FIBROLYTIC ENZYMES IN RUMINANT NUTRITION AND THEIR EFFECT ON FORAGE CELL WALL INTEGRITY

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DECLARATION

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DATE: February 2011

ABSTRACT

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Exogenous fibrolytic enzymes (EFE) as additives in ruminant feeds are being researched worldwide. Promising effects on dry matter intake (DMI), digestibility and production in especially dairy cows, but also feedlot steers and even sheep have been observed. However, lack of or negative effects are also reported and the need arises for clarity on the mode-of-action of EFE. Forages are characterised as being highly heterogenic and contain varying concentrations of fibre. The fibre, in turn, varies greatly in digestibility, due to the chemical as well as anatomical build-up of this complex carbohydrate. Fibre, however, presents a major source of potential energy for ruminant animals and EFE is a viable option to increase the digestibility of forages. Therefore, a study with the aim of establishing whether EFE can affect the digestibility of forages, how it affects the digestibility and the clarification of the mode-of-action was drafted. From the literature, the first objective was readily attained and clear indications exist that EFE can indeed improve animal performance when their diets are treated with such enzymes. From the current study, it was shown that EFE can alter the rate and extent of gas production of certain forages (lucerne, kikuyu and weeping love grass) and also improve the *in vitro* digestibility thereof ($P < 0.05$). This is in agreement with other research findings and it was concluded that these effects were likely exerted during the early stages of digestion. A complete feed for sheep, when treated with the EFE, showed positive effects on the *in sacco* digestibility, as well as on the digestion kinetics of the feed ($P < 0.05$). The *in vitro* digestibility of the complete feed was also improved due to EFE treatment ($P < 0.05$). The observations on *in vitro* digestibility were less marked when a purified xylanase, obtained from the partial purification of the EFE cocktail, was used as the sole fibrolytic enzyme treatment. It is apparent, therefore, that enzyme specificity plays a major role in obtaining positive effects on digestibility of forages

and feeds. In agreement with the literature, it is proposed that the approach to improve the digestibility of forages should be to use EFE cocktails containing various enzymes, matching the complexity of the substrate. The major aim of the study was, however, an in depth investigation of the mode-of-action of EFE. This aim was approached by observing changes in plant tissue material at the histological level upon treatment with EFE and incubation in buffered rumen fluid. Results showed that EFE had subtle, yet significant effects on cell wall material for the various tissues studied ($P < 0.05$). The major effect observed here was that EFE had a thinning effect on the cell wall thickness ($P < 0.05$). It was deduced that as EFE affected the cell wall of the plant material, earlier access by microorganisms could be achieved. Also, nutrients caught in the cell wall matrix could then be released for digestion. Therefore, observations that EFE increases the rate of digestion, as well as the extent of digestion of not only fibre, but also protein, were explained by the enzyme's action on cell wall material. It was concluded that there is definite merit in the use of EFE to improve the digestibility of ruminant feeds and that this is partly related to effects on the cell walls of the forages. The effects can be expected to occur during the early stages of digestion, thereby potentially increasing the passage rate of digesta from the rumen. Additionally, the effect of the EFE is not limited to fibre and increased digestibility of all nutrients can be expected, thereby increasing the overall digestibility of the feed. Future research should further elucidate the mode-of-action of EFE using advanced technologies routinely employed in the plant sciences. Additionally, the main potential advantage of EFE treatment lies in improving the digestibility of poor quality roughages. Unfortunately, this is an area where limited positive effects are observed and in depth investigations should be undertaken to classify the specificity and optimum conditions of EFE to better match the complexity of the substrate being treated.

SAMEVATTING

FIBROLITIESE ENSIEME IN HERKOUERVOEDING EN DIE EFFEK DAARVAN OP PLANTSELWAND INTEGRITEIT

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Eksogene fibrolitiese ensieme (EFE) word tans wêreldwyd ondersoek vir die gebruik daarvan as voerbymiddels vir herkouers. Belowende effekte op DMI, verteerbaarheid en produksie van vernaam melkbeeste, maar ook voerkraalbeeste en selfs skape is al gerapporteer. Swak en selfs negatiewe effekte word egter ook waargeneem en daarom is 'n deeglike ondersoek na die metode van werking van EFE van belang. Ruvoere word gekenmerk deurdat dit heterogeen van aard is en bevat variërende vlakke van vesel. Vesel maak op sy beurt 'n wesenlike deel uit van die ruvoer, maar varieer baie in verteerbaarheid weens die chemiese sowel as anatomiese samestelling van hierdie komplekse koolhidraat. Ruvoer verteenwoordig egter 'n goeie bron van potensiële energie vir herkouers en EFE word voorgestel as 'n haalbare behandeling om die verteerbaarheid daarvan te verhoog. Dus is 'n studie beplan met die doelwit om die effekte van EFE te definieer, hoe dit verteerbaarheid beïnvloed en die metode van werking daarvan te ondersoek. Vanuit die literatuur is dit duidelik dat daar wel baie positiewe effekte is waar ruvoere met EFE behandel is en dat diereproduksie wel bevoordeel word daardeur. Vanuit die studie is dit getoon dat die tempo en hoeveelheid gasproduksie van sekere ruvoere (lusern, kikuyu en oulandsgras) verbeter word deur EFE behandeling ($P < 0.05$). Hierdie bevinding was ondersteun deur verbeterde *in vitro* verteerbaarheid van die ruvoere ($P < 0.05$). Dit is in ooreenstemming met literatuur en die gevolgtrekking is gemaak dat hierdie effekte tydens die vroeëre stadia van vertering verwag kan word. 'n Volledige skaapvoer wat met EFE behandel is, het positiewe effekte op *in sacco* verteerbaarheid en verterings kinetika data gehad ($P < 0.05$). Weereens is die *in vitro* verteerbaarheid van die voer verbeter ($P < 0.05$). Waarnemings op *in vitro* verteerbaarheid was veel minder opvallend wanneer 'n gesuiwerde xylanase as enigste fibrolitiese ensiem behandeling gebruik is. Dit is dus duidelik dat

ensiem spesifisiteit 'n belangrike rol speel in die verkryging van positiewe resultate in die verteerbaarheid van ruvoere en veevoere. In ooreenstemming met literatuur word dit voorgestel dat ensiemmengsels wat verskeie ensieme bevat as EFE gebruik behoort te word ten einde aan die kompleksiteit van die substraat te voldoen. Die hoof doelwit van die studie was egter 'n indiepte ondersoek na die metode van werking van EFE. Hierdie doelwit is benader deur die effekte van EFE op selwand strukture van plantmateriaal op 'n histologiese vlak te ondersoek. Die ruvoere was vooraf met EFE behandel en *in vitro* geïnkubeer in rumen vloeistof. Die resultate het getoon dat EFE 'n matige, dog betekenisvolle effek op die selwand materiaal van die onderskeie weefsels gehad het ($P < 0.05$). Die belangrikste waargeneemde effek was dat EFE 'n verdunningseffek op die selwande gehad het. Dit is afgelei dat as EFE die selwand kan beïnvloed, mikro-organismes vroeër toegang tot die inhoud kan kry. Verder, nutriënte vasgevang in die selwand matriks raak ook beskikbaar vir vertering. Hierdie afleiding en die effek van EFE op selwande verklaar waarnemings dat EFE die tempo van vertering sowel as die vlak van vertering van nie net vesel, maar ook proteïen kan bevoordeel. Die gevolgtrekking is gemaak dat daar definitiewe meriete is in die gebruik van EFE om die verteerbaarheid van herkouervoere te verbeter en dat dit verband hou met die ensiem se werking op selwande van die ruvoere. Die effekte kan verwag word tydens die vroeë stadia van vertering om dus deurvloeitempo van digesta te verbeter. Die effek van die EFE is verder nie beperk tot vesel nie, maar positiewe effekte op ander nutriënte kan verwag word en vervolgens 'n algehele verhoging in die verteerbaarheid van die voer. Navorsing behoort in die toekoms verder die metode van werking van EFE te ondersoek deur gebruik te maak van gevorderde tegnologie wat alledaags gebruik word in die Plantwetenskappe. Die belowendste aanwending van EFE lê in die verbetering in vertering van swak kwaliteit ruvoere. Dit is ongelukkig juis hier waar min positiewe resultate gerapporteer word en indiepte navorsing moet onderneem word om ensiem spesifisiteit en optimum kondisies te definieer sodat EFE beter opgewasse is teen die kompleksiteit van die substraat.

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CONFERENCE CONTRIBUTIONS

Oral Presentations

- Effect of exogenous fibrolytic enzymes on *in vitro* gas production and IVTD, 41st Congress of the South African Society for Animal Sciences, April 2006, Bloemfontein, South Africa.
- Exogenous fibrolytic enzymes: unlocking nutrients from fibre for ruminant animal production. Faculty of AgriSciences research day, December 2010, Stellenbosch, South Africa.

Poster Presentations

- Morphological evaluation of forage degradation *in vitro*. 61st annual meeting of the European Association for Animal Production, August 2010, Heraklion, Greece.

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CHAPTER 1

General Introduction

Enzymes are used worldwide in numerous industrial applications, ranging from:

- the food industry for clarification of juices, beers and wines (Grassin and Fauquembergue, 1996a & b; Galante *et al*., 1998a & b),
- the textile and laundry industry for the bio-staining and stonewashing of denim garments (Galante *et al*., 1998a),
- in washing powders to improve colour brightness and touch of garments,
- the pulp and paper biotechnology,
- in the ethanol fuel industry for the conversion of starch to glucose,
- in the synthesis of drugs, antibiotics and speciality chemicals, and
- in the animal feed industry to improve nutrient utilization (Bhat, 2000; Beg *et al.*, 2001).

The reader is referred to the review paper of Bhat (2000) for a comprehensive discussion on the applications of enzymes. It is, however, the application of enzymes to animal feeds that is of particular interest to this study, especially those used in ruminant feeds. In animal feed biotechnology, enzymes are added to monogastric feeds to eliminate anti-nutritional factors, improve the nutritional value of the feeds by degrading cereal components or to supplement the enzymes lacking in the animals digestive system (Galante *et al*., 1998b). Cellulases, hemicellulases and even pectinases are used in ruminant feed biotechnology to improve feed utilization, affect production of milk or meat and to improve the digestibility of certain feed components. As discussed by Bhat (2000), many research findings several decades earlier have already shown an improvement in feed digestibility and animal production using exogenous enzymes (Burroughs *et al*., 1960; Rust *et al*., 1965) while negative effects have also been shown in these early studies (Theurer *et al*., 1963; Perry *et al*., 1966). Today, renewed research report very similar positive effects (Beauchemin *et al*., 1995, 2003) but with inconsistencies in research findings still being prevalent. Great strides in our understanding of the enzymes and their application have however been made, as is evident in the host of exogenous fibrolytic enzymes (EFE) commercially available. A major field of research in addition to the application of enzymes is a better understanding of the mode of action of fibrolytic enzymes and forms the core of this study. In short, our understanding of EFE at present entails the following:

1. There appears to be a pre-feeding effect which is related to an enzyme-substrate pre-incubation interaction period. The enzyme requires an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Forwood *et al*., 1990; Beauchemin *et al*., 2003);
2. Another pre-feeding effect would be the rate of enzyme applied to the feed prior to feeding and Eun *et al*. (2007) points to the importance of determining the optimum dose rate (DR). Jalilvand *et al.* (2008) also states the optimum DR as essential for enzymes to efficiently alter fibre digestion;
3. According to Pinos-Rodriguez *et al*. (2002) the effects of exogenous fibrolytic enzymes are substrate-related. White *et al*. (1993) indicated that for enzymes to be effective in altering forage degradation the enzyme activities must be specific to the chemical composition of the targeted substrate. Except for enzymes being substrate specific, their action is also reliant on substrate temperature and pH. Of the post feeding effects of enzymes, the ruminal pH appears to be one of the most important factors (Colombatto *et al*., 2007);
4. Alvarez *et al*. (2009) reports that due to the increased dry matter (DM) and crude protein (CP) soluble fractions of diets upon fibrolytic enzyme addition, the reducing sugars produced would provide energy that would lead to rapid microbial growth. Increased ruminal bacteria numbers could lead to increased microbial colonization of the feed particles;
5. Furthermore; Giraldo *et al*. (2008) suggested an alteration in the fibre structure due to the enzyme effects. This, coupled with the increased colonization would shorten the lag time prior to the initiation of digestion by the rumen microbes (Yang *et al*., 1999). Indeed, by enzymes acting on the structures of plant cell walls, the access of the microbes to the potentially fermentable fibre is enhanced (Sutton *et al*., 2003; Elwakeel *et al*., 2007);
6. There is a synergistic effect of EFE with the microbial enzymes produced in the rumen, hence the hydrolytic activity within the rumen is increased (Morgavi *et al*., 2000), and
7. Laboratory results suggest that it is important to consider the combined effect of enzyme type, enzyme level, and forage moisture condition when forage is treated with enzymes.

These contributing factors will be dealt with in more detail in the subsequent sections of this document and expanded on in the research conducted in this study.

Objectives

The objectives of this study were threefold. The first research question posed was whether EFE can alter fibre digestibility and therefore have an impact on animal performance. To a large extent, the answer to this question lies in the host of published research available and is addressed in the literature review (Chapter 2). Secondly, if EFE indeed alters fibre digestibility, the question arises on how it affects fibre digestion. This objective is addressed in the first part of the dissertation wherein the effects of EFE on various substrates is discussed based on *in vitro* and *in situ* studies (Chapter 4 and 5). Finally, the objective was set to further elucidate on how EFE affects fibre digestion. This third objective forms the distinguishing feature of this study and is related to establishing the mode-of-action of EFE. The answer to this question not only lies in research conducted within this study, but also in the research already published during the last couple of decades. This objective was approached by observing and quantifying the histological changes to forage plant material due to EFE treatment at histological level, as is discussed in Chapter 7.

Understanding the mode-of-action of the exogenous fibrolytic enzymes will better equip us to utilize and apply these exogenous enzymes that are otherwise limiting the rate of the hydrolysis reaction of fibrolytic feed components for commercially important ruminants.

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CHAPTER 2

Literature review

Limitations of forages to rumen degradation

The use of relatively high fibre, low energy diets as ruminant feeds in comparison to other domesticated animal diets is common practise in the nutrition of sheep, dairy and beef cattle and in feedlot finishing of animals. Increasing the digestibility of the often poor quality forages has been a topic of research for many years. It is clear that forages play an important role in the animal industry worldwide. The fibre (cell wall) portion makes up to 300 to 800 g/kg of forage dry matter and represents a major source of nutritional energy for ruminants, but, unfortunately less than 50 % of this fraction is readily digested and utilized by the animal (Hatfield *et al.,* 1999).

The accessibility of the plant cell wall to ruminal microorganisms is complex and is described by Boon *et al.* (2005) to consist of three components. The first is the accessibility of a tissue particle. This is related to the size of the particle with large particles having only outer cell walls available for fermentation, hence the slow initial rate of fermentation of these often lignified and poorly degradable tissue (Engels and Schuurman, 1992). Mastication of course plays a major role in overcoming this limitation (Wilson 1990) as does processing of forages. The second component is the accessibility of the cell wall and the third component is the accessibility of the plant cell wall components by ruminal microorganisms. These components are related to structural factors such as cell wall thickness. For instance, sclerenchyma cells increase their cell wall thickness to such an extent that the lumen diameter of the cell becomes so limited that the space available is only sufficient for one microbe at a time (Boon *et al*., 2005). Finally, the highly digestible cell wall contents can be encrusted by indigestible lignin, making it almost impossible for the microorganisms or even their enzymes to find access to such components.

The plant's first line of defence against microbial degradation in the rumen is the outer layers of epicuticular waxes, the cuticle and pectin (Forsberg and Cheng, 1992). The cuticle, however, is disrupted by mastication and pretreatment of the feedstuffs. The plant cells are connected by lamellae composed of pectin which in turn is formed by a backbone of α-1, 4 linked residues of D-galacturonate. Pectin is digested in the rumen by pectinolytic species

or by species producing pectinases and xylanases (Cheng *et al*., 1991; Gordon and Phillips, 1992).

The plant cell wall is comprised primarily of fibrils of cellulose which accounts for 20-30% of the dry weight of primary cell walls (McNeill *et al*., 1984). Hemicellulose is another major component of both stem and leave cell walls and is comprised mainly of a backbone of β-1, 4 linked xylose residues (xylans). The structure of hemicellulose is complicated by attachment of the various side chains, consisting of acetic acid, arabinose, coumaric acid, ferulic acid and glucuronic acid to the xylose residues (McNeil *et al*., 1984). Xylan polymers may be cross-linked to other hemicellulose backbones or to lignin. Structural proteins (extensins) are also commonly found in dicotyledonous cell walls which entrap other polymers within the wall (Fry, 1986). Thus the plant cell wall is an interwoven matrix of polymers (Selinger *et al*., 1996). In certain species, a secondary cell wall is deposited interior to the primary cell wall, allowing for structural strength of the plant. These cell walls form a formidable barrier against microbial invasion (Somerville *et al*., 2004).

As stated by Somerville *et al*. (2004) plant cell walls are a complex and dynamic structure consisting of mainly polysaccharides, highly glyosylated proteins and lignin. Weimer (1996) listed the cell-wall structure as a major constraint to penetration by non-motile cellulolytic microbes into the lumen. This constraint is related to the matrix interactions between biopolymers of the cell wall and the low substrate surface area. One potential strategy of ruminal cellulolytic bacteria and fungi to support rapid rates of cellulose hydrolysis is the synthesis of large amounts of fibrolytic enzymes, particularly cellulase. This is predominantly the strategy of ruminal fungi and has been used by microbiologists to produce hypercellulolytic strains for enzyme production (Montenecourt and Eveleigh, 1977 as cited by Weimer, 1996). In addition, ruminal fungi will also produce lower amounts of fibrolytic enzymes, but of high specific activity (Wood *et al*., 1986). Ruminal cellulolytic bacteria on the other hand utilize a strategy in which the enzyme activity is predominantly located at the cell surface that facilitates adhesion to and degradation of the cellulose microfibril. Fibrous cells can only be digested by bacteria from the interior (lumen) because the middle-lamella primary wall region is indigestible as stated by Wilson and Mertens (1995) after extensive research on cell wall accessibility and cell structure limitations to microbial digestion of forages. This is consistent with the “inside-out” theory of plant digestion as described by Cheng *et al.* (1991). The most readily digestible plant tissues are located inside the plant and therefore intact plants are digested slowly. For microorganisms to digest the plant cell contents, access is only gained via the stomata and therefore mechanical disruption of plant material, such as chewing or grinding improves microbial access to the nutrient-rich inner

tissues. In addition Wilson and Mertens (1995) state that as little as 20% of the wall thickness would typically be degraded within the average residence time of fibre particles in the rumen.

In addition to the chemical and architectural composition of fibre, the rumen environment is also an important contributing factor to fibre digestion. In this regard it is mostly the ruminal pH that can serve as constraint to cellulose digestion. The optimal pH for ruminal bacteria is near neutrality and ruminal cellulolytic bacteria in particular appear to be sensitive to pH<6.0 (Russell and Dombrowski, 1980). In addition, Weimer (1996) discussed microbial interactions as a second environmental factor of importance for fibre digestion. There is significant competition between individual species of ruminal cellulolytic bacteria and other bacteria for nutrients.

In summary, the limitations of forages appear to be related to three factors: 1) the chemical composition of the fibrous source, 2) the spatial orientation and crystalline architecture of the fibre and 3) the rumen environment. Weimer (1996) concluded that the upper limit in the rate of cellulose digestion in the rumen environment is close to its potential due to the aggressive cellulose digestion capabilities of predominantly ruminal cellulolytic bacteria. Consequently, to further improve the rate of fibre digestion other avenues than improving these bacterial strains need to be researched. These avenues are likely linked to feeding management strategies that prevent unfavourable rumen conditions and to improving the extent of digestion by removing matrix interactions among forage cell wall biopolymers (Weimer, 1996).

Exogenous fibrolytic enzymes in animal nutrition

Exogenous fibrolytic enzymes have been studied extensively in the last couple of decades and a summary of the most important findings thereof is given in Table 2.1 at the end of this chapter. The main aim of the inclusion of these enzymes is to increase the fibre digestibility of the diets fed, with the subsequent improvements in feed intake and animal production, amongst other. Preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fibre (Feng *et al*., 1996). Hristov *et al.* (1998) in a review paper on the mode of action of exogenous enzymes, defined enzymes as “proteins that catalyze chemical reactions in biological systems”. In the context of animal feeds, exogenous enzymes catalyze the degradative reactions of feedstuffs in order to release nutrients such as glucose for utilization by the microorganisms or host animal itself. The

complete breakdown of any feedstuff is a complex process and literally requires hundreds of enzymes (Hristov et al., 1998).

Bhat in 2000 wrote a research review paper on cellulases and related enzymes in biotechnology. In that paper, the author details the use of these enzymes in both monogastric and ruminant feeds, showing that cellulases and hemicellulases have a wide range of potential applications in these livestock systems. In monogastric nutrition, hydrolases are the main class of enzyme used to eliminate anti-nutritional factors, degrade certain cereal components to improve the value of the feed or to supplement the animal's endogenous enzymes that might be limiting in the utilization of their feeds. Of particular interest to this study is the use of β-glucanases and xylanases to hydrolyse non-starch polysaccharides (NSP) commonly found in barley, wheat and other cereals fed to pigs and poultry. In the review, Bhat (2000) reported on the interest in using enzyme preparations in ruminant feeds. The successful use of enzymes depends on their stability in the feed, the ability of the enzymes to hydrolyse the plant cell wall components and the ability of the animal to utilize the resultant products efficiently. As is often reported in the literature, the author pointed to the inconsistent results obtained and ascribed this mainly by the presence of the hydrophobic cuticle, lignin and its close association with the cell wall components (Bhat, 2000). Since Bhat's review in 2000, many research groups have been studying effects of fibrolytic enzymes in ruminant diets and although the results can still be regarded as inconsistent, our understanding of the action of these enzymes has greatly improved. Other explanations for the variability include the types and activities of enzymes which is caused to a large extent by the organism from which it is produced, the substrate used for its growth and the culture conditions used (Considine and Coughlan, 1989; Gashe, 1992). There is evidence that biodegradable substrates such as sugar cane bagasse yield higher enzyme activities than submerged fermentation (Gerardo *et al*., 2009). Also, the composition of the substrate used, the method of enzyme application and the portion of the diet the enzymes is added to, confounds results (Beauchemin and Rode, 1996; Hristov *et al*., 1998).

It is of importance to identify the key enzymatic activities (Eun and Beauchemin, 2007) and as noted by Wallace *et al.* (2001), many of the enzymes used in ruminant studies were developed for other applications. Hence, the key activities are likely to differ from those needed for fibre degradation. Technology, however, exists in which certain strains can be grown to produce fibrolytic enzyme cocktails as an alternative to using commercial products not necessarily designed for ruminant nutrition. In this regard, Gerardo *et al*. (2009) showed that when two strains of white-rot fungi were cultivated on sugar cane bagasse, the resultant

enzymatic extracts contained xylanases and cellulases, with additional laccases that could be beneficial in fibre modification. In fact, research has been reported where such novel enzyme preparations (produced from Abo 374) have been used to improve body weight (BW) gains of Dohne Merino lambs (Cruywagen and Goosen, 2004; Cruywagen and Van Zyl, 2008). Earlier recommendations by Hristov *et al.* (1998) are in agreement with those made by Beauchemin *et al.* (2003b) and the authors recommended the way forward in three categories: 1) the site of enzyme action needs to be defined, 2) the enzymes have to match the feed/substrate and 3) the cost of the enzyme should be economically justified regarding the effects expected from its inclusion.

Exogenous fibrolytic enzymes in ruminant nutrition

The challenge in successful farming with ruminants, be it dairy cattle, beef cattle or sheep and goats lies in the effective utilization of feed resources, as feeding costs present the largest component of production costs. Of the feeds typically utilized, forage composes the largest part and hence presents a logical area of research for the improvement thereof. Exogenous fibrolytic enzymes present one way of improving fibre digestibility (Johnston, 2000). Many authors have reported on the successful use of this technology and will be highlighted in the following sections. In the past, advances in the use of exogenous enzymes have been far greater in the nutrition of monogastric animals than of ruminants. Concerns were that the fibrolytic activity of the rumen was such that EFE would not be effective. Also, many believed that the EFE would be deactivated either in the feed manufacturing process or by proteolysis in the rumen itself. These concerns have been thoroughly addressed and positive effects of EFE have been demonstrated in beef and dairy cattle (Beauchemin *et al*., 1995; 2003a) and even small stock such as sheep (Cruywagen and Goosen, 2004).

Alvarez *et al*. (2009) studied the effect of two commercially available fibrolytic enzyme products on rumen digestibility in steers fed high fibre diets. They found that both the DM and CP soluble fractions (a) of the high fibre diet were increased due to exogenous enzyme addition. These results were ascribed to the pre-incubation interaction time of 24h that were allowed. Other researchers have also previously suggested that pre-incubation of the diet with the enzyme is of importance (Forwood *et al*., 1990; Elwakeel *et al*., 2007; Krueger and Adesogan, 2008). The enzyme requires an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Forwood *et al.,* 1990; Beauchemin *et al*., 2003a). The resultant stable enzyme-feed complex can then potentially degrade the relevant tissue in the rumen (Kung *et al*, 2000). Alvarez *et al*. (2009) however

did not find any benefit of the exogenous fibrolytic enzyme treatment on wheat middlings or oat straw and an increase in the *a*-fraction was only reported for the prepared high fibre diet. Of interest is that this research group reported a higher total disappearance of CP. This suggests that the effect of the enzyme was not only limited to the fibre components. In fact, in that study there were no improvements on NDF or ADF disappearance. Kohn and Allen (1995) suggested that the fibrolytic enzymes facilitate the degradation of cell wall bound proteins.

In large ruminants, positive effects due to exogenous fibrolytic enzyme treatment of the substrate/diet have been reported. These results were obtained with pre-treatment of the feed with the enzyme, but direct-fed enzymes have also shown increases in NDF digestion in bulls (Murillo *et al*., 2000) and in dairy cattle (Lopez-Soto *et al*., 2000). Average daily gain increases have been reported for steers (Ware *et al.,* 2002). Some EFE preparations result in improved cell wall digestibility *in vitro* (Colombatto *et al*., 2003) or *in vivo* (Schingoethe *et al.*, 1999). Recent studies also indicated increases in milk production of lactating dairy cows (Tricarico *et al*., 2008) or improvements in the energy balance of transition cows (DeFrain *et al*., 2005). Increases in forage utilization, production efficiency and reduced nutrient excretion have been reported (Beauchemin *et al*., 2003a). Giraldo *et al*. (2007) reported that treating a high-forage substrate with EFE from *T. longibrachiatum* increased the microbial protein synthesis (MPS) (measured as $^{15}N\text{-}NH_3$ after 6 hours of incubation in Rusitec fermenters) and improved fibre degradation. These authors concluded that EFE stimulated the initial phase of microbial colonization. This supports the hypothesis that EFE subtly erode cell wall structure allowing ruminal microbes to obtain earlier access to fermentable substrate during the initial phase of digestion (Colombatto *et al*., 2003). However, some results have also been published showing no effect of exogenous polysaccharide degrading enzyme preparations on ruminal fermentation, polysaccharide degrading activities or apparent digestion of nutrients in dairy cattle (Hristov *et al*., 2008). Similarly, exogenous fibrolytic enzyme treatments do not always result in positive effects on fibre digestion (Vicini *et al*., 2003).

In the semi-arid and arid regions of the world, sheep and goats are increasingly produced due to their adaptation to these climates (Bala *et al*., 2009). Studies using goats to ascertain the effect of exogenous fibrolytic enzymes have been limited and results poor due to the goat's ability to utilize fibre being superior to that of large ruminants (Bala *et al*., 2009). In addition, information on the effects of ruminal fermentation in small ruminants is scarce (Pinos-Rodriquez *et al*., 2002). Yang *et al*. (2000) could not ascertain any effects of fibrolytic enzymes fed to goats. However, in a study done by Bala *et al.* (2009) positive results on

DM, OM, NDF, ADF, CP and total carbohydrate digestibility were reported when cross bred lactating Beetle-Saanen goats were fed a concentrate supplement fortified with cellulases and xylanases. They also reported positive effects on milk yield in the third quarter of lactation along with a decrease in feed intake of up to 7%.

In another study on small ruminants by Cruywagen and Goosen (2004) with Döhne Merino lambs, no effects of exogenous enzyme inclusion were observed on feed intake of a completely mixed roughage based diet (NDF 443 g/kg DM). Animal performance was, however, improved in that lambs gained significantly more body weight when fed the enzyme treated diet, and had improved feed conversion ratios. It has to be noted that in this study a novel enzyme cocktail was used and was produced by fermentation of the fungal strain, Abo 374. The enzyme cocktail was extracted from the fermentation media and the supernatant added to the wheat straw component of the feed 18h prior to feeding, to allow for an enzyme-substrate interaction period. In a subsequent study, the enzyme containing supernatant was stabilised and included in the diet in the liquid lyophilized or fresh supernatant form. In this study, no pre-incubation interaction period was used and diets were only equilibrated for 15 min prior to being fed to lambs. In both the high (920 g/kg forage) and low (600 g/kg forage) forage diets, the enzyme treatment resulted in improved BW gains and feed conversion ratios (Cruywagen and van Zyl, 2008). This is in concurrence with earlier findings (Cruywagen and Goosen, 2004). Giraldo et al. (2008a) also reported positive effects of an exogenous fibrolytic enzyme (endoglucanases and xylanases) on ruminal activity in sheep. Even though they did not find an effect on diet digestibility (70 grass hay: 30 concentrate), they reported increases in the ruminally insoluble potential degradable fraction of grass hay DM as well as its fractional rate of degradation. The molar proportion of propionate was increased and the aceate:propionate ratio lowered. These findings are of particular interest as no enzyme substrate interaction period was allowed and enzymes were directly delivered into the rumen of the sheep.

When EFE treated lucerne or rye-grass based diets were directly fed (no pre-treatment period allowed) to lambs, positive results were obtained (Pinos-Rodriguez *et al*., 2002). The EFE was a commercially available fibrolytic product supplied by Alltech Inc., Nicholasville, KY (Fibrozyme) and supplied directly via the ruminal cannula daily at 5g per animal. For both substrates, the DMI, OMI and CP intake were increased; however, fibre (ADF and NDF) intake was not affected. The apparent digestibility of CP, NDF and hemicellulose were increased for the lucerne treatment only. The N balance was improved for both hays due to enzyme inclusion as more N was retained by the lambs. The total VFA concentration measured at 3 and 6h after enzyme treatment was increased for both hays (Pinos-Rodriguez

et al., 2002). In a later study by Pinos-Rodriguez *et al.* (2008), a total mixed ration (TMR) treated with fibrolytic enzymes were fed to lambs. The same enzyme product as in the previous study was used at a rate of 2g EFE/kg DM TMR, with the difference that the TMR was treated with the EFE 24h prior to feeding. As stated by Beauchemin *et al.* (2003a), an enzyme is not necessarily appropriate for all feed ingredients, but an approach would be to include an enzyme that is relatively suitable for most. Therefore, three forage to concentrate ratios were used in the TMR's; 400:600, 500:500 or 600:400 kg/kg to best match the activity of the EFE product. The EFE increased the soluble fraction of DM as well as the DM and NDF ruminal *in situ* disappearance rates. In that trial though, no effects were observed on feed intake or N balance and ruminal fermentation patterns were unchanged (Pinos-Rodriguez *et al*., 2008). Further results with Fibrozyme indicated that the enzyme preparation stimulated the *in vitro* fermentation of substrates at 5 and 10h of incubation, but that the effect diminished at 24h (Ranilla *et al*., 2008). Again, it was reported that effects were dose dependant and varied with substrate used (particularly the presence of neutral detergent soluble components in the substrate).

The abundance of research on exogenous fibrolytic enzyme application appears to have been done using either *in vitro* or *in situ* studies. Dean *et al.* (2008) evaluated four different commercial exogenous enzyme products on the ruminal degradation of coastal bermudagrass hay or Pensacola bahiagrass hay (12-week re growths, tropical grasses). The enzyme treatments were Promote®, Biocellulase X-20®, CA® and Biocellulase A-20®. These products were found to contain cellulase at 33.7, 22, 0 and 51.3 filter paper U/g and xylanase at 5190, 7025, 0 and 3530 µmol xylose released/min/ml, respectively. Although CA® showed zero cellulase or xylanase activity, it contained some fibrolytic activity. Results showed that Promote hydrolysed NDF into water soluble carbohydrates (WSC), decreased ADF levels and had higher 6h IVDMD. The other enzyme treatments also resulted in decreased NDF concentrations and increased 6h IVDMD, but only for Bermudagrass hay. Only enzyme X-20 resulted in an increased 48h IVDMD of both substrates. The enzymes also resulted in higher 6h IVADFD, with the exception of CA and Promote. The enzymes had negligible effects on the extent of fibre digestion and *in situ* DM degradation as no responses were observed in the maximal degradable (*b), a+b* or potentially degradable (P) fractions. It appears that the enzymes therefore exhibited their effects mostly in the initial and 48h stages of DM digestion. It is of interest to report that the feed substrates were also evaluated after ammoniation and that results were far superior to that obtained by the enzyme treatments. However, application of exogenous enzyme products is far less costly in terms of infrastructure, storage of treated substrate or hazards associated with ammoniation (Dean *et al*., 2008).

Commercial enzymes for monogastric nutrition can be used in ruminant diets as shown in the results of Jalilvand *et al.* (2008b) where Cellupract AS 130, Natuzyme and Endofeed DC were evaluated *in situ*. These enzyme products are typically added to monogastric diets to reduce the negative effects associated with NSP's present in the diet. Cellupract showed postive effects evident in the increased rapidly soluble fraction (*a*), potentially degradable fraction (*b*) and effective degradability (ED) for DM of all the forages studied (lucerne hay, maize silage and wheat straw) in Blochi ewes.

Giraldo *et al.* (2008b) used different ratios of grass hay to concentrate (0.7:0.3; 0.5:0.5 and 0.3:0.7) to evaluate the effect of three fibrolytic enzymes produced by *Trichoderma viride, Aspergillus niger* and *Trichoderma longibrachiatum*. All enzyme treatments increased the *in vitro* degradability of the substrate DM and the total VFA as well as acetate and propionate production were increased. This was accompanied by an increase in the *in vitro* gas production. These effects were greatest at 8h incubation, with effects remaining but less pronounced at 24h. The enzymes were used at two inclusion rates, but it was found that little differences occurred due to dose rate of enzyme (40 or 80 enzyme units/g substrate)(Giraldo *et al*., 2008b). This would appear to be in contrast with recommendations by other researchers such as Eun *et al*. (2007a) to determine the optimal dose rate. Further elucidation is validated though.

Rusitec fermenters can be used to determine the effect of exogenous fibrolytic enzymes on fibre digestion. Giraldo and co workers (2007) have used this system in their laboratory to evaluate EFE and other treatments (such as fumarate) on methane production, fermentation, VFA production and microbial production. In one such study they found that mixed fibrolytic enzymes from *Trichoderma longibrachiatum* resulted in daily increases in the production of acetate, butyrate and methane as well as substrate DM and fibre disappearance. The daily flow of microbial-N and microbial colonisation of substrate was affected only at 6h of the total incubation of 48h, resulting in enhanced fibre degradation. Of interest here is that enzyme treatment resulted in similar effects on rumen fermentation to than when enzyme was fed in combination to fumarate. Giraldo *et al*. (2007) stated that fumarate is included in ruminant diets for the purpose of decreasing methane production. Methane represents an energy loss to the animal and contributor to global warming. In addition, it has been shown to stimulate the production of VFA and increase diet degradation (Lopez *et al*., 1999; Carro and Ranilla, 2003; Garcia-Martines *et al*., 2005). It therefore appears that the fumarate enzyme combination used in their study had no additional benefits on rumen fermentation, compared

to the enzyme treatment alone (Giraldo *et al*., 2007), indicating that fumerate addition was not advantageous.

Ferulic acid esterases can be used to release ferulic acid bound to arabinose side chains of hemicellulose (Faulds and Williamson, 1994). Upon the release of ferulic acid, the cell walls may be further degraded by other polysaccharidases. Hence, if ferulic acid esterases were to be used in combination with fibrolytic enzymes, such as cellulases and xylanases, synergistic effects might be expected (Faulds and Williamson, 1994). Ferulic acid esterase was therefore used in various combinations with cellulase and xylanase to determine the best combination for the degradation of fibre in bahiagrass (Krueger and Adesogan, 2008). These combinations were tested in either the absence or presence of rumen fluid. Results showed that combinations of these enzymes can result in increases in DM disappearance (24h incubation) even in the absence of rumen fluid. In the subsequent experiment, the combinations were tested in the presence of rumen fluid and assayed for its effects on bahiagrass using in vitro gas production over 24 or 96h incubation. For the 24h incubation, no effect was noticed on DM or NDF digestibility or on gas production, but the acetate concentration was decreased whilst the propionate and butyrate concentrations were increased. For the 96h incubation, DM and NDF digestibility as well as gas production and fermentation rate were again not affected, but the lag phase decreased due to use of any of the combinations of enzymes (Krueger and Adesogan, 2008).

A very useful *in vitro* technique to measure effects of exogenous enzyme treatment of forages is the i*n vitro* gas production technique, in which head space gas production can be measured throughout the incubation. Eun and Beauchemin (2007) evaluated 13 endoglucanases and 10 xylanases in this manner and were able to show increased gas production (GP) and OMD (18h) with many of these enzymes when applied to lucerne hay. Based on these initial screenings, two superior enzymes of each category were further evaluated, also in combination with each other. The authors found that the enzymes were effective in improving GP and OMD, but that the combination of the two types of enzymes did not lead to any further improvement (Eun and Beauchemin, 2007).

Although the focus of this document is on the use of exogenous fibrolytic enzymes, mention needs to be made regarding research on α-amylase as dietary supplements for ruminant diets. As starch form a major component of dairy cattle feeds, any improvement in its digestion can have marked effects on animal performance. During the process of starch hydrolysis, α-amylase plays an important role in cleaving starch polymers into oligosaccharides and eventually maltotriose and maltose. Therefore, the addition of α-

amylase to the diet of highly productive animals such as dairy cattle and beef animals can liberate additional starch hydrolysis products, bar the availability of substrate. Tricarico *et al*. (2008) found that although α-amylase did not increase ruminal starch digestion in dairy cows or steers, it did result in increased butyrate and decreased propionate molar proportions in the rumen. In addition, supplemental α -amylase fed to dairy calves resulted in improved epithelium growth as this tissue preferentially utilises butyrate as an energy source. Rumen development is stimulated by the production of VFA by microorganisms and especially by butyrate and propionate (McLeod and Baldwin, 2000). Most of the ruminal butyrate is absorbed by rumen tissue, providing energy for rumen wall thickening and development of papillae (Weigand et al., 1975). The supplementation of α-amylase also supported the rapid growth of bacteria that otherwise grow slowly, or not at all, on starch. These included *Butyrivibrio fibrosolvens, Selenomonas ruminantium* and *Megasphaera elsdenii*. The beneficial effects of the enzyme addition to diets resulted in higher weight gains and longissimus muscle area in feedlot cattle. In dairy cattle, increased milk yield and reduced milk fat proportion without reducing milk fat yield was recorded in 45 commercial herds (Tricarico *et al*., 2008).

Exogenous fibrolytic enzymes in monogastric nutrition

Exogenous enzymes are routinely used in modern monogastric diets. They are abundantly used for their hydrolytic activity to eliminate anti-nutritional factors, degrade certain cereal components to enhance the nutritional value of the feed or to supplement the animal's endogenous enzymes that might be limiting in the utilization of their feeds (Classen *et al*., 1991). Of particular interest is the use of β-glucanases and xylanases to hydrolyse non-starch polysaccharides (NSP) commonly found in barley, wheat and other cereals fed to pigs and poultry (Bhat, 2000). Castanon *et al*. (1997) found that NSP degrading enzyme preparations have two associated effects on cereal NSP. The first is the solubilisation of the insoluble NSP followed by the hydrolysis of this solubilised NSP along with the original soluble NSP present in the feed. This is in concurrence with the findings of Rouau and Moreau (1993) that reported that most insoluble NSP (arabinoxylans) of wheat were first solubilised prior to being hydrolysed to low molecular weight polysaccharides. The subsequent hydrolysis of the solubilised NSP then appears to be limited by the amount of available NSP degrading enzyme (Bedford and Classen, 1992). Therefore, findings that low levels of NSP degrading enzyme result in increases in the amount of solubilised NSP are not uncommon and increased digesta viscosity in the hind-gut of the bird could exist in some situations.

Choct (2006) refers to the use of enzymes to remove the anti-nutritional effects of NSP's, such as arabinose and β-glucan as the first phase in the development of this technology that has now been in use for more than 20 years. According to this researcher the scope of enzyme application expanded in the 1990's from removing anti-nutritional factors and improving digestibility of NSP containing substrates. One of the best examples lies in the use of phytase to liberate P from the unavailable phytic acid form thereof. This has the additional advantage of reducing P excretion in the faeces and therefore alleviating the environmental burden thereof. The next phase is described as the shift in focus to obtain highly effective enzymes for the non-cereal component of monogastric feeds. Of particular interest is the inclusion of enzymes to improve the utilization of vegetable proteins for pigs and poultry. Ongoing research in the field of enzyme application has yielded new areas of application. These include the use of glycanases to degrade carbohydrates as an alternative to antibiotics used in feeds.

Results with the use of exogenous enzyme products in wheat based diets with broiler chickens include improved apparent protein digestion, apparent fat digestion, and improved overall nutrient digestibility and increased apparent metabolizable energy (AMEn) and are reflected in improved weight gain and feed conversion efficiency (FCE) of the chickens (Steenfeldt *et al*., 1998). The major limiting component in wheat fed to chickens is its relatively high content of arabinoxylans (50-80 g/kg DM) (Annison, 1990). Using appropriate enzymes to degrade the xylan backbone of arabinoxylan has been shown to be effective in increasing the nutritive value of diets containing wheat for chickens (Annison, 1992; Choct *et al*., 1995). Few studies have, however, indicated that the degradation of the cell wall NSP to smaller fragments due to EFE treatment lead to the increase in the utilization thereof (Annison, 1992). Steenfeldt, *et al*. (1998), however, concluded that since the pH measured in the caeca of the chickens was lowered as a consequence of enzyme supplementation; part of the degraded NSP was available for microbial fermentation.

Choct (2006) regards further developments in enzyme technology to be dependent on better characterization of substrates used, the gut microflora and the immune system.

Mode-of-action

For the complete breakdown of any feedstuff into its components, literally hundreds of enzymes are required. Hristov *et al*. (1998) in a review paper describes the complexity of

digestion of plant cell walls since plant cell walls contain numerous chemical bonds. Pectin holds the plant cells together and is composed of a backbone of D-galacturonate, interspersed with rhamnose, with methyl ester and sugar side chains. Pectin itself is, however, readily digestible. The primary cell walls are composed of cellulose, a chain of D-glucose monomers (Chafe, 1970). Cellulose has structural properties linked to its crystalinity. The higher the crystalinity, the more resistant the cellulose is to digestion. Cellobiohydrolase, endoglucanase and cellobiase are needed for the breakdown of cellulose. Hemicellulose is the most complex structure, composed mainly by a backbone of xylose monomer residues. The bonds between cellulose and hemicelluloses are relatively weak hydrogen bonds, not covalent bonds. Disrupting these bonds is probably a non-enzymatic process. The xylan polymer backbone in turn is bonded to the cellulose fibrils and this structure is further complicated by side-chains of acetic acid, arabinose, glucaronic acid etcetera (McNeil *et al*., 1984). Xylan polymers may be further cross-linked to other hemicellulose backbones, or to lignin. This structural complexity of hemicellulose obviously requires many enzymes for its digestion. Therefore, based on this simple explanation of a cell wall, it is clear that a major challenge lies in identifying the rate-limiting step in digestion. Ruminant animals, however, have a dynamic array of microbial fibrolytic enzymes to cleave fibrous structures (Hristov *et al*., 1998). Limitations thereof can theoretically be overcome by the addition of exogenous fibrolytic enzymes to complement the rumen microbial system. Theoretically, to positively influence feed digestion, exogenous enzymes would have to contain enzymatic activities that are limiting the rate of the hydrolysis reaction (Morgavi *et al*., 2000b). Herein lies the challenge of exogenous enzyme application. Exogenous enzyme activities are calculated to represent less than 15% of the total ruminal activity, which makes it difficult to envisage exogenous enzymes enhancing fibre digestion through direct hydrolysis alone (Beauchemin *et al*., 1997). Morgavi *et al*. (2000b) indicate that there is substantial synergism between exogenous and ruminal enzymes, such that the net hydrolytic effect is much greater than previously believed. They found co-operation in the degradation of carboxymethylcellulose (CMC) between rumen and exogenous enzymes, particularly at low pH, which could explain, at least in part, the positive results observed with dairy and feedlot cattle.

Another scenario in which feed digestion could benefit from the addition of exogenous enzymes occurs when the rumen pH is sub-optimal for efficient fibre digestion (Morgavi *et al*., 2000). For example, fibre digestion is inhibited because of the depression of the ruminal cellulolytic bacteria when ruminal pH drops below 6.0, but ruminal pH in dairy and feedlot cattle fed high-energy diets is often below 6.0 for much of the day. The optimum pH for the exogenous enzymes produced from *Trichoderma* and *Aspergillus* cultures is lower than the

optimum pH of the rumen, and when high-energy diets are fed, the rumen pH drops significantly and becomes optimal for the exogenous enzymes, thus positively influencing fibre digestion under these conditions.

Evidence exist that the mode of action of exogenous enzymes in ruminants is a combination of pre- and post-feeding effects (McAllister *et al*., 2001; Colombatto *et al*., 2003). The pre-feeding effects include an enzyme-substrate pre-incubation interaction period. Alvarez *et al*. (2009) reported that several researchers had previously suggested that pre-incubation of the diet with the enzyme is of importance (Forwood *et al*., 1990; Elwakeel *et al*., 2007; Krueger and Adesogan, 2008). The enzyme requires an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Forwood *et al*., 1990; Beauchemin *et al*., 2003). The resultant stable enzyme-feed complex can then potentially degrade the relevant plant tissue in the rumen (Kung *et al*, 2000). When enzymes are directly infused into the rumen instead of inclusion via the feed, no improvements in degradation were observed (Kopecny *et al*., 1987; Lewis *et al*., 1996) which serves as further justification for allowing a pre-incubation interaction period. Indeed, Moharrery *et al*. (2009) reported improved *in vitro* DMD and aNDFom digestibility after 8h incubation in rumen fluid where forages were pre-treated (24h prior to incubation) with EFE. The most pronounced effects were on the *a*-value which increased after enzyme pre-treatment. The b-value, however, decreased; therefore no effect was seen on the potential degradability of the forages (a+b). The lag time for aNDFom was also reduced. When no pre-treatment enzyme substrate interaction time was allowed, none of the reported effects mentioned earlier were observed (Moharrery *et al*., 2009), supporting the recommendations of other research groups that a pre-treatment period should be allowed.

Proteolysis of exogenous enzymes, however, seems to not be the sole reason why a pre-incubation interaction time should be allowed as several studies have reported that fibrolytic enzymes are resistant to rumen proteolysis for a significant (6h) time (Hristov *et al*., 1998; Morgavi *et al*., 2000, 2000b). Morgavi *et al*. (2000) found that endoglucanase and xylanase (both from *A.niger* extract) were stable for at least 6h in the rumen, whilst β-glucosidase and β-xylosidase activities were more labile and deactivated after 1h. Different feed enzyme additives were reported to be more stable in the rumen than was previously thought possible, and this stability has been reported to depend on origin and type of activity (Hristov *et al*., 1998). Glycosylation of exogenous enzymes of fungal origin appear to instill sufficient protection for the enzymes in monogastric animals (Chesson, 1993) and indeed in ruminant animals where enzymes are found to be stable for up to 6h in the rumen (van de Vyver *et al*., 2004), or even throughout the whole incubation period (Hristov *et al*., 1998). Where

enzymes are not stable in the rumen, technologies do exist for their stabilization. These include treatment with albumin which increased the half-life of β-glucosidase from 0.5 to 3h or proteins extracted from plant materials, particularly soybean 7S globulin (Morgavi *et al*., 2000).

In the study of Giraldo *et al*. (2008) it was found that direct-fed fibrolytic enzymes positively affected fibrolytic activity in the rumen of sheep and increased the growth of cellulolytic bacteria without a pre-feeding substrate enzyme interaction period. They reported increases in the ruminally insoluble potential degradable fraction of grass hay DM, as well as its fractional rate of degradation. However, the enzyme supplementation did not affect diet digestibility even though molar proportions of propionate were greater and acetate: propionate was lower.

Another pre-feeding effect would be the rate of enzyme application. Responses to enzyme application rate in ruminant studies have been inconsistent (Colombatto *et al*., 2007), but mostly reported as quadratic or non-linear responses (Beauchemin *et al*., 2003b). A good example where low rates of application can lead to adverse effects can be found in work done on NSP degrading enzymes in monogastric nutrition. Castanon *et al*. (1997) reported that low levels of NSP degrading enzyme result in increases in the amount of solubilised NSP, and increased digesta viscosity in the hind-gut of the bird could exist in some situations. This is due to the two-fold action of the NSP degrading enzyme; 1) solubilisation of insoluble NSP and 2) hydrolysis of all soluble NSP (that produced from 1 as well as the original soluble NSP present in the diet). The latter is reliant on enzyme dose level (Bedford and Classen, 1992) and if insufficient it is not uncommon to find increased levels of soluble NSP present in the faeces of the chicken.

Similar effects can undoubtedly occur in ruminant nutrition. Eun *et al*., (2007b) points to the importance of determining the optimum dose rate (DR) for ruminant diets. In their experiment, two substrates (corn silage and lucerne hay) were treated with various exogenous fibrolytic enzymes (containing mainly endoglucanase and xylanase activities). The enzymes were applied at three different dose rates. In that particular experiment, they observed that two of the EFE treatments resulted in significantly higher GP and degradation in either lucerne or corn silage fibre. The optimum DR was 1.4 mg/g of DM for this particular experiment. At this DR, NDF degradability was increased by 20% for lucerne hay and by an astounding 60% for corn silage (Eun *et al*., 2007b). As was reported earlier by these researchers (Eun and Beauchemin, 2007), no additional benefit was observed when the EFE's were used in combination (decreased endoglucanase to xylanase ratio). The

important finding though is that the optimal dose rate has to be determined for each EFE and substrate.

Jalilvand *et al*. (2008) was in agreement with this finding. In their trial they treated three substrates (lucerne hay, maize silage and wheat straw) with an exogenous fibrolytic enzyme, Natuzyme, at three dose rates (3, 6 or 9 g enzyme/kg DM substrate). The results on gas production measured over a 96h period clearly showed that only the 3g/kg DM inclusion rate increased volume of gas produced for the wheat straw only, measured in calibrated syringes. No effects of enzymes were reported on the lucerne hay or maize silage. They concluded that adding enzyme at higher dose rates may not be beneficial (Jalilvand *et al*., 2008). The period of incubation is also of importance and Eun *et al.* (2007b) recommend a 24h incubation time for *in vitro* assays.

According to Pinos-Rodriguez *et al*. (2002) the effects of exogenous fibrolytic enzymes appear to be substrate related. Except for enzymes being substrate specific, their action is also reliant on environmental temperature and pH. Of the post feeding effects of enzymes, the ruminal pH appears to be one of the most important factors. Colombatto *et al*. (2007) tested fibrolytic enzymes in rumen fluid incubation medium with different initial pH ranges (pH 5.4 to 6.8) and found that for the specific conditions of their experiment, the enzymes worked best at pH close to ruminal pH conditions of 6.8. For this particular experiment, it was found that the positive effects of the enzyme treatment on *in vitro* digestion parameters were independent of the pre-treatment period (no interaction time or 20h interaction time).

As reported by Alvarez *et al*. (2009), the reducing sugars released due to fibrolytic enzyme addition would provide energy that leads to rapid microbial growth. In turn, increased ruminal bacteria numbers could lead to increased microbial colonization of the feed particles. Furthermore, Giraldo *et al*. (2008) suggested an alteration in the fibre structure due to the enzyme effects. This, coupled with the increased colonization, would shorten the lag time (Yang *et al*., 1999). Indeed, by enzymes acting on the structures of plant cell walls, the access of the microbes to the potentially fermentable fibre is enhanced (Sutton *et al*., 2003; Elwakeel *et al*., 2007).

Earlier mention was made to the specificity of enzymes for their substrate and White *et al*. (1993) indicated that for enzymes to be effective in altering forage degradation, the enzyme activities must be specific to the chemical composition of the targeted substrate. Studies using intact forages treated with EFE for their digestion characteristics *in vitro,* supplied evidence toward the specificity between enzyme activity and feed composition (Eun *et al*.,

2007a; Giraldo *et al*., 2007; Ranilla *et al*., 2008). Results reported by Ranilla *et al*. (2008) showed that the presence of neutral detergent (ND) soluble components of the substrate is of importance in the interaction between enzyme and substrate. This is a component not previously studied and preliminary results indicate that ND soluble components of the substrates tested can influence the response to the EFE supplement (Ranilla *et al*., 2008). In addition, there is evidence that EFE work in synergy with the microbial enzymes produced in the rumen, hence the hydrolytic activity within the rumen is increased (Morgavi *et al*., 2000). Similarly, laboratory results suggest that it is important to consider the combined effect of enzyme type, enzyme level, and forage moisture condition when forage is treated with enzymes. *In vivo* data indicate improved intake, digestibility, particulate passage, and ruminal degradability when fibrolytic enzymes are added to dry grass immediately before feeding (Feng *et al*., 1996). The beneficial activities of exogenous fibrolytic enzymes are related to their ability to enhance the initial degradation of plant structural carbohydrates and complement normal enzymatic activities associated with ruminal microorganisms (Dawson and Tricarico, 1999).

Microscopic investigations on fibre digestion in the rumen

Forage heterogeneity contributes largely to the variation in degradability of plant material even in those with similar chemical composition but with differences in anatomy. This heterogeneity originates from the distribution of cell wall material between plant cell types, amongst other (Travis *et al*., 1997). It is important therefore to recognize the botanical characteristics of plant material as factor determining its nutritional value (Walters, 1971).

This is in agreement with Weimer (1996) that states: “The architecture of the plant cell may be just as important as its chemistry”. McManus and Bigham in 1973 already said that the distribution pattern of lignin, for instance, rather than the total amount of lignin can mask the potentially digestible cell walls of forages (Travis *et al*., 1997). Marked interactions exist between the cell wall thickness, lignification and other anatomical characteristics of forages and their digestibility (Wilson, 1993). Therefore investigations on the anatomical structure of forages are of importance in determining the digestibility potential of forages.

Akin (1982) reported a method termed the “section to slide” technique where forage tissues can be anatomically analysed. In addition, this method can be employed to digest forage samples *in vitro* in rumen fluid and the samples rapidly evaluated by light microscopy for digestibility.

The use of such a technique showed normal digestion patterns of the various forages studied (orchard grass and bermuda grass), with mesophyll being degraded rapidly and prior to other tissues. Indeed, Wilson (1993) states that mesophyll cell walls are not lignified and are rapidly digestible (Akin, 1989), with complete digestion in under 12h (Chesson *et al*., 1986). Along with mesophyll, phloem was also digested before the other tissues. Extensive digestion of tissues was observed after 17h of incubation, with mostly indigestible tissue remaining after 24h.

This technique is propagated as a simple and rapid technique for studying large numbers of tissue samples and to study effects of various treatments on forage anatomy and digestibility thereof. Furthermore, to quantify the results, software that allows for cross section area measurements can be employed.

Twidwell *et al*. (1989) describes such a method. The objective of that method is to measure plant tissue degradation using computer-based image analysis instead of the subjective visual appraisal. Plant material was degraded in buffered rumen fluid prior to sections being made; therefore the section-to-slide technique of Akin was not employed in this study. After incubation in rumen fluid for 48h, sections of the material were prepared with a thickness of 2µm. The tissues in the cross section were measured with an optical image analysis system and each tissue graded as undigested, partially digested or completely digested (Twidwell *et al*., 1989). This method was one of the earliest developments in the quantification of plant tissue degradation using image analysis. Today, more precise software exists and automated image analysis techniques, such as applied in the plant sciences (Kolukisaoglu and Thurow, 2010), could be adapted for use in investigating the degradation of plant material by microorganisms.

Exciting technology, such as laser-assisted microdissection (LAM), could also be applied to isolate specific microscopic regions from tissue sections. Specific tissues, for instance the epidermis, or even organelles from sectioned forage plant material could be isolated and studied in great detail (Day *et al*., 2005). These new and powerfull technologies should be investigated further for future studies on the degradation of plant material and how treatments, such as EFE, can alter the plant material.

One of the limitations of Akin's technique are the thick sections (50-100 µm) needed to maintain the structural integrity of the tissue during degradation. These thick sections result in poor images with conventional light microscopy images (Travis *et al*., 1997). Confocal

laser scanning microscopy (CLSM) offers an alternative method for estimating the amount of cell wall material present in tissue sections of forages before and after digestion and visualization of the tissue using three-dimentional image reconstruction (Travis *et al*., 1997).

Jung and Engels (2001) used this technique to evaluate the rate and extent of cell-wall thinning of lucerne stem tissues during ruminal degradation. The degree of removal of tissues were determined using light microscopy, while cell wall thickness over certain time periods within a 96h incubation period was measured using scanning electron microscopy as a determinant of cell wall thinning. The rate and extent of cell-wall degradation was then calculated. Results showed that the thin-walled tissues such as xylem parenchyma were degraded at a rate of 0.04µm/h while thick walled colenchyma degraded at a rate of 0.11 µm/h. The non-lignified primary phloem fibres required up to 24h for complete degradation, whereas lignified tissues (pith parenchyma and secondary xylem fibres) were only partially degraded after 96h of incubation in rumen fluid. Some tissues, such as the primary and secondary xylem vessels were completely undegraded (Jung and Engels, 2001). Similarly, Jung *et al.* (2004) examined the degradation of lucern stem walls by different bacterial species as compared to ruminal degradation over 24h and 96h incubation periods. Again they were successful in describing the degradation patterns of the various cell wall components.

Scanning electron microscopy (SEM) offers another option in visualizing the three-dimentional surfaces of plant sections (Grenet, 1989), but is regrettably flawed in that it is difficult to obtain quantitative information from such images (Travis *et al*., 1997). However, it is a valuable tool for the study of the anatomical configuration of tissues, the susceptibility of the plant tissue to microbial attack and the identification of the microorganisms involved in the initial degradation of the plant (McManus *et al*., 1976; Akin, 1986 as cited by Horn *et al*., 1989).

It can be concluded that more sophisticated methods and aids (for example computer software) can greatly improve our ability to describe and quantify the effect of EFE on plant tissue at histological level.

Conclusion

In conclusion, due to the complexity of forages typically used in ruminant diets, not only in terms of chemical composition, but also the architecture of the plant cell wall, there is great potential in the use of exogenous fibrolytic enzymes to improve the uitilzation thereof. As is summarized in Table 2.1, many positive effects in using EFE in ruminant diets have been reported, but a clearer understanding of the mode-of-action of these EFE is of importance. A project with the aim of investigating the method by which exogenous fibrolytic enzymes can improve the efficiency of nutrient utilization by ruminants was therefore established. The focus of the project was on the effect of the exogenous fibrolytic enzymes on digestion kinetics of certain economically important roughages used in the formulation of ruminant diets. The mode of action of the exogenous fibrolytic enzymes, how it affects the digestion of the relevant plant cell wall constituents in the rumen (*in vitro*) and methods to quantify the efficiency of the enzymes formed the basis of the research and was nestled in methodology applied in histological studies on forages (Grabber *et al*., 1992; Jung and Engels, 2001).

Table 2.1. Summary table on the enzyme types, application methods and main effects of various feeds and roughages treated with exogenous fibrolytic enzymes

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Alvarez *et al*., 2009	Xylananes (43.4IU) Cellulase (31.0 IU)	24h	High fibre diet (>500g/kg DM NDF)	Steers	*In sacco*: Increased DM and CP fraction a. No effect on DM b or c. Increased CP c. No effect on NDF or ADF disappearance	No effects reported on DMI, ADG or feed conversion. Pre-incubation effects suggested.
Alvarez *et al*., 2009	Xylananes (43.4IU) Cellulase (31.0 IU)	24h	Wheat middlings and oat straw	Steers	Increased disappearance of ADF (Wheat middlings) and NDF and ADF (Oat straw)	Pre-incubation effects suggested.
Bala *et al*., 2009	Cellulase Xylanase		Enzyme added to concentrate supplement fed at 500g/d	Lactating cross bred Beetle-Sannen goats	Increased digestibilities of DM, OM, CP, NDF, ADF and total carbohydrates Increased microbial protein reported (NS).	Improved milk yield, fat and solubles-non-fat (SNF). Decreased feed intake. Improved body weight.
Burroughs *et al*., 1960	Amylase, protease, cellulase, hemicellulase	NA	High corn or roughage diets evaluated with EFE applied to protein concentrates	Feedlot cattle and digestibility trial	Higher ADG (7%), DMI (1%) and improved FCR (6%). No effect reported for total tract digestibility.	

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Cruywagen and Goosen, 2004	Fibrolytic enzyme cocktail	18h	Roughage based formulated diet (443 g/kg NDF)	Dohne Merino lambs	No effect observed on DMI. Lambs gained significantly more body weight. Improved feed conversion ratios.	Improved weight gains. Improved feed conversion ratio's (FCR).
Cruywagen and Van Zyl, 2008	Lyophilized and fresh fibrolytic enzyme cocktail	None	High (920 g/kg forage) and low (600 g/kg forage) forage diets	Dohne Merino lambs		Improved body weight (BW) gains and FCR. Fresh enzyme cocktail were reported to be superior.
Dean *et al*., 2008	Commercial enzymes: Promote® Biocellulase X-20®, CA and Biocellulase A-20®	Substrate treated 3 weeks prior to feeding and stored in plastic containers	Tropical grasses: Coastal bermudagrass hay and Pensacola bahiagrass hay	Buffered rumen fluid from non-lactating cows	X-20 and A-20 resulted in reduced fibre concentrations, increased initial (a) and later phases (48h) of IVDMD. No improvement in extent of digestion	Higher application rates increased effects. Ammoniation yielded superior results to enzyme treatment.
Eun and Beauchemim, 2007	13 Endoglucanases (END) 10 Xylanases (Xyl)	3h	Fresh low quality lucerne hay	Buffered rumen fluid from lactating Holstein cows	Improved *in vitro* ruminal degradation Increased Gas production and OMD	Combination of END and Xyl did not yield additional effects.

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Eun *et al*., 2007a	Various EFE (endoglucanases and xylanases)	Pre-incubation allowed	Lucerne hay corn silage	Buffered rumen fluid lactating Holstein cows	Improved NDF degradability of 20 and 60% for lucerne hay and corn silage, respectively	Superior results were obtained with the optimum dose rate.
Eun *et al*., 2007b	Two proteolytic enzyme products 3 Fibrolytic EFE products (endoglucanase, xylanase)	3h	Lucerne hay	Buffered rumen fluid lactating Holstein cows	Improvements in *in vitro* digestibility with one of the proteases and two of the EFE products	Recommend 24h incubation time for *in vitro* assays (GP).
Giraldo *et al*., 2008	Fibrolytic enzyme containing endogluconase and xylanase from *Trichoderma longibrachiatum*	0h	70 grass hay: 30 concentrate	Sheep	Increases in the ruminally insoluble potential degradable fraction DM and fractional rate of degradation. Increased propionate and decreased ace:prop	Positive effects obtained without an enzyme substrate interaction period.

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Giraldo *et al*., 2008b	Fibrolytic enzymes from *Trichoderma viride, Aspergillus niger* and *Trichoderma longibrachiatum*	24h	700, 500 and 300 g forage/kg diet DM. Grass hay.	Buffered rumen fluid from Merino sheep in Rusitec fermentors	Increased true degradability of substrate DM. Increased total VFA, acetate and propionate.	Reported effects were the greatest at 8h incubation.
Giraldo *et al*., 2007	Mixed fibrolytic enzymes from *Trichoderma longibrachiatum* and Fumarate	24h	Grass hay and concentrate (600 and 400 g/kg DM, respectively)	Buffered rumen fluid from Merino sheep in Rusitec fermentors	Increased acetate, butyrate and methane. Increased substrate DM and fibre disappearance (6 and 48h)	No additional benefit on rumen fermentation due to Fumarate addition to enzyme treatment.
Jalilvand *et al.*, 2008	Natuzyme at DR of 3, 6, 9 g enzyme/kg DM substrate	24h	Lucerne hay maize silage wheat straw	Buffered rumen fluid from Taleshi steers	Increased cumulative gas production (syringes) at 6 and 12h and fractional fermentation rate for Wheat straw only. Asymptotic gas production not affected	Optimal dose rate determined as 3g enzyme/kg DM substrate.

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Jalilvand *et al*., 2008	Cellupract AS 130, Natuzyme and Endofeed DC (enzymatic products for poultry feeds)	24h	Lucerne hay maize silage wheat straw	*In situ* analysis with Blochi ewes	Cellupract resulted in increased *a, b* and effective degradability (ED) of all substrates	Fibrolytic enzyme products for poultry feeds have potential in ruminant nutrition.
Krueger and Adesogan, 2008	Combinations of Ferulic acid esterase, cellulase and xylanase	<1h	Bahiagrass hay	Incubation in the absence of rumen fluid or with buffered rumen fluid from non-lactating Holstein cows	Increased DMD (absence of RF) Decreased ace and increased prop and but concentrations. Decreased lag phase (presence of RF).	Ferulic acid esterase evaluated in combination with other fibrolytic enzymes.

Researchers	Enzyme description	Pre-incubation enzyme substrate interaction	Substrate/feed	Animals studied	Digestion effects reported	Production effects and general comments reported
Moharrery *et al*., 2009	Two cellulase containing enzyme treatments	0h or 24h	Grasses (ryegrass and festulolium) and legumes (clover, red clover and lucerne)	Buffered rumen fluid from dry Holstein cows	Improved *in vitro* DMD and aNDFom digestibility (8h). Increased *a* and decreased *b* value. Reduced lag time for $aNDF_{om}$.	Effects were only observed when a 24h pre-treatment period was allowed.
Pinos-Rodriguez *et al*., 2002	Fibrozyme	0h	Lucerne or rye-grass based diets	Criollo lambs (ruminally cannulated)	Increased apparent digestibility of CP, NDF and hemicellulose (lucerne). Increased total VFA concentration (both hays at 3 and 6h).	Increased DMI, OMI and CP intake (both hays). Improved N balance (both hays).
Pinos-Rodriguez *et al*., 2008	Fibrozyme	24h	TMR (forage:concentrate ratios of 40:60, 50:50 or 60:40)	Ramboulliet lambs (ruminally cannulated)	Increased soluble fraction (*a*) of DM. Increased DM and NDF ruminal *in situ* disappearance rates.	No effects observed on feed intake or N balance.
Ranilla *et al*., 2008	Fibrozyme	0h	Lucerne hay, grass hay, barley straw and isolated cells walls (as NDF)	Buffered rumen fluid from Merino sheep	Increased VFA and acetate concentration (5h). *In vitro* fermentation stimulated at 5 and 10h.	Dose response noted. Presence of neutral detergent soluble components of importance.

NA: Not applicable

a: soluble fraction, b: fraction of potential disappearance, c: rate of DM disappearance, ab: total disappearance

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CHAPTER 3

General Materials and Methods

Ethical clearance for animal use

This study was completed with ethical clearance from the Stellenbosch University Care and Use Committee (SU ACUC), reference number: 2006B03005.

Introduction

Increasing the digestibility of poor quality forages has been a topic of research for many years. It is clear that forages play an important role in the animal industry worldwide. As the structural fibre (cell wall) portion contributes from 300 to 800 g/kg of forage dry matter and represents a major source of nutritional energy for ruminants, any improvement in the digestibility thereof can be of great benefit. Unfortunately, less than 50 % of the fibre fraction is readily digested and utilized by the animal (Hatfield *et al*., 1999). Exogenous fibrolytic enzymes (EFE) have been studied extensively in the last couple of decades as a viable means of improving the digestibility of forages typically used in ruminant nutrition. Preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fibre (Feng *et al*., 1996). Many authors have reported on the successful use of this technology as extensively described elsewhere in this document. However, many inconsistent findings have also been reported and the variability in results is related to the types and activities of enzymes which are caused to a large extent by the organism from which they are produced, the substrate used for its growth and the culture conditions used (Considine and Coughlan, 1989; Gashe, 1992). Also, the composition of the substrate used, the method of enzyme application and the portion of the diet to which the enzymes are added, confound results (Beauchemin and Rode, 1996; Hristov *et al*., 1998). The current study had the overall objective of investigating the mode of action of EFE's and in the first part of the study popular methodologies were employed. These included an establishment of the *in vitro* gas production profile of EFE treated forages, *in vitro* digestibility thereof, as well as the *in sacco* degradation characteristics. These general methodologies will be discussed in length at the subsequent sections.

Exogenous fibrolytic enzyme cocktail description and enzymic activity

The exogenous fibrolytic enzyme cocktail used in this study was isolated from fungi obtained from soil in South Africa and was used as an extracellular enzyme supernatant in the fresh form. The exogenous enzyme supernatant was produced by the Department of Microbiology, Stellenbosch University, South Africa. This extracellular enzyme supernatant of the patented fungal strain, ABO 374, grown on wheat straw, was characterized as a fibrolytic enzyme cocktail, containing mostly xylanase (296 ± 0.07 U/mg protein) but also cellulase (1.44 ± 0.39 U/mg protein) and mannanase (1.10 ± 0.37 U/mg protein) activity (Cruywagen and Van Zyl, 2008). The activities of these enzymes were determined using 10 g/kg birchwood xylan in 50mM sodium citrate buffer, pH 5.3 for β-xylanase, 10g/kg CMC (carboxymethyl cellulose) as substrate in 0.05M sodium acetate buffer, pH 4.8 for cellulase (endoglucanase) and 10 g/kg locust bean gum for β -mannanase activity determination. In most of the experiments reported in this document, fresh extracellular supernatant was used as the EFE.

Nutrient composition of forages, feed and samples

Lucerne (*Medicago sativa)*, kikuyu (*Penisetum clandestinum*), weeping love grass (*Eragrostis curvula*) and a formulated feed were used in the different experiments, as described in subsequent chapters. These feeds were analyzed for moisture, DM, ash, crude protein, ether extract and NDF.

Dry matter was determined according to the official method of AOAC International, method 934.01, fat (crude) or ether extract according to the AOAC Official Method 920.39 and ash according to method 942.05. Crude protein was measured according to the combustion method (Method 990.03, AOAC, 2002) for nitrogen on a Leco FP-428 Nitrogen and Protein analyzer (Leco Corporation, St. Joseph, MI, USA). Neutral detergent fibre (NDF) was determined according to the procedures described by ANKOM with the aid of the ANKOM^{220} Fiber Analyzer (ANKOM Technologies, Fairport, NY, USA), using the ANKOM F 57 filter bags.

Animals and diets

Six adult Dohne-Merino wethers were used in the *in sacco* trial and as donors of rumen liquor in the *in vitro* trails. All animals were fitted with rumen cannulae from Bar Diamond

Industries (Bar Diamond, Inc., Parma, ID, USA) by a qualified veterinarian under Ethical Clearance from the Stellenbosch University ethics committee (reference number: 2006B03005). Sheep were maintained on a 50:50 mixture of oat hay and lucerne *ad libitum* and supplemented with a commercial sheep concentrate at a daily level of 100 g/animal for the *in vitro* study. The roughage basal diet supplied 135 g/kg CP, 525 g/kg NDF, 16.2 g/kg crude fat and 80.0 g/kg ash (DM basis). For the *in sacco* study, animals were fed a formulated diet based on the specification of the Nutrient Requirements of Sheep (National Research Council, 1985). The composition and nutrient content of this diet is presented in Table 3.1 and was fed to animals during the *in sacco* trail and as basal diet in other experiments, where indicated. Animals were adapted to this diet for a 10 d period before the experimental phase wherein animals were randomly assigned to two groups (enzyme and control group) in a 2 x 3 cross over experiment with a 10 d adaptation period in between. Sheep received the respective treatment diets for a period of four days. All sheep therefore received both treatments.

Table 3.1. Formulation and chemical composition (DM-basis) of a balanced diet for sheep based on NRC requirements for sheep (National Research Council, 1985).

Raw material	Inclusion, g/kg
Maize meal	450
Wheat	150
Lucerne meal	120
Oat hay	200
Cotton seed oilcake meal	50
MuttonGainer premix[1]	30
TOTAL	**1000**
Chemical composition	g/kg
Moisture	132
DM	868
Crude protein	166
NDF	349
Ether extract	11.3
Ash	46.5

[1]: Product supplied by FORMUFEED cc, Villiersdorp, South Africa and manufactured by NUTEC (Pty) Ltd, Willowton, South Africa.

Forages and diet characteristics

Forages evaluated and used in the experiments of this project study were lucerne hay (*Medicago sativa*), freshly cut and dried (dried at 50^{o}C for 72h) kikuyu (*Pennisetum clandestinum*), weeping love grass hay (*Eragrostis curvula*) and wheat straw. The nutrient composition of these forages is presented in Table 3.2.

Table 3.2. Nutrient composition of forages used in the *in vitro* assays reported on a DM basis

Nutrient content	Lucerne hay (*Medicago sativa)*	Dried kikuyu (*Pennisetum clandestinum)*	Weeping lovegrass hay (*Eragrostis curvula*)	Wheat straw (*Triticum spp.)*
Moisture, g/kg	102.0	165.8	116.8	107.9
DM, g/kg	898.0	834.2	883.2	892.1
Ash, g/kg	86.1	55.4	57.5	74.9
Crude fat, g/kg	12.7	17.4	14.8	18.2
Crude protein, g/kg	238.1	143.9	123.2	64.5
NDF, g/kg	463.3	635.7	714.2	817.7

The four forages, as well as the complete diet were all treated in a similar manner before any analysis. The samples were milled through a 2mm screen using a laboratory scale hammer mill (Scientech RSA, Hammer mill Ser. No. 372, Cape Town, RSA). The milled samples were then sieved through a 124 µm sieve on an automated horizontal shaker for 5 minutes (Retsch AS 200, Retsch Gmbh, Germany) and the coarse sample retained for analysis. All samples were stored in glass screw top bottles at 4^{o}C untill required for analysis. The sieving step was included to eliminate the presence of particles small enough to pass through the polyester *in sacco* or *in vitro* bags as to limit over estimating the soluble (a) fraction (Cruywagen *et al*., 2003). The pore size of the F57 *in vitro* filter bags (Ankom® Technology Corp., Fairport, NY, USA) and the polyester *in sacco* bags (Ankom forage bags) were 25 µm and 50 µm respectively, as indicated by the manufacturer.

Collection of rumen fluid from donor sheep

The rumen fluid inoculum was collected from ruminally cannulated Döhne-Merino wethers fed a basal diet of roughage (*ad libitum*, Table 3.1) supplemented daily with a commercial concentrate (100g/sheep). The collection of the rumen fluid inoculum was only done after animals were adapted to the diet for a period of at least 10 d. The donor animals were fed *ad libitum* with the first feeding in the morning (approximately 07:30) and topped up in the late afternoon to ensure the availability of fresh feed. Concentrate supplementation was done once daily during the morning feeding. Rumen fluid was always collected at least 30 minutes after the morning feeding and any digesta other than the rumen liquor removed, was replaced. Post-feeding rumen collection was done to ensure a high concentration of micro-organisms present in the liquid (Mauricio *et al*., 1999).

The rumen content of each animal was hand mixed before sampling and depending on the amount of inoculum needed, at least two to four sheep were sampled. Some of the rumen contents were then removed from the ventral rumen (Weimer *et al*., 1999) and strained through two layers of cheesecloth. Approximately 500 ml of the ruminal fluid were collected from each donor animal, mixed and transferred to pre-heated thermos flasks. The flasks were filled completely to eliminate any oxygen (Mauricio *et al*., 1999) while being transported back to the laboratory. In the laboratory, the flasks were emptied into an industrial blender (Waring Commercial® Heavy Duty Blender, Waring® Corporation, New Hartford, CT, USA) while being purged with CO_2 to maintain anaerobic conditions (Grant and Mertens, 1992). The blender was sealed and the contents blended for 1 minute on the pulse setting of the blender.

Once blended, the rumen fluid was again squeezed through a single layer of cheesecloth to remove any remaining large solid particles and transferred to a large glass beaker inside a 39^{o}C water bath. The blended rumen fluid was then allowed to stand for up to 10 minutes to allow for the breakdown of the frothy layer that formed due to the blending step while being continuously purged with CO_2 as recommended by Goering and Van Soest (1970). Thereafter, the required amount of rumen fluid was added to the buffer solution in the respective incubation vessels at a ratio of one part rumen fluid to four parts buffer solution.

Preparation of the buffer solution

The rumen fluid collected from the donor Döhne-Merino wethers was mixed with a reduced buffer solution as described by Goering and Van Soest (1970), with slight modifications. This buffer solution was used for the *in vitro* gas production determination as well as the *in vitro* true digestibility analysis. According to Mertens and Weimer (1998), this phosphate-bicarbonate buffer is capable of maintaining a pH of above 6.2 for 40 to 60 mg of fermented substrate when gases are not released during a 96 h fermentation. As recommended by Tilley and Terry (1963), a relatively large volume of buffer solution was added (four parts), to maintain a pH level within the usual limits for digestion, and to ensure that the final acid concentration would be comparable to and not exceeding that found in the rumen. The composition of the buffer solution is presented in Table 3.3.

Table 3.3. Composition of the buffer solution used in the *in vitro*- GP and digestibility trials.

Reagent	**Quantity added**
Rumen buffer solution:	
Deionised water	2.0 l
NH_4HCO_3	8.0 g
$NaHCO_3$	70.0 g
Macro-mineral solution:	
Deionised water	2.0 l
NaH_2PO_4 (anhydrous)	11.4 g
KH_2PO_4 (anhydrous)	12.4 g
$MgSO_4 \cdot 7\ H_2O$	1.17 g
Micro-mineral solution:	
Deionised water	100 ml
$CaCl_2 \cdot 2\ H_2O$	13.2 g
$MnCl_2 \cdot 4\ H_2O$	10.0 g
$CoCl_2 \cdot 6\ H_2O$	1.0 g
$FeCl_3 \cdot 6\ H_2O$	8.0 g
Cysteine sulphide reducing agent:	
Deionised water	48 ml
Cysteine hydrochloride	312 mg
1 N NaOH	2 ml
$Na_2S \cdot 9H_2O$	312 mg
Final buffer solution (per litre):	
Deionised water	500 ml

Rumen buffer solution	250 ml
Macro-mineral solution	250 ml
Resazurin (0.2%, w/v)	2 ml
Micro-mineral solution	0.12 ml
Tryptose	1.25 g
Reducing buffer solution:	
Final buffer solution	570 ml
Cysteine sulphide reducing agent	30 ml

The rumen buffer solution, macro mineral solution and micro mineral solution were prepared in large quantities and utilized as needed. The micro mineral solution was stored in a dark glass bottle as to maintain the quality of the solution. On the morning before commencement of the experiment, the appropriate amounts of deionised water, rumen buffer solution, macro and micro mineral solutions were mixed with the tryptose and freshly prepared resazurin. The cysteine sulphide reducing solution was also prepared fresh and only added to the rest of the solution once all chemicals were dissolved. As soon as the reducing agent was added, the buffer solution was placed in a 39^{o}C water bath and bubbled with CO_2 until the optimal pH of 6.8 was obtained. The glass vial was then sealed with a rubber stopper and left at 39^{o}C until the buffer solution was clear, indicating that the solution was sufficiently reduced. A purple or pink colour was regarded as indicative of the buffer being in the oxidized form (Goering and Van Soest, 1970). Only after the solution was completely reduced were the appropriate amounts dispensed into the various *in vitro* glass vials already containing the substrates and treatment material under CO_2 phase, capped and placed in the 39^{o}C incubator. As discussed in the following section, rumen fluid was added to the glass vials containing the substrate, EFE or dH_2O and reduced buffer solution shortly before the experiment was initiated. This protocol was regarded as sufficient to limit shock to the microbes when added to the buffer solution as optimal pH, temperature and reduced conditions were achieved. The trace minerals, macro minerals, ammonia and Tryptose (T2813, Sigma-Aldrich, St. Louis, MO, USA), a pre-digested source of amino nitrogen and branched chain fatty acid precursors prevented any deficiencies of nutrients to the microorganisms (Mertens and Weimer, 1998).

In vitro gas production measurement

In this study, two gas production systems were used. In the early stage of the work, a semi-automated system was used whereas later on a fully automated system was used. The semi-automated system consisted of a digital pressure gauge from SenSym ICT (Honeywell Inc., Morris, NJ, USA) fitted with a luer lock adapter and disposable 21 gauge needle. In the

latter studies, fully automated mobile gas pressure sensors were used (MadgeTech, West Warner, NH, USA). These sensors can be programmed to initiate the recording of gas pressure at a pre-determined time and at pre-determined intervals for the whole incubation period. Sensors were fitted with 21 gauge needles which were inserted through the rubber stoppers on the *in vitro* gas production vials. After the experiment, sensors were removed and pressure data down loaded to a computer, exported to the MadgeTech data recorder software, version 2.00 program (MadgeTech, West Warner, NH, USA) and converted to gas volume as described in the following section.

For the determination of gas production 500 mg samples of the test material were incubated in buffered rumen fluid under anaerobic conditions at 39^{o}C. The samples were incubated for the appropriate incubation period, and depending on the system, gas pressure was recorded manually or automatically at pre-determined time intervals. The gas production systems used here were based on the Reading Pressure Technique (RPT) and was similar to the system described by Theodorou *et al*. (1994) with the exception that semi-automated or automated gas pressure devices instead of syringes were used to log the gas pressure at the relevant time intervals.

Glass vials with a nominal volume of 120 ml were used for the *in vitro* gas production measurements. Feed samples were prepared as described earlier and weighed accurately into the vials prior to the start of the experiment. In this study, amounts of up to 0.5 ± 0.01 g substrate ("as is" basis) were used per vial. The substrates were pre-treated with the EFE at least 12h prior to adding the buffered rumen fluid. This was to allow for an enzyme-substrate interaction time as the enzyme requires an adsorption and binding time to the substrate to allow for protection against proteolytic breakdown in the rumen (Forwood *et al*., 1990; Beauchemin *et al*., 2003). The precise amounts of EFE or dH_2O added and substrate used are described in the following chapters. Fourty millilitres of buffered rumen fluid and 10 ml of strained and blended rumen fluid were added to each vial shortly before initiating the experiment. Each vial therefore contained no more than 50% of its total volume as substrate, EFE and buffered rumen fluid, allowing for sufficient head space for gas production. However, pressure was released every three hours by inserting a 21 gauge needle through the rubber stopper.

Digital gas pressure readings were converted to gas volumes produced in millilitres by a pre-determined calibration curve developed in our laboratory, as described by Goosen (2004). This was done by setting up a correlation between pre-determined gas volumes added to the vials and consequently recording the pressure readings, taking into account the temperature

at which the gas was added as well as the head space of each individually marked glass vial. The equation used to convert gas pressure to volume was y = 0.0977x (R^2 = 0.99) where x = net pressure (psi) and y = volume (ml).

The following two non-linear models (model 1: no lag phase calculation and model 2: lag phase calculation) were used to determine the kinetic coefficients for gas production. The Solver function of Excel was used to fit the models to the gas production data (nett gas production, ml/g DM).

Model 1: $$Y = b\left(1 - e^{-ct}\right)$$

Model 2: $$Y = b\left(1 - e^{-c(t-L)}\right)$$

Where:
- Y = gas volume at time t
- b = total gas production
- c = rate of gas production
- t = incubation time
- L = lag time

Determination of neutral detergent fibre content (NDF)

The procedure described by Ankom® was used in the determination of NDF of fresh feed and forage samples, as well as for samples after fermentation in buffered rumen fluid. Apparatus used were the Ankom® $^{200/220}$ Fiber analyzer (Ankom® Technology Corp., Fairport, NY, USA). Neutral detergent fibre when treated with α-amylase, yields similar average fibre concentrations in the Ankom fibre analyzer with filter bags compared to using porous Gooch crucibles as was reported by Ferreira and Mertens (2007).

ANKOM filter bags (F57) were prepared in acetone as per manufacturer instructions to remove the waxy surfactant layer which could interfere with microbial digestion. The bags were then placed in a forced draught oven at 100^{o}C for 24h, labelled and used as needed.

After weighing the labelled bags, the substrates were weighed into the bags at 0.5 ± 0.01 g per bag, or less if stated so elsewhere. The bags were then sealed using an impulse heat sealer (Ankom® 1915/1920 Heat sealer; Ankom® Technology Corp., Fairport, NY, USA). One bag per every 23 analyzed was also heat sealed without any substrate to serve as blank and to determine the correction factors. The sealed bags were then placed on the trays (three per tray on eight trays), inserted into the fibre analyser chamber and weighted down with the weight supplied with the system. The neutral detergent solution (2000ml) was added to the chamber and the agitation button activated. Twenty grams of sodium sulphite and 4.0 ml heat stable α-amylase were added to the neutral detergent solution before pouring the solution into the chamber to allow for solubilisation of the sodium sulphite. The chamber was then sealed and the heat button activated and allowed to run for 75 min. After the 75 minute period, agitation and heating were deactivated, the solution drained from the chamber and opened. Two litres of hot water containing 4.0 ml α-amylase (for the first two rinses) were poured into the chamber and bags were allowed to rinse with agitation activated for three minutes. This was repeated another two times, with the last rinse containing hot water only. Bags were removed from the chamber, excess water removed by gently squeezing the bags and placed in a 100°C oven for 24h to dry. The bags were then weighed and NDF corrected for DM calculated as per formula.

Neutral detergent solution (NDS):

Deionised water	1.0 l
Sodium lauryl sulphate	30.0 g
Ethylenediaminetetraacetic disodium salt, dihydrate	18.61 g
Sodium tetraborate decahydrate	6.81 g
Sodium phosphate dibasic, anhydrous	4.51 g
Triethylene glycol	10 ml

Neutral detergent fibre:

$$a = ((b - (c * d)) * 100) / e$$

Where:

a = neutral detergent fibre (%)

b = dried bag weight (post-NDF treatment) (g)

c = original bag tare weight (g)

d = DM corrected blank bag (g)

e = sample dry matter weight (g)

***In vitro* digestibility**

The Ankom® DAISY^{II} *in vitro* fermentation system (Ankom® Technology Corp., Fairport, NY, USA) was used for the estimation of *in vitro* true digestibility (IVTD) and was done according to the protocol as described by the manufacturers. Slight modifications were, however, made in the current study. This included using the buffer solution of Goering and Van Soest (1970), containing Tryptose as protein source, as described earlier. Also, the buffer solution was partially replaced by the EFE or dH_2O in the flasks, in order for the flasks to contain the same final volume as prescribed by the manufacturers. This consisted of replacing 200ml of the Goering and Van Soest buffer with EFE or dH2O. To this, 1400 ml of reduced buffer solution and 400 ml of strained and blended rumen fluid inoculum were added.

The DAISY^{II} Incubator consists of a large incubator that can be maintained at 39^{o}C. Four large (4 L) flasks can be accommodated in the incubator and constantly rotated to improve access of microorganisms to the substrate and limit sedimentation of feed particles present in the buffered rumen fluid. Substrate is added to the flasks by weighing substrate into F57 fibre filter bags and heat sealing the bags. The F57 filter bags have a pore size of 25 µm allowing for access of microorganisms and substantially limiting the wash out of fine sample particles. As samples were milled and sieved as described earlier, it was regarded that the over-estimation of the soluble fraction (*a*) would be minimal (Cruywagen *et al*., 2003). Each flask may accommodate 25 substrate bags, which includes one blank bag for correction purposes in bag weight changes (Holden, 1999).

As described by the manufacturer, multi-layer polyethylene polyester cloth bags (Ankom® F57 filter bag; Ankom® Technology Corp., Fairport, NY, USA.) were pre-rinsed in acetone for three minutes to remove the waxy surfactant coating that may inhibit access of microorganisms through the pores of the bag. Bags were then dried in a forced draught oven (100^{o}C, 24h) before being weighed and marked. Samples were accurately weighed into the bags at amounts ranging from 0.25 ± 0.01g (according to manufacturer specifications) to 0.5 ± 0.01 g as indicated in the relevant chapters and heat sealed with an impact heat sealer (Ankom® 1915/1920 Heat Sealer; Ankom® Technology Corp., Fairport, NY, USA). A blank bag containing no substrate was also prepared. The blank bag served as correction for any weight changes in bag weight due to microbial adhesion or particulate contamination due to the incubation in buffered rumen fluid. The sealed bags were then placed in the Ankom® DAISY^{II} Incubator digestion jars, ensuring that the bags were evenly distributed on both sides of the digestion jar divider.

As for the *in vitro* gas production, the substrate filled F57 bags were treated either with 200ml EFE or dH_2O at least 12h prior to incubation with buffered rumen fluid whilst being inside the flasks. This was again to allow for an enzyme-substrate interaction time before the addition of buffered rumen fluid. The reduced Goering and Van Soest (1970) buffer solution was added to each flask (1400ml) shortly before the experiment commenced, purged with CO_2 and kept under anaerobic conditions inside the pre-warmed incubator (39^{o}C). Buffered rumen fluid (400ml) was added to the flasks under CO_2 phase when the experiment was started. Bags were removed after pre-determined incubation intervals as indicated in the relevant chapters. Three bags per treatment were never included in the incubation flasks and served as 0h values. These bags, along with the bags shortly after removal were gently rinsed under running tap water until the water was clear. Bags were retrieved from the flasks by removing the flasks from the incubator, opening the lids and purging the flask with CO_2 while the relevant bags were removed. The washed bags were then placed either in a drying oven (60^{o}C) for 24 h to allow for the determination of DM disappearance or frozen (-18^{o}C) if only the *In vitro* true digestibility was to be determined. On completion of the drying period the bags were removed and weighed to determine the DM disappearance of substrate. Thereafter, these bags or the frozen bags were placed in the Ankom$^{®200/220}$ Fiber Analyzer (Ankom$^{®}$ Technology Corp., Fairport, NY, USA) and the procedure for determination of NDF (as described by the manufacturer) was followed. On completion of the NDF procedure the bags were dried at 100˚C for 24h, and then weighed.
The *in vitro* true digestibility corrected for dry matter ($IVTD_{DM}$) was calculated according to the formula given by Ankom (Ankom® Technology Corp., Fairport, NY, USA). The *in vitro* dry matter digestibility and *in vitro* NDF digestibility were calculated for the different treatments. The calculations used to obtain these values are shown below:

In vitro dry matter digestibility (%):

$$a = 100 - ((b - (c * d / e)) / f * 100)$$

Where:

a = *in vitro* dry matter digestibility (%)

b = dried bag weight (post-incubation) (g)

c = original bag tare weight (g)

d = blank bag weight (post-incubation) (g)

e = blank bag tare weight (g)

f = sample dry matter weight (g)

In vitro neutral detergent fibre digestibility (%):

$$a = 100 - (((b - (c * d / e)) - (f)) / (* h) * 100)$$

Where:

a = *in vitro* neutral detergent fibre digestibility (%)

b = dried bag weight (post-NDF treatment) (g)

c = original bag tare weight (g)

d = blank bag weight (post-NDF treatment) (g)

e = blank bag tare weight (g)

f = crucible weight

g = sample dry matter weight (g)

h = NDF proportion of an untreated sample (%)

In vitro true digestibility corrected for dry matter (%):

$$a = 100 - (((b - (c * d / e)) - (f)) / g * 100)$$

Where:

a = *in vitro* neutral detergent fibre digestibility (%)

b = dried bag weight (post-NDF treatment) (g)

c = original bag tare weight (g)

d = blank bag weight (post-NDF treatment) (g)

e = blank bag tare weight (g)

f = crucible weight (g)

g = sample dry matter weight (g)

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CHAPTER 4

The effect of an exogenous fibrolytic enzyme cocktail on *in vitro* gas production and *in vitro* digestibility of forages

Abstract

The aim of the study was to determine the effect of treatment of forage substrates with an exogenous fibrolytic enzyme cocktail (EFE, containing xylanase and cellulase activities) on *in vitro* gas production and *in vitro* true digestibility (IVTD). Rumen liquor, obtained from cannulated sheep fed a lucerne-oat hay based diet, was used as inoculum. Substrates used in the fermentations were lucerne hay (*Medicago sativa*) and two C4 grasses: weeping love grass (*Eragrostis curvula*) hay and kikuyu (*Pennisetum clandestinum)* grass dried at 50ºC for 24h. Gas production was measured in 120 ml glass vials with the aid of a digital pressure gauge, following anaerobic fermentation in an incubator at 39ºC after 6, 12, 18, 24, 48 and 72 hours. Pressure values were converted to volume using a pre-determined regression equation. *In vitro* true digestibility was measured after 24 hours of fermentation with the aid of an Ankom Daisy II incubator. The gas production fermentation kinetic values upon applying a non-linear model to the data showed significant effects on the total gas production (*b*) as well as the rate of gas production (*c*). The rate of gas production (*c*) was 0.049 and 0.054 h^{-1} for lucerne control and EFE treatement, respectively and 0.016 and 0.019 h^{-1} for kikuyu control and EFE treatment, respectivley. The non-linear model did not fit the weeping love grass data and therefore only a linear regression line was fitted to the data. The potential total gas production (*b*) was increased for EFE-treated lucerne, regardless of whether the model was used with or without a lag phase. For kikuyu, the potential gas production (*b*) was increased due to EFE treatment, but only in the non-linear model that included an estimation for lag. Exogenous fibrolytic enzyme treatment resulted in higher rates of gas production (*c*) for both lucerne and kikuyu, and in both models used, but had no effect on the lag time of either lucerne or kikuyu. In contrast with the fermentation kinetic values, enzyme treatment had no effect on cumulative gas production (ml/g DM) of lucerne or weeping love grass as substrate. However, with kikuyu as substrate, EFE treatment resulted in a 12 % increase in cumulative gas production ($P < 0.05$) after 24h of fermentation which was carried over to the rest of the incubation period. This is in agreement with the higher *b* value predicted by the models ($P < 0.05$). This increase was observed for the remainder of the fermentation period at each subsequent time point ($P < 0.05$). *In vitro* true digestibility, as measured after 24h of fermentation, was higher for lucerne than for the C4 grasses (love grass or kikuyu). Enzyme treatment increased 24h *IVTD* ($P<0.05$), but only for

lucerne and kikuyu hay. In this study, EFE treatment resulted in a higher rate of gas production (c), higher potential gas production (b) and an improved IVTD for both kikuyu and lucerne. Cumulative gas production was increased for EFE treated lucerne only ($P < 0.05$).

Introduction

The use of exogenous enzymes in animal nutrition has in the recent past received renewed attention. This is to an extent due to modern and improved technologies, making the commercial production of such enzymatic products cost effective. Therefore, these products are increasingly being used, not only in monogastric nutrition, but also in ruminant nutrition. Cellulases, hemicellulases and even pectinases are used in ruminant feeding to improve feed utilization, increase production of milk or meat and to improve the digestibility of certain feed components. As reported by Bhat (2000), many research findings have reported on the improvement of feed digestibility and animal production (Burroughs *et al*., 1960; Rust *et al*., 1965), while negative effects have also been shown in these early studies (Perry *et al*., 1966; Theurer *et al*., 1963). These findings also had been reported several decades earlier. Today, renewed research in effect has reported very similar positive effects (Beauchemin *et al*., 1995, 2003), including increased forage digestibility, improvements in the efficiency of nutrient utilization, increased *in vitro* NDF digestion and more (Morgavi *et al*., 2000; Chesson, 1993). However, inconsistencies in research findings are still prevalent. These inconsistencies are related to several factors regarding the application of the EFE, as well as our understanding of the mode of action of such enzymes. In terms of the application of EFE, there appears to be an enzyme-substrate pre-treatment effect, indicating the importance of pre-treating the substrate with the EFE (Forwood *et al*., 1990; Beauchemin *et al*., 2003). Another pre-feeding effect is the rate of enzyme application, or dose rate. The optimal dose rate has to be determined for each enzyme preparation (Eun and Beauchemin, 2007; Jalilvand *et al*. 2008) in addition to its specificity (White *et al*., 1993). Post-feeding effects include ruminal pH with EFE in general being less efficient at lower pH levels (Colombatto *et al*., 2007).

It appears that EFE has its major action on the fibre structure (Giraldo *et al*., 2008) and could possibly affect the structures of plant cell walls. Additionally, Alvarez *et al.* (2009) reports that due to the increased DM and CP soluble fractions of diets resulting from fibrolytic enzyme addition, the reducing sugars produced would provide energy that would lead to rapid microbial growth. Increased ruminal bacteria numbers could lead to increased microbial colonization of the feed particles. This is consistent with views that the action of

EFE is due to a synergistic effect with rumen microbes and therefore the hydrolytic activity within the rumen is increased (Morgavi *et al.,* 2000).

From the literature, it is evident that exogenous fibrolytic enzymes indeed have an effect on fibre digestion. However, results suggest that it is important to consider the combined effect of enzyme type, enzyme level and substrate, in addition to factors such as pre-treatment effects discussed above. Therefore, the first objective of this study was to establish the effect of an EFE cocktail, allowing for pre-treatment effects and different substrates, on gas production kinetics and *in vitro* digestibility of lucerne, kikuyu and weeping love grass. The EFE cocktail was produced by the Microbiology Department of Stellenbosch University.

Materials and Methods

Samples of lucerne (*Medicago sativa)* hay, dried kikuyu (*Penisetum clandestinum*) (50^{o}C) and weeping love grass (*Eragrostis curvula*) hay were milled through a 2 mm screen. The material was then sieved with an orbital shaker (Retsch AS 200, Retsch Gmbh, Germany) for 5 minutes through a 128 µm sieve to reduce variance in particle size by removing the extremely fine particles. For the *in vitro* gas production technique, 0.5 ± 0.01 g of the relevant material was weighed into 120 ml serum vials of which the volume of each had previously been determined accurately. Four vials were used per treatment. To each vial a magnetic stir bar was added to facilitate mixing during the incubation period. A pre-incubation time of 12h was allowed for the exogenous enzyme to interact with the substrate. The enzyme cocktail was diluted 200 fold and 1 ml thereof added to each experimental vial. Control bottles contained 1 ml distilled water to allow for similar changes in the headspace of the vials. Rumen fluid was collected from adult donor Dohne-Merino wethers fed a lucerne-oat hay based diet supplemented daily with 100 g of a commercial concentrate for sheep. Collection was in the morning shortly after the 07:30 feeding and rumen fluid was strained through two layers of cheese cloth into a pre-warmed thermos flask and sealed. In the laboratory the rumen fluid was blended for 2 minutes before being added to the incubation bottles in a ratio of one part rumen fluid to four parts buffer. The buffer was prepared according to the method of Goering and Van Soest (1970), with slight modifications as discussed in the general materials and methods chapter. Anaerobic principles were adhered to and bottles were sealed under CO_2 gas phase with rubber stoppers and crimp seal tops and placed in the 39^{o}C incubator. The stirrer plates were activated and continuous, slow (20 rpm/min) stirring allowed. Gas pressure (psi) was measured at 3, 6, 12, 18, 24, 36, 48 and 72 h with a manual gas pressure gauge from SenSym ICT (Honeywell Inc., Morris, NJ,

USA). After each gas pressure measurement gas build-up was released by inserting a 21 gauge needle through the rubber stopper. Gas pressure was converted to gas volume with a pre-determined regression equation taking into account the headspace of each particular bottle.

For the determination of *in vitro* true digestibility (IVTD) the Ankom® DAISY^{II} *in vitro* fermentation system (Ankom® Technology Corp., Fairport, NY, USA) was used and the procedure followed as described by the manufacturers. The prescribed Ankom buffer was, however, replaced by the Goering and Van Soest (1970) buffer. The F57 filter bags containing 0.25 (± 0.01) g substrate were incubated for 24 h in rumen fluid inoculum with buffer at a ratio of 1:4. The same enzyme dilution (1ml in 200 ml distilled water) as for the gas production trial was used and 40 ml added to the two treatment incubation jars. Two jars served as control with 40 ml distilled water added instead of the enzyme cocktail. Bags were duplicated in each jar (2x2 replications). After the incubation period, the bags were removed and *in vitro* true digestibility (IVTD) determined according to the Ankom procedure, as explained in Chapter 3. The whole experiment (gas production and *in vitro* digestibility) was duplicated in a second run using rumen fluid collected in exactly the same manner and from the same animals as during the first run.

The enzyme cocktail used was the extracellular enzyme supernatant of the patented fungal strain, ABO 374, produced by the Department of Microbiology, Stellenbosch University. It is characterised as a fibrolytic enzyme cocktail and contains xylanase (296 ± 0.07 U/mg protein), cellulase (1.44 ± 0.39 U/mg protein) and mannanase (1.10 ± 0.37 U/mg protein) as major enzyme activities (Cruywagen and Van Zyl, 2008). This cocktail was added to the experimental vessels at a dilution rate of 1 in 200 ml distilled water, as discussed previously.

The following two non-linear models (model 1: no lag phase calculation and model 2: lag phase calculation) were used to determine the kinetic coefficients for gas production. The solver function of Microsoft Excel was used to fit the models to the gas production data (nett gas production, ml/g DM). The exponential equation presented by Model 2 is also proposed by other researchers as a fitting model for the determination of gas production kinetic values (France *et al*., 2000; Jalilvand *et al*., 2008)

Model 1: $$Y = b\left(1 - e^{-ct}\right)$$

Model 2: $$Y = b\left(1 - e^{-c(t-L)}\right)$$

Where: Y = cumulative gas volume at time t (ml)

b = asymptotic total gas production

c = rate of gas production ($.h^{-1}$)

t = incubation time (h)

L = lag time (h)

Due to limitations in the manual gas production system, only eight incubation times were observed during the 72h period and therefore the data was not additionally fitted to a two-compartmental model to distinguish effects of EFE on the rapidly soluble fraction.

Statistical analysis

The experiment was a completely randomized design and conducted in duplicate runs. Fractional gas production at each time interval and *in vitro* true digestibility data were subjected to a main effects ANOVA, using Statistica 8.1 (2008). Significant forage * treatment interactions were detected and data pertaining to the respective forages were further subjected to a one way ANOVA. Total cumulative gas production data were subjected to a repeated measures ANOVA and the Bonferroni distribution of the post-hoc comparisons. If interactions were observed, the data was subjected to a one-way ANOVA to determine cumulative gas production effects at specific time intervals. For the gas production kinetic data, the *b* and *c* (model without lag) and *b, c* and L (model with lag) kinetic values were subjected to a factorial ANOVA with the factors substrate and enzyme, using Statistica 8.1 (2008). This was done for all the non-linear parameters. If no interaction was observed, the main effects were interpreted.

Results

Roughages

The proximate analysis results of the three forages used in the experiments of this chapter are presented in Table 4.1 on a DM basis and compared to typical values for South African produced hays. The composition of our test substrates compared well to long term average South African values (determined in the Nutrition laboratory of the Department of Animal

Sciences, Stellenbosch University), with the exception of NDF that differed from the norm for all the substrates and crude fat that differed greatly from the norm, only for weeping love grass. The differences in fibre content can be attributed to various factors as stated by Van Straalen and Tamminga (1990). These are likely due to differences in the stage of cutting and hay making, but also to cultivar and species differences, season and weather conditions.

Table 4.1. Nutrient composition (on a DM basis) of forages used in the evaluation of exogenous fibrolytin enzymes (EFE) and its effects on gas production and IVTD

Forage	Moisture, g/kg	DM, g/kg	Ash, g/kg	Crude fat, g/kg	Crude protein, g/kg	NDF, g/kg
Lucerne hay (*SA average)*	102.0	898.0 (887)	86.1 (62.1)	12.7 (18.6)	238.1 (141)	463.3 (362.9)
Dried Kikuyu (*SA average)*	165.8	834.2 (820)	55.4 (96.3)	17.4 (15.6)	143.9 (123.0)	635.7 (630.5)
Weeping lovegrass hay (*SA average*)	116.8	883.2 (869)	57.5 (31)	14.8 (40.1)	123.2 (91)	714.2 (CF379)

Values in brackets indicate the typical average nutrient content determined with South African forages in the Nutrition laboratory of the Department of Animal Sciences.

CF: Crude fibre. The average crude fibre value is given where insufficient data was available for the determination of the average NDF content.

Gas production

When net gas production is expressed as the volume of gas produced (ml/g DM) in consecutive 6 h gas production periods (termed fractional gas production, with removal of gas produced after each measurement), it is evident that the EFE cocktail had varying results on the three substrates evaluated as seen in Figures 4.1, 4.4 and 4.7.

As indicated in Figure 4.1, kikuyu incubated with EFE showed improved gas production during the second, third and fifth 6 h consecutive incubation periods, corresponding to 12 h, 18 h and 30 h incubation times respectively ($P < 0.05$). This effect disappeared during the last 6 h incubation period (corresponding to the 36 h incubation time) and indeed no further enzyme effects were noted in the remainder of the incubation period (48 h and 72 h). Figure 4.2 indicates the cumulative gas production of kikuyu over the 72 h incubation period. The addition of EFE resulted in greater net gas production with the kikuyu substrate as determined by a Bonferroni test from 12 h onwards ($P < 0.05$). This cumulative gas production data was then fitted to two non-linear models (with and without a lag phase) to

determine the gas production kinetic digestion values. The estimated gas production of the model containing a lag phase is presented in Figure 4.3. From Table 4.2 it is clear that EFE treatment of kikuyu resulted in a higher total gas production (*b*-value; *P* = 0.039) and increased rate of gas production (*c*-value; *P* = 0.001) when the model with a lag phase was used. The model without lag phase did not result in a higher *b*-value (*P* = 0.16), but is consistent with the first model in that the rate of gas production was also positively influenced (*P* = 0.002). Lag time was not decreased due to enzyme treatment of kikuyu (*P* = 0.99). Although the total gas production (*b*) was improved by enzyme addition, it appears from Figure 4.1 that this effect was related to a carry-over effect, as fractional gas production was mostly improved during the earlier stages of fermentation and disappeared after incubation times exceeding 30 h.

Table 4.2. Gas production fermentation kinetics of lucerne hay or kikuyu treated with EFE and incubated in buffered rumen fluid for 72 h

Lucerne hay	**Control**	**EFE cocktail**	**SEM**	***P***
		Model with lag		
b	108.87	103.86	1.162	0.013
c	0.049	0.054	0.00089	0.005
Lag	0.373	0.307	0.039	0.260
		Model without lag		
b	109.84	104.56	1.151	0.009
c	0.047	0.052	0.00082	0.002
Dried kikuyu	**Control**	**EFE cocktail**	**SEM**	***P***
		Model with lag		
b	156.32	165.94	2.873	0.039
c	0.016	0.019	0.00029	0.001
Lag	1.40	1.40	0.027	0.990
		Model without lag		
b	175.43	183.41	3.740	0.160
c	0.013	0.015	0.00030	0.002

b: asymptotic total gas production, c: rate of gas production (per h), lag: lag time (h)

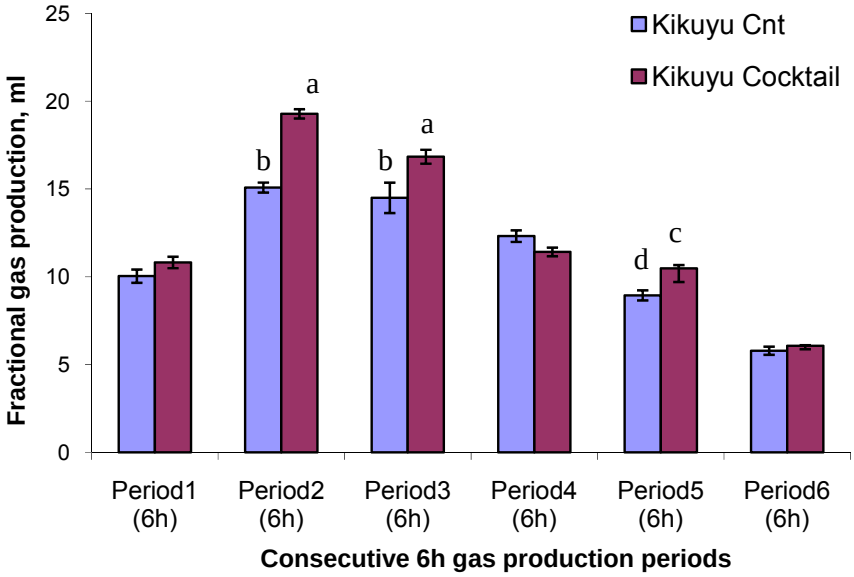

Figure 4.1. Fractional gas production of kikuyu with or without EFE in six consecutive 6 h gas production periods (36 h incubation period). Error bars represent the SEM. Different superscripts (a, b, c or d) indicate significant differences within a specific 6h period (P < 0.05).

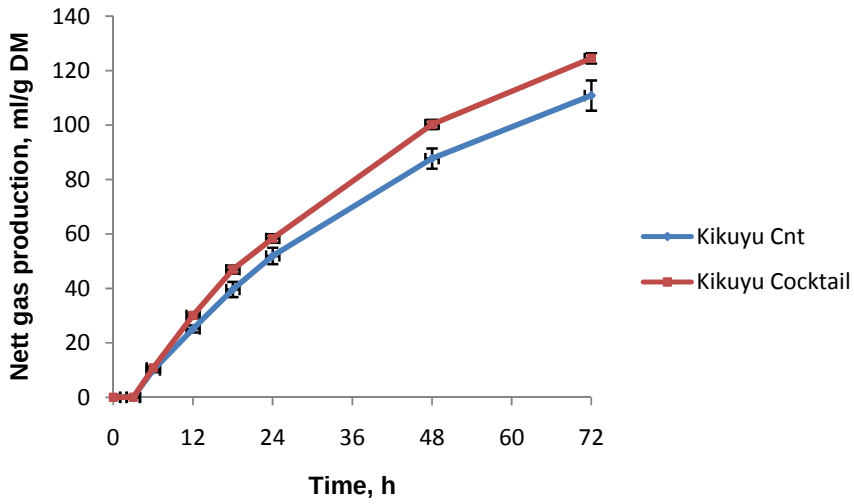

Figure 4.2. Cumulative gas production of kikuyu treated with or without EFE over a 72h incubation period in buffered rumen fluid. Error bars represent the SEM.

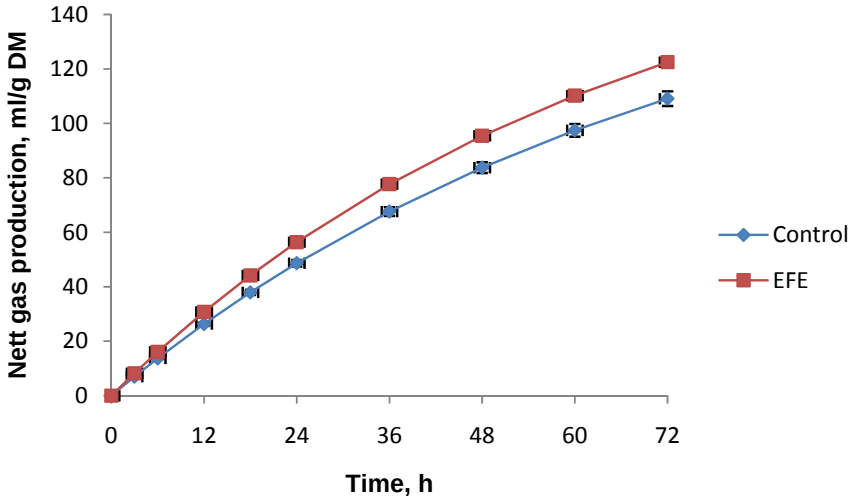

Figure 4.3. Gas production of kikuyu treated with EFE or distilled water over a 72h incubation period in buffered rumen fluid using estimate values with data fitted to a non-linear model (with lag). Error bars represent the SEM.

Far less positive effects were observed when lucerne was used as substrate. Neither the fractional gas production (Figure 4.4) nor the cumulative gas production (Figure 4.5) of EFE treated lucerne was positively influenced ($P > 0.05$). The cumulative gas production data was again fitted to the two models described earlier and results presented in Table 4.2. For both models, the *b*- and *c*- values were significantly improved due to EFE treatment ($P < 0.05$). Exogenous fibrolytic enzyme treatment, however, had no effect on the lag time ($P = 0.26$). It therefore appears that EFE treatment consistently improved the potential gas production and rate of gas production, but in the case of lucerne, no explanation can be given as to when this effect occurred.

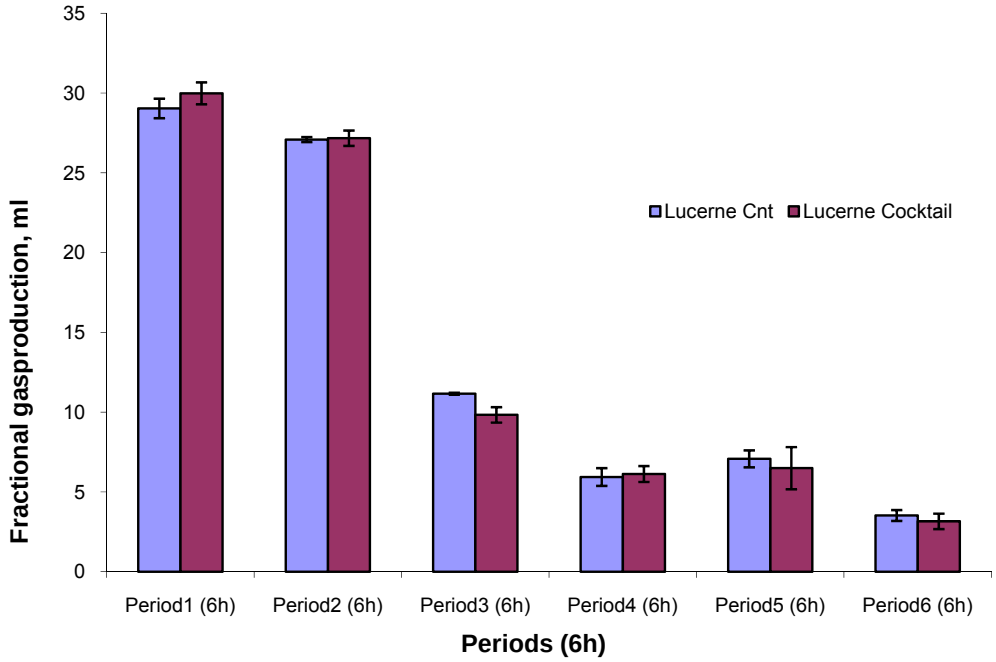

Figure 4.4. Fractional gas production of lucerne hay with or without EFE in six consecutive 6 h gas production periods (36 h incubation period). Error bars represent the SEM.

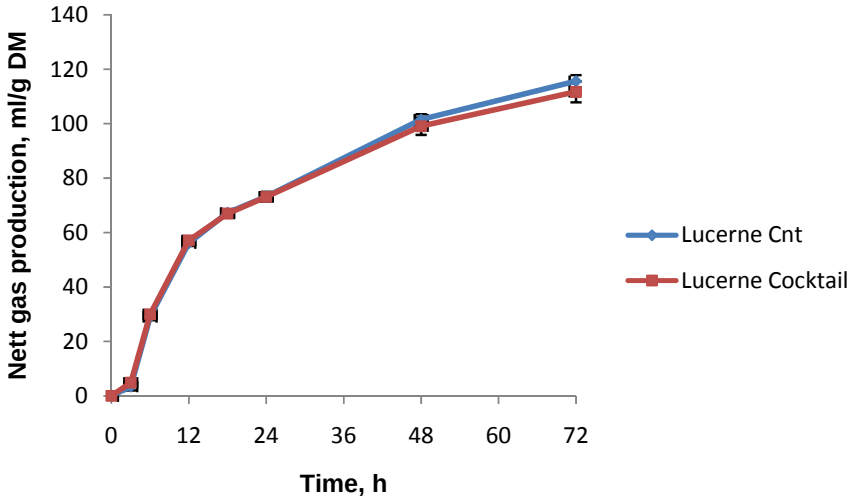

Figure 4.5. Cumulative gas production of lucerne treated with or without EFE over a 72 h incubation period in buffered rumen fluid. Error bars represent the SEM.

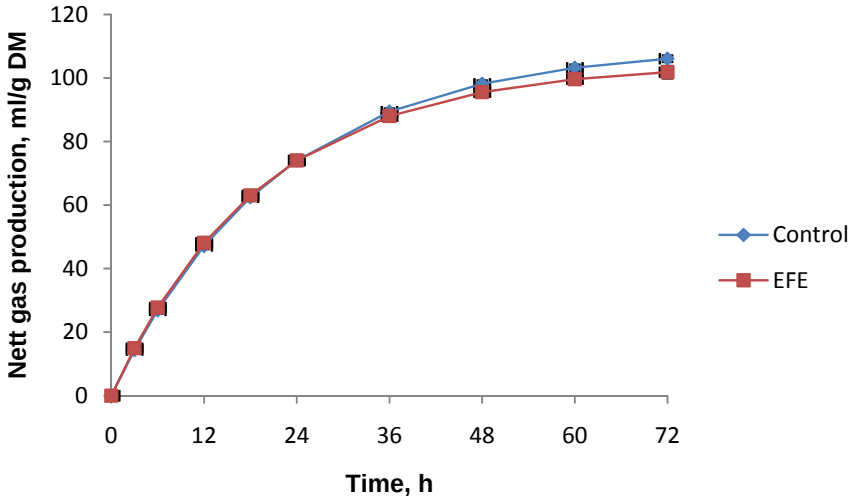

Figure 4.6. Gas production of lucerne treated with EFE or distilled water over a 72 h incubation period in buffered rumen fluid using estimate values with data fitted to a non-linear model (with lag). Error bars represent the SEM.

With the weeping love grass as substrate (Figure 4.7), an increased gas production due to EFE addition was observed during the early hours of incubation, similar to that observed for kikuyu. Fractional gas production was higher in period 2 and period 4, corresponding to 12 h and 24 h incubation times ($P < 0.05$) for the EFE treated weeping love grass. During period 3 the EFE treated weeping love grass, however, produced significantly less net gas than the control ($P < 0.05$). However, with data expressed as cumulative gas production there was only an increased gas production due to EFE treatment at 12 h (Figure 4.8, $P = 0.03$) and the control treatment never exceeded the EFE treatment. From Figure 4.8 it is evident that weeping love grass were still being degraded in a linear fashion and therefore the data could not be fitted to the two non-linear models used for kikuyu and lucerne. A linear regression was therefore fitted to the data to get an indication of the rate of gas production. The trend lines fitted the data well ($R^2 > 0.99$) but the rate of production (taken as the slope of the trend lines) did not differ significantly between treatments.

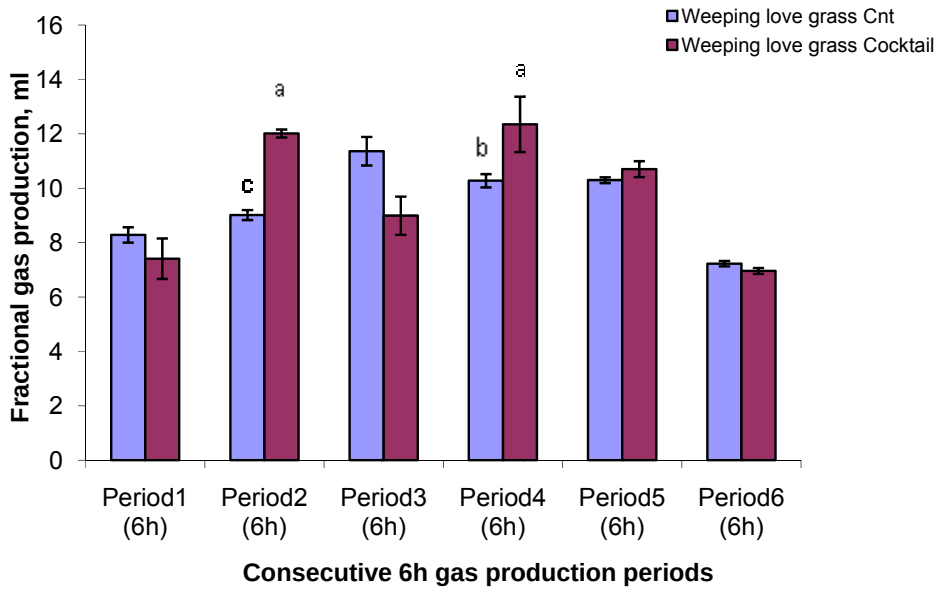

Figure 4.7. Fractional gas production of weeping love grass with or without EFE in six consecutive 6 h gas production periods (36 h incubation period). Error bars represent the SEM. Different superscripts (a, b or c) indicates significant differences ($P < 0.05$).

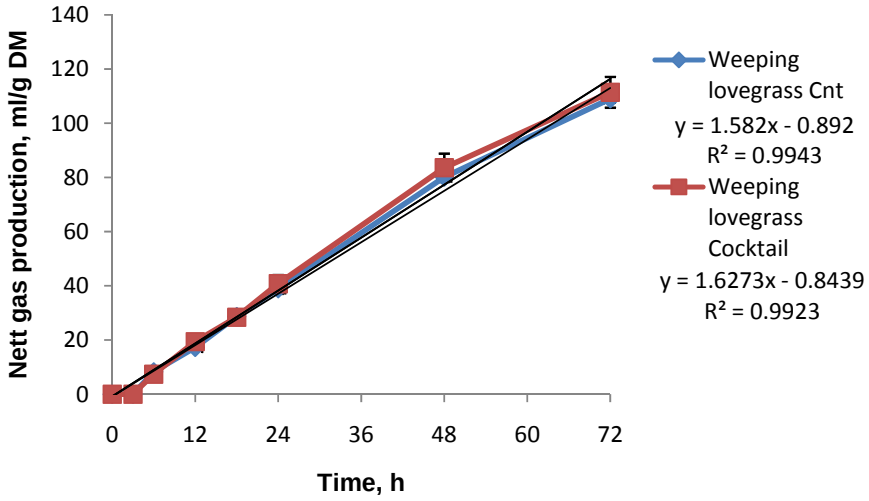

Figure 4.8. Cumulative gas production of weeping love grass treated with or without EFE over a 72 h incubation period. Error bars represent the SEM.

In vitro true digestibility

Relative to the respective forages without enzyme treatment, *in vitro* true digestibility at 24 h was greater for both EFE-treated lucerne ($P = 0.044$) and EFE-treated kikuyu ($P = 0.004$) but

not for EFE-treated weeping love grass (P = 0.17, Figure 4.9). The increase was 3.42 % and 3.52 % for the lucerne and kikuyu, respectively. The kikuyu digestibility results are in accordance with the net gas production results where a 12 % increased gas production was observed at the 24 h incubation time (P < 0.05). However, there appears to be a poor relation between the gas production data and the *in vitro* digestion data as effects differed in magnitude. Yet, the IVTD data supports the earlier findings on gas production in that a higher total gas production is indicative of higher digestibility. The IVTD data did not support the small improvement in gas production observed due to EFE treatment of weeping love grass.

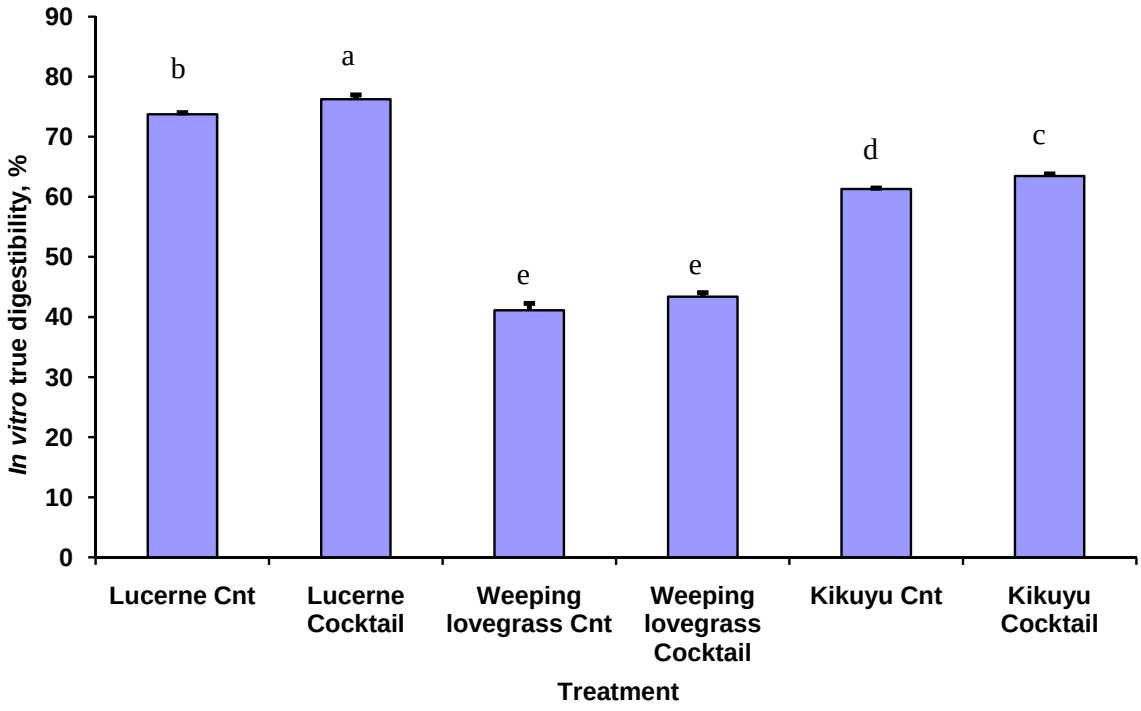

Figure 4.9. *In vitro* true digestibility of three forages treated with EFE after 24h incubation in buffered rumen fluid. Error bars represent the SEM. Different superscripts (a, b, c, d or e) indicate significant differences upon pairwise comparisons (P < 0.05).

Discussion

The proximate analysis of the three forages used compares well with the average nutrient values of South African produced hays, with expected differences in fibre content noted. The forages were therefore regarded as acceptable test substrates.

In this study we reported significant effects of EFE treatment on fractional gas production during the early hours of incubation. Fractional gas production was measured to eliminate the carry-over effect of higher gas production at the early stages of the incubation to the later stages of fermentation. Indeed, for kikuyu the EFE resulted in higher gas production at 12, 18 and 30 h of the incubation. For the incubation periods thereafter gas production was not improved by EFE. Similar effects were observed for weeping love grass in which EFE treatment increased the fractional gas production at 12 and 24 h of fermentation. The finding that EFE affected the kikuyu and weeping love grass at the earlier incubation times is in agreement with findings from Jalilvand *et al.* (2008) that reported effects of their EFE on stimulation of ruminal fermentation during the early hours (6 h) of incubation, but not thereafter. Giraldo *et al.* (2008) also noted the effect of EFE at 8 h of the fermentation of different ratios of forages to concentrates. Tricarico (2001) found that exogenous fibrolytic enzymes did not alter final gas production or extent of fibre digestion when extended periods of incubation were evaluated and is in agreement with the statement of Colombatto *et al.* (2007) that the final gas production is often not increased by EFE as ruminal microorganisms degrade material anyway, albeit at a later stage. Generally, it is accepted that the enzyme exert its effect within the first 6 – 12 h of fermentation. Unfortunately, in this study the time of EFE effect on lucerne could not be determined as no differences were observed in fractional gas production for lucerne.

The cumulative gas production data showed a significantly increased net gas production over the 72 h incubation period for kikuyu. This is in agreement with the model predicted total gas production (*b*) which was also increased for the EFE treated kikuyu. For lucerne, somewhat different results were obtained. Although *b* was also increased due to EFE treatment, no significant difference was observed for the cumulative net gas production. The increased total gas production (*b*) for kikuyu and lucerne is in agreement with findings that EFE can improve total GP of lucerne (Eun and Beauchemin, 2007), mixed forage and concentrate diets (Giraldo *et al.*, 2008) or wheat straw (Jalilvand *et al*., 2008). However, other researchers have reported no effects on total GP. Colombatto *et al*. (2003b) found that the final gas production value of lucerne was not increased by the two levels of two enzymes

tested by them and this corresponds to findings of Jalilvand *et al*. (2008) whom also reported no effects on total GP of lucerne.

For both lucerne and kikuyu, the rate of gas production (*c*) was increased in this study. This is in stark contrast with findings from Jalilvand *et al.* (2008) who reported that the fermentation rate was adversely affected by enzyme addition, and the level of enzyme addition, as their results showed lower fractional gas production rates and higher half times as enzyme level increased. They reported relatively high maximum fermentation rates (approximately 0.08 h^{-1}) for lucerne which is close to the theoretical first order rate constants for cellulose digestion of 0.05 to 0.08 h^{-1}, as suggested by Weimer (1996). Weimer (1996) reported these values for rate constants of cellulose and suggests that ruminal cellulolytic bacteria have evolved to digest cellulose within this narrow range when digesting structurally ordered, insoluble polymers of fibrous sources. It could be hypothesized that there are similar theoretical maximum rates above which EFE addition could not further improve the fermentation rate of lucerne and indeed any forage. In our study, much lower gas production rates (c: 0.05 h^{-1} and 0.016 h^{-1} for lucerne and kikuyu, respectively) were observed than in the study of Jalilvand *et al.* (2008) following that there was scope for improvement by EFE. This was indeed the observation and EFE resulted in higher gas production rates for both lucerne and kikuyu, regardless of the model used for the determination of the kinetic values. Most of the exogenous fibrolytic enzymes tested by Eun and Beauchemin (2007) resulted in increased initial rates of gas production and supports findings of this study. These researchers suggested that the increased rate of gas production supports the hypothesis of Colombatto *et al.* (2003a) that the enzyme treatment of forages lead to subtle changes in the cell wall structure, thereby allowing ruminal microbes earlier access to the highly digestible cell contents.

Volatile fatty acids (VFA) were not measured on the rumen fluid inoculum after fermentation as it was assumed that the EFE would not alter the ratio of the VFA from the same substrates and thereby affecting the gas production. As a future prospect, it will be of value to confirm this assumption by measuring the effect of the EFE treatment of similar substrates on the total VFA production as well as on the VFA ratio.

In vitro true digestibility (IVTD) was significantly higher for both lucerne and kikuyu when treated with EFE. The increase amounted to approximately 3.5% which is lower than values reported by Beauchemin *et al.* (2003) who listed the top ranked enzyme products for their ability to affect the IVDMD of lucerne or corn silage. Although lower, the finding that EFE treatment of forages can increase the IVTD thereof is in agreement with other researchers.

Dean *et al.* (2008), similarly to Beauchemin *et al.* (2003), reported improved IVDMD at 6 h for enzyme treated tropical grass hay, but not at 48 h. Giraldo *et al.* (2008) further indicated that the effect of enzymes on *in vitro* true DM degradability became less marked as the fermentation progressed. This again is in agreement with earlier findings that EFE exerts its effects during the earlier stages of fermentation. The IVTD results obtained here is in agreement with the gas production results and it appears as if EFE had the most consistent effects with kikuyu and lucerne, in this trial.

Conclusion

It was deemed important to characterise our enzyme for its particular effects on gas production and IVTD of lucerne, kikuyu and weeping love grass hay. Results indicated that, especially for kikuyu, EFE can have a marked effect on the total gas production, as well as on the rate of gas production. For future studies, however, two-compartmental models should be employed to determine the effect of the EFE on both the rapidly and slowly digesting pools present in forages.

The gas production findings were supported by the increased *in vitro* digestibility (DMD) of kikuyu, but not for lucerne or weeping love grass where discrepancies were observed between GP and DMD. These discrepancies are not well understood but can be related to the experimental protocol followed and two probable explanations are offered. First of all, no attempt was made to measure VFA on the rumen fluid inoculum during or after fermentation. Increased VFA production or altered VFA ratios could have affected the gas production and should be addressed in future studies. Additionally and more likely, the pre-treatment of the substrate with the EFE could have resulted in altered reducing sugar content in the substrate when inoculated 12h later on, affecting GP differently to DMD. For GP, the measurement does not include any of these pre-treatment effects, while for DMD; the pre-treatment effects are included in the calculation of digestibility.

Limited indications exist and are in agreement with other researchers that the enzyme effect is likely exerted during the early phases of fermentation. Results with lucerne were very similar to kikuyu regarding the gas production fermentation kinetic parameters, with increased total gas production and enhanced rate of gas production. Weeping love grass showed very little potential in improving its digestibility with EFE treatment.

It is concluded that there is sufficient evidence that the EFE cocktail used in this study can affect the digestibility characteristics of especially kikuyu- and lucerne hay and further studies are warranted. Such studies will be discussed in the subsequent chapter and includes information on *in sacco* degradability as opposed to merely evaluating gas production and IVTD.

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CHAPTER 5

The effect of an exogenous fibrolytic enzyme cocktail on *in sacco* and *in vitro* digestibility of a complete feed for sheep

Abstract

Exogenous fibrolytic enzymes (EFE) show potential in improving the digestibility of forages, not only *in vitro* and *in situ*, but also in terms of the production of ruminant animals. Many research publications point to improved rates of DM, NDF and/or CP disappearance, potential disappearance of these nutrients, decreased lag times for digestion and effective degradability. These results have, however, been inconsistent. In this study, a novel exogenous fibrolytic enzyme cocktail, applied 12 h prior to incubation as a supernatant from the fermentation of fungal strain ABO 374 was evaluated *in sacco* (up to 96 h) using six Döhne-Merino sheep. Data were fitted to two non-linear models differing only in terms of the inclusion of a calculation for lag time. Parallel to this trial the *in vitro* digestibility of the complete feed was also evaluated (up to 72 h) for its susceptibility to EFE. The complete feed contained a substantial amount of NDF (349 g/kg DM basis) and was further formulated according to the specifications for growing lambs. Results were in agreement with other research findings on EFE treated feeds or feedstuffs. It was observed that the rate of DM, CP and NDF disappearance and the effective degradability of each nutrient was significantly improved due to EFE treatment. The two models yielded similar results with the exception that EFE resulted in a shorter lag time for NDF only. Contradictory to expectations, EFE treatment of the complete feed resulted in a significantly lower soluble and rapidly degradable NDF fraction. The *in vitro* digestibility results substantiated the findings that EFE treatment of the complete feed can result in the improved digestibility thereof. Also, in agreement with findings of other groups, it was demonstrated that, although the EFE contained only fibrolytic enzyme activity, its effects were not limited to NDF. Based on these results on nutrient digestibility, it is speculated that EFE partly results in subtle changes to the cell wall structure, allowing microorganisms earlier access to the cell contents. In addition, these effects may also be related to the enhanced attachment of microorganisms to the plant cell wall and by the synergistic effect with enzymes produced in the rumen, therefore affecting the *in sacco* disappearance of CP in addition to improving the overall digestibility of the feed.

Introduction

The use of high fibre, low energy diets as ruminant feeds is common practise in the nutrition of sheep, dairy and beef cattle and in feedlot finishing of animals. Increasing the digestibility of the often poor quality forages has been a topic of research for many years. It is clear that forages play an important role in the animal industry worldwide. The fibre (cell wall) portion makes up to 300 to 800 g/kg of forage dry matter and represents a major source of nutritional energy for ruminants, but, unfortunately less than 50 % of this fraction is readily digested and utilized by the animal (Hatfield *et al*., 1999). Exogenous fibrolytic enzymes and preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fibre (Feng *et al*., 1996) and are proposed as a means of unlocking this source of nutritional energy for ruminants (Johnston, 2000). Indeed, research findings indicate positive effects of enzyme treatment of forages and ruminant feeds in beef and dairy cattle (Lewis *et al*., 1999; Rode *et al*., 1999; Yang *et al*., 1999; Beauchemin *et al*., 1995; 2003) and even small stock such as sheep (Cruywagen and Goosen, 2004).

The abundance of research on exogenous fibrolytic enzyme application appears to be done either in *in vitro* or *in situ* studies. Dean *et al*. (2008) evaluated four different commercial exogenous enzyme products on the ruminal degradation of Coastal bermudagrass hay or Pensacola bahiagrass hay (12-week re growths, tropical grasses). The enzyme treatments contained mainly xylanase and cellulase enzymes, with the exception of one treatment that contained other fibrolytic activity. Results showed that one preparation (Promote) hydrolysed NDF into water soluble carbohydrates (WSC), decreased ADF levels and had higher 6 h IVDMD. The other enzyme treatments also resulted in decreased NDF concentrations and increased 6h IVDMD of bermudagrass hay. The enzymes had negligible effects on the extent of fibre digestion and *in situ* DM degradation as no responses were observed in the *b, a+b* or potentially degradable (P) fractions. It appears that the enzymes therefore exhibited their effects mostly in the initial and 48 h stages of DM digestion.

Similarly, Pinos-Rodriguez *et al.* (2008) reported that EFE increased the soluble fraction (*a*) of DM as well as the DM and NDF ruminal *in situ* disappearance rates of TMR diets containing varying proportions of concentrate to forage. Moharrery *et al.* (2009) recently also reported on increased *a*-values for NDF with a decreased *b*-value. Commercial enzymes for monogastric nutrition can be used in ruminant diets as shown in the results of Jalilvand *et al*. (2008) where Cellupract AS 130, Natuzyme and Endofeed DC (enzymatic products for poultry feeds) were evaluated *in situ*. Cellupract showed increased rapidly

soluble fraction (*a*), potentially degradable fraction (*b*) and effective degradability (ED) for DM of all the forages studied (lucerne hay, maize silage and wheat straw) in Blochi ewes. Reported results on EFE effects regarding *in sacco* digestion kinetics, however, do not seem to be consistent and therefore a study was proposed in which these digestion kinetics could be determined for our EFE cocktail. The exogenous fibrolytic enzyme used in this study was isolated from soil in South Africa and used as an extracellular enzyme supernatant in the fresh form. This extracellular enzyme supernatant of the fungal strain, ABO 374 was characterized as a fibrolytic enzyme cocktail, containing mostly xylanase (296 ± 0.07 U/mg protein) but also cellulase (1.44 ± 0.39 U/mg protein) and mannanase (1.10 ± 0.37 U/mg protein) activity (Cruywagen and Van Zyl, 2008). The activities of these enzymes were determined using 10 g/kg birchwood xylan for β-xylanase, 10 g/kg CMC (carboxymethyl cellulose) as substrate for cellulase (endoglcanase) and 10 g/kg locust bean gum for β -mannanase activity determination.

The objective of this study was to extend the characterization of the novel EFE cocktail to its effects on the *in sacco* and *in vitro* digestibility of a complete feed for sheep.

Materials and Methods

In this study, a complete total mixed ration was formulated on the specifications for growing lambs as per the nutrient requirements of small ruminants (NRC, 2007). The diet contained approximately 30% roughage and 65% concentrate, with 166 g/kg CP and 348 g/kg NDF. The fibre component of this diet was deemed high enough for the exogenous fibrolytic enzyme (EFE) to have a postive effect on digestion. Table 5.1 shows the formulation and proximate analysis of the feed. In chapter 3, positive results were achieved with treating various forages with EFE. In this chapter, the decision was taken to evaluate the effect of EFE in a total mixed ration, containing relatively high levels of forages typically used in sheep diets.

Table 5.1. Formulation and chemical composition (DM-basis) of a balanced diet for growing lambs used in the *in sacco* and *in vitro* digestibility studies

Raw material	Inclusion, g/kg
Maize meal	450
Wheat	150
Lucerne meal	120
Oat hay	200
Cotton seed oilcake meal	50
MuttonGainer premix[1]	30
Chemical composition	**g/kg**
Moisture	132
DM	868
Crude protein	166
NDF	349
Ether extract	11.3
Ash	46.5

[1]: Product supplied by FORMUFEED cc, Villiersdorp, South Africa and manufactured by NUTEC (Pty) Ltd, Willowton, South Africa.

Six ruminally cannulated Döhne-Merino wethers were randomly assigned to two groups and adapted to the diet as described in Table 5.1 over a 10 day period. The diet of three sheep (treatment group) was treated with the EFE at a rate of 5 ml EFE supernatant per kg of feed. The enzyme was applied and thoroughly mixed with the feed daily 12 h prior to feeding. The control diet was treated with the same amount of distilled water daily and fed to three control sheep. After the first 96 h collection period, the diets were switched over in a cross over design study and animals adapted to the diet before commencing with the second period of the study. Each animal therefore received each treatment in either of the two periods. Each animal then received 2 kg/day of the relevant fresh feed.

A sample of the feed was ground through a 2 mm screen (Hammer mill 372, Cape Town, SA) and sieved through a 124 µm screen for 5 minutes to remove the extremely fine particles as described by Cruywagen *et al*. (2003). An amount of 8 g (± 0.01g) of the milled and sieved sample was accurately weighed into 10 x 20 cm polyester bags (Ankom forage bags, Ankom Technology Corp., NY, USA). The bags were closed with 0.44 mm fishing twine with a fisherman's knot and attached to a circular stainless steel weight. Ten bags were attached to each circular weight and one thereof inserted into the rumen of the relevant

animals. The bags were inserted simultaneously into sheep fed either the control or EFE treated TMR and one bag per animal removed after 3, 9, 12, 24, 36 and 48 h. After 72 and 96 h two bags per time increment was removed to insure sufficient residue for chemical analysis. After the 96 h incubation was completed, the circular weight with the blank bag was removed. The animals were then adapted to the switched diet in period 2 in order for each animal to receive each treatment.

Prior to insertion of the bags into the rumen of the animals, the bags were pre-treated with either distilled water (Control) or EFE. The same enzyme dilution was used as for the treatment of the feed (1 ml exogenous enzyme supernatant per 200 ml distilled water) and 1 ml thereof applied per gram of feed in the *in sacco* bags. Therefore, xylanase and cellulase were added to the substrate at levels of 0.8 U/mg xylanase per g substrate and 0.05 U/mg cellulase per g substrate. The bags were allowed to soak overnight in either the 8ml EFE dilution or 8ml distilled water before insertion into the rumen of the animals at 08:00 the following day. The 0 h bags were treated in the same manner, but were not inserted into the rumen of any animal. The 0 h bag served as correction for material lost via the pores of the bag not ascribed to the digestion process. Upon removal, bags were rinsed in ice cold water and frozen at -4^{o} C until analysis.

Simultaneously with the *in sacco* study, an *in vitro* study was performed. The *in vitro* true digestibility assay is reported to be being highly repeatable in the results observed as well as comparable to *in situ* results (Spanghero *et al*., 2003). Rumen fluid (RF) was collected from each sheep at the time of insertion of the *in sacco* bags. Approximately 200 ml rumen fluid was collected from each animal, strained through four layers of cheesecloth and the rumen fluid of the sheep fed the EFE diet or the control then pooled. The rumen fluid was handled anaerobically and transferred to the laboratory in a pre-heated thermos flask. Upon arrival in the laboratory the rumen fluid of the sheep were blended in a high-speed mixer for 1 minute under CO_2 gaseous phase. The rumen liquor was mixed with the pre-heated (39^{o} C), reduced buffer solution as described in Chapter 3 at a ratio of 1 part RF and 4 parts buffer solution. The same milled and sieved sample as for the *in sacco* trial was used and 1g (± 0.01g) sample weighed into Ankom F57 filter bags (Ankom Technology Corp., NY, USA). Bags were pre-treated overnight with either 200 ml EFE or 200 ml distilled water in the Incubation flasks as described for the *in sacco* bags. Sixteen bags were added per flask and four flasks per treatment (two flasks per treatment per period as for the *in sacco* study) were allowed (two flasks for the EFE treatment and two flasks for the dH2O treatment). Before collection of the RF, 1400ml pre-warmed reduced Goering and Van Soest (1970) buffer was added to each flask, purged with CO_2 and placed in the incubator. The RF solution (400 ml)

was added to the flasks containing the enzyme pre-treated bags, purged with CO_2 and placed in the pre-heated incubator (39^{o}C) for up to 72 h. Upon retrieval of the bags, the flasks were removed from the incubator, opened and bags removed while purging with CO_2. Bags were removed after 3, 12, 24, 36, 48 or 72 h incubation and rinsed in ice cold water until the water was clear, and kept at -4^{o} C until analysis for *in vitro* digestibility according to the ANKOM procedure. Six bags with substrate were also prepared but did not undergo *in vitro* digestion. These bags were termed 0 h bags and served as correction for fine material lost due to dissolving and washing out of the bags.

Upon analysis, the *in sacco* bags were thawed and washed in a washing machine on a gentle cycle in cold water for three cycles of 5 minutes each, with rinses in between. The 0 h bags were included in the washing process to correct for any soluble material lost during the incubation and washing processes. After washing, the *in sacco* bags along with the F57 *in vitro* bags were dried in a forced draught oven at 55^{o} C for 48 h. On completion of the drying process, all bags were removed and weighed.

The *in sacco* bags were emptied and the residual matter transferred to air tight containers for storage. The residual matter was then analyzed for DM, NDF and CP and the DM disappearance, NDF disappearance and CP degradation subsequently calculated. As described in Chapter 3, NDF was determined according to the Ankom procedure (Ankom Fibre Analyzer 200/220, Ankom Technology Corp., NY, USA) and CP was analyzed on the Leco (Leco corporation, St. Joseph, MI, USA).

The disappearance of DM, NDF and CP were calculated as:

Nutrient D = ((a-b)/a)*100 where:

Nutrient D = disappearance of the relevant nutrient

a = total mass of DM, NDF or CP before incubation (g)

b = total mass of DM, NDF or CP after incubation (g)

The DM-, NDF- and CP- disappearance values of the *in sacco* trial were fitted to a non-linear model as described by Ørskov and McDonald (1979) to calculate the a, b and c- values. As proposed by McDonald (1981), a lag phase was also included in the model as shown in Model 2 below.

Degradation rate model:

Model 1: $Y = a + b(1-exp^{-ct})$

Model 2: $Y = a + b(1-exp^{-c(t-L)})$ where:

Y = degradation

a = soluble and readily degradable fraction

b = potentially degradable fraction

c = degradation rate of fraction b

t = incubation time

L = Lag time

The effective degradability (EFF Degr) was calculated with the obtained a, b and c-values according to the formula of Ørskov and McDonald, 1979:

Eff Degr = a + ((bc)/(c+k)) where:

Eff Degr = Effective degradability

a = soluble and readily degradable fraction

b = potentially degradable fraction

c = degradation rate of b

k = rate constant of digesta passage

The rate constant (k) was assumed to be 0.05/h (Ørskov and McDonald, 1979).

All the *in vitro* bags were subjected to the Ankom Fibre Analyzer 200/220 (Ankom Technology Corporation, NY, USA) to determine the *in vitro* true digestibility corrected for dry matter ($IVTD_{DM}$) (%) as described in Chapter 3.

Statistical analysis

The *a, b, c* and Lag derivatives and the effective degradability were subjected to a main effects ANOVA with the aid of Statistica 9.0 (2009), with the main effects being time and treatment. For the IVTD data, the data were subjected to a Repeated measures ANOVA, also with the aid of Statistica 9.0 (2009). Interactions with period were tested and where no significant interactions were declared, the main effects were interpreted. Least square means were determined and significance declared at $P<0.05$.

Results

The *in sacco* fermentation kinetics of the EFE treated and control feeds are presented in Tables 5.2 to 5.4 for DM and NDF disappearance and CP degradation data. In each table, data yielded by either the model with or without a lag component build in, is given.

For DM disappearance, the EFE treatment of a complete feed containing 868 g/kg DM did not result in an increase of the soluble and rapidly fermentable fraction (a) or in the potential degradable fraction (b). However, the degradation rate of the degradable fraction was 6.2%.h^{-1} for EFE treated feed as opposed to 4.2%.h^{-1} for the control diet. This increase in degradation rate was highly significant (P = 0.0002). Effective DM degradability was also positively increased with the EFE treated feed having an 11.5 % higher effective DM degradability than the control (P = 0.0006). Lag time was not influenced by the treatments and when the model without lag was applied, the same effects were observed. Again, the rate of degradation and the effective degradability were increased, although the magnitude of increase was slightly smaller than in the former case. Figure 5.1 indicates the mean DM disappearance of the model fitted data (with lag). The preparation of the models was based solely on the data yielded by the models, with a lag calculation included.

Table 5.2. *In sacco* DM disappearance fermentation kinetics of a complete feed treated with EFE or distilled water when fitted to a model with or without lag time calculation

DMD	Control	EFE	SEM	*P*
Model with lag calculation				
a	22.06	21.64	0.472	0.540
b	60.97	62.89	0.840	0.140
c	0.042	0.062	0.002	0.0002
Lag	0.086	0.085	0.079	0.900
Eff Degr	49.89	57.40	1.041	0.0006
Model without lag calculation				
a	21.83	21.22	0.462	0.380
b	61.20	63.64	0.970	0.110
c	0.042	0.059	0.003	0.0009
Eff Degr	49.76	55.80	0.491	0.0001

a = soluble and readily degradable fraction

b = potentially degradable fraction

c = degradation rate of b

Eff Degr = effective degradability

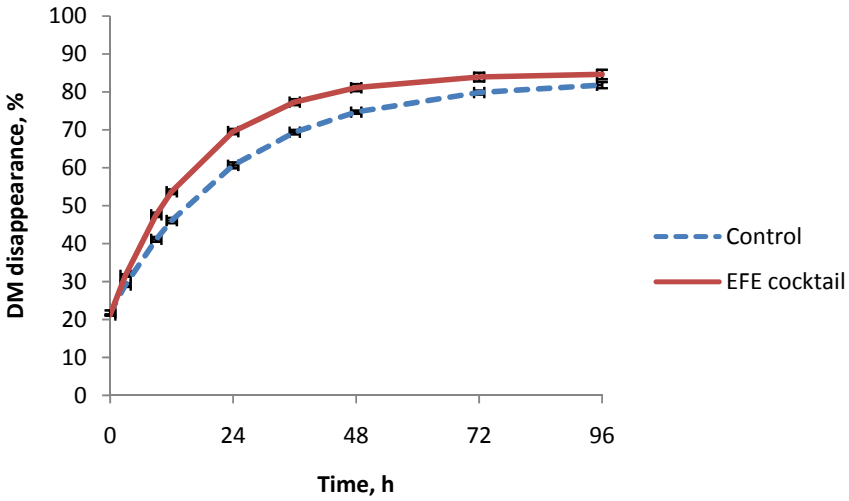

Figure 5.1. Mean disappearance of DM of a complete feed incubated *in sacco* in sheep when data were fitted to the model with a lag time calculation. Error bars indicate the SEM.

The soluble and rapidly fermentable CP fraction (a) was not increased due to EFE treatment of the complete feed. However, with DM disappearance, the potential degradable fraction (b) was increased (P = 0.008), as was the rate of CP degradation (c) (P = 0.015) and the effective degradability of CP (P = 0.0037). Lag time was unaffected by treatment. The same differences followed when data was fitted to a model without lag with b, c and effective degradability being increased for EFE treated feed (P < 0.05). Figure 5.2 indicates the mean CP degradation of the model fitted data (with lag).

Table 5.3. *In sacco* CP degradation fermentation kinetics of a complete feed treated with EFE or distilled water when fitted to a model with or without lag time calculation

CP degradation	Control	EFE	SEM	P
Model with lag calculation				
a	30.20	29.47	0.491	0.32
b	38.31	41.13	0.592	0.01
c	0.061	0.091	0.007	0.02
Lag	0.14	0.14	0.011	0.93
Eff Degr	50.88	55.91	0.913	0.04
Model without lag calculation				
a	31.04	28.93	0.903	0.13
b	37.47	41.67	1.362	0.06
c	0.061	0.091	0.006	0.02
Eff Degr	51.33	55.72	0.691	0.01

a = soluble and readily degradable fraction

b = potentially degradable fraction

c = degradation rate of b

Eff Degr = effective degradability

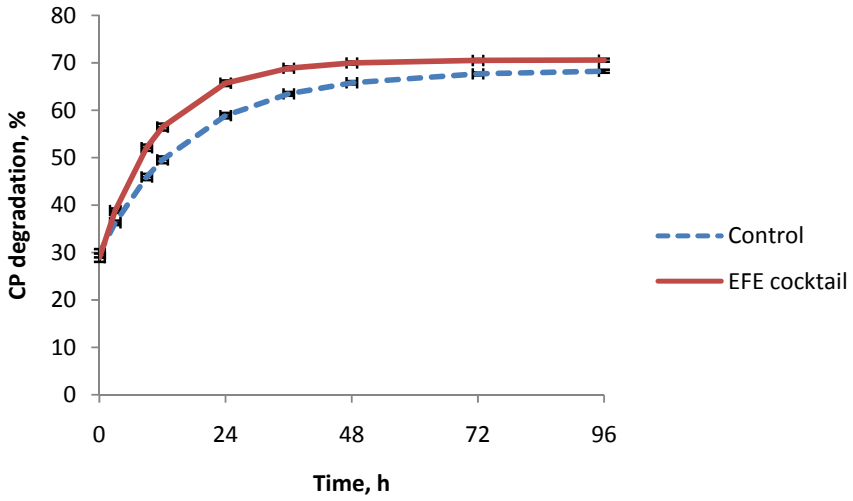

Figure 5.2. Mean CP degradation of a complete feed incubated *in sacco* in sheep when data were fitted to the model with a lag time calculation. Error bars represent the SEM.

The enzyme used in the study, as discussed in detail earlier, was an exogenous fibrolytic enzyme cocktail capable of digesting xylan and cellulose, as its main function. Therefore and because NDF consists mainly of hemicellulose, cellulose and lignin (McDonald *et al*., 2002) this is where the direct effects of the EFE can be expected. As can be seen in Table

5.4, EFE treatment of the complete feed (containing 349 g/kg NDF) resulted in a number of significant observations. First of all, the degradation rate of the NDF degradable fraction was more than doubled (110 % increase) (P = 0.003) even though the potential degradable fraction itself was not increased. Additionally, the lag time for fermentation was shorter for the EFE treated feed. Surprisingly though was the observation that the EFE treated feed had a lower (P = 0.008) soluble and rapidly degradable fraction. The effective NDF degradation, as for DM and CP was increased by EFE treatment of feed. The same effects were observed for the data fitted to the model excluding a lag phase calculation. Figure 5.3 indicates the mean NDF degradation of the model fitted data (with lag).

Table 5.4. *In sacco* NDF disappearance fermentation kinetics of a complete feed treated with EFE or distilled water when fitted to a model with or without lag time calculation

NDF disappearance	Control	EFE	SEM	*P*
Model with lag calculation				
a	19.30	16.39	0.611	0.01
b	61.12	60.60	4.332	0.93
c	0.019	0.040	0.004	0.01
Lag	0.27	0.10	0.022	<0.01
Eff Degr	35.17	42.82	0.741	<0.01
Model without lag calculation				
a	18.97	16.33	0.623	0.02
b	65.46	60.88	6.262	0.62
c	0.019	0.039	0.003	<0.01
Eff Degr	34.88	42.45	0.671	<0.01

a = soluble and readily degradable fraction

b = potentially degradable fraction

c = degradation rate of b

Eff Degr = effective degradability

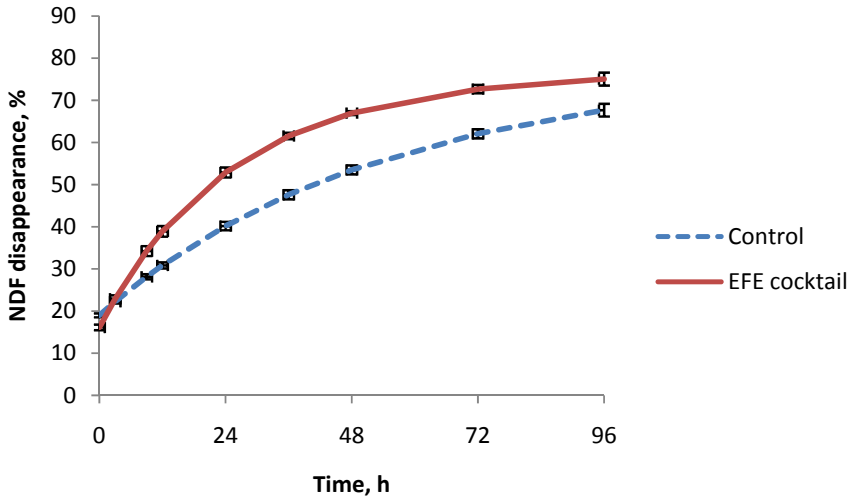

Figure 5.3. Mean NDF disappearance of a complete feed incubated *in sacco* in sheep when data were fitted to the model with a lag time calculation. Error bars represent the SEM.

The *in vitro* true digestibility data, as can be seen from Table 5.5 or Figure 5.4 substantiates the findings reported for NDF degradation. No significant interactions were noted and therefore main effects were interpreted. From this data it is apparent that EFE treatment of the feed increased the *in vitro* digestibility of the samples at each of the time points ($P < 0.05$), with the exception of the IVTD at 0 h and 36 h. The biggest percentage increase in IVTD was seen at incubation times 3 h and 12 h with a 3.1 % and 4.3 % increase in IVTD over the control feed, respectively. Thereafter, although still significant, the increase was smaller and well below 3 %.

Table 5.5. *In vitro* true digestibility (IVTD, %) of a complete feed incubated for up to 72 h in buffered rumen fluid (n=8)

Time, h	EFE cocktail	Control	*P*-value
0	32.8 [a] ± 0.33	31.8 [a] ± 0.26	0.082
3	54.7[a] ± 0.55	53.1 [b] ± 0.44	0.004
12	59.4 [a] ± 0.72	56.9 [b] ± 0.42	< 0.001
24	64.2 [a] ± 0.32	62.7 [b] ± 0.35	0.009
36	68.4 [a] ± 0.40	67.7 [a] ± 0.32	0.181
48	70.6 [a] ± 0.37	69.1 [b] ± 0.18	0.007
72	71.5 [a] ± 0.27	70.1 [b] ± 0.16	0.016

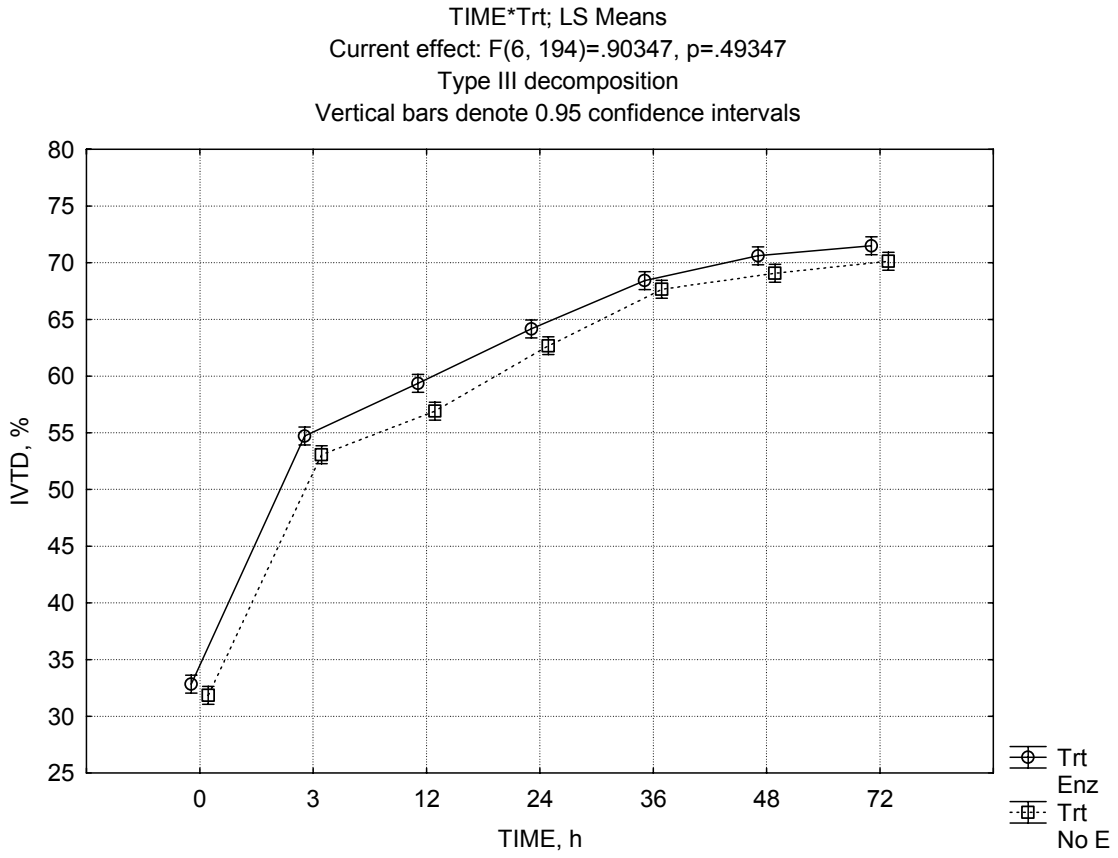

Figure 5.4. *In vitro* digestibility of the complete feed treated with EFE or distilled water and fermented in buffered rumen fluid in Daisy^{II} Incubator for pre-determined incubation periods. The graph was obtained after the repeated measure ANOVA and generated in Statistica 9.0 (2009). Error bars indicate the SE.

Discussion

The EFE used in this study was characterized as an exogenous fibrolytic enzyme cocktail obtained from the fermentation of ABO 374 on wheat straw as growth medium. As ABO 374 is of fungal origin, it secretes the enzymes into the medium and therefore the supernatant of the growth medium can be used as the source of exogenous enzymes. The major enzymatic activity of the supernatant was xylanase, with lower amounts of cellulase and mannanase also produced (Cruywagen and Van Zyl, 2008).

Many reports have been published wherein EFE have been observed to increase the fibre digestibility of diets or feedstuffs (Elwakeel *et al*., 2007; Eun and Beauchemin, 2007; Pinos-Rodríguez *et al*., 2008). When a complete feed formulated based on the specifications of the NRC Sheep (National Research Council, 1985) for growing lambs was treated with this exogenous fibrolytic enzyme cocktail and evaluated *in situ* and *in vitro*, results indicated definite benefits in the use thereof. The fermentation kinetic values for DM, CP and NDF all revealed that EFE treatment can substantially increase the rate of fermentation (c). This finding, for the most part is consistent with other researchers including Alvarez *et al*. (2009), Pinos-Rodriguez *et al*. (2008), Feng *et al*. (1996) and Wang *et al*. (2004). Alvarez *et al*. (2009) reported an increased rate of CP degradation of the feed studied by them as well as an increased rate of ADF and NDF disappearance for wheat middlings. Similarly, Pinos-Rodriguez *et al*. (2008) found improvements in the disappearance rates of DM and aNDF when total mixed rations were fed to sheep. This is in agreement with our findings where sheep were also used.

Exogenous enzyme activities are calculated to be less than 15 % of the total ruminal activity, which makes it difficult to envisage exogenous enzymes enhancing fibre digestion through direct hydrolysis alone (Beauchemin *et al*., 1997). Probably, the effect is related to the enzymes effect on the substrate physical properties, i.e. cell wall thickness and accessibility of the cell contents to microorganisms and warrants further investigation. Indeed, Colombatto *et al.* (2003a) indicated that EFE treatment results in subtle changes to the cell wall structure, allowing microorganisms earlier access to the highly digestible cell contents, including protein. In addition, these effects can also be related to the enhanced attachment of microorganisms to the plant cell wall (Nsereko *et al*., 2000; Wang *et al*., 2001) and by the synergistic effect with enzymes produced in the rumen (Morgavi *et al*., 2000).

In our study, it was consistently observed that the effective degradability was increased for DM, CP and NDF. These results are indicative that EFE can improve fibre digestion and the rate of digestion of various feedstuffs, feeds or TMR's. This is possibly related to the improvement in the attachment of microorganisms to the plant cell wall as proposed by Nsereko *et al*. (2000) and Wang *et al*. (2001) and by the synergistic effect of EFE with the enzymes produced in the rumen (Morgavi *et al*., 2000).

Interestingly though, it was observed that the EFE was not limited to NDF or DM, but that CP digestion was also positively influenced. This is not a new finding and it has been reported before that EFE effects are not limited to the dietary component it is applied to (Pinos-Rodriguez *et al*., 2008). Beauchemin *et al*. (2003) states this argument as the reason why the digestibility of the non-fibre carbohydrate fraction can be improved in addition to improvements in fibre digestibility when the concentrate portion of diets is treated with EFE. In agreement with the higher digestibility of CP reported by us, Pinos-Rodriguez *et al.* (2002 found that a xylanolytic enzyme increased the apparent digestibility of CP in addition to the increased apparent NDF digestibility of lucerne. Supposedly, as the EFE results in subtle changes to the cell wall structure and microorganisms gain earlier access to the cell contents (Colombatto *et al*., 2003a), the CP located within the cell is digested quicker (greater *c*-value) and to a larger extent. Also, CP entrapped in the complex crystalline structure of cellulose as described earlier, may be unlocked by the EFE's effect on cellulose, as seen by the improved NDF digestibility reported here.

If fibre and other nutrients are digested faster and to a larger extent as reported here, it follows that DMI of animals will be positively influenced. In a study by Cruywagen and Goosen (2004) using the exact same EFE as in this study, unfortunately no improvement in DMI was observed in Döhne-Merino lambs. However, effects on DMI have been reported in literature on numerous occasions and mostly in dairy cattle. Beauchemin *et al.* (2003) listed across 20 studies using 41 treatments, that the average DMI increase observed was 1.0 ± 1.3 kg/d for dairy cattle and that milk production was increased on average by 1.1 ± 1.5 kg/d.

Although DMI was not improved in the study of Cruywagen and Goosen (2004), animal performance was improved in that animals gained more body weight and had improved feed conversion ratios ($P < 0.05$). The improved animal performance is linked to the improved FCR and with results from this study, it can also be stated that animal performance can be improved due to higher digestibility of various nutrients found in EFE treated feeds. Iwaasa *et al.* (1997) (as cited by Beauchemin *et al.*, 2003) for instance related the increased feed efficiency observed by them in beef cattle to an increase in the diet digestibility.

The *in vitro* digestibility data in our study confirmed the improved digestibility of especially fibre reported in the *in sacco* study.

Conclusion

In this study, the aim of establishing the effects of a locally produced novel fibrolytic enzyme cocktail was continued. It was shown in the previous study that the EFE can affect the *in vitro* gas production kinetic values of various forages, as well as the *in vitro* digestibility of the forages. When the EFE was applied to a complete feed for sheep and evaluated *in sacco* and *in vitro*, results demonstrated positive effects on fibre digestibility. These results included improved rates of degradation of nutrients as well as increased *in sacco* and *in vitro* digestibility. A two compartmental model can also be used to determine the effects of the EFE on both the rapidly digesting and the slowly digesting pools of the mixed ration. However, upon plotting the data, lines were relatively smooth and there was no indication that there were two pools being digested. For comparison purposes, it was therefore digested to base results on the one compartmental non-linear models.

In the *in sacco* trial, a 15, 10 and 22 % increase in the effective degradability of DM, CP and NDF, respectively, was observed when a complete feed was treated with EFE. The *in vitro* improvement of digestibility was much smaller but significant and appeared to be greater during the earlier hours of incubation.

Overall though, the increase in digestibility is higher than can be attributed to the additional EFE activity alone as a very small amount of either xylanase or cellulase was added to the inoculum. It follows then, that in addition to the direct effect of the EFE, the effect of the enzyme can also be on another level than merely adding the limiting enzyme activity. This is based on the effects of EFE not being limited to fibre alone, as indicated in this study and by other groups.

It was concluded that the EFE evaluated here shows sufficient positive effects to further be evaluated for its mode-of-action in altering the digestibility of feeds and feedstuffs.

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CHAPTER 6

Partial purification of an exogenous fibrolytic enzyme cocktail and the effects thereof on *in vitro* gas production

Abstract

The exogenous fibrolytic enzyme cocktail produced from ABO 374 is characterised as a fibrolytic enzyme cocktail, containing mainly xylanase, but also cellulase and mannanase activity. The fresh supernatant was used in previous studies as the source of enzyme for the treatment of forages or feeds and positive results regarding *in vitro* and *in sacco* digestibility were reported. However, there is variation between batches of enzymes produced in terms of enzyme activity and digestibility effects. The fresh supernatant used in this study were markedly different from the activities reported before using the same fungal strain for the production of the exogenous fibrolytic enzyme cocktail. A simple purification step was therefore investigated to isolate the main fibrolytic enzyme from the cocktail, namely xylanase. The supernatant was applied to a glass column packed with porous beads with a molecular weight cut-off of 40,000 Daltons. On a continuous basis, 2 ml aliquots were collected and analyzed for protein content on a spectrophotometer at 280 nm. All aliquots within the same peak were pooled and its enzyme activity determined. The pooled aliquot from Peak 2 contained 8.51 times the xylanase activity of the cocktail (1351.8 and 158.9 U/mg protein, respectively) and was further tested for its ability to alter fibre digestion. No cellulase activity was detected. As positive results have been obtained with EFE treatment of lucerne hay and kikuyu, these forages were used as substrates in determining the effect of the partially purified xylanase on the *in vitro* gas production of the forages. *In vitro* gas production was measured with a completely automated system over a 48 h incubation period. The fermentation kinetic values were then determined by fitting the gas production data to two non-linear models. No significant effects were observed in either of the forages treated in either of the two models used. There was only a tendency for EFE to increase the total gas production of lucerne hay (P = 0.075) and to decrease the lag time for digestion of kikuyu (P = 0.08). These results were far inferior to those obtained with the enzyme cocktail in previous studies and the conclusion was made that the partially purifed xylanase was incapable of significantly increasing *in vitro* digestion and no further studies were conducted. These findings are likely explained by the complexity in composition of forages and the specificity of enzymes. Recommendations are that for EFE to positively affect digestion, various types of enzymes need to be simultaneously added to the complex plant substrates.

Introduction

The exogenous fibrolytic enzyme cocktail (EFE) used in this study was isolated from soil in South Africa and used as an extracellular enzyme supernatant in the fresh form. This extracellular enzyme supernatant of the fungal strain, ABO 374 was characterized as a fibrolytic enzyme cocktail, containing mostly xylanase (296 ± 0.07 U/mg protein) but also cellulase (1.44 ± 0.39 U/mg protein) and mannanase (1.10 ± 0.37 U/mg protein) activity (Cruywagen and Van Zyl, 2008). The activities of these enzymes were determined using 10 g/kg birchwood xylan for β-xylanase, 10 g/kg CMC (carboxymethyl cellulose) as substrate for cellulase (endoglucanase) and 10 g/kg locust bean gum for β-mannanase activity determination. Generally, cellulolytic fungi such as ABO 374 produce large numbers of enzymes capable of the decomposition of plant cell wall polysaccharides (Wood, 1989). The three major types of cellulolytic enzymes are endoglucanase (endo-1,4-β-glucanase; hydrolyzing 1,4-β bonds on the interior of the cellulose chain), cellobiohydrolase (exo-1,4-β-glucanase; cleaves cellobiosyl units from the non-reducing ends of the cellulose chains) and β-glucosidase (cleaves glucosyl units from the non-reducing ends of cello-oligosaccharides) (Ye *et al*., 2001).

In the previous chapters of this document, fresh extracellular supernatant harvested from ABO 374 produced on wheat straw as growth substrate was used as the EFE. In this chapter, however, it was attempted to partially purify the cocktail using gel filtration chromatography to isolate the xylanase and cellulase for further studies. Porous beads were used as the chromatographic support and a glass column was packed with the beads. Such a packed column has two measurable liquid volumes, the external volume, consisting of the liquid between the beads and the internal volume, consisting of the liquid within the pores of the beads. Large proteins (larger than the molecular-mass-cut-off of the beads) are excluded from the internal volume and therefore emerge first from the column while smaller protein molecules, which can access the internal volume, emerge later (Stellwagen, 1990). Fractions or aliquots of the emerging liquid are collected and analysed for protein content. Those fractions containing protein are then further subjected to enzymatic activity determination. Royer and Nakas (1991) purified two xylanases from the fungus, *Trichoderma longibrachiatum* and indicated that the fungus generated a highly active xylanase enzyme of approximately 20 kDa and a less active enzyme of approximately 30 kDa. More complex purification schemes exist, such as the scheme used by Chen *et al*. (1997). In their experiment a xylanase from *Trichoderma longibrachiatum* was purified by subjecting the culture supernatant to a four-step purification scheme involving ultra filtration,

ammonium sulphate precipitation and cation exchange and gel filtration chromatography. They produced a homogenous purified protein that migrated as a single sharp band (M_r 18.6 kDa) on SDS-PAGE. Zymogram analysis proved that the single protein band was active on oat spelt xylan. This purification scheme gave about a 56-fold overall purification and approximately 5.1 % recovery of activity. The aim of the experiment in this chapter was to produce and characterise purified xylanase and cellulase from the extracellular enzyme supernatant from ABO 374. Fractions showing xylanase or cellulase activity were then further evaluated for their potential in improving fibre digestion using the *in vitro* gas production system as described earlier (Chapter 3).

Materials and Methods

Gel filtration chromatography

A portion (5.0 ml) of a 0.5 % (v/v) fresh enzyme cocktail from ABO 374 were prepared in 0.1 M sodium citrate (pH 5.3) and applied to a 90 x 0.75 cm glass column packed with Bio-Gel P-30 polyacrylamide gel (150-4150, Bio-Rad) and equilibrated with 0.1 M sodium citrate buffer, pH 5.3. The exclusion limit of the porous beads is listed by the manufacturer as 40,000 Daltons (nominal). The Bio-Gel P-30 gel beads were prepared according to the manufacturer's instructions. The Bio-Gel P-30 beads were hydrated for 12 h at 20^{o} C and half of the supernatant decanted. The solution containing the beads was then degassed by vacuum for 5 to 10 min and washed with degassed buffer to remove > 90 % of the fine particles before the even slurry was poured into the column and allowed to pack. Elution was at a flow rate of 5 ml/h and 2 ml fractions were continuously collected with an automatic fraction collector and analysed for the presence of protein by absorbance at 280 nm. After the absorbance data were plotted on a graph, all the fractions with absorbance within the same peak on the graph were pooled together, and named Peak 1, 2 and so forth. The pooled aliquots were then analysed for xylanase and cellulase activity and the protein concentration determined by the Bradford method (1976).

Xylanase activity

Xylanase present in the partially purified and fresh extracellular enzyme preparations were determined by measuring reducing sugars released from xylan with dinitrosalicylic acid (DNS). Xylanase was assayed using 10 g/kg (w/v) birchwood xylan (X-0502, Sigma Chemical Co.) in 50 mM sodium citrate buffer, pH 5.3 as substrate. Assay conditions were adapted from those described by Bailey and Poutanen (1989). Xylan substrate (1.8 ml) was equilibrated in a water bath at 50^{o}C for 5 min in 16 x 125 mm glass tubes. Duplicate tubes

were used for enzyme samples, enzyme blanks and substrate blank. Appropriate serial enzyme dilutions were made using the citrate buffer. After equilibration and at precise time intervals, enzyme dilutions (0.2 ml) were added to one test tube of a pair containing the xylan substrate to start the reaction. The other test tube served as an enzyme blank. The DNS solution (3.0 ml) was added to each test tube after exactly 5 minutes to stop the reaction. At this time, the enzyme dilution was added to the blank test tube and buffer to the substrate blank. The DNS contained the following (per litre): 10 g dinitrosalicylic acid, 16 g NaOH and 300 g potassium-sodium tartrate. All test tubes were removed from the water bath, mixed and capped. Samples, blanks and standards were placed in a boiling water bath for exactly 5 minutes, removed and cooled in tap water. Absorbance was read at 540 nm against the substrate blank. Net absorbance was calculated by subtracting the absorbance measured in the enzyme blank tube from that of the tube containing the enzyme. Xylose served as the standard and the relationship between absorbance and xylose concentration was determined from a standard curve using xylose at 3.0, 5.0, 10.0, 15.0 and 20.0 µmol/ml. Xylanase activity was calculated from the amount of xylose released in each sample and was expressed as international units (U) per ml or g of enzyme preparation. One xylanase U releases 1 µmole xylose per minute under the particular assay conditions.

Cellulase activity was measured in a similar fashion and was determined using 10 g/kg carboxymethyl cellulose (CMC) as substrate. This assay was adapted from the procedure described by Miller *et al.* (1960). The suitable enzyme dilution (1.0 ml in 0.05 M sodium acetate buffer, pH 4.8) was equilibrated to 50^{o} C. To this, 1.0 ml CMC substrate was added and incubated at 50^{o} C for 10 min. The reaction mixture was then removed and 3.0 ml DNS added before being boiled for exactly 5 minutes, cooled in an ice water bath and reading the absorbance of the enzyme samples and blanks at 540 nm against distilled water. The standard curve was prepared from the stock glucose solution in deionised water to contain 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml and assayed along with the enzyme samples. One carboxymethyl cellulose unit (CMC unit) of activity liberates 1 µmol of reducing sugar (expressed as glucose equivalents) in one minute under the particular assay conditions.

Protein

Protein concentration was determined by the method described by Bradford (1976). The protein sample or standard (250 µl) were added to duplicate 16 x 125 mm glass test tubes. To this 50 µl of 0.03 % desoxycholate in water and 50 µl of 20 % phosphoric acid was added and gently mixed without bubble formation and incubated at room temperature. After exactly 10 min, 650 µl distilled water and 1000 µl Coomassie brilliant blue R-250 reagent was

added. The dye reagent was prepared by diluting 1 part dye reagent concentrate (500-0006, Bio-Rad) with 4 parts distilled, deionised (DDI) water and filtered through Whatman #1 filter paper to remove particles. After gentle mixing, the absorbance was read at 595 nm using the 0 µg/ml standard as blank. The relationship between absorbance and protein concentration was determined from a standard curve using bovine serum albumin (A-7906, Sigma Chemical Co.) at concentrations of 0, 25, 50, 75 and 100 µg/ml.

Gas production

The *in vitro* gas production technique was used as a simple, rapid screening method to evaluate the partially purified enzyme for its potential to affect fibre digestion. Three glass serum vials per substrate (lucerne hay or dried kikuyu) treated either with purified EFE (containing xylanase) or distilled water (control) was used per run. The experiment was then done in triplicate (three runs) on different days under exactly the same experimental conditions. Into each bottle, 0.5 ± 0.01 g of the relevant substrate was weighed ("as is" basis) and treated with the purified EFE or distilled water 12 h prior to incubation with buffered rumen fluid. Rumen fluid was collected from cannulated Döhne-Merino wethers on a standard diet, as described in Chapter 3. Reduced buffer solution (Goering and Van Soest, 1970) was added to each vial (40ml) prior to adding strained and blended rumen fluid. The vials were sealed with rubber stoppers and crimp tops and the automatic gas pressure loggers attached with 21 gauge needles. Bottles were placed in the 39^{o} C incubator and the experiment started after 30 minutes equilibration and removal of gas pressure. Three bottles per run was also included that contained no substrate to serve as correction of gas pressure produced due to the buffered rumen fluid alone. Gas pressure was recorded every 10 minutes for a period of 48 h. After the gas pressure values were converted to volume gas produced (ml/g DM), the data were fitted to a non-linear model to determine the b (potential total gas production, ml/g DM) and c (potential gas production rate, ml/h) kinetic coefficients. A model with and without a calculation for lag phase was used and the kinetic coefficients determined using the solver function of Microsoft Excel.

The models used were:

Model 1: $$Y = b\left(1 - e^{-ct}\right)$$

Model 2: $$Y = b\left(1 - e^{-c(t-L)}\right)$$

Where: Y = gas volume at time t

b = total gas production

c = rate of gas production

t = incubation time

L = lag time

Statistical analysis

For the gas production kinetic data, the *b* and *c* (model without lag) and *b, c* and L (model with lag) kinetic values were subjected to a factorial ANOVA with the factors substrate and enzyme, using Statistica 9.0 (2009). This was done for all the non-linear parameters. If no interaction was observed, the main effects were interpreted.

Results

The results obtained here regarding the xylanase and cellulase activities of the extracellular enzyme cocktail from ABO 374 is different to those reported earlier by Cruywagen and Van Zyl (2008). The xylanase was assayed here as containing only 158.9 ± 0.32 U/mg protein and cellulase as 10.3 ± 0.21 U/mg protein. In both cases this is substantially different from the values reported for the same enzyme cocktail by Cruywagen and Van Zyl (2008) where activities of 296 ± 0.07 U/mg protein for xylanase and 1.44 ± 0.39 U/mg protein for cellulase were reported. This can be explained by the production method of the enzyme and that the fresh supernatant of different batches of fermentation on wheat straw was used here and in other studies of Goosen (2004) and Cruywagen and Van Zyl (2008). Therefore, there is a definite need for a step in the production process to ensure minimum xylanase, cellulase and mannanase activity. Also, it has to be pointed out that assay conditions were not exactly the same between the two studies.

The elution of the fresh ABO 374 enzyme cocktail as aliquots from the gel filtration column is shown in Figure 6.1. Two distinct peaks of protein were observed, dubbed Pool 1 and 2. All aliquots within the same peak were pooled and assayed for xylanase and cellulase activity. The enzymatic activity of the fresh supernatant and the two partially purified pools obtained after gel filtration chromatography is shown in Table 6.1. The purification procedure in itself resulted in the dilution of the total protein. Therefore, enzymatic activity is indicated as U/ml and as specific activity (U/mg protein) and the purification factor based solely on the change in the specific activity of the enzymes. Pool 2 had superior xylanase activity to both the original cocktail and Pool 1. It contained 8.51 times the concentration of xylanase than the

fresh enzyme cocktail. No cellulase activity was detected in this fraction, or in pool 1 and therefore no cellulase effects were evaluated for. Pool 1 also contained xylanase activity at 1.86 times the specific activity of the fresh enzyme cocktail. However, Pool 1 had a low protein content (1.34 µg/ml) and was too diluted to be used as EFE. The decision was therefore made to use Pool 2 as the sole source of enzyme for the *in vitro* gas production analysis to determine whether this enzyme would be beneficial in altering fibre digestion of kikuyu or lucerne hay. The analysis was limited to these two substrates as they were shown earlier to be positively affected by EFE treatment.

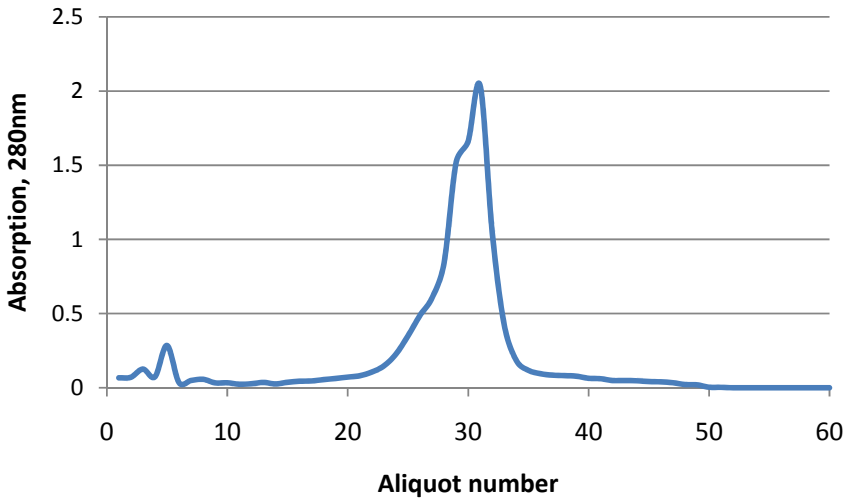

Figure 6.1. Purification of the fresh exogenous fibrolytic supernatant of ABO 374 by gel electrophoresis and the determination of the presence of protein by measuring aliquots for absorbance at 280 nm on a spectrophotometer.

Table 6.1. Purification of an extracellular enzyme cocktail from the fungal strain ABO 374 using gel filtration chromatography

Aliquot	Enzymic activity	Total protein (µg/ml)	Activity (U/ml) [a]	Specific activity[a] (U/mg)	Purification (fold)
ABO 374 supernatant	Xylanase	362.7	57.663	158.9	1.00
	Cellulase	362.7	3.728	10.3	1.00
Pool 2	Xylanase	4.5	6.086	1351.8	8.51
	Cellulase	4.5	ND	ND	ND
Pool 1	Xylanase	1.34	0.396	295.1	1.86
	Cellulase	1.34	ND	ND	ND

[a]Xylanase activity was assayed using birchwood xylan and cellulase using CMC with reducing sugar detection by the DNS method. Protein was measured by the Bradford method.

In vitro gas production results using Pool 2 as EFE treatment of lucerne hay or kikuyu is shown in Figures 6.2 and 6.3. The purified EFE did not result in any significant changes to the fermentation kinetic values for gas production as indicated in Table 6.2 (from model simulated data). There was only a tendency for the purified EFE to increase the total gas production of lucerne hay (P = 0.075) and decrease the lag time of dried kikuyu (P = 0.08). Earlier results on GP indicated that the enzyme cocktail is capable of improving the potential (total) gas production (b-value) and rate of gas production (c-value) of both kikuyu and lucerne hay and was therefore regarded as the superior enzyme treatment hence forth.

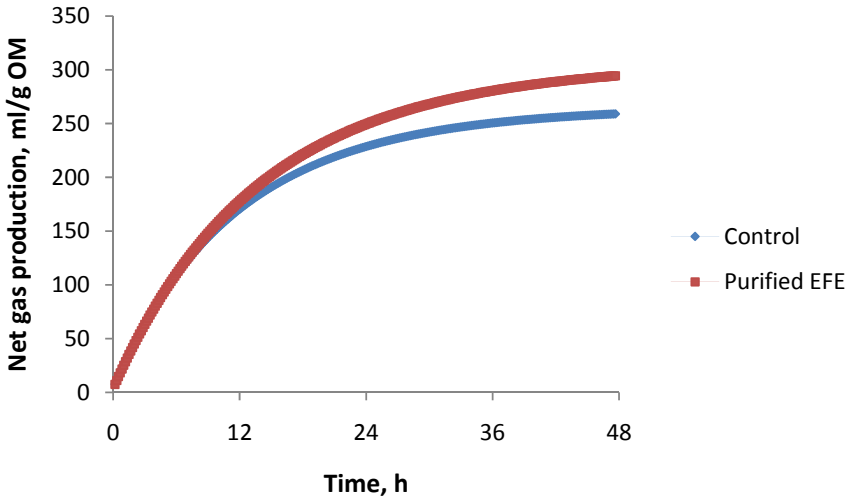

Figure 6.2. Gas production of lucerne hay treated with purified EFE (xylanase) or distilled water as control and incubated in buffered rumen fluid for a period of 48 h.

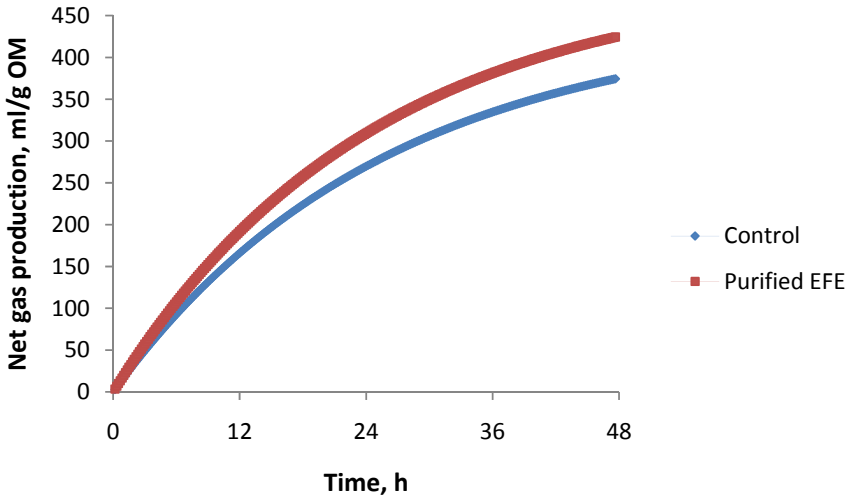

Figure 6.3. Gas production of dried kikuyu treated with purified EFE (xylanase) or distilled water as control and incubated in buffered rumen fluid for a period of 48 h.

Table 6.2. Digestion kinetics of EFE treated lucerne hay or dried kikuyu from gas production of substrates incubated in buffered rumen fluid for 48 h

Lucerne hay	**Control**	**Purified EFE**	**SEM**	***P***
		Model with lag		
b	259.09	298.44	8.09	0.075
c	0.106	0.086	0.0052	0.12
Lag	1.13	1.97	0.034	0.31
		Model without lag		
b	264.14	306.23	7.69	0.06
c	0.076	0.066	0.0027	0.12
Dried kikuyu	**Control**	**Purified EFE**	**SEM**	***P***
		Model with lag		
b	410.39	452.59	10.31	0.10
c	0.047	0.049	0.0040	0.76
Lag	1.72	0.92	0.17	0.08
		Model without lag		
b	438.43	467.83	11.91	0.22
c	0.045	0.045	0.0009	0.85

b = total gas production

c = rate of gas production

Discussion

The EFE cocktail used in the different experiments up to this point of this dissertation was characterised as a fibrolytic enzyme cocktail as it contained xylanase, cellulase and mannanase activities. In the current study however, a purification step was included to isolate the major fibrolytic enzyme, namely xylanase and determine its effect as purified enzyme on the *in vitro* digestion kinetics of two forages. Gas production data, used as a simple screening method, clearly showed that the purified enzyme was far inferior to the EFE cocktail used in the previous studies as no significant improvements in digestion kinetics were observed. With the exogenous fibrolytic enzyme cocktail used up to this point, significant effects in the rate of digestion and even the extent of digestion have been observed.

The explanation of the lack of positive effects on digestion likely lie in the complexity of the substrates tested coupled with the specificity of the enzymes for their substrate (White *et al.,*

1993). Cell wall polysaccharides are complex components and vary widely in physical properties, chemical composition and nutritional effects in ruminants (Graham *et al*., 1990). The structure of these cell wall polysaccharides can consist of more than 100 different monosaccharides of which 10 dominate quantitatively in cell walls of higher plants. These are arabinose and xylose (pentoses); glucose, galactose and mannose (hexoses); rhamnose and fucose (6-deoxy-hexoses); galacturonic, glucuronic and 4-O-methyl-glucuronic acid (uronic acids) (Aman, 1993) These monosaccharides form the building blocks of three main groups of polysaccharides, namely: cellulose, hemicellulose and pectin (Lagaert *et al*., 2009) and constitutes the largest (90%) component of primary cell walls with the remaining 10% made up of proteins. The composition varies greatly between species, especially in grasses but typical composition values are 30% cellulose, 30% hemicellulose and 35% pectin (Cosgrove, 1997). The primary cell wall in turn forms the first barrier of plants against penetration by ruminal microorganisms (Weimer, 1996).

It is therefore evident that the fibre component of forages not only forms one of the major parts of the plant, but it also characterised by great variation. As ruminant feeds contain several types of forages, enzyme feed specificity is problematic in the formulation of such diets (Graminha *et al*., 2008). For exogenous fibrolytic enzymes to exert their maximal effect on digestion, a host of different enzyme types and sources is thus needed for the successful treatment of ruminant feeds and roughages as pointed out by Beauchemin *et al*. (2003). This is in agreement with the view of other researchers such as Eun *et al.* (2006) and EFE cocktails or purified EFE should be carefully characterised regarding its substrate specificity (Bhat, 2000). The partially purified xylanase used in the current study, although containing a higher xylanase activity than the cocktail were inadequate purely because it contained xylanase as its sole fibrolytic activity.

Conclusion

The partially purified xylanase enzyme did not alter the *in vitro* digestion kinetics of kikuyu or lucerne hay and therefore not further elucidated in the following experiments. The enzyme cocktail contained various fibrolytic activities and it was thus decided to use only this cocktail in further studies. This decision was taken based on the two-fold approach suggested by Beauchemin *et al.* (2003) to:

1. use an enzyme that is relatively suitable for most forages or substrates and
2. to include a host of different enzyme types for the successful treatment of ruminant feeds.

However, the recommendation of Bhat (2000) that EFE cocktails or purified EFE should be carefully characterised regarding its substrate specificity should be considered in the further characterisation of the enzyme cocktail. The determination of the specificity and action of each of the enzymes present in the EFE cocktail is warranted. This should include a description of the molecular weights of the different enzymes in the cocktail, their optimal pH and temperature ranges in addition to its specificity towards nutrients. Additionally, any enzyme product and in particular the EFE tested here, should be tested under assay conditions closer to that of the rumen environment (39^{o}C and pH 6.8) to precisely characterise the potential of the product.

Finally, purification cannot be ruled out as a sensible step in ensuring enzyme activity levels in commercial EFE products, but was not regarded as beneficial to this study.

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CHAPTER 7

Histological evaluation of forages treated with exogenous fibrolytic enzymes in buffered rumen fluid, *in vitro*

Abstract

In the preceding chapters of this dissertation, it has been shown that there is definite potential in the use of EFE in improving the digestibility of forages. This has been indicated with *in vitro* and *in sacco* studies and results were in agreement with literature. Additionally, the literature also reports production responses *in vivo*. However, there is a need for a better understanding of the mode-of-action of EFE and therefore it forms the core objective of this experiment. Four forages, treated with EFE, were evaluated *in vitro* and at microscopic level, in an attempt to determine the effect of EFE on tissue degradation. For the histological evaluation, weeping love grass and kikuyu leaf material and lucerne and wheat straw stem material were used. Simultaneously, lucerne hay, weeping love grass hay, dried kikuyu and wheat straw were used as substrates and incubated in rumen fluid inoculated media for the determination of the effect of EFE on *in vitro* digestibility. Substrates were either pre-treated with an EFE cocktail or distilled water (Control) 12 h prior to incubation. *In vitro* digestibility (after 24 h) was determined using the ANKOM Daisy incubation system. The main focus, however, was a quantitative assessment of the degradation of the plant tissue at histological level over a 24 h period. The section to slide technique was used to mount plant tissues on microscope slides for incubation in buffered rumen fluid media. Images were acquired using the Olympus Cell R system coupled to a MT 20 illumination apparatus (Olympus Biosystems GMBH, Muenster, Germany). Degradation of cell wall components were quantified using image analysis software of the same system. *In vitro* true digestibility was higher for EFE treated lucerne and kikuyu at 24 h of incubation ($P < 0.05$). Clear histological differences were observed for all tissue types over the incubation period, but limited significant effects were observed. Cell wall of the metaxylem of both kikuyu and weeping love grass leaf material were significantly thinner for the EFE treated samples at 12 h of incubation ($P < 0.05$). There was also a significant thinning effect of EFE on the cell wall of phloem at 12 h of incubation for kikuyu as well as the adaxial epidermis at 24 h (kikuyu). The abaxial epidermis at 12 h was thinner for weeping love grass due to EFE treatment. Excluding the thinner epidermis of EFE treated lucerne (at 12 h incubation, $P < 0.05$), no further significant effects of EFE on cell wall material or total surface area of lucerne or wheat straw stem material was observed. It was concluded that image analysis can be useful to quantify changes in cell wall over an incubation period and that the addition of exogenous enzymes could be quantified by this

system. There was a definite, although subtle thinning effect of EFE on cell wall thickness of plant material which could be indicative of the mode-of-action of EFE.

Introduction

Exogenous fibrolytic enzymes (EFE) as additives in ruminant feeds have been researched worldwide. Promising effects on DMI, digestibility, feed utilization and production in especially dairy cows and feedlot cattle have been demonstrated (Beauchemin, 2003, Eun *et al*., 2007). However, research also points to varied responses of ruminants to EFE treatment of their diets. A better understanding of the mode-of-action of EFE is of importance (Colombatto *et al*., 2003) as responses vary due to many factors, including experimental conditions, dose rate of EFE, method of application and so forth (Beauchemin *et al*., 1995, 2003).

Forage heterogeneity contributes largely to the variation in degradability of plant material even with similar chemical composition but varied anatomy. This heterogeneity originates from the distribution of cell wall material between plant cell types, amongst other (Travis *et al.,* 1997). It is important therefore to recognize the botanical characteristics of plant material as a factor determining its nutritional value (Walters, 1971). As early as 1973, McManus and Bigham stated that the distribution pattern of lignin for instance rather than the total amount of lignin can mask the potentially digestible cell walls of forages (Travis *et al.*, 1997). Marked interactions exist between the cell wall thickness, lignification and other anatomical characteristics of forages and their digestibility (Wilson, 1993). Therefore investigations on the anatomical build-up of forages are of importance in determining the digestibility potential of forages.

Akin (1982) reported a method termed the “Section to slide” technique where forage tissues can be anatomically analysed. In addition, this method can be adapted to digest forage samples *in vitro* in rumen fluid and the samples rapidly evaluated by light microscopy for digestibility. The use of this technique showed normal digestion patterns of the various forages studied (orchard grass and Bermuda grass), with mesophyll being degraded rapidly and prior to other tissues. The method is propagated as a simple and rapid technique for studying large numbers of tissue samples and to study effects of various treatments on forage anatomy and digestibility thereof. Furthermore, to quantify the results, software that allows for cross section area measurements and the determination of cell wall thickness can be employed. Today, precise software exists and automated image analysis techniques,

such as applied in the plant sciences (Kolukisaoglu and Thurow, 2010), can be adapted for use in investigating the degradation of plant material by microorganisms. Exciting technology, such as laser-assisted microdissection (LAM), can also be applied to isolate specific microscopic regions from tissue cross sections. Specific tissues, for instance the epidermis, or even organelles from cross-sectioned forage plant material can be isolated and studied in great detail (Day *et al*., 2005). These new and powerful technologies should be investigated further for future studies on the degradation of plant material and how treatments, such as EFE, can alter the plant material.

One of the limitations of Akin's technique, however, is the thick sections (50-100um) needed to maintain the structural integrity of the tissue during degradation. These thick sections result in poor images with conventional light microscopy images (Travis *et al.,* 1997). Confocal laser scanning microscopy (CLSM) offers an alternative method for estimating the amount of cell wall material present in tissue sections of forages before and after digestion and visualization of the tissue using three dimentional image reconstruction (Travis *et al.,* 1997). However, in the current study the decision was made to use the "section to slide" technique but to limit tissue section thickness to 20 µm.

Scanning electron microscopy (SEM) offers another option in visualizing the three-dimensional surfaces of plant sections (Grenet, 1989), but is regrettably flawed in that it is difficult to obtain quantitative information from such images (Travis *et al*., 1997).

Limited research is available on the effect of exogenous enzymes on forage tissue at the histological level, although some research findings points to the effect of EFE at the cellular level. Recently, it has been reported that EFE alters the fibre structure of plant material (Giraldo *et al.,* 2008). Indeed, Senthilkumar *et al.* (2007) reports that the solubility effect of EFE on feeds is likely related to the removal of structural barriers (cell wall) of digestion. Similarly, Krause *et al.* (1998) states that enzymes applied to feed result in the partial solubilisation of cell wall components, thereby making more nutrients available for ruminal digestion (Hristov *et al.,* 1998).

Therefore, the objectives of this study was to determine the effect of EFE on *in vitro* digestibility of kikuyu (*Pennisetum clandestinum)* hay, weeping love grass (*Eragrostis curvula)* hay, lucerne (*Medicago sativa)* hay and wheat (*Triticum spp.*) straw and to quantitatively assess the degradation of the plant tissue at the histological level when treated with EFE and incubated *in vitro* in buffered rumen fluid. The effect of EFE on the *in vitro* digestibility of these forages was also determined.

Materials and Methods

For the histological study, leaf material from freshly cut weeping love grass and kikuyu and stem material from freshly cut lucerne and wheat straw were collected and prepared for sectioning by infusion of tissue with tissue freezing medium (Cryo-M-Bed, embedding compound, Bright Instrument Company Limited, Huntingdon, England) prior to cryo-freezing in liquid nitrogen. Infusion of the tissue involved a step-wise protocol to prevent freeze damage to cell structures. First, the collected leaf (cut from 3-week regrowth of kikuyu or weeping love grass) and stem (3-week regrowth of lucerne or baled wheat straw) were placed in a 5% (w/v) sucrose solution in phosphate buffered saline (PBS) at 4^{o}C for 3 to 4 h. Material was transferred to a 20% (w/v) sucrose solution for an overnight infusion period (4^{o} C) and then finally in a 50:50 mixture of 20 % sucrose and tissue freezing medium for another 24 h at 4^{o} C. The leaf or stem material was then frozen in liquid nitrogen and stored at -4^{o} C until sectioning.

Cross sections (20 µm) were made on a cryostat and fixed to microscope slides (Lasec™) by means of clear double-sided tape, as described by Akin (1982). Specimens were sectioned in a cryostat (Leica, CM1100) set at -23° C after an equilibration time of the specimens in the chamber for 5 minutes. After each section was made, numerically labelled and fixed to the slide, the following section was labelled with the same number followed by an x and fixed to another slide, indicating that samples were collected adjacent to each other. This protocol was followed to allow for comparison between EFE or distilled water treated specimens. Prepared slides were stored overnight at 4^{o} C in closed Coplin jars to prevent the material from drying out. Each slide contained three sections of the same forage and for experimental purposes two slides were incubated back to back in the large glass test tubes. Two test tubes were allowed per incubation time and per treatment (EFE or Control), and the whole experiment was duplicated. Incubation times were 0 h (no incubation to set base values for tissues), 6 h, 12 h and 24 h in buffered rumen fluid.

The EFE were prepared in the same manner as for the *in vitro* digestibility study and consisted of diluting the ABO 374 supernatant (1 ml) in 200 ml distilled water and applying 1 ml to each slide in large glass test tubes (50ml). Slides were pre-treated with EFE or distilled water for 12 h in glass tubes sealed with rubber stoppers prior to incubation in buffered rumen fluid. To this, 40 ml buffer (pH 6.8) and 10 ml filtered and blended rumen fluid was added under strict anaerobic conditions. The tubes were sealed with rubber stoppers under CO_2 gas phase with one way valves to release the build-up of gas and incubated in a

shaking water bath at 39° C. After the appropriate incubation period, slides (two per treatment) were randomly removed from the test tubes, rinsed in ice cold water and stained with Toluidine blue for 5 minutes, rinsed and covered with a cover slip to prevent the sample from drying out. Samples were observed on an Olympus Cell^R system attached to an IX-81 inverted microscope equipped with a F-view-II cooled CCD camera (Soft Imaging Systems). Images were acquired by using Olympus 40x (leaf material) and 4x objectives (stem material) and the Cell^R imaging software. Images were analysed using the Cell^R software (Olympus Biosystems GMBH, Muenster, Germany).

In vitro digestibility was evaluated according to the method for "*In vitro* true digestibility using the DAISY incubator" as described by ANKOM Technology, Fairport, NY. The F57 filter bags containing 0.5 g substrate were incubated for 24 h in rumen fluid inoculum with buffer (Goering and Van Soest, 1970) at a ratio of 1:4. The supernatant of ABO 374 was used as EFE in this study and 40 ml of a 1 in 200 dilution added to the two treatment incubation jars 12 h prior to incubation with buffered rumen fluid. Two jars served as control with 40 ml distilled water added instead of the EFE. Bags were duplicated in each bottle (2x2 replications) and the experiment duplicated along with the histological study. After the 24 h incubation period the bags were removed and *in vitro* true digestibility (IVTD) determined according to the ANKOM procedure.

Rumen fluid was collected from two cannulated adult Döhne-Merino wethers fed a lucerne-oat hay based diet supplemented daily with 100 g concentrate, as described in Chapter 3. Collection was in the morning after the 07:00 feeding and fluid strained through two layers of cheese cloth into a pre-warmed thermos flask and sealed. In the laboratory, the rumen fluid was blended for 2 minutes prior to being added to the incubation jars (DAISY jars or large test tubes containing the "section to slide" microscope slides) in a ratio of one part rumen fluid to four parts buffer. The buffer (pH 6.8, 39° C) was prepared according to the method of Goering and Van Soest, (1970), with slight modifications. Anaerobic principles were adhered to and bottles were sealed under CO_2 gas phase and placed in the 39° C incubator. Four forage types, as described above, pre-treated (12 h prior to incubation) with the EFE cocktail or distilled water were used as incubation substrates for both the determination of *in vitro* digestibility and tissue degradation.

Statistical analysis

The *in vitro* digestibility data were subjected to a factorial ANOVA, using Statistica 8.1 (2008). Significant forage x treatment interactions was detected and data pertaining to the respective forages were further subjected to a one way ANOVA. Histology data were

analyzed with either a Bonferroni or Newman-Keuls multifactorial test where significant interactions were observed. Main effects were otherwise interpreted. Significance was declared at $P < 0.05$.

Results

The identification of the various tissues measured for cell wall thickness (CWT) and/or surface area is indicated in Figure 7.1 and 7.2. Labelling of the tissues was done with the assistance of a botanist (Dr B. Marais, personal consultation, Stellenbosch University) and according to the description given by Wilson (1993). The digestion process can easily be observed subjectively from Figures 7.4 to 7.6. These figures indicate the multiple measurements made using imaging software and shows examples of EFE or control specimens at different incubation times.

Rapid disappearance of mesophyll, phloem and xylem and the remainder of sclerenchyma and other lignified vascular tissue was noted after 24 h for kikuyu (or 12 h for weeping love grass) incubation in buffered rumen fluid, regardless of treatment. Unfortunately, due to the thickness of the sections, weeping love grass and wheat straw material did not stay attached to the double-sided tape on the slide for periods longer than 12 h incubation periods in rumen fluid. Few samples of weeping love grass and wheat straw remained thereafter and the decision was taken not to include the 24 h data in this report due to the limited number of samples available for statistical analysis.

For the histological data, the factorial ANOVA showed significant interactions of treatment with substrate ($P = 0.033$). Subsequently, a one way ANOVA was done on each substrate at the incubation times. The cell wall thickness- and surface area measurements of kikuyu and weeping love grass of the various tissues studied is given in Table 7.1. The adaxial epidermis thickness (kikuyu after 24 h) and abaxial epidermis thickness (weeping love grass after 12 h) was thinner due to EFE treatment and incubation in buffered rumen fluid ($P < 0.05$). Metaxylem cell wall thickness was decreased due to EFE treatment for both kikuyu and weeping love grass at 12 h of incubation. Phloem cell wall thickness was only thinner for kikuyu after 12 h incubation ($P < 0.05$). Metaxylem expressed as a percentage of the unincubated (0 h) kikuyu specimen was also decreased due to EFE treatment ($P < 0.05$). For the stem material investigated (Table 7.2), the only significant observation ($P < 0.05$) was a decrease in the epidermal cell wall thickness of lucerne at 12 h of incubation in buffered rumen fluid with EFE.

Based on the numerous measurements made and reported in Table 7.1 and 7.2, relatively few significant findings can be reported. However, overall there was a tendency towards a subtle thinning of cell wall thickness of the various tissues studied. This is based on the calculation of the percentage reduction in cell wall thickness from the unincubated specimens (0 h) after the appropriate incubation times with EFE compared to the control treatment. No significant differences were however observed other than where the cell wall thickness was affected by the EFE treatment as reported in the previous paragraph.

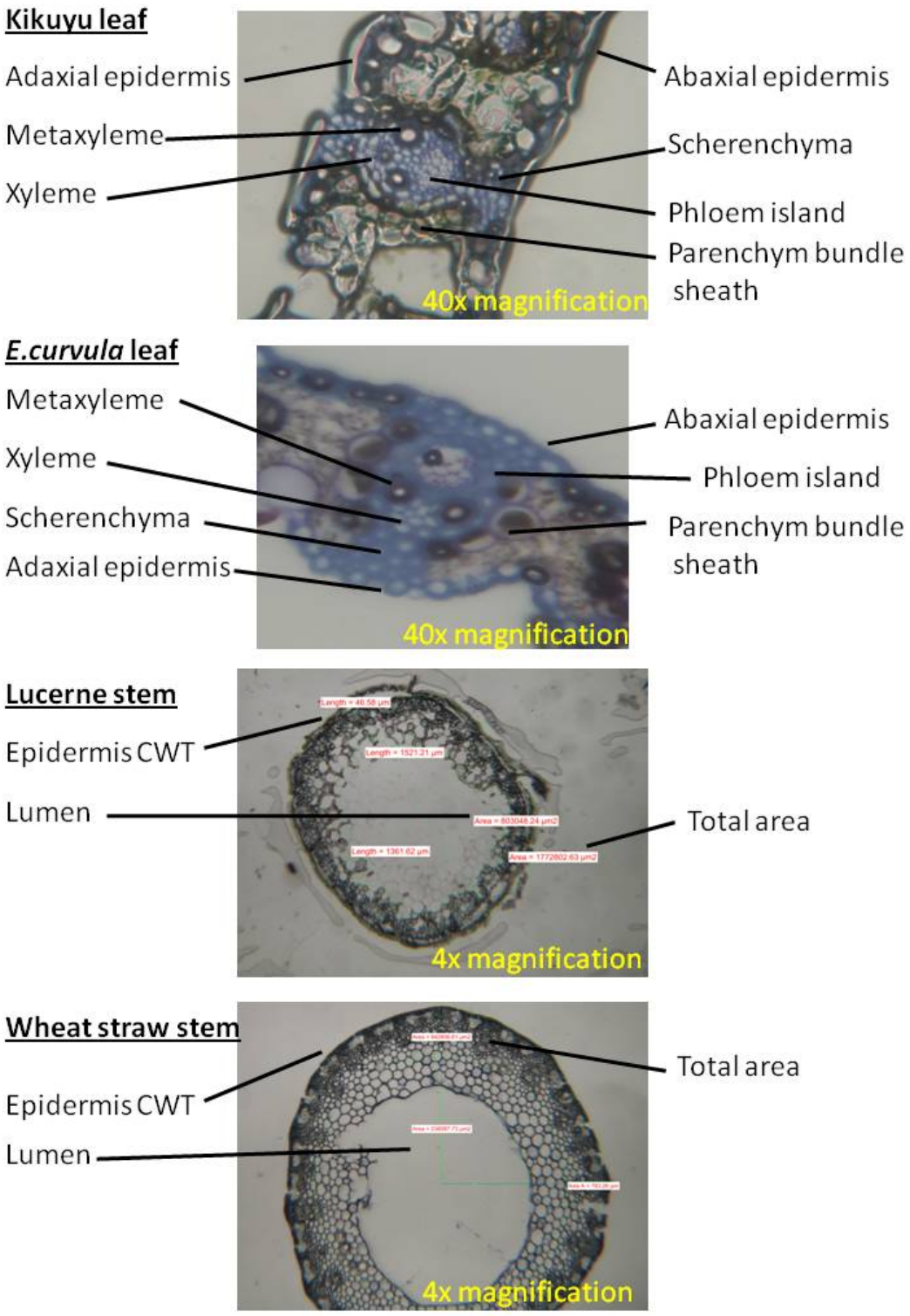

Figure 7.1. Histology of kikuyu and weeping love grass leaf material and lucerne and wheat straw stem material determined under 40 x or 4 x magnification lenses of a conventional light microscope (Olympus) of undigested material.

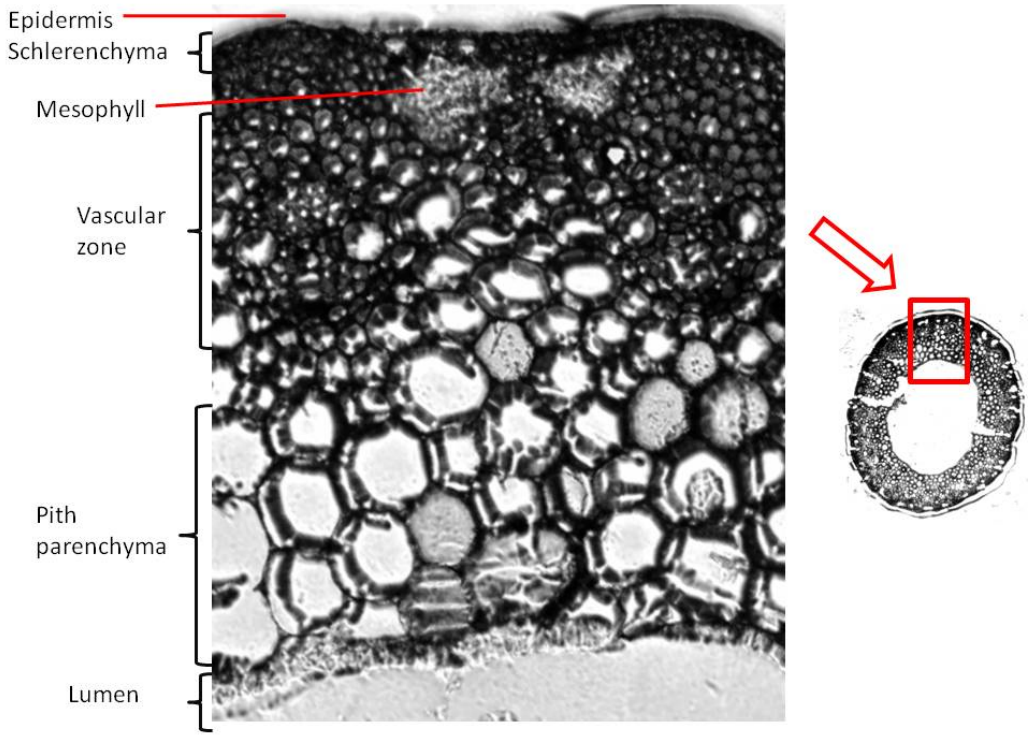

Figure 7.2. Anatomy of wheat straw stem material (adapted from Wilson, 1993).

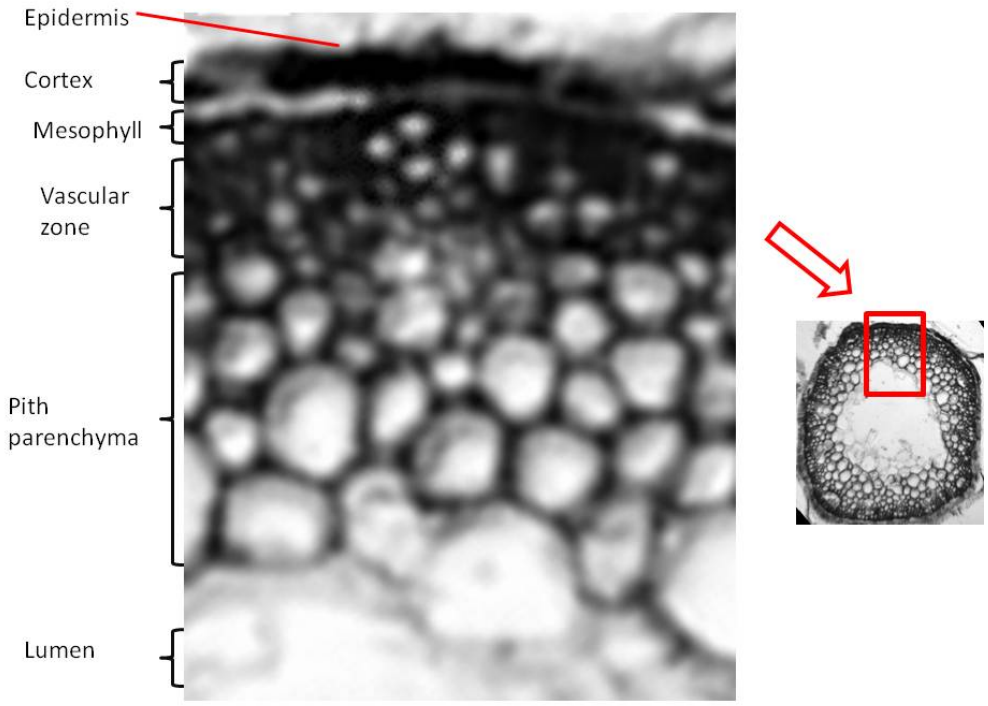

Figure 7.3. Anatomy of lucerne stem material (adapted from Wilson, 1993).

Table 7.1. Cell wall thickness (CWT) of EFE-treated kikuyu or weeping love grass tissues after *in vitro* digestion in buffered rumen fluid

Treatment and incubation time	Ad. Epi. CWT, µm	% Red. in CWT from 0h	Ab. Epi. CWT, µm	% Red. in CWT from 0h	Xyl. CWT, µm	% Red. in CWT from 0h	Metaxyl. CWT, µm	% Red. in CWT from 0h	Phl. CWT, µm	% Red. in CWT from 0h	Xyl. surface area, µm^2	% Red. in AOI from 0h	Metaxyl. surface area, µm^2	% Red. in AOI from 0h	Phl. surface area, µm^2	% Red. in AOI from 0h
Kikuyu cell wall thickness													**Surface area measurement**			
Cnt 0h	1.4 ± 0.05	0	1.6 ± 0.07	0	1.5 ± 0.13	0.00	1.5 ± 0.17	0.00	1.4 ± 0.20	0	271.3 ± 29.52	0.00	306.0 ± 40.49	0.00	671.3 ± 78.35	0.00
Cnt 6h	1.4 ± 0.07	-1.53	1.7 ± 0.01	-5.64	1.8 ± 0.29	-22.70	1.3 ± 0.09	11.66	1.3 ± 0.10	12.7	292.4 ± 27.03	-7.79	372.8 ± 61.23	-21.84	448.6 ± 46.82	33.17
EFE 6h	1.3 ± 0.35	6.95	1.5 ± 0.01	8.5	1.4 ± 0.25	5.38	1.4 ± 0.13	7.66	1.3 ± 0.08	6.2	214.8 ± 54.72	20.80	364.6 ± 57.09	-19.16	439.7 ± 20.00	34.50
Cnt 12h	1.3 ± 0.11	7.82	1.6 ± 0.14	-3.47	1.8 ± 0.144	-17.25	**1.4^a** ± 0.05	4.96	**1.4^a** ± 0.09	1.3	188.7 ± 10.11	30.43	240.9 ± 65.06	21.24	438.9 ± 59.09	34.63
EFE 12h	1.3 ± 0.02	6.92	1.7 ± 0.00	-7.14	1.3 ± 0.053	14.62	**1.0^b** ± 0.01	30.69	**1.1^b** ± 0.20	21.6	189.2 ± 17.73	30.24	232.1 ± 63.55	24.16	436.2 ± 66.42	35.02
Cnt 24h	**1.4^a ± 0.09**	5.75	1.4 ± 0.15	13.58	1.4 ± 0.16	8.77	1.2 ± 0.10	21.12	1.0 ± 0.08	29.7	263.5 ± 38.35	2.85	**288.4^a ± 92.85**	5.75	442.9 ± 59.74	34.02
EFE 24h	**1.1^b ± 0.01**	22.4	1.3 ± 0.01	16.44	1.2 ± 0.17	21.68	1.1 ± 0.10	29.20	1.0 ± 0.20	28.7	237.6 ± 78.05	12.42	**176.9^b ± 20.09**	42.17	449.3 ± 48.93	33.08
Weeping love grass cell wall thickness													**Surface area measurement**			
Cnt 0h	1.6 ± 0.16	0	1.8 ± 0.29	0	ND	ND	1.1 ± 0.09	0.00	1.2 ± 0.00	0	472.7 ± 139.51	0.00	249.4 ± 9.10	0.00	416.1 ± 71.73	0.00
Cnt 12h	1.4 ± 0.04	9.9	**1.9^a ± 0.33**	-7.3	ND	ND	**1.1^a** ± 0.01	2.00	1.1 ± 0.26	4.1	444.3 ± 4.30	6.00	241.1 ± 39. 19	3.31	452.1 ± 36.57	-8.65
EFE 12h	1.5 ± 0.00	4.3	**1.4^b ± 0.50**	22.3	ND	ND	**0.7^b** ± 0.00	37.37	0.9 ± 0.16	16.9	292.9 ± 2.90	38.03	243.9 ± 22.87	2.20	484.5 ± 3.67	-16.42

Ad. = adaxial, Ab. – abaxial, Epi. = epidermis, CWT = cell wall thickness, Xyl. = xyleme, Metaxyl. = metaxyleme, Phl. = phloem, AOI = area of interest.

Values with different superscripts differed significantly ($P < 0.05$)

Table 7.2. Cell wall thickness (CWT) and surface area measurements of EFE-treated lucerne and wheat straw tissues after *in vitro* digestion in buffered rumen fluid

Cross sections of stems	Treatment and incubation time	Epidermis CWT, µm	% Reduction in CWT from 0h	Inner mambrane CWT, µm	% Reduction in CWT from 0h	Material surface area as % of Total	% Reduction in material surface area from 0h
Cell wall thickness						Surface area measurement	
Lucerne	Cnt 0h	13.0 ± 0.48	0	7.6 ± 0.25	0.00	66.6 ± 2.41	0
	Cnt 6h	9.2 ± 0.83	28.9	7.6 ± 1.35	0.71	52.1 ± 6.98	21.8
	EFE 6h	9.4 ± 0.43	27.9	6.8 ± 0.19	11.11	47.4 ± 1.19	28.9
	Cnt 12h	**8.8 [a] ± 0.46**	32.5	7.4 ± 0.19	2.82	29.9 ± 3.27	55.1
	EFE 12h	**7.4[b] ± 0.59**	43.1	6.8 ± 0.21	10.87	30.9 ± 1.79	53.6
	Cnt 24h	8.5 ± 0.53	34.7	6.5 ± 0.99	14.37	23.7 ± 0.84	64.4
	EFE 24h	7.9 ± 0.17	39.5	6.5 ± 0.34	14.71	23.7 ± 2.17	64.4
Wheat straw	Cnt 0h	13.0 ± 1.22	0	11.9 ± 1.20	0.00	70.9 ± 0.72	0
	Cnt 12h	9.3 ± 0.70	28.8	8.7 ± 0.78	26.59	66.7 ± 1.38	5.9
	EFE 12h	9.1 ± 0.91	29.9	6.3 ± 0.99	47.23	66.7 ± 0.90	6
	Cnt 12h			ND	ND	57.9 ± 0.21	18.3
	EFE 12h			ND	ND	58.2 ± 1.66	18

CWT = cell wall thickness. Values with different superscripts differed significantly ($P < 0.05$)

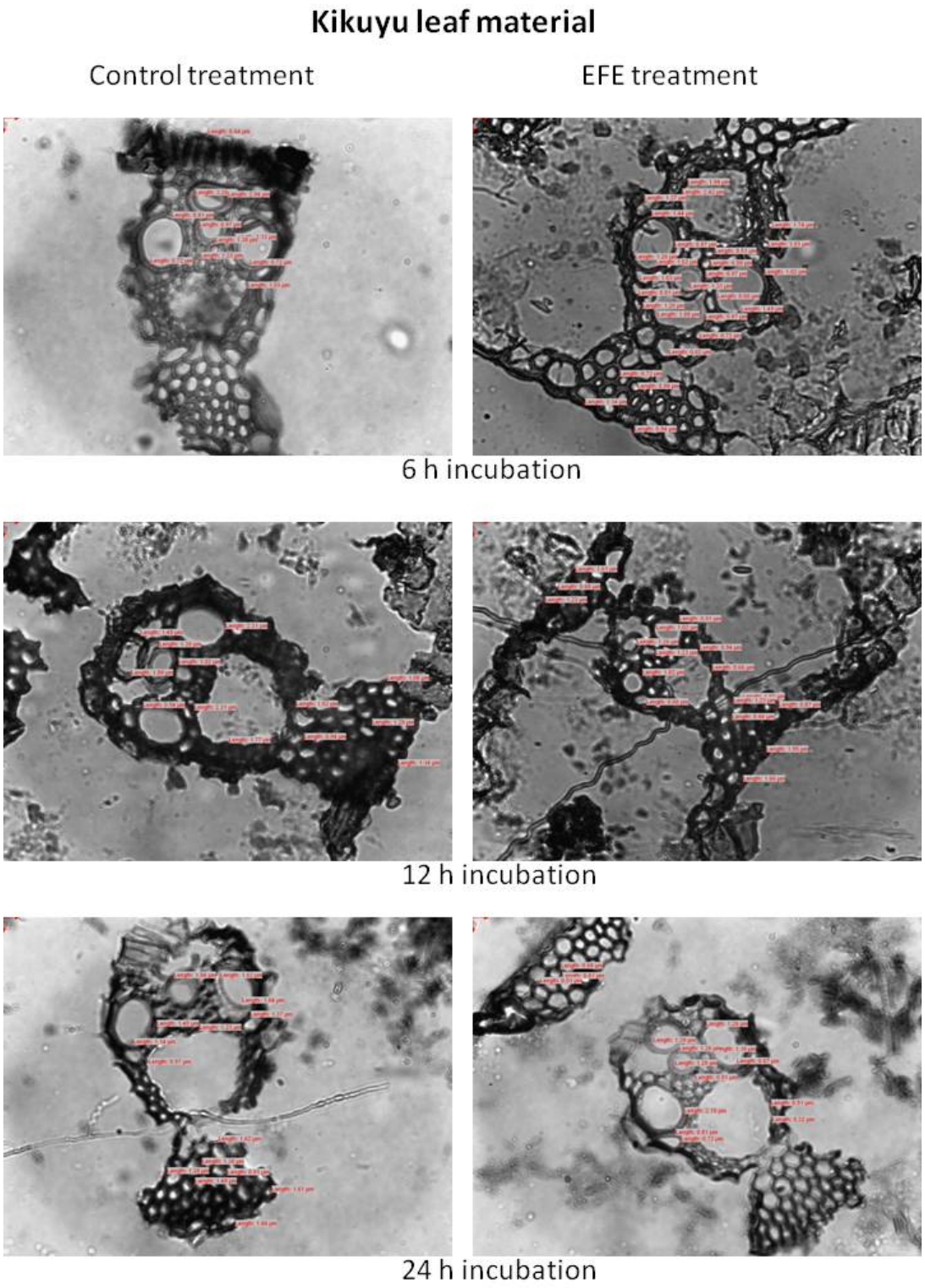

Figure 7.4. Degradation of kikuyu leaf material treated with EFE or dH2O after 6, 12 or 24 h incubation in buffered rumen fluid. The cell wall thickness and tissue surface area was determined using Cell^{R} imaging software (Soft Imaging Systems).

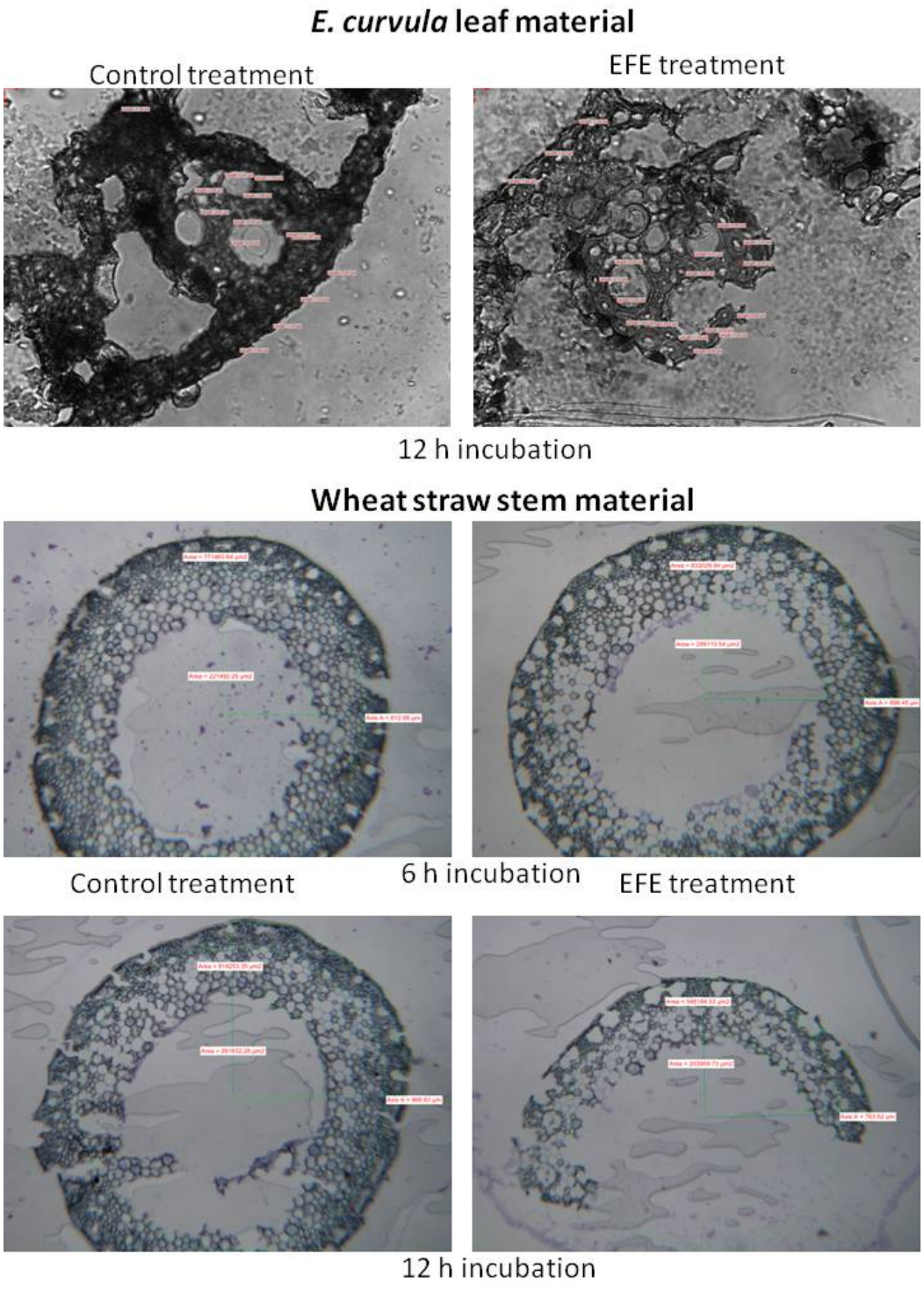

Figure 7.5. Degradation of weeping love grass leaf material after 12 h or wheat straw stem material after 6 or 12 h of incubation in buffered rumen fluid. Specimens were treated with EFE or dH2O and the cell wall thickness and tissue surface area determined using Cell^{R} imaging software (Soft Imaging Systems).

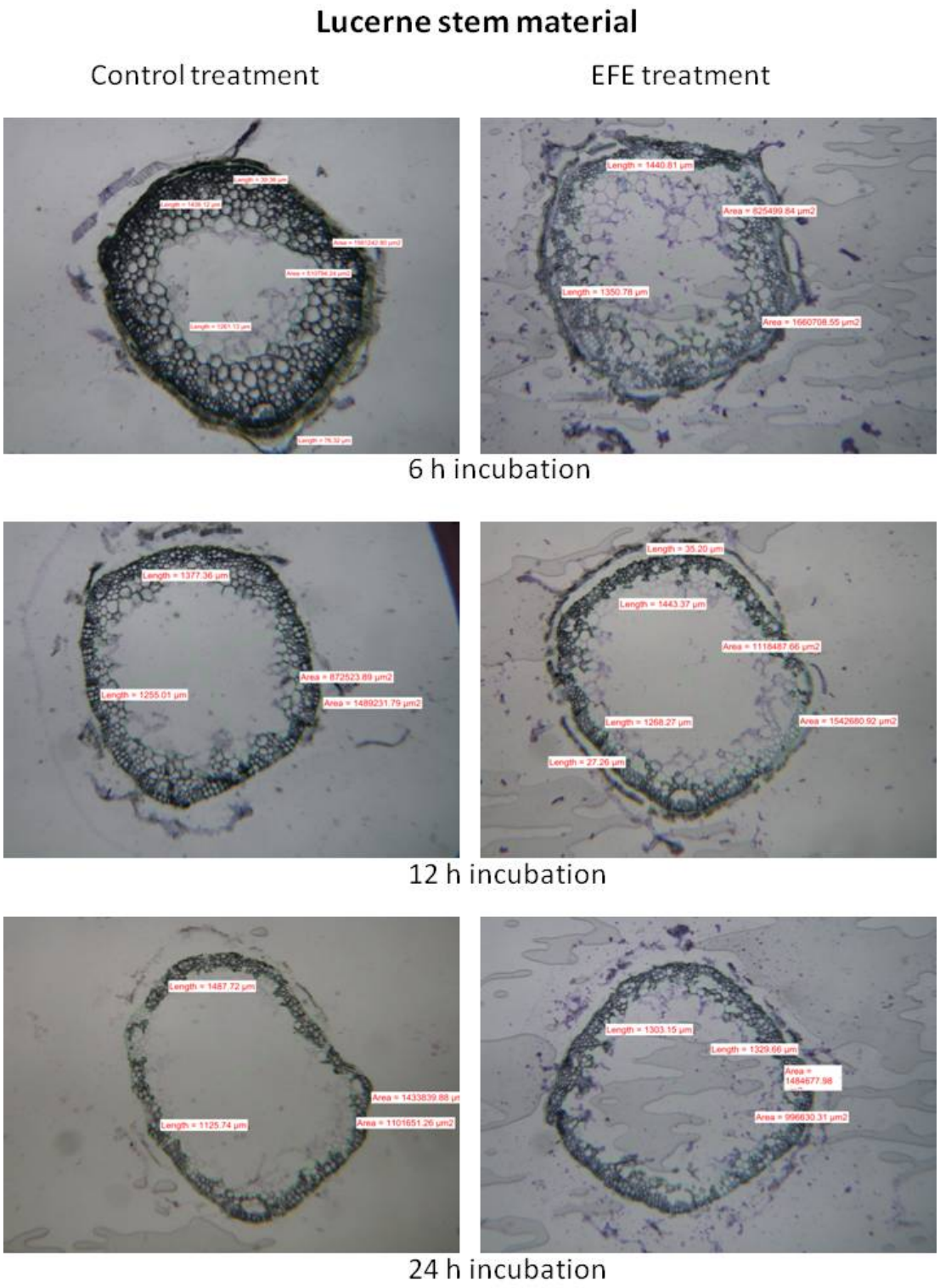

Figure 7.6. Degradation of lucerne stem material after 6, 12 or 12 h of incubation in buffered rumen fluid. Specimens were treated with EFE or dH2O and the cell wall thickness and tissue surface area determined using Cell^{R} imaging software (Soft Imaging Systems).

The *in vitro* digestibility values (after 24 h of incubation) of EFE treated weeping love grass, dried kikuyu, lucerne hay and wheat straw is given in Table 7.3 and graphically represented by Figure 7.7.

Table 7.3. *In vitro* digestibility of forages treated with EFE or distilled water and incubated in buffered rumen fluid for 24 h

IVTD, %	EFE treatment	Control	SEM	Significance, *P*
Weeping love grass hay	42.3	41.5	0.695	0.486
Dried Kikuyu	65.4[a]	61.9[b]	0.655	0.004
Lucerne hay	77.0[a]	74.7[b]	0.488	0.008
Wheat straw	42.9	44.3	1.200	0.411

Values within rows with differing superscripts differed significantly ($P < 0.05$)

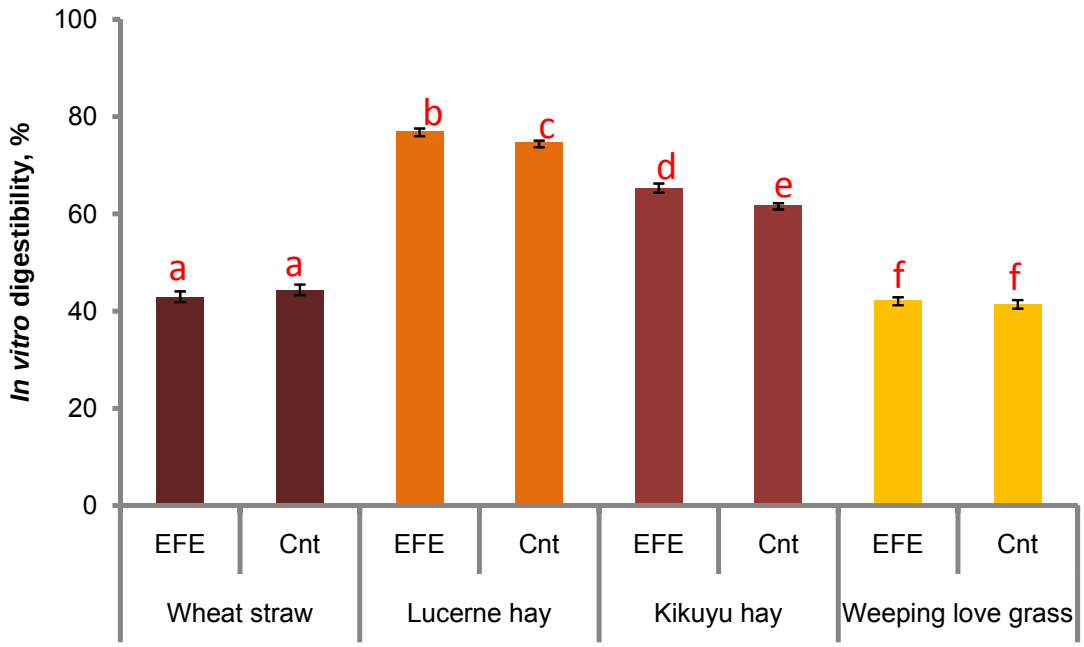

Figure 7.7. *In vitro* digestibility of forages treated with EFE or distilled water and incubated in buffered rumen fluid for 24 h. Error bars represent the SEM. Different superscripts depicted at the bars indicate significance at $P < 0.05$.

Exogenous fibrolytic enzyme treatment of wheat straw and weeping love grass was not effective in improving the *in vitro* digestibility of these relatively poor quality roughages. Lucerne hay and kikuyu hay both however showed increased *in vitro* digestibility after 24 h incubation in buffered rumen fluid due to EFE treatment.

Discussion

No evidence could be found that the methodology followed in this study has been applied before in the evaluation of the effect of EFE on tissue degradation of forages at histological level. Direct comparisons with literature therefore are limited. However, many researchers have reported the effect of EFE on cell wall structures as part of the mode-of-action of enzymes in altering the digestibility of forages and feeds and will be discussed later on.

Valuable information has, however, been gained on the degradation of forage tissue regardless of treatment and will be briefly discussed first. Using image analysis software, the degradation pattern of the different tissues studied can be followed. As is evident from the quantitative data and by subjective evaluation of the microscope slides, it is clear that the non-lignified tissues such as mesophyll, xylem and phloem were rapidly degraded. Highly lignified tissue such as sclerenchyma and structural tissues such as pith parenchyma and epidermal tissue were far from being completely digested after 24 h. These observations were in close agreement with other researchers such as Buxton and Readfearn (1997) who summarized the digestibility of various plant tissues (Table 7.4). Indeed, Wilson (1993) states that mesophyll cell walls are not lignified and are rapidly digestible (Akin, 1989), with complete digestion in under 12 h (Chesson *et al*., 1986). Along with mesophyll, phloem was also digested before the other tissues.

Extensive digestion of tissues was observed after relatively short periods of incubation (17h), with mostly indigestible tissue (lignified vascular tissue) remaining after 24 h. These findings on *in vitro* tissue disappearance are in agreement with other more recent research articles. Horn *et al.* (1989) listed the susceptibility of leaf tissue of wheat forage at various stages of maturity as mesophyll and parenchyma bundle sheath > phloem > epidermis > schlerenchyma > lignified vascular tissue. For stem material, small losses of phloem, cortex and parenchyma tissue were reported with cortex being the most susceptible to degradation in rumen fluid.

Interestingly, the susceptibility of the various tissues to ruminal degradation was found to be similar regardless the stage of maturity of the wheat forage. The total cell wall content of forages increases with maturity (Morris, 1984) and the stage of maturity is a major factor in the nutritive value of plants. However, it appears that it is the extent of digestion more than the type of tissue degraded that is influenced by the stage of maturity of leaf material. For this study, care was taken to collect specimens of similar maturity to minimise any potential maturity effects.

Table 7.4. Summary of plant tissues and their relative digestibility (from Buxton & Readfearn, 1997)

Tissue	Function	Digestibility	Comments
Mesophyll	Contain chloroplasts	High	Thin wall, no lignin. Loosely arranged in legumes and C3 grasses.
Parenchyma	Metabolic	Moderate to high	In midrib of grass and main vein of legume leaves, leaf sheath, and stem of grasses, and petiole and stem of legumes. Highly digestible when immature.
Collenchyma	Structural	Moderate to high	In legume leaves and stems. Thick wall, not lignified.
Parenchyma bundle sheath	Contain chloroplasts	Moderate to high	Surrounds vascular tissue in C4 leaf blades. Wall moderately thick and weakly lignified.
Phloem fibre	Structural	Moderate	In legume petioles and stems. Often does not lignify.
Epidermis	Dermal	Low to high	Outer wall thickened, lignified, and covered with cuticle and waxy layer.
Vascular tissue	Vascular	None to moderate	Comprises phloem and xylem. Major contributor to indigestible fraction.
Sclerenchyma	Structural	None to low	Up to 1200 mm long and 5–20 mm in diameter, thick, lignified wall.

The focus of this study was however to evaluate and quantify the effect of EFE on cell wall structures. It was hypothesised that EFE can result in the thinning of cell wall structures, thereby allowing earlier access of microbes to the cell contents. This hypothesis is strongly based on findings from several research groups. Senthilkumar *et al*. (2007) reported that EFE improved *in vitro* gas production as well as stimulating microbial production. They related their findings on the solubility effect of EFE on feeds to the removal of structural barriers of digestion, thereby releasing more nutrients to support the production of the

bacterial glycocalyx, which improves the colonization of plant cell walls and the activity of rumen microbes. This is in agreement with Hristov *et al.* (1996) who reports that exogenous enzymes applied to feed can randomly release reducing sugars and possibly make more nutrients available. Krause *et al.* (1998) also reported that enzymes applied to feed result in the partial solubilisation of cell wall components, thereby making more nutrients available for ruminal digestion. Similarly, Giraldo *et al.* (2008) reported that enzymes can alter the fibre structure of plant material and indeed concluded earlier that EFE stimulated the initial phase of microbial colonization (Giraldo *et al.,* 2007), in agreement with the above researchers. The effect of the exogenous enzyme might also be exerted prior to incubation with rumen fluid, during the pre-treatment interaction time as indeed was observed with lucerne fractions becoming more amenable to degradation by rumen microorganisms (Nsereko *et al*., 2000).

In this study, the cell wall thickness of the different tissues were studied and as reported earlier, subtle yet significant thinning effects were observed for both adaxial and abaxial epidermis of kikuyu and weeping love grass and the epidermis and cortex of lucerne due to EFE treatment. EFE treatment also had a thinning effect on the metaxyleme cell wall of kikuyu and weeping love grass, as well as the phloem cell wall of kikuyu ($P < 0.05$). This supports the hypothesis that EFE subtly erode cell wall structures allowing ruminal microbes to obtain earlier access to fermentable substrate during the initial phase of digestion (Colombatto *et al*., 2003). Should the cell wall structures be weakened or altered in such a way as to allow earlier access of microbes to the cell contents as proposed here, it follows that the digestibility should be positively influenced. This was indeed the case in this study (and in previous chapters) wherein it was observed that the 24 h *in vitro* digestibility of kikuyu and lucerne was increased.

This experiment was, however, conducted over a relatively short 24h incubation period due to limitations in measuring histological effects after longer incubation periods. However, from the literature it has been reported that enzymes degrade material in the rumen that would have been degraded in the rumen anyway, only at a later time (Colombatto *et al.,* 2007) and this would have been interesting to determine at the histological level by allowing for longer incubation periods. The implication of such a finding is that enzymes exert their multiple effects at a different level than by supplementing limiting enzymatic activity in the rumen. One of the limitations of the section to slide technique is that it is difficult to obtain suitable specimens after longer incubation periods, more so with specimens of younger maturity (Unknown author, from: “12. Fibers, fibre products and forage fibre”). Additionally, when sectioning, the cell walls aren’t necessarily cut at a straight angle; thereby over-estimating the cell wall thickness upon measurement (Boon *et al.,* 2005). This is a likely

explanation for the large variation found in our dataset. Regardless of these limitations, the section to slide technique proved to be a valuable tool to evaluate EFE effects at histological level of various forages.

In this study, no attempt was made to quantify the level of lignification of the various structures examined as the EFE tested was not evaluated for enzymatic activity capable of digesting lignin. In fact, identifying such an enzyme remains one of the biggest challenges in truly affecting fibre digestion. As a future prospect, more precise and differential staining techniques can be investigated to determine the binding of EFE to plant tissue at histological level.

Conclusion

Results from this study suggest that enzymes have a thinning effect on the cell wall components of plant material which was substantiated by the improved *in vitro* digestibility of EFE treated samples. Although limited significant effects were reported in terms of the cell wall thinning of the various tissues studied, the key to the observations were that subtle effects were indeed observed. This is in strong agreement with literature where effects on cell wall digestion have been proposed by several researchers as a contributing factor to the mode-of-action of EFE. It was concluded that EFE altered fibre cell wall structures, thereby positively influencing fibre digestion and fibre components.

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CHAPTER 8

General Conclusion and future prospects

Exogenous fibrolytic enzymes (EFE) as additives in ruminant feeds is a topic being researched worldwide. Enzymes are included in animal diets to improve feed utilization and many positive effects have been reported and are often related to improvements in DMI and increased digestibility of feeds and nutrients. Improved feed utilization and production responses, in dairy cows especially and feedlot cattle, but also in sheep and goats, have been reported. The literature available, reporting on such effects, serve as evidence that EFE can indeed be included in ruminant diets to improve feed digestibility.

However, research also points to varied responses of ruminants to EFE treatment of their diets. The inconsistencies in research findings necessitate investigations into establishing how EFE exert its effects. The first part of this study therefore focussed on establishing how EFE can alter feed digestibility. In this study, a novel enzyme cocktail preparation (ABO 374), cultivated from a patented fungal strain found in South African soil was used as the EFE. This cocktail was produced locally and characterised as a fibrolytic enzyme cocktail as it contained xylanase, cellulase and mannanase activities. Upon treatment of lucerne, kikuyu or weeping love grass, it was observed that the EFE had marked effects on the total volume of gas produced and the rate of gas production of lucerne and kikuyu ($P < 0.05$). The *in vitro* digestibility of the kikuyu was also improved ($P < 0.05$). Of particular interest, and in agreement with other researchers, it was observed that the greatest effects were achieved during the early hours of incubation (< 12 h). The *in sacco* results of a complete feed treated with the EFE, substantiated findings that enzymes can improve the digestibility of feeds. An increase in effective degradability of nutrients of up to 20 % was observed, in addition to an increased rate of degradation of DM, CP and NDF. Of importance though, was the finding that the effect of the EFE was not only limited to the fibre component of the feed, but also affected CP degradability. Supposedly, the EFE resulted in changes to the cell wall, albeit subtle, and therefore allowing microorganisms earlier access to the highly digestible cell contents. Additionally, as cell walls are likely altered, the protein embedded in the cell wall matrix becomes available for degradation. Hence, results indicating increased rates of degradation and increased levels of digestion of not only NDF, but also CP, are to be expected.

Findings on the gas production kinetic and *in sacco* digestibility values were based on fitting the data to a one-compartmental non-linear model. As a future prospect the data can also

be evaluated by a two compartmental model. This will allow for the evaluation of the EFE on the rapidly digesting pool as well as on the slowly digesting pool. All substrates evaluated will likely show two-compartmental digestion kinetics. Unfortunately though, the initial gas production data did not allow for evaluating such a model as data points were too few and all subsequent findings were therefore compared using the same model one-compartmental model. The latter results on automated gas production and on *in sacco* digestibility effects will however be fitted to a two-compartmental model, as a future prospect. Results from this could then be combined into 6h intervals to allow for comparison with the early findings on gas production.

The novel approach of the dissertation was aimed at investigating the effects of EFE treatment of forages on cell wall structures. Forage heterogeneity contributes largely to the variation in degradability of plant material even when of similar chemical composition. This heterogeneity originates from the distribution of cell wall material between plant cell types, amongst other. The statement of Weimer (1996) that: “The architecture of the plant cell may be just as important as its chemistry” cannot be overlooked when attempting to elucidate the effects of EFE on cell wall material. In the latter part of the study, investigations were made on the effect of EFE treated forages on the histology of cross sections of the forages. Effects were limited, but significant effects were observed and there was a thinning effect of the EFE on the cell wall of the less digestible tissues such as the epidermis, metaxylem and phloem of both kikuyu and weeping love grass. The emphasis of these findings lies in the subtle changes observed at histological level being sufficient to allow earlier access by microorganisms and freeing nutrients for digestion. It then follows that nutrients will be digested faster, and to a greater extent; therefore improving animal performance.

Additionally, the reported results should be related in terms of the EFE’s studied and the characteristics of enzymes should be noted. Enzymes are obtained from various sources and differ in their pH and temperature sensitivity. Also, enzyme specificity might form one of the major limitations in observing positive effects. As forage and fibre composition is complex, it is of importance to treat it with enzymatic cocktails with similar diversity if positive effects are to be observed. In the literature, poor reporting of enzyme specificity and enzyme characteristics exist and this needs to be improved in future studies. Limitations do exist in reporting the detail of enzyme products as many of these products are commercially available and protected by patent rights. This was indeed the case in our study and limited specific information on the fungal strain and production methods could be reported. Regardless, in evaluating enzymes, detailed information on its activity is of great importance. Low enzyme activity and too narrow enzyme specificity, compared to a mixture of enzymes

in an enzyme cocktail, is likely accountable for limited responses as was observed when the purified xylanase was used as sole enzyme source. Therefore, enzyme types should be diverse enough, and match the substrate closely enough to truly affect digestion to a great extent, as is typically found in the dynamic rumen environment. Also, application of the enzyme directly to the feed and prior to ingestion should be noted. This being said, continued investigations of the mode-of-action of EFE is necessary and might it be important to use new and exciting technology to isolate certain tissues, for instance the cell walls of the epidermis, to determine the effect of EFE at histological level and cell type by cell type.

In this study and per chapter, at least two methods of assessing enzyme effects on forage digestibility were reported. This allowed us to support findings in *in vitro* and *in sacco* studies. The methods were always performed simultaneously under similar conditions, using exactly the same rumen fluid inoculum and substrates. Even so, discrepancies were observed between the results of the methods, with special reference to discrepancies between *in vitro* gas production and *in vitro* digestibility. These discrepancies are probably related to the pre-treatment of the substrate with the enzyme mixture. Pre-treatment of the substrate, from literature, is reported as an important contributing factor in the ability of exogenous enzymes to alter fibre digestibility. Therefore, the pre-treatment of substrate with the enzyme formed part of our protocol. However, the methods used to assess the enzyme effects on forage digestibility could have been affected by this protocol in different manners. Pre-treatment of the substrate can result in altering the reducing sugars present in the substrate. For the measurement of gas production, this change in reducing sugars is not accounted for as gas production measurement is only recorded after the pre-treatment interaction time. On the other hand, where *in vitro* digestibility is measured, the effects of pre-treatment is included in the analysis; therefore leading to discrepancies in results. However, the different methods studied were successful in supporting one another regarding the conclusions drawn from the results.

The main focus of the use of EFE lies in improving the digestibility of poor quality roughages, such as wheat straw. Unfortunately, limited positive effects have been reported to date and future investigations into EFE application should include clear and definite information on factors such as the experimental conditions, the characteristics of the enzymes used regarding specificity and pH and temperature optimal and so forth. Continued research on the mode-of-action of EFE is necessary and new technology to isolate certain cell types or employing differential staining techniques to evaluate plant tissue and cell components in depth, to clearly indicate enzyme effects, might be beneficial in broadening our knowledge on EFE.

In summary, it can be concluded that there is definite merit in the use of EFE in improving the digestibility of ruminant feeds and feedstuffs. The effects of such EFE treatments can be expected to be exerted during the early stages of digestion, thereby increasing the passage rate of digesta. Additionally, the effect of the EFE is not limited to fibre and increased digestibility of all nutrients can be expected, thereby increasing the overall digestibility of the feed. This increased digestibility is likely related to subtle changes to the cell wall material of the forages, hence allowing earlier access of the microorganisms to the cell content and freeing nutrients from the fibrous complex for digestion. For these effects to be observed, the enzyme specificity should be diverse enough and should closely match the targeted substrate.