.0 - 6.0 (Wood, 1985). Endoglucanases, cellobiohydrolases, xylanases, and glucosidases from mesophilic fungi are optimally active between 40-55°C (Bhat et al., 1989; Coughlan et al., 1993). In addition, most xylanases have low acidic pH optima (Wong et al., 1988). Ruminal conditions are often very different from these optima, with a relatively constant temperature of 39°C and a pH approximating 6.0 (Van Soest, 1994). Therefore, many commercial EFE have suboptimal enzymatic activities when analyzed under ruminal conditions or added to ruminant diets (Kung et al., 2002; Vicini et al., 2003). Attempts have been made to standardize methods of determining enzyme activity (Colombatto and Beauchemin, 2003); however, they are based on using pure substrates. Therefore, they do not accurately indicate how much of plant substrate will be degraded by the EFE (Beauchemin et al., 2004). More research is needed to develop realistic cell wall models to analyze activities of EFE that will be added to ruminant feeds.

c) Specificity to the substrate

Enzyme-substrate specificity is a widely-accepted phenomenon in the literature (Nelson and Cox, 2008). On a fundamental level, the complexity of plant cell walls complicates targeting EFE to such substrates. Ideally, each component of the cell wall (e.g.cellulose, hemicelluloses, etc.) should be targeted by one or more enzymes but this is difficult because the concentration of each cell wall component varies with tissue type; moreover, the proportions of each tissue vary with plant species and growth conditions. For instance an EFE treatment (Xylanase B, Biovance Tech. Inc., Omaha, NE, and Spezyme CP, Genencor, Rochester, NY, combination) improved ADG of alfalfa and timothy hay, but had no similar effect on barley silage (Beauchemin et al., 1995). Also, EFE that were most effective at improving the 18-h DMD of alfalfa hay differed from those that were most effective on corn silage (Colombatto et al., 2003). These and other studies indicate strong enzyme-feed specificity. Lignin and its crosslinking to hemicelluloses can conceivably obscure the specificity of EFE to plant cell walls because it prevents enzyme accessibility to digestible polysaccharides(Wang et al., 2004). Pretreatments with enzymes from aerobes like β-etherases, ligninases, laccases, glucose oxidase or chemicals may be required for proper hydrolysis of such substrates.

d) Influence of the animal

Animal factors like low ruminal pH and high passage rates that compromise ruminal fiber digestion are common in high-producing early-lactation dairy cows. Such cows are typically in negative energy balance and therefore need all the energy that they can assimilate to cope with milk production. In these critical situations where fiber digestion is depressed and energy requirement is greatest, response to added EFE is

usually greatest (Beauchemin et al., 2004). Greater intake, body-weight gain and milk yield responses to EFE treatments have been reported in early-lactation cows compared to those from their mid- to late-lactation counterparts (Schingoethe et al., 1999; Knowlton et al., 2002). Consequently, Zheng et al. (2000) recommended dosing with EFE soon after parturition. In summary, it seems that currently available EFE are only capable of improving digestibility and animal performance in situations when normal fiber digestion is compromised. Therefore, research on more effective EFE is needed to facilitate their use in production situations where digestion of fiber is not compromised by animal factors, but by forage factors like lignification.

Method of providing EFE. The effects of EFE will be influenced by the different forms of providing EFE as described next.

a) Time of application

Little or no time is required for EFE to attach to substrates (Beauchemin et al., 2004). Lewis et al. (1996) reported no difference in response to applying EFE to diets immediately before feeding or 24-h earlier. Some in vitro studies support this report (Colombatto, 2000), but others revealed that a 24-h EFE-pretreatment period was advantageous for improving forage digestibility (Krueger, 2007). Therefore, more research is needed in this area.

b) Method of EFE application

Spraying in liquid form on the feed is a more effective way of applying EFE than adding the powder form (Beauchemin et al., 2004). Infusing EFE in the rumen was not effective compared to spraying EFE on the feed (Lewis et al., 1996). Beauchemin et al. (2004) stated that close association between the substrate and EFE is needed for EFE

to bind strongly enough and to prevent its removal and destruction by proteolytic activity in the rumen. Consequently, spraying is the most widely used method of applying EFE.

A related question is which fraction of the diet should be treated with the EFE. Based on their higher cell wall concentration, the forage component should be the target; however, contradictory reports exist in the literature. Digestibility and milk production were improved when EFE was applied to the concentrate fraction instead of the TMR (Yang et al., 2000). Others found no difference between applying EFE to dry forage or dry forage plus concentrate (Yang et al., 1999; Dean, 2005). Such counterintuitive results may reflect the presence of non-fibrolytic activities in the EFE.

c) Rate of enzyme supplementation

An enzyme product that has the ideal enzymatic activities for the target substrate can be ineffective if applied in excessive or insufficient amounts (Sanchez et al., 1996; Beauchemin et al., 2004). Kung et al. (2000) reported that cows fed a low level of enzyme tended ($P < 0.10$) to produce more milk than those fed a high level of enzyme. Others have mentioned that in vivo responses to increasing levels of dietary EFE are typically non-linear (Beauchemin, 2004). A possible explanation was given by Nsereko et al. (2002), who documented a quadratic response in total bacterial numbers in ruminal fluid with increasing levels of enzyme application. These authors speculated that application of a moderate level of EFE to ruminant feeds caused some beneficial disruption of the surface structure of the feed either before or after ingestion. Whereas the high EFE rate decreased this benefit because excess EFE attached to the feed and thereby restricted microbial adhesion and digestion. Adesogan (2005) proposed an

alternative explanation that negative feedback inhibition of enzymes by hydrolysis products may explain poorer efficacy at high application rates.

Summary. This first half of this review discussed the C_4 photosynthetic pathway of warm-season grasses, and the chemical and anatomical features that limit the digestibility of such forages by ruminants. The second half summarized the benefits and challenges of improving the quality of such forages with ammonia and subsequently discussed the mode of action of EFE and factors that have led to inconsistent effects of EFE on forage quality and animal performance. Most of the studies on EFE application to feeds involved addition of EFE to diets fed to dairy cows or feedlot cattle. Little attention has been paid to using EFE to improve the performance of beef cattle fed warm-season grasses in cow-calf production systems that are common in the Southeast. Therefore the objective of this study was to evaluate the effect of applying an exogenous fibrolytic enzyme or anhydrous ammonia to bermudagrass hay [*Cynodon dactylon* (L.) Pers.] on its nutritive value, voluntary intake, digestion kinetics and the growth of beef cattle.

CHAPTER 3
EXOGENOUS FIBROLYTIC ENZYME OR ANHYDROUS AMMONIA EFFECTS ON THE NUTRITIVE VALUE, INTAKE, AND DIGESTION KINETICS OF BERMUDAGRASS AND THE GROWTH OF BEEF CATTLE

Introduction

Forages are the major feed source for ruminant animals and they represent approximately 61 and 83% of the ration of dairy and beef cattle in the US, respectively (Barnes and Nelson, 2003). In the southeastern US warm-season grasses are the basis of livestock production (Pitman, 2007). For Florida in particular, all beef cattle operations rely on forages as the primary source of nutrients (FASS, 2000).

Warm-season grasses have lower nutritional value than cool-season grasses because they are less digestible and contain less CP (Minson, 1980). Several methods have been proposed to improve forage quality (Fahey et al., 1993) and anhydrous ammonia application is one of the most effective (Berger et al., 1995). Ammoniation has improved the nutritive value of cool- (Wanapat et al., 1985; Flachowsky et al., 1996; Wang et al., 2004) and warm-season forages (Brown, 1988; Brown and Kunkle, 2003; Krueger et al., 2008) but it is not widely used because of its cost, potential toxicity and caustic nature (Rotz and Shinners, 2007). Recent research has focused on using exogenous fibrolytic enzymes (EFE) to improve forage nutritive value and animal performance. Supplementing dairy cow and feedlot cattle diets with EFE has improved cell wall digestion and animal performance (McAllister et al., 1999; Beauchemin et al., 2003; Arriola et al., 2007). Some EFE also have been used to enhance the digestibility of warm-season grasses (Dean, 2005; Krueger et al., 2008). However, results of EFE on forage nutritive value and animal performance have been equivocal (McAllister et al., 2001; Wang and McAllister, 2002; Adesogan, 2005). Thus, more research is needed to

develop consistently effective enzyme products and improved application strategies, particularly for warm-season forages. Krueger et al. (2008) reported that when applied at cutting instead of at hay baling or feeding, an EFE was more effective at improving the intake and digestibility of a 5-wk regrowth of bermudagrass hay. Limited information is available on whether EFE-induced increases in forage intake and digestion depend on forage regrowth interval. If EFE efficacy is independent of regrowth interval, application to higher-yielding mature forages would be advisable.

The first objective of this study was to evaluate the effect of applying an EFE or anhydrous ammonia on the nutritive value, voluntary intake, and digestion kinetics of bermudagrass [*Cynodon dactylon* (L.) Pers.) hay harvested at two regrowth intervals. A second objective was to determine the effect of the treatments on the growth of beef cattle. The first hypothesis was that application of anhydrous ammonia and EFE would improve the nutritive value and voluntary intake of bermudagrass hay and the growth of beef cattle. The second hypothesis was that ammoniation would be more effective in the more mature bermudagrass, whereas the EFE would be more effective in the less mature bermudagrass.

Materials and Methods

Forage Treatments

An established stand of Coastal bermudagrass (*Cynodon dactylon* [L.] Pers) owned by a local hay producer in Alachua County, Florida was staged in August, 2007 by mowing to a 4-cm stubble and removing the residue. The field subsequently was fertilized with N (69 kg / ha) and the grass in each half of the field was allowed to regrow for 5 or 13 wk concurrently such that the respective harvest dates were 28 September and 17 November,, 2007. On each harvest date, the grass was mowed in 1 d to a 4-cm

stubble with a New Holland 617 mower conditioner (New Holland North America, New Holland, PA). Average precipitation (mm), temperature (°C) and solar radiation (watts/m^2) in Alachua during the growth periods were obtained from University of Florida Forage Research Unit weather database (FAWN, 2007) and are shown in Figure 3-1. Mowed forage in two of every three windrows was untreated (CON) and the third windrow was sprayed (10 g/ton) with an exogenous fibrolytic enzyme product (ENZ; Biocellulase A20, Loders Croklaan, Channahon, IL) using a tractor-mounted 57-L continuous flow sprayer (FIMCO, North Sioux, SD) fitted with a three-nozzle boom. The enzyme had previously increased the in vitro digestibility of bermudagrass (Dean, 2005) and intake and in vivo digestibility of bermudagrass in beef cattle (Krueger et al., 2008),. Endoglucanase and xylanase activities of the enzyme were determined under ruminal conditions (pH 6 and 39°C), according to Colombatto and Beauchemin (2003), were 71.3 nmol of glucose released/min per mg and 206.5 nmol of xylose released/min per mg of enzyme powder, respectively. Hays were stored in round bales in a covered shed.

For ammoniation, untreated round bales were stacked on each other, covered with 0.15-mm plastic, and treated with anhydrous ammonia (4% of DM) as described by Brown (1988) and Brown and Kunkle (2003). The forage was allowed to react with the ammonia for 6 wk and then vented to release ammonia gas.

Experiment 1

Cattle and diets

The Institutional Animal Care and Use Committee of the University of Florida approved the animal protocol for this experiment. Six yearling, ruminally cannulated Brangus steers (216 ± 6 kg of BW) were dewormed with Ivomec (Merial, Duluth, GA)

and assigned to six treatments arranged in a 3 (additives) x 2 (regrowth intervals) factorial arrangement in a experiment with a 6 x 6 Latin square design. Each of the six, 27-d periods consisted of a 14-d adaptation period, followed by 7-d of voluntary intake and digestibility measurements (total feces collection method), 4-d of in situ degradability, 1-d without measurements and 1-d of rumen parameters. Steers were housed in individual 4 x 12 m pens in an open-sided barn equipped with continuous lighting.

After storage for 4 mo, each hay bale was chopped to 15-cm lengths using a tub hay grinder (Roto Grind, model 760, Burrows enterprises, Greely, CO). Steers were fed 110% of the previous day's intake to allow ad libitum intake at 1000 and 1600 h. A supplement formulated to meet the maintenance energy needs of a 250-kg steer gaining 0.58 kg/d (NRC, 2000) was fed in a separate container at 0900 h. The supplement consisted of 2 kg of sugar cane molasses (DM = 83%; CP = 11.7%), 0.8 kg of dried distillers grains with solubles (NDF= 37.3%; CP = 30%) and 0.06 kg of a mineral - vitamin mix (Lakeland Animal Nutrition, Lakeland, FL) containing 21% NaCl, 13% Ca, 6% P, 1% Mg, 0.95% Zn, 0.8% K, 0.4% S, 0.4% Fe, 0.22% Mn, 0.2% Cu, 800 mg/kg of Fe, 200 mg/kg of Co, 175 mg/kg of I, 48 mg/kg of Se, 45,454 IU /kg of vitamin A and 9,091 IU /kg of vitamin D_3 (DM basis). Feed grade urea (Southeastern Minerals Inc., Bainbridge, GA) containing 46% N was added and mixed (0.03 kg/hd/d) with the supplements of steers fed CON and ENZ hays to minimize the risk of N deficiency in the rumen.

Sampling and analysis

Feces were collected using fecal bags twice a day (0900 and 1700h) during the 7-d digestibility trial. A subsample was taken after weighing and stored. Daily samples of

hay, supplements, refusals, and feces taken during the 7-d digestibility trial collection period were analyzed for DM (48 h at 60°C). Dried samples were composited by steer within each period, ground to pass a 1-mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) and analyzed for ash (600°C at 2 h, AOAC, 2000), NDF and ADF (Van Soest, 1991) using an ANKOM 200 Fiber Analyzer (ANKOM, Macedon, NY). Amylase was used for NDF analysis but sodium sulfite was not used. Hays also were analyzed for ADL according to Van Soest (1991) and NDF, NDS (neutral detergent solubles, 100-NDF), and ADL results were expressed on an organic matter basis. Cellulose (ADF minus ADL) and hemicellulose concentrations (NDF minus ADF) and apparent digestibilities of DM, OM, NDF, hemicellulose, ADF and cellulose were calculated. Crude protein concentration of hays and distillers grain were calculated as N x 6.25 after N concentration was determined using a Vario MAX CN Macro Elementar Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) by the Dumas combustion method (AOAC, 2000). Hay ADF residues were also analyzed for CP to estimate ADICP concentration.

In situ ruminal degradability

The in situ ruminal DM disappearance of each hay was determined in triplicate on d 22-25 within steers consuming the same hay in that period. Hay samples taken representatively at the beginning of each period were dried for 48 h at 60°C, ground to pass a 4-mm screen using a Wiley mill and weighed (5 g DM) into 10 x 20 cm ANKOM R1020 in situ bags (ANKOM, Macedon, NY), which were tied with rubber bands. The bag pore size was 50 μm and the sample size to free bag surface area ratio was 12.5 mg/cm^2. Bags were attached to a rope with clips, placed in the ventral sac of the rumen for 0, 3, 6, 9, 12, 18, 24, 48, 72, and 96 h and removed simultaneously. Upon removal

from the rumen, bags were washed with cold water to remove adherent particles and bacteria and then washed in a commercial washing machine (Kenmore, Benton Harbor, MI) using a cool-wash cycle without soap. Washed bags were dried for 48 h at 60°C and weighed. The model of Mertens (1977) was fitted to the DM degradation data using the NLIN procedure of SAS, version 9.1 (2009, SAS Inst., Inc., Cary, NC). The model is of the form:

$$R_{(t)} = D_i \times (e^{-k_d(t-L)}) + I_o$$

where $R_{(t)}$ = Total indigested residue at any time t, D_i = potentially digestible fraction, k_d = fractional rate of digestion of D_i, t = time incubated in the rumen in h, L = discrete lag time in h, and I_o = fraction not digested after 96h of incubation. Effective degradability was calculated using the Orskov and McDonald (1979) formula:

$$\text{Effective degradability} = A + D \times [k_d / (k_d + k_p)]$$

Where A = washout fraction, D = potentially digestible fraction, k_d = fractional rate of digestion, k_p = assumed ruminal passage rates of 0.046,.according to Pond et al. (1989)

Rumen fermentation parameters

Samples (100 ml) of ruminal fluid were taken by aspiration at 0800 h (1 h before feeding) and hourly thereafter until 2000 h on d 27. The samples were immediately filtered with 2 layers of cheese cloth, and analyzed for pH using a calibrated electrode (Accumet, model HP-71, Fisher Scientific, Pittsburgh, PA). Subsequently, the ruminal fluid was acidified to pH < 2 with 50% H_2SO_4 and then frozen at -40°C for further analysis. Thawed samples were centrifuged at 8000 x g for 20 min at 4°C and the supernatant was analyzed for VFA and lactate (Muck and Dickerson, 1988) using a High Performance Liquid Chromatograph (Hitachi, FL 7485, Tokyo, Japan) coupled to a

UV detector. Ammonia-N concentration was measured using an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification on a Technicon Auto Analyzer (Technicon, Tarrytown, NY).

Ruminal fluid volume and liquid rate of passage

Ruminal fluid volume and dilution rate were measured on d 27 using samples taken at the same time as those analyzed for VFA. Cobalt EDTA was prepared according to Uden et al. (1980). Twenty eight grams of a cobalt chelate (4 g of Co) were diluted in 1 L of distilled water and dosed into the rumen. Rumen contents were hand thoroughly mixed to aid equilibration of the marker. Ruminal fluid samples were taken 3 min later and processed in the same way as those reserved for VFA analysis. The cobalt concentration of the ruminal fluid supernatant was measured with a Perkin Elmer 5000 (Wellesley, MA) atomic absorption spectrometer. Ruminal fluid dilution rates were calculated as the slope of the natural logarithm of cobalt concentration on time post-dosing and are expressed as percentage of volume/hour. Ruminal fluid volumes were calculated by dividing the Co dose by the extrapolated Co concentration at time 0 (dosing).

Experiment 2

The hays and supplements used in Experiment 1 also were used in Experiment 2. Ninety Angus and Brangus steers (308 ± 37 kg) were stratified by weight, dewormed with Ivomec and randomly allocated to 18 1.01-ha pens (5 steers per pen) containing dormant bahiagrass (*Paspalum notatum* Flügge) pasture. Forage mass was not measured in the pens because the bahiagrass was minimal and dormant during the experiment. Three pens were assigned randomly to each treatment. The CON, ENZ, and AMN hays from both regrowth intervals were weighed and fed in round-bale

feeders to cattle in respective pens in quantities sufficient to ensure *ad libitum* access for 55 d. Steers were adapted to diets for 6 d, and full body weights were obtained on two consecutive days at the beginning (d 7 and 8) and end (d 54 and 55) of the measurement period. Weekly supplement allocations were fed in one open trough in each pen in equal amounts three times per week and they were consumed completely. Refused hay was collected and weighed on d 30 and 55.

Statistical Analysis

Data from Experiment 1 were analyzed using the MIXED procedure of SAS v9.1 (2009, SAS Inst., Inc., Cary, NC).

The model used to analyze intake and digestibility data was:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + r_k + c_l + \varepsilon_{ijkl}$

Where:

μ = general mean

α_i = effect of additive i

β_j = effect of regrowth interval j

$\alpha\beta_{ij}$ = effect of additive i × regrowth interval j interaction

r_k = effect of period k

c_l = random effect of steer l

ε_{ijkl} = experimental error

A similar model that excluded the steer effect was used to analyze hay composition data. Rumen fermentation data was analyzed with the GLIMMIX procedure of SAS and a repeated measures statement that used the autoregressive [ar(1)] covariate structure. The model was similar to the intake model but included effects of time and interactions with time.

Experiment 2 had a completely randomized, split-plot design. The main and sub-plots were the additive × regrowth interval interaction and breed, respectively, and pen was the experimental unit. The initial and final weight and ADG were analyzed using the MIXED procedure of SAS v9.1 and the model was:

Y_{ijkl}: $\mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \alpha\gamma i_k + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \varepsilon_{ijk}$

Where:

μ = general mean

α_i = effect of additive i

β_j = effect of regrowth interval j

$\alpha\beta_{ij}$ = effect of additive i × regrowth interval j interaction

γ_k = effect of breed k

$\alpha\gamma_{ik}$ = effect of additive i × breed k interaction

$\beta\gamma_{jk}$ = effect of regrowth interval j × breed k interaction

$\alpha\beta\gamma_{ijk}$ = three way interaction effect of additive i, regrowth interval j, and breed k

ε_{ijkl} = error effects

Main plot (additive × regrowth interval) error was additive × regrowth interval and sub-plot error was the residual. A similar model was used for estimating treatment effects on intake and gain to feed ratio (G:F) but the breed effect and its interactions were omitted because steers were group-fed in pens.

Contrast statements were used to determine the effects of enzyme treatment (ENZ vs. CON), ammoniation (AMN vs. CON), and regrowth interval (5 vs. 13 wk) and the interactions of regrowth interval with the respective additives. Significance was declared at $P \leq 0.05$, and only significant interactions effects are discussed.

Results and Discussion

Nutritional Composition

The 5-wk hay had greater ($P < 0.001$) concentrations (% DM) of NDF (77.7 vs. 72.7), hemicellulose (36.8 vs. 33.4), ADF (40.9 vs. 39.4), cellulose (34.4 vs. 32.4) and CP (14.3 vs. 11.5) and less ($P < 0.001$) NDS (Neutral detergent solubles) (22.3 vs. 27.3), ADL (6.42 vs. 6.98) and ADICP ($P = 0.03$; 9.82 vs. 10.37 % of total CP) than the 13-wk hay (Table 3-1). Although fiber concentrations often increase with plant maturity, higher fiber concentrations in bermudagrass with shorter regrowth intervals compared to longer regrowth intervals have been reported in the literature on some occasions, as is the case in this study (Joliff et al., 1979; Mandebvu et al., 1999 and Scarbrough et al., 2006). Chronological age effects on bermudagrass nutritional value can be confounded with environmental conditions influence on plant growth and development changes during growing period (Joliff et al., 1979). In this experiment, differences between the average temperatures during the growth of the 5- and 13-wk hays (25.2 vs. 21.7^{o}C; Figure 3-1) partly explain the greater fiber concentrations of the 5-wk hay.

As reported in other studies on tropical grasses (Krueger et al. 2008), enzyme application had no effect on fiber fractions or lignin. However, enzyme application increased CP concentration of the 5-wk but not the 13-wk hay (enzyme x regrowth interval interaction, $P = 0.03$) but had contrasting effects on ADICP of 5- and 13-wk hays (enzyme × regrowth interval interaction, $P = 0.004$). The enzyme used in this study had increased the CP concentration of guineagrass hay (*Panicum maximum* Jacq.; Tous et al., 2006) but had no effect on CP of bermudagrass hay (Krueger et al., 2008). The mechanism by which enzyme application increases CP concentration is unclear particularly because quantities added in the form of the enzyme were minute.

Ammoniation resulted in reduced NDF, hemicellulose, ADICP, and ADL concentration and greater, NDS, cellulose, and CP concentrations and these effects were more pronounced in the 13-wk hay (regrowth interval × ammonia interaction, $P < 0.05$). Others have reported that the efficacy of ammoniation increased with maturity (Fahey et al., 1993; Brown and Kunkle, 2003; Sollenberger et al., 2004). The reduction in NDF by ammoniation reflects solubilization of hemicellulose (Saenger et al., 1983; Haddad et al., 1995) and hydroxycinnamic compounds (Fahey et al., 1993; Chesson, 1993; Wang et al., 2004) in the alkali due to breakage of ester bonds. As reported previously (Haddad et al., 1995; Krueger et al., 2008), ammoniation did not affect ADF concentration yet it reduced ADL concentration particularly for the 13-wk hay (regrowth interval × ammonia interaction, $P = 0.04$). Similar effects of ammoniation on lignin have been attributed to solubilization of α-aryl ether linkages within the lignin macromolecule (Chesson, 1993). The increase in CP caused by ammoniation agrees with other studies (Wanapat et al., 1985; Flachowsky et al., 1996; Krueger et al., 2008). The reason for the greater CP response to ammoniation in the more mature forage is unclear but the outcome is particularly beneficial for ruminants fed warm-season grasses, which have greater yields and lower CP concentrations as they mature (Moser et al., 2004).

Voluntary Intake

Intakes (kg/d) of total diet DM, OM, CP, NDF, hemicellulose, ADF and cellulose ($P \leq 0.008$) were greater by steers fed the 5-wk than those fed the 13-wk hay (8.0 vs. 7.5; 7.3 vs. 6.9; 1.3 vs. 1.1, 4.3 vs. 3.7; 2.1 vs. 1.8, 2.2 vs. 1.9 and 1.8 vs. 1.6, respectively; Table 3-2). Intakes of hay DM, OM, CP, NDF, hemicellulose, ADF and cellulose ($P \leq 0.008$) were greater in the 5-wk than the 13-wk hay (5.1 vs. 4.6; 4.9 vs. 4.4; 0.7 vs. 0.5, 4.0 vs. 3.4, 1.9 vs. 1.6, 2.1 vs. 1.8, 1.8 vs. 1.5 respectively). Enzyme application did not

affect these intake measures but ammoniation decreased ($P \leq 0.05$) total intakes of DM, OM, NDF and hemicellulose (7.6 vs. 8.0, 6.9 vs. 7.3, 3.7 vs. 4.2 and 1.7 vs. 2.1, respectively). Ammoniation also decreased hay NDF and hemicellulose (3.4 vs. 3.9 and 1.5 vs. 1.9) and tended ($P < 0.08$) to decrease intake of total ADF (2.0 vs. 2.1) and hay DM, OM and ADF (4.7 vs. 5.1, 4.5 vs. 4.9 and 1.9 vs. 2.0). Ammoniation increased total and hay CP intake ($P < 0.001$; 1.3 vs. 1.1 and 0.8 vs. 0.5). In other studies, ammoniation of hay increased (Flachowsky, 1996; Krueger et al., 2008) or did not affect (Ward and Ward, 1987; Wang et al., 2004) DMI. However, ammoniation has decreased hay intake particularly when applied at rates of 3% or greater to forages with 30% moisture or more due to the strong smell and reduced acceptability of the ammoniated hay (Streeter et al., 1981; Grovum and Chapman, 1988; Brown and Kunkle, 2003). The use of partially enclosed feedbunks that concentrated the odor of the ammoniated hay may have caused the adverse effects of ammoniation on intake.

In vivo apparent digestibility

Digestibility (%) of CP, NDF, hemicellulose, ADF and cellulose were greater ($P \leq 0.002$) in the 5-wk hay compared to the 13-wk hay (67.5 vs. 64.6, 58.1 vs. 51.2, 69.8 vs. 63.2, 46.8 vs. 40.2 and 58.3 vs. 50.9; Table 3-3). However, there was no difference between 5-wk and 13-wk DM and OM digestibilities.

Enzyme application did not affect digestibility (%) of DM, OM, CP, ADF and cellulose but increased NDF and hemicellulose digestibility of the 5-wk hay (57.8 vs. 53.2 and 69.3 vs. 62.3, respectively) and decreased that of the 13-wk hay (46.7 vs. 49.6 and 56.7 vs. 61.1; enzyme × regrowth interval interaction, $P \leq 0.009$). When the same enzyme was applied to a 5-wk regrowth of Coastal bermudagrass hay, digestibility of NDF by steers also was increased (Krueger et al., 2008). The improvement in NDF-

digestibility may have only occurred in the 5-wk hay because it contained less lignin than the 13-wk hay. The response probably reflects a pre-ingestive hydrolytic effect of the enzyme on the forage.

Ammoniation increased digestibility ($P \leq 0.007$) of DM (66.9 vs. 63.2%), OM (66.4 vs. 62.5%), NDF (60.3 vs. 51.4%), hemicellulose (74.8 vs. 61.7), ADF (47.7 vs. 41.2%) and cellulose (61.0 vs. 50.5). Ammoniation cleaves ester linkages and solubilizes hydroxycinnamic acids, hemicellulose, and acetyl groups in the cell wall (Wang et al., 2004), thereby disrupting the lignin-hemicellulose matrix and facilitating cell wall degradation by microbial enzymes (Jung and Allen, 1995). Greater ADF and cellulose digestibility with ammoniation probably reflect reduced encrustation of cellulose by lignin and increased exposure of the cellulose polymer to microbes (Fahey et al., 1993). However, ammoniation decreased ($P < 0.001$) CP digestibility (62.2 vs. 67.5), which contradicts the results of Krueger et al. (2008) on a 5 wk regrowth of bermudagrass hay. These different responses may be due to use of a lower ammoniation rate (3% of hay DM) in the latter study.

In Situ Rumen Digestion Kinetics

Discrete lag times were unaffected by additives but were longer ($P < 0.001$) for the 5-wk (2.8 h) compared to the 13-wk hay (1.5 h; Table 3-4). Lag time is influenced by factors such as the rate of hydration of the substrate, microbial attachment, and nutrient limitations (Lopez, 2005). The lower concentration of the digestible, nutrient-rich NDS fraction in the 5-wk hay likely reduced the rate of substrate colonization causing a longer lag phase.

Compared to values for the 13 wk hay, in situ DM digestion of the 5 wk hay was less ($P < 0.05$) during the first 6 h of digestion, similar at 12, 18, and 24 h, and greater

($P <$ 0.05) between 36 and 96 h (Figure 3-2; Table 3-5). Enzyme application did not affect in situ DM digestion but ammoniation consistently increased ($P < 0.05$) in situ digestion and the response was greater in the 13-wk hay during the first 48 h of incubation (ammonia × regrowth interval interaction, $P < 0.05$).

The 5-wk hay contained less ($P < 0.05$) soluble (12.7 vs. 13.6%) and indigestible (40.9 vs. 43.5%) fractions and more ($P < 0.001$) of the potentially digestible fraction (46.4 vs 42.9%) than the 13-wk hay. These results reflect the greater NDF and ADF concentrations and lower lignin concentration of the 5-wk hay (Table 3-1).

As in the study of Krueger et al. (2008), enzyme application to bermudagrass hay, had no effect on in situ digestibility of DM. McAllister (2001) stated that in most studies, enzymes have not increased the potentially digestible fraction, but they have increased the rate of digestion (Feng et al., 1996). Such increases in the digestion rate occurred mainly in reports on cool-season or temperate forages, which have considerably lower NDF concentrations than warm-season grasses.

Ammoniation increased the soluble fraction and effective degradability of the hays to a greater extent in the 13-wk versus 5-wk hay (ammonia × regrowth interval interaction, $P = 0.002$). Ammoniation also increased ($P < 0.001$) the potentially digestible (50.3 vs. 42.7%) fraction and decreased ($P < 0.001$) the indigestible fraction (34.6 vs. 45.3%) of 5 and 13-wk hays, whereas it only increased the degradation rate of the 13-wk hay (ammonia × regrowth interval interaction, $P = 0.04$). Ammoniation may have increased the soluble fraction by weakening the physical structure and properties of the cell wall (Harbers, 1982) thereby increasing its fragility (Selim, 2002, 2004). Effects on the potential and indigestible fractions likely reflect hydrolysis of ester and

ether linkages and solubilization of cell wall components (Chesson, 1993). The greater degradation rate and effective degradability responses to ammoniation of the 13-wk hay agree with reports that the alkali is more effective on more mature, lignified forages (Fahey et al., 1993; Brown and Kunkle, 2003; Sollenberger et al., 2004).

Rumen Fermentation Parameters

Ruminal pH was unaffected by regrowth interval or enzyme or ammonia treatment (Table 3-6). When applied to forages, enzymes have not affected ruminal pH (Lewis et al., 1996; Yescas-Yescas et al., 2004; Dean, 2005). Also, ammoniation of hay has not affected ruminal pH in previous studies (Zorrila-Rios, 1985; Vagnoni et al., 1995).

Ruminal ammonia concentration was not affected by maturity or enzyme application. In contrast, ammoniation increased (P = 0.004) ruminal ammonia-N concentration (15.1 vs. 11.4 mg/dl) when compared to control, reflecting increased supply of ruminally available N from the ammoniated hay (Fahey et al., 1993). In agreement with Vagnoni et al. (1995), ammoniation reduced the initial rate of release and subsequent rate of decrease in ruminal ammonia-N concentration (Figure 3-3; treatment × time interaction, P < 0.001). A more gradual release of ruminal ammonia may benefit rumen microbes due improved stability of N supply. Mean ruminal ammonia-N concentrations of all diets exceeded the level (5 mg /dl; Satter and Slyter, 1974) that limits microbial protein synthesis. However, those of control and enzyme diets were slightly below the threshold prior to feeding the concentrate in the morning and after feeding the hay in the evening due to the rapid solubilization of the supplemented urea..

Enzyme application and regrowth interval did not affect total VFA concentration or VFA molar proportions except that the 5-wk hay had more (P = 0.002) acetate (58.3 vs.

56.5 mol / 100 mol) and less (P = 0.001) butyrate (16.1 vs. 17.8) than the 13-wk hay, reflecting the greater NDF concentration and digestibility of the 5-wk hay. Higher acetate proportions and lower butyrate proportions also were reported when less mature tall fescue hay was fed to steers instead of mature hay (Fieser and Vanzant, 2004). Relatively high proportion of butyrate in ruminal fluid in this study reflect the fermentation of the supplementary molasses (Wing et al., 1988) and distillers grains (Leupp et al., 2009) in the steer diets. Ammoniation increased ($P \leq 0.02$) total VFA concentration (155.4 vs. 144.3 mM) as reported previously in beef cattle fed ammoniated bermudagrass hay (Wyatt et al., 1989) or tall fescue (Chestnut et al., 1987). Such results are probably a consequence of greater digestion and fermentation of ammoniated hay. Ammoniation also decreased ($P \leq 0.006$) the molar proportion of propionate (17.9 vs. 19.1) and increased ($P \leq 0.02$) the acetate to propionate ratio (3.4 vs. 3.2).

Ruminal Fluid Volume and Liquid Rate of Passage

Ruminal fluid volume, dilution rate, turnover time and passage rate were unaffected by additive treatment or regrowth interval (Table 3-7). Judkins and Stobart (1988) noted that enzyme treatment of an alfalfa hay - based diet did not affect rate of dilution or ruminal fluid volume in lambs. In addition, Beauchemin et al. (1999) reported that enzyme treatment of a barley silage – based TMR did not affect liquid passage rate in dairy cows. However, Zorrilla-Rios (1985) reported that ammoniation of wheat straw increased liquid passage rate in steers. Ammoniation may have not increased liquid passage rate in this study because it reduced DMI and tended (P = 0.13) to reduce ruminal fluid volume. Rate of fluid passage values (%/h) observed in this trial (14 - 15.7) were relatively high but similar to those of beef steers fed old world bluestem

(*Bothriochla inermedium*) (14.7 %/h; Coleman et al., 1984) or tall fescue (*Festuca arundinacea*) plus soy hulls (13 %/h; Richards et al., 2006) for ad libitum intake.

Growth Performance of Steers

Enzyme treatment only increased hay and total DMI when the 5-wk hay was fed (enzyme × regrowth interval interaction, $P \leq 0.007$; Table 3-8). In contrast, ammoniation increased hay and total DMI at both regrowth intervals but the increase was greater by steers fed the 5-wk hay (ammonia x regrowth interval interaction, $P < 0.05$). Ammoniation also tended ($P = 0.11$) to increase final BW (347 vs. 342 kg) and ADG (0.79 vs. 0.70 kg/d), but enzyme treatment had no effect on measures of growth. Ammoniation has improved the ADG of beef cows and steers fed low quality forages (Ward and Ward, 1987; Brown,1988; Flachowsky et al., 1996) by improving intake and digestion (Flachowsky et al., 1996), whereas, enzyme application has had equivocal effects on the intake, digestion, and ADG of ruminants fed low quality forages (Beauchemin et al., 1995; Robison et al., 2001; Krueger et al., 2008).

Ammoniation did not affect gain to feed ratio, but enzyme application tended to reduce the ratio in the 5-wk hay and increase it in the 13-wk hay. As in this study, Krueger et al. (2008) reported greater intake of enzyme-treated 5-wk hay without a corresponding ADG increase. These results suggest that the increased nutrient supply resulting from enzyme treatment was insufficient for improving gain or was used inefficiently. Therefore, future studies on enzyme treatment of forages or diets should estimate nutrient partitioning and retention.

Differences in intake responses to treatments between Experiments 1 and 2 reflect the different feeding strategies used. Contrasting intake responses to ammoniation reflect use in Experiment 1 of feedbunks that concentrated the ammonia odor to a much

greater extent than the hay ring feeders used in Experiment 2. That enzyme treatment increased intake of the 5-wk hay in Experiment 2, but not in Experiment 1, suggests that chopping hays before they were fed in Experiment 1 may have reduced benefits of enzyme treatment because chopping forages increases DMI (Fahey et al., 1993; Berger et al., 1995).

Conclusions

The 5-wk hay had more NDF, hemicellulose, ADF, cellulose, and CP and less lignin than the 13-wk hay, but the NDF, hemicellulose, ADF, cellulose and CP digestibility of the 5-wk hay was greater. Ammonia treatment improved the nutritional composition of bermudagrass hay by reducing NDF, hemicellulose, and lignin concentrations and increasing cellulose and CP concentrations particularly in the 13-wk hay. Ammoniation increased DMI in the pasture-based experiment but reduced hay DMI in the confined feeding experiment likely due to concentration of the ammonia odor. Ammoniation improved the extent of total tract apparent digestibility and extent and rate of DM digestion of the hays in the rumen and tended to increase the ADG and final BW of steers.

Except for slightly increasing CP concentration, enzyme treatment did not improve the nutritive value or in situ rumen digestion of bermudagrass hay. However, enzyme treatment increased the NDF and hemicellulose digestibility of the 5-wk hay and increased the DMI of this hay when offered at pasture, but this did not result in improved growth performance. As hypothesized, ammoniation had more pronounced effects on the more mature (13 wk) hay, whereas enzyme application only resulted in beneficial effects when applied to the 5-wk hay.

Table 3-1. Chemical composition of untreated (CON), enzyme-treated (ENZ) and ammoniated (AMN) hay harvested at two regrowth intervals (RI) (n=6)

Component,	5-wk regrowth			13-wk regrowth			SE	*P* values				
% of DM	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZ×RI	AMN×RI
OM	95.6	94.7	95.6	95.5	95.7	95.6	0.1	0.006	0.01	0.9	<0.001	0.7
NDS	21.4	20.9	24.7	25.0	25.3	31.4	0.5	<0.001	0.84	<0.001	0.40	0.004
NDF	78.6	79.2	75.2	75.0	74.7	68.6	0.5	<0.001	0.84	<0.001	0.40	0.004
Hemicellulose	38.1	38.4	34.1	35.7	35.3	29.1	0.5	<0.001	0.95	<0.001	0.47	0.01
ADF	40.6	40.8	41.2	39.3	39.4	39.5	0.3	<0.001	0.66	0.17	0.85	0.43
Cellulose	34.1	34.3	34.9	32.0	32.1	33.1	0.2	<0.001	0.43	<0.001	0.98	0.47
ADL	6.47	6.49	6.29	7.32	7.23	6.40	0.2	<0.001	0.82	0.003	0.73	0.04
CP	12.2	12.9	17.8	9.1	9.2	16.3	0.2	<0.001	0.01	<0.001	0.03	<0.001
ADICP (% of CP)	10.1	9.9	9.4	10.9	11.9	8.3	0.3	0.03	0.20	<0.001	0.04	0.004

SE values represent the variability of samples collected daily for 7d from hay offered to each steer and composited by period.

Table 3-2. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on the voluntary intake of steers

Item	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZx RI	AMNx RI
Total Net intake, kg/d												
DM	8.12	8.10	7.81	7.80	7.45	7.29	0.24	0.007	0.34	0.05	0.43	0.62
OM	7.47	7.40	7.16	7.16	6.83	6.67	0.23	0.008	0.29	0.05	0.50	0.63
CP	1.19	1.22	1.35	1.00	0.97	1.20	0.03	<0.001	0.90	<0.001	0.20	0.28
NDF	4.42	4.45	4.03	3.98	3.71	3.34	0.18	<0.001	0.39	0.002	0.31	0.40
Hemicellulose	2.21	2.24	1.90	1.96	1.83	1.52	0.09	<0.001	0.48	<0.001	0.26	0.34
ADF	2.21	2.20	2.13	2.02	1.88	1.83	0.09	<0.001	0.33	0.08	0.37	0.48
Cellulose	1.86	1.85	1.80	1.64	1.53	1.54	0.08	<0.001	0.38	0.23	0.45	0.72
Hay intake, kg/d												
DM	5.25	5.22	4.96	4.92	4.57	4.44	0.24	0.007	0.35	0.06	0.43	0.62
OM	5.01	4.94	4.74	4.70	4.37	4.24	0.23	0.008	0.29	0.06	0.50	0.63
CP	0.64	0.67	0.89	0.45	0.42	0.74	0.28	<0.001	0.90	<0.001	0.20	0.28
NDF	4.12	4.15	3.73	3.68	3.41	3.04	0.19	<0.001	0.39	0.002	0.31	0.40
Hemicellulose	2.00	2.03	1.68	1.74	1.62	1.30	0.09	<0.001	0.48	<0.001	0.26	0.34
ADF	2.13	2.12	2.04	1.94	1.79	1.75	0.10	<0.001	0.33	0.08	0.37	0.48
Cellulose	1.79	1.78	1.73	1.57	1.46	1.47	0.08	<0.001	0.38	0.23	0.45	0.72

Table 3-3. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on in vivo apparent digestibility by steers

Digestibility, %	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
DM	63.0	64.9	67.4	63.3	62.9	66.4	0.86	0.14	0.31	<0.001	0.14	0.40
OM	62.4	64.3	67.0	62.7	62.4	65.8	0.88	0.16	0.31	<0.001	0.15	0.32
CP	68.1	70.2	64.0	66.9	66.6	60.3	0.80	<0.001	0.30	<0.001	0.15	0.14
NDF	53.2	57.8	63.2	49.6	46.7	57.4	1.43	<0.001	0.46	<0.001	0.004	0.35
Hemicellulose	62.3	69.3	77.9	61.1	56.7	71.6	2.54	<0.001	0.50	<0.001	0.009	0.20
ADF	44.1	46.0	50.1	38.3	36.9	45.3	2.43	0.002	0.91	0.007	0.45	0.82
Cellulose	54.8	57.6	62.4	46.2	47.0	59.6	1.80	<0.001	0.29	<0.001	0.55	0.09

Table 3-4. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on kinetics of in situ ruminal DM digestion.

Measure	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
Lag phase, h	2.7	2.7	3.0	1.2	1.3	2.0	0.4	<0.001	0.84	0.12	0.91	0.41
Soluble fraction, % of DM	12.1	12.3	13.8	11.8	12.6	16.4	0.4	0.02	0.23	<0.001	0.48	0.002
Potentially digestible fraction, % of DM	43.9	43.3	52.1	41.5	38.8	48.4	1.2	<0.001	0.12	<0.001	0.32	0.52
Fractional digestion rate, %/h	6.12	6.74	6.08	5.56	5.89	7.43	0.48	0.96	0.28	0.05	0.74	0.04
Indigestible fraction, % of DM	44.1	44.5	34.1	46.6	48.6	35.2	1.0	<0.001	0.18	<0.001	0.38	0.39
Effective[a] degradability	37.1	37.9	43.3	34.2	34.4	46.1	0.9	0.07	0.53	<0.001	0.65	0.002

[a]Effective degradability calculated assuming a passage rate of 0.046.

Table 3-5. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on in situ ruminal DM digestion after different incubation periods.

Hour	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
0	12.1	12.3	13.8	11.8	12.6	16.4	0.4	0.02	0.23	<0.001	0.48	0.002
3	14.2	14.3	16.0	15.4	16.3	20.0	0.5	<0.001	0.19	<0.001	0.36	0.003
6	19.9	20.7	22.4	21.3	22.0	28.0	0.9	0.001	0.30	<0.001	0.99	0.007
12	31.0	32.2	35.6	30.3	30.7	41.2	1.0	0.12	0.32	<0.001	0.65	0.002
18	38.6	39.9	44.8	36.6	36.9	49.6	1.2	0.91	0.44	<0.001	0.58	0.003
24	43.9	45.1	51.2	41.2	41.2	54.9	1.2	0.24	0.56	<0.001	0.54	0.003
36	50.1	50.8	58.6	46.8	46.3	60.5	1.1	0.01	0.90	<0.001	0.50	0.006
48	53.1	53.4	62.3	49.7	48.9	62.9	1.0	0.001	0.70	<0.001	0.46	0.02
72	55.3	55.1	65.0	52.2	50.8	64.4	1.0	<0.001	0.32	<0.001	0.44	0.13
96	55.8	55.5	65.7	53.0	51.3	64.7	1.0	0.001	0.23	<0.001	0.41	0.27

Table 3-6. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on rumen fermentation parameters

Parameter	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
pH	6.54	6.53	6.44	6.50	6.53	6.52	0.06	0.70	0.87	0.36	0.65	0.19
Ammonia (mg/dl)	11.4	12.2	16.3	11.5	10.9	13.9	1.5	0.26	0.91	0.004	0.55	0.33
Total VFA (mM)	142.7	147.0	161.7	145.9	141.3	149.1	5.9	0.20	0.98	0.02	0.33	0.10
VFA (mol / 100 mol)												
Acetate	58.3	58.3	58.3	56.0	56.3	57.3	0.8	0.002	0.85	0.39	0.81	0.35
Propionate	19.0	19.1	17.7	19.1	19.8	18.1	0.5	0.24	0.38	0.006	0.53	0.69
Butyrate	15.7	15.4	17.3	18.1	17.2	18.2	0.7	0.001	0.34	0.19	0.64	0.23
Isobutyrate	3.1	3.5	3.0	3.0	2.9	3.2	0.2	0.25	0.41	0.93	0.26	0.36
Isovalerate	3.8	3.7	3.8	3.8	3.7	3.3	0.2	0.43	0.65	0.24	0.89	0.22
Acetate : Propionate	3.2	3.2	3.4	3.1	3.0	3.3	0.1	0.17	0.55	0.02	0.91	0.80

Table 3-7. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on ruminal fluid volume and fluid kinetics

Measure	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
Ruminal fluid volume, L	45.1	48.1	38.1	45.2	48.3	42.9	3.9	0.48	0.30	0.13	0.99	0.42
Dilution rate, %/h	15	14.4	15.7	14	14.3	14.6	1.2	0.37	0.87	0.51	0.64	0.97
Rate of fluid passage, L/hr	6.6	6.4	5.9	6.3	6.5	6.1	0.5	0.96	0.94	0.21	0.51	0.42
Turnover time, h	6.8	7.4	6.5	7.4	7.4	7.0	0.7	0.41	0.60	0.51	0.59	0.93

Table 3-8. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on growth performance of steers

Parameter	5-wk regrowth			13-wk regrowth			SE	*P* values				
	CON	ENZ	AMN	CON	ENZ	AMN		RI	ENZ	AMN	ENZxRI	AMNxRI
Total DMI	6.1	6.8	7.1	6.9	6.7	7.2	0.15	0.04	0.10	<0.001	0.007	0.02
Hay DMI	3.5	4.2	4.3	4.2	4.0	4.5	0.12	0.02	0.05	<0.001	0.003	0.03
Initial wt, kg	305	306	306	309	306	311	3.3	0.27	0.73	0.70	0.56	0.79
Final wt, kg	343	341	346	340	341	348	3.9	0.98	0.84	0.11	0.60	0.42
ADG, kg/d	0.77	0.70	0.82	0.64	0.73	0.77	0.06	0.23	0.84	0.11	0.18	0.50
Gain:feed ratio	0.12	0.10	0.12	0.09	0.11	0.11	0.01	0.12	0.82	0.49	0.10	0.27

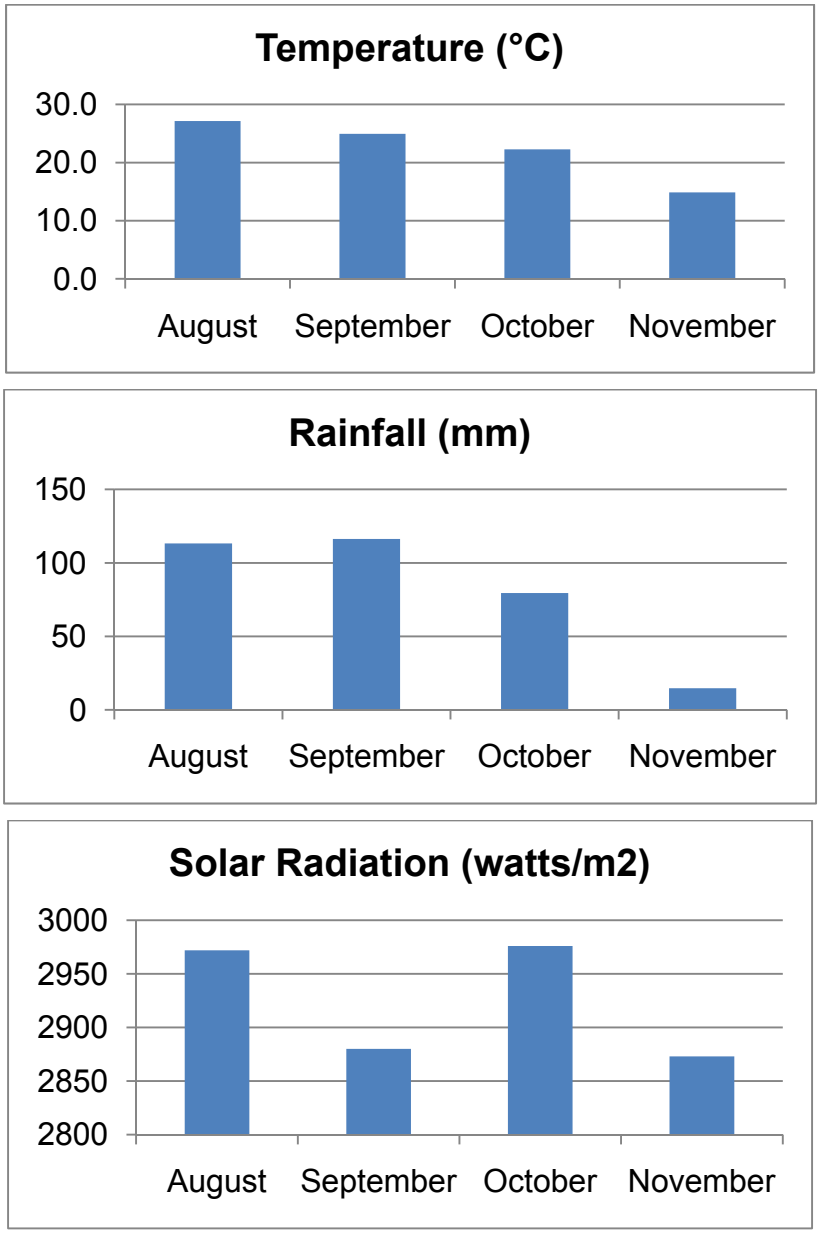

Figure 3-1. Temperature (°C), rainfall (mm) and solar radiation (watts/m^2) during the growing period of the hays (Florida automated weather network, 2009).

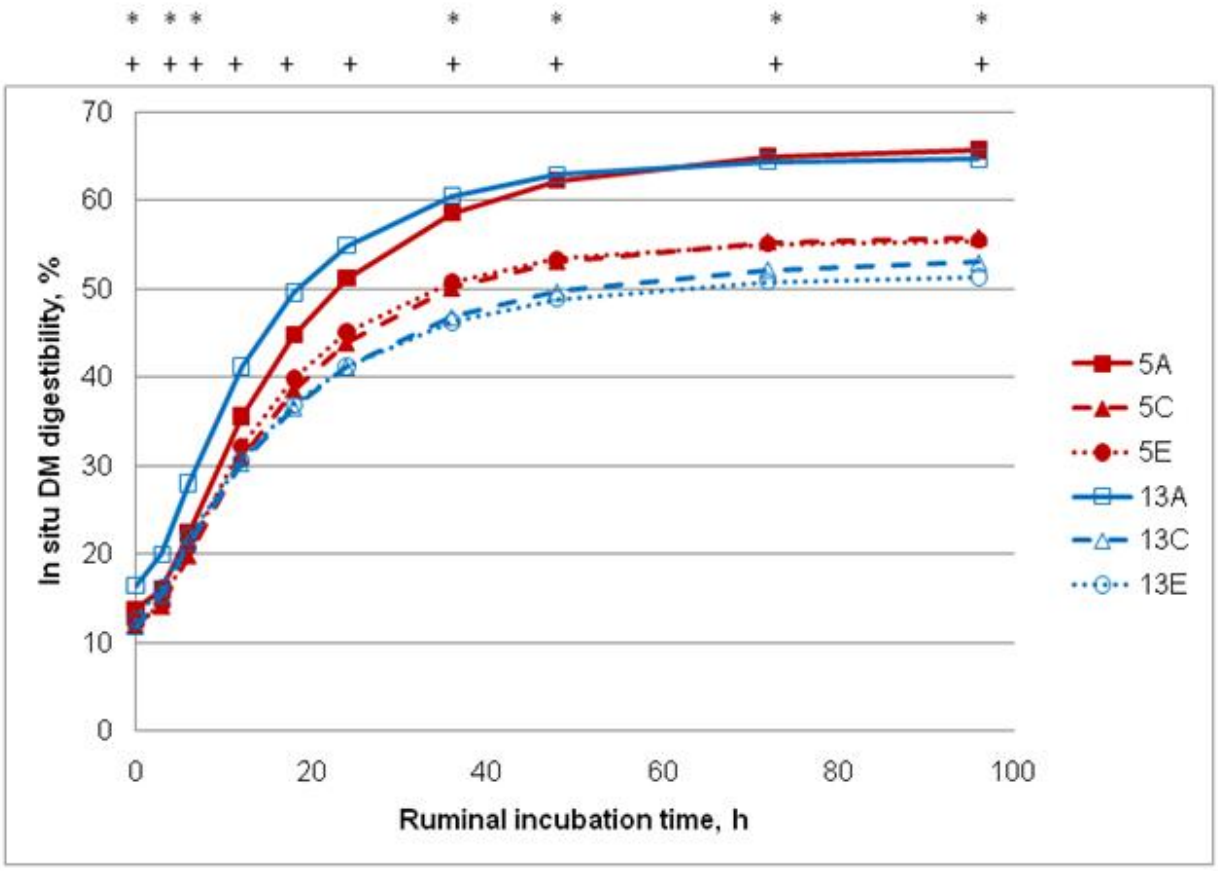

Figure 3-2. In situ DM digestibility of 5- and 13-wk regrowth of bermudagrass hay that were untreated (C) or treated with enzymes (E) or ammonia (A).* and + indicate significant effects of maturity and ammoniation at that incubation time.

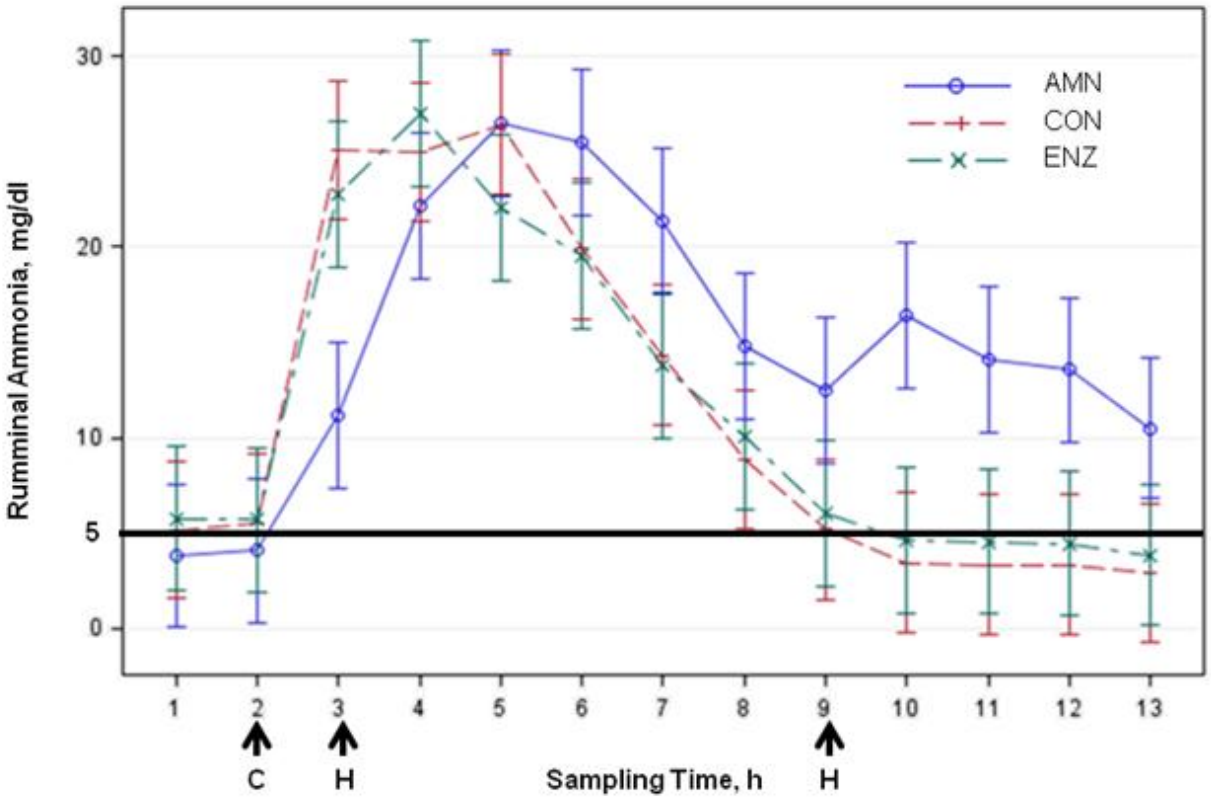

Figure 3-3. Effect of treating bermudagrass hay harvested at two regrowth intervals with a fibrolytic enzyme or ammonia on ruminal-N ammonia concentrations of steers.. Error bars indicate the 95% confidence interval of the means; treatment × time interaction, $P < 0.001$. Arrows labeled “C” and “H” indicate times when concentrate and hay were fed respectively.

CHAPTER 4
GENERAL SUMMARY AND RECOMMENDATIONS

In the southeastern U.S., warm-season grasses are the basis of livestock production (Pitman, 2007), but they have intrinsically low nutritional value. Several methods have been proposed to improve their quality, and ammonia application is one of the most effective. However, ammoniation is costly, potentially toxic and caustic, therefore it is not used widely. Recent research has focused on enzyme treatments to improve forage quality but this effort has had inconsistent results and has been used primarily on cool-season grasses. Consequently, two experiments were initiated to evaluate the effect of applying an exogenous fibrolytic enzyme or anhydrous ammonia to Coastal bermudagrass hay harvested as 5- or 13-wk regrowths on forage nutritive value and growth of beef cattle.

In Experiment 1, 6 ruminally-cannulated Brangus steers (BW 216 ± 6 kg) were used in an experiment with a 6 x 6 Latin square design with a 3 (additives) x 2 (regrowth intervals) factorial arrangement of treatments. Each period consisted of 14 d of adaptation, 7 d of digestibility measurements, 4 d of in situ degradability, 1 d of rumen rest and 1 d of ruminal fluid fermentation measurements. Steers were housed individually and fed hay ad libitum. A supplement consisting of sugar cane molasses, distillers grain and a mineral and vitamin premix was fed at a rate that met maintenance energy requirements (NRC, 2000). Urea (0.03 kg/hd/d) was mixed with the concentrate and offered to steers fed control and enzyme-treated hays to ensure that N deficiency did not limit rumen function.

Ammonia treatment improved the nutritional composition of the hay and this effect was more pronounced in the more mature and lignified 13-wk hay. Enzyme did not

affect most nutritional components but slightly increased CP concentration of 5-wk hay. Enzyme application did not affect intake measures, but ammoniation decreased intake probably because the enclosed feedbunks concentrated the strong ammonia odor. Ammoniation increased digestibility of DM, OM, NDF, hemicellulose, ADF and cellulose across regrowth intervals, but enzyme application only increased NDF and hemicellulose digestibility of the 5-wk hay. Ammoniation also improved the DM degradation in the rumen but enzyme treatment did not.

In Experiment 2, 90 Angus and Brangus steers (308 ± 37 kg) were stratified by weight and randomly allocated to 18, 1.01-ha pens located on dormant bahiagrass pasture. Three pens were assigned randomly to each treatment. The same hays and supplement fed in Experiment 1 were fed in Experiment 2. The treated hays from both regrowth intervals were weighed and fed in round-bale feeders to cattle in respective pens in quantities sufficient to ensure *ad libitum* access for 55 d. Steers were adapted to diets for 6 d, and full body weights were obtained on two consecutive days at the beginning (d 7 and 8) and end (d 54 and 55) of the measurement period. Weekly supplement allocations were fed in open troughs in equal amounts three times per week and they were completely consumed. Refused hay was weighed on d 30 and 55. Ammoniation increased hay DMI and tended to increase final BW and ADG. Enzyme treatment increased DMI of the 5-wk hay but had no effect on growth performance.

In summary, ammoniation improved the forage quality of the hays and enhanced the growth of steers. Enzyme treatment had few beneficial effects on the nutritional value of the hays and did not improve the growth performance of the steers.

Ammoniation had more pronounced effects on the more mature (13 wk) hay, whereas enzyme application only resulted in beneficial effects when applied to the 5-wk hay.

Like others in the literature, this study indicates that much work needs to be done to improve the consistency and efficacy of enzyme treatment of forages and diets before it can be recommended for routine use by cattle producers. Enzyme treatment of forages differs from chemical treatment because the enzymes involved are not a simple chemical entity, instead they are a myriad of complex proteins that only work when certain conditions are met (Nelson and Cox, 2008). As mentioned by Wang and McAllister (2002), measuring animal responses to commercial enzyme products without a proper understanding of the mechanisms underlying their action will only confuse and delay the practical adoption of this technology. Therefore, additional research on animal responses to enzyme addition to diets should be delayed until a mechanistic investigative approach is implemented that describes the full array of enzymes needed for cell wall hydrolysis and outlines the conditions required for synergistic effects that result in through degradation of cell walls. Also, presence in appropriate proportions of subtypes of xylanase and cellulase enzymes that are critical for thorough cell wall hydrolysis should be verified in addition to measuring cellulase and xylanase activities.

Since enzymes are expected to release oligosaccharides and monosaccharides as a consequence of their catalytic actions, the Van Soest detergent system (Van Soest et al., 1967, 1991) should be considered inadequate for evaluating improvements in enzyme potency at cell wall hydrolysis because it does not measure sugars. Rather, techniques like the Uppsala dietary fiber system should be used (Theander et al., 1995). This system uses a gas chromatographic-colorimetric-gravimetric method for

determination of total dietary fiber as neutral sugar residues, uronic acid residues, and Klason lignin. This approach is more labor intensive and costly than the Van Soest system but it has been used by the lignocellulosic degradation industry, partly because it reflects enzyme effects on cell wall components more accurately. Hydroxycinnamic compounds in forages should also be measured routinely when evaluating the impact of enzyme treatments because they impede enzymatic cell wall degradation.

Finally, the impacts of anatomic structures and tissue arrangement of forages on cell wall digestibility should be considered in order to develop improved enzyme cocktails that hydrolyze cell walls consistently and thoroughly. In particular, future research should evaluate effects of adding cutinases, suberinases, esterases, and etherases to xylanase – cellulase enzyme cocktails in order to enhance the removal of specific anatomical and chemical features that impede plant cell wall digestion.

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BIOGRAPHICAL SKETCH

Juan Jose Romero was born in Lima, Peru in 1984. He graduated from San Agustin High School in 2001 and his favorite courses were biology, chemistry and history. He was raised in a family deeply involved in Agricultural Sciences research; therefore, he was exposed to the agricultural challenges in Peru at a very early age. He realized that there was a big gap between the modern agriculture industry on the coast of Peru and the agriculture of subsistence in the Andes and he promised himself that he would endeavor to improve agriculture in the Andes. His parents tackled the challenges of farming in the Andes from a Crop Science perspective, but he always liked cows and llamas more, so he decided to enroll in the Animal Science program at Universidad Nacional Agraria La Molina (UNALM). During his studies he came across a paper titled 'Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes'. Prior to that time, had no knowledge about improving the quality of straw but knew that cereal straw was an abundant resource in the Andes. Consequently, he presented a seminar on the subject to his classmates at the University and conducted his undergraduate thesis research on the subject. He submitted his Literature Review on the subject to the 2007 Alltech Young Animal Scientist Contest for Latin America and was awarded the prize for the second best report in the region. Afterwards, he received his B.S. from UNALM in 2007 and finished at the top of his Animal Science class. Soon after his graduation, he was admitted to UF to pursue an M.S. in Animal Science under the supervision of Dr. Bill Brown. The fact that he was going to work on improving forage quality was particularly exciting to him. After Dr. Brown moved to the University of Tennessee, he was supervised by Dr. Adesogan until he completed his M.S. program. During his time at UF, his understanding of constraints of, and methods to

improve forage digestibility was greatly enhanced and he presented his research findings at the Annual Meeting of the American Society of Animal Science in Montreal in 2009. He plans to continue working on the subject so that he can develop a practical solution for improving digestibility of low-quality forage in the Andes.