The effect of glycosylation on the stability of exogenous xylanase under *in vitro* proteolytic conditions similar to the rumen

Submitted in partial fulfillment of the
requirements for the degree

M.Sc.(Agric.) Animal Science

Department of Animal and Wildlife Sciences
Faculty of Natural and Agricultural Sciences
University of Pretoria

By

Wilhelmus Francois Joubert van de Vyver

September, 2002

ABSTRACT

The effect of glycosylation on the stability of exogenous xylanase under *in vitro* proteolytic conditions similar to the rumen

The aim of this study was to evaluate the effect of glycosylation on exogenous xylanase stability when incubated under proteolytic conditions.

Xylanase produced by *Trichoderma longibrachiatum*, was purified using gel filtration chromatography, ammonium sulfate salt precipitation and dialysis. A partially purified xylanase with Mr of 20- and 10 kDa was identified and contained > 65% of the original xylanase activity. Glycoproteins present in the xylanase were identified by thymol sulfuric acid staining or by the FITC-labeled lectin method, specific for glycoproteins. This naturally glycosylated xylanase was enzymatically deglycosylated with one of two endo-N-glycosidases: PNGase F or Endo H. Efficiency of deglycosylation was determined with electrophoresis by observing protein mobility shifts or by staining with FITC-labeled lectin. The effect of glycosylation on the stability of the exogenous xylanase was tested by incubating the glycosylated or deglycosylated xylanase with rumen fluid (Rf), *Prevotella ruminicola* culture supernatant (Pr) or a commercial protease from *Bacillus subtilis* (Bs) for 0, 3, 6, 9 and 24h at 37°C. Results indicated that glycosylated xylanase was significantly more stable ($P<0.05$) against proteolytic inactivation under the relatively low protease conditions of Rf and Pr (0.018 and 0.046 mg azocasein degraded/ml/h, respectively), but not under high proteolytic conditions of Bs (1.009 mg azocasein/ml/h). Also, the glycosylation effect was observed earlier when incubated with the numerous proteases of Rf (3h), than with Pr (9h). These results indicate that glycosylation enhances xylanase stability and therefore is an important characteristic for exogenous enzyme supplements for ruminants.

OPSOMMING

Die effek van glikosilasie op die stabiliteit van eksogene xylanase onder *in vitro* proteolitiese kondisies soortgelyk aan die rumen

Hierdie studie het ten doel gehad om die effek van glikosilasie op die weerstandbiedendheid van eksogene xylanase teen proteolitiese degradasie te evalueer. Xylanase, vanaf *Trichoderma longibrachiatum*, is gesuiwer d.m.v. jelfiltrasie chromatografie, ammoniumsulfaat-presipitasie en dialise. 'n Gedeeltelik gesuiwerde xylanase met Mr van 20- en 10 kDa proteïene is geïdentifiseer en het >65% van die oorspronklike xylanase aktiwiteit bevat. Thymol-swaelsuur-vlekking asook FITC-gemerkte lektien, spesifiek vir glikoproteïene, is gebruik om die glikoproteïene teenwoordig in die xylanase te identifiseer. Deglikosilasie van die natuurlik-geglikosileerde xylanase is verkry deur gebruik te maak van chemiese (TFMS)- of ensiematiese deglikosilasie metodes. Twee endo-N-glikosidases, PNGase F en Endo H is vir die ensiematiese deglikosilasie metode gebruik. Die doeltreffendheid van deglikosilasie is geëvalueer deur veranderinge in proteïenmotiliteit in SDS-PAGE jels waar te neem of deur gedeglikosileerde proteïene met FITC-gemerkte lektien te vlek. Die effek van glikosilasie op eksogene ensiemstabiliteit is ondersoek deur die geglikosileerde- of ensiematiese gedeglikosileerde xylanase te inkubeer met rumenvloeistof (RF), *Prevotella ruminicola* kultuur supernatant (Pr) of 'n kommersiële protease vanaf *Bacillus subtilis* (Bs), vir 0, 3, 6, 9 en 24 ure by 37°C. Die bevinding was dat geglikosileerde xylanase betekenisvol meer stabiel ($P<0.05$) was teen proteolitiese degradasie onder die relatief swak proteolitiese kondisies van RF en Pr (0.018 en 0.046 mg azokaseïen gedegradeer per ml per uur, onderskeidelik). Onder die sterk proteolitiese invloed van Bs (1.009 mg azokaseïen/ml/h) was daar egter geen verskil in stabiliteit tussen die geglikosileerde- en gedeglikosileerde xylanase nie. Verder was die effek van glikosilasie vroeër waargeneem wanneer die ensieme geïnkubeer is met die verskeie proteases van RF (3h) in vergelyking met Pr (9h), waar proteases slegs deur *P.ruminicola* geproduseer word. Vanuit die resultate word die gevolgtrekking gemaak dat glikosilasie wel eksogene xylanase stabiliteit verbeter en dus 'n belangrike eienskap van eksogene ensiemsupplemente vir herkouers is.

Acknowledgments

I want to express my gratitude to Dr. T.P.Lyons and Alltech Biotechnology Inc. for giving me the opportunity to do research at their research centre in Kentucky, USA and for providing financial support during the internship program. In particular, I want to thank Dr. Karl Dawson, Dr. Juan Tricarico and Dr. Gary Spedding for their guidance, without which I would not have succeeded. For financial aid and their contribution in more ways than one to the success of my M.Sc., I would like to thank THRIP. Appreciation also goes to Professor Norman Casey and the faculty members of the Department of Animal and Wildlife science at the University of Pretoria, who supplied me with an outstanding bachelors and masters' education.

I would like to thank my family and friends, in particular my parents, for their sacrifices, commitment and support during my University years.

Also, I would like to thank our Heavenly Father for equipping me with the tools necessary to pursue my dreams.

Table of Contents

Acknowledgements 3
List of abbreviations 7
List of tables 8
List of figures 9
Chapter 1 11
Literature review 11
Background 11
The use of exogenous enzymes in ruminant diets 11
Stability of exogenous fibrolytic enzymes in the rumen 15
Glycosylation and characteristics of enzymes that contribute to their stability 18
Objective 21
Chapter 2 22
General Materials and Methods 22
Enzyme assays 22
Protease activity 22
Xylanase activity 22
Protein 23
Electrophoresis 24
Chapter 3 25
Purification of a xylanase from *Trichoderma longibrachiatum* 25
Introduction 25
Materials and Methods 25
Gel filtration chromatography 25
Ammonium sulfate precipitation 26
Dialysis 26
Results 27
Discussion 29
Chapter 4 31

Glycoprotein detection and deglycosylation of a xylanase produced by *Trichoderma longibrachiatum* 31

Introduction 31

Materials and Methods 31

Glycoprotein detection 31

Thymol-sulfuric acid staining 32

Fluorescein Isothiocyanate-labeled lectin method 32

Deglycosylation 33

Trifluoromethanesulfonic acid method (TFMS) 33

Enzymatic deglycosylation 33

PNGase F 34

Non-denaturing protocol 34

Endo-β-N-acetylglucosaminidase H 34

Results 35

Glycoprotein detection 35

Enzyme activity recovery 37

Efficiency of deglycosylation 38

Discussion 40

Chapter 5 42

Stability of a partially purified xylanase from *Trichoderma longibrachiatum* in rumen fluid and *Prevotella ruminicola* culture supernatant 42

Introduction 42

Materials and Methods 43

Stability trials 43

General xylanase stability methods 43

Study 1 43

Study 2 44

Study 3 44

Study 4 44

Protease sources 44

Strained rumen fluid 44

Culture supernatant 44
Results 45
Protease activity 45
Xylanase stability 45
Discussion 52
Chapter 6 55
Stability of a partially purified xylanase from Trichoderma longibrachiatum in a commercial proteolytic solution and buffer 55
Introduction 55
Materials and Methods 55
Study 1 55
Study 2 55
Study 3 55
Protease source 55
Enzyme assays 56
Results 56
Discussion 60
Chapter 7 61
General discussion and summary 61
References 63

List of abbreviations

CMC	Carboxymethylcellulose
CMC-ase	Carboxymethylcellulase
CP	Crude protein
DM	Dry matter
DNS	Dinitrosalicylic acid
Endo H	Endo-β-N-acetylglucosaminidase H
EPDE	Exogenous polysaccharide degrading enzymes
FITC	Fluorescein isothiocyanate
IVNDF	*In vitro* neutral detergent fibre
Mr	Relative molecular mass
NDF	Neutral detergent fibre
NPN	Non-protein nitrogen
PNGase F	Peptide-N-glycosidase F
RGCA	Rumen fluid glucose cellobiose agar
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TFMS	Trifluoromethanesulfonic acid
VFA	Volatile fatty acids

List of tables

Table 3.1. Purification of a xylanase from *Trichoderma longibrachiatum* 28

Table 4.1. Enzyme activity recovery after deglycosylation 38

Table 5.1. Enzyme activities of native and PNGase F-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 47

Table 5.2. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 48

Table 5.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 50

Table 5.4. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 51

Table 6.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 57

Table 6.2. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 58

Table 6.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with citrate buffer at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates 59

List of figures

Figure 3.1. Protein concentration determination by absorbance at 280 nm. Separation of xylanase by gel filtration chromatography on Bio-Gel P-30 polyacrylamide gel 28

Figure 3.2. Molecular mass determination of the collected fractions in SDS-PAGE after GFC. The gel contained 15% acrylamide and proteins were stained with Coomassie brilliant blue. Lane 1, low molecular mass standards; lane 2, crude xylanase (4%); lane 3-4, unpooled fractions from pool B; lane 5-7, unpooled fractions from pool A 29

Figure 4.1. **A**: Thymol sulfuric acid staining of glycoproteins. Lane 1, negative control; lane 2, high molecular mass proteins (60 kDa); lane 3, low molecular mass proteins (20 & 10 kDa); lane 4, positive control. **B**: Coomassie blue stained proteins indicating low and high molecular mass proteins after purification. Lane 1, low molecular mass standards; lane 2, crude xylanase; lane 3-4, high molecular mass proteins (60 kDa); lane 5-7, low molecular mass proteins (20 & 10 kDa) 36

Figure 4.2. **A**: Glycoprotein detection by FITC labeled Concanavalin A method. **B**: Coomassie brilliant blue counterstain of the proteins. Lane 1, molecular mass standards; lane 5, high molecular mass proteins (60 kDa); lane 6, low molecular mass proteins (20 & 10 kDa); lane 8, crude xylanase. Arrows indicate faint yellow bands of the low molecular mass proteins 37

Figure 4.3. Coomassie blue stained proteins for assessing the extent of deglycosylation. Lane 1, low molecular mass standards; lane 2, control xylanase; lane 3, enzymatically deglycosylated xylanase (**A:** PNGase F deglycosylation and **B**, Endo H deglycosylation). 39

Figure 5.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 47

Figure 5.2. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 48

Figure 5.3. Contribution of rumen fluid to the total xylanase activity over a 24 h incubation period. Error bars represent SEM of three replicates 49

Figure 5.4. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with *P.ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 50

Figure 5.5. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 51

Figure 6.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 57

Figure 6.2. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 58

Figure 6.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with citrate buffer at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates 59

Chapter 1

Literature review:

Background:

Several groups have developed exogenous enzyme systems that can be used to enhance digestive processes in the rumen (Beauchemin *et al.*, 1997; Feng *et al.*, 1996). However, the success of these enzymes is largely dependent on the ability of these proteins to withstand the proteolytic activities of the microbial populations that inhabit the rumen. Some researchers believe that the post-translational glycosylation inhibits proteolysis and infers some protection for enzymes in the rumen (Chesson, 1993; Chu *et al.*, 1978). This study will examine the effects of the glycosylation process on the stability of a specific exogenous enzyme in the rumen.

The use of exogenous enzymes in ruminant diets:

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, 1999).

To optimize the performance of ruminants, nutritional strategies with the following objectives are required:

- Maximized fermentation of carbohydrates, which are unable to be digested and absorbed in the small intestine.
- Minimized fermentation of carbohydrates, which are digested and absorbed in the small intestine.
- Maximum synthesis of microbial protein from NPN.
- Minimal breakdown of dietary protein (Annison, 1997), allowing for the minimum requirements for rumen degradable protein.

Enzyme containing supplements are used widely in poultry and swine feed industries, but their inclusion in ruminant diets is still under development. In monogastric animals, the

key enzyme activities have been identified and the mode of action has been, at least partially, explained. However, in ruminants the mechanisms of exogenous enzymes are still poorly understood. A more detailed knowledge of exogenous enzyme interactions with the feed, the host and the rumen microorganisms and enzymes is necessary to apply this technology successfully (Morgavi *et al.*, 2000).

Preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fibre (Feng *et al.*, 1996). A number of studies have shown that the addition of such enzymes to grass or lucerne before ensiling reduced the concentration of plant structural carbohydrates compared with untreated silages (Feng *et al.*, 1996). However, there is little data available involving the application of fibrolytic enzymes to forages that are not subsequently ensiled. 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 & Tricarico, 1999). Morgavi and co-workers (2000) cited articles by Varel *et al.* (1993) and Yang *et al.* (1999) that show that exogenous enzymes enhance the numbers of fibre-digesting rumen microbes *in vitro* and *in vivo*. In addition, enzymes have the potential to hydrolyze feed in the rumen. However, the magnitude of this effect has not been quantified. 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.*, 2000 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.

Studies of a number of enzyme preparations clearly show differences in the way individual enzymes and mixtures of enzymes alter ruminal microbial activities. For example, use of enzyme preparations based on high levels of xylanase activity have been shown to enhance total VFA production and overall carbohydrate utilization by a ruminal microbial population in batch cultures provided with ground fescue hay as a substrate (Dawson & Tricarico, 1999). This is consistent with the overall effects observed in studies that demonstrate enhanced dry matter digestion and fibre digestion. However, enzyme preparations that contain measurable levels of fungal cellulase are more inclined to alter the relative proportions of VFA produced by a population of ruminal bacteria (Dawson & Tricarico, 1999). This reflects dramatic changes in the physiological activities of the ruminal bacteria and can lead to a more efficient fermentation. This results in the production of more propionate and butyrate and less acetate by the microbial population (Dawson & Tricarico, 1999; Howes *et al.*, 1998). Hristov *et al.* (1998b) also reported that treating the diet with exogenous polysaccharide-degrading enzymes (EPDE) prior to feeding tended to increase the ruminal acetate to propionate ratio, the butyrate concentration and to increase ruminal reducing sugar concentrations.

As indicated by this work, changes in the rumen suggest that certain types of enzyme preparations cannot only have a significant impact on the digestive process, but may also have dramatic effects on the overall fermentation activity in the rumen resulting in improvements in the efficiency of nutrient utilization. There is still, however a major question as to the possibility of enzymes surviving in the rumen for sufficient time and at sufficient activity to give a consistent effect on fibre digestibility.

Theoretically, to positively influence feed digestion, exogenous enzymes would have to contain enzymatic activities that limit the rate of the hydrolysis reaction. 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 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 dramatically and becomes optimal for the exogenous enzymes, thus positively influencing fibre digestion under these conditions.

Beauchemin *et al.* (1997) reported that fibrolytic enzymes could be used to improve the digestibility of barley-based diets, but not corn-based diets. The lower energy content of barley compared with corn should result in lower performance for cattle fed barley. The reason is largely due to its relatively indigestible hull. Also, barley grain-based feedlot diets can produce high ruminal β-glucan concentrations. If poorly utilized in the rumen, β-glucan may impair digestion and depress nutrient absorption in the small intestine by altering pH, rate of transit, and viscosity of digesta, by affecting digestive enzymes, or by interacting with the intestinal surface (Hristov *et al.*, 2000). A number of studies showed that using exogenous polysaccharide degrading enzymes in ruminant diets substantially increased feed digestibility and animal performance, although other studies reported no effects, and even negative responses (Beauchemin *et al.*, 1997).

Fibrozyme is an enzyme supplement for ruminants prepared from the fermentation extracts of *Aspergillus niger* and *Trichoderma longibrachiatum,* developed and marketed by Alltech Biotechnology, Inc. It has been formulated on the basis of its ability to degrade the xylans (contains 100 U of xylanase activity/g) found in the hemicellulose fraction. It aids in the breakdown of the fibrous matrix in plant structural carbohydrate complexes and exposes many of the bound nutrients to digestive activities in the rumen. The enzyme preparation also contains measurable levels of cellulase and protease activities that may augment other enzymatic activities in the rumen. As a result, its digestive activities are not limited to attack on hemicellulose (Howes *et al.*, 1998).

In a study performed by Johnston (2000) this dry commercial enzyme supplement, Fibrozyme, is discussed as a feed supplement rather than a forage treatment. The product in this study was evaluated through measurement of its effects on *in vitro* NDF digestion

(IVNDF) of corn silage. The results indicated that Fibrozyme increased the disappearance of NDF across corn silage hybrids and planting rates within the rumen at least for the first 6 hours of *in vitro* fermentation. Fibrozyme is active and thus stabile against proteolysis in the rumen for the first 6 hours after ingestion. The reason for this protection against proteolysis is believed to be due to post-translational glycosylation of the enzymes produced from *Aspergillus* and *Trichoderma* cultures.

With increased commercial use of feed enzymes in processed feeds the question of enzyme stability becomes more and more of an issue. For instance, the ability of the enzymes to withstand the various heat treatments now being employed in feed manufacturing is seriously questioned. Inactivation of enzymes occurs during processing, such as pelleting. The magnitude of the inactivation depends on the processing conditions employed, with higher temperatures and prolonged conditioning times increasing inactivation (Inborr & Bedford, 1994). A treatment condition, such as heat during ensiling is known to affect the degradability of proteins and therefore might influence the stability of enzymes. Due to the denaturation of the enzymes, their activity is expected to decrease under such treatments, but it is unsure what the effect of processing would be on the stability of the exogenous enzymes in the rumen.

Furthermore, once the enzymes enter into the gastrointestinal tract, where the enzyme action occurs, they are subjected to a range of conditions that can cause denaturation, thus rendering them inactive (Inborr & Bedford, 1994). A potential problem is that protein in the diet, especially soluble protein, is usually degraded rapidly in the rumen. Thus, if dietary enzymes are to be effective as modifiers of rumen fermentation, the enzymes must resist proteolysis by rumen microorganisms for a time sufficient to effect digestion (Morgavi *et al.*, 2000).

Stability of exogenous fibrolytic enzymes in the rumen:

McDonald *et al.* (1995) reported that when proteins enter into the rumen they are hydrolyzed to peptides and amino acids by rumen microorganisms, but some amino acids are further degraded to organic acids, ammonia and carbon dioxide. The ammonia

produced, together with some small peptides, free amino acids and carbon sources, is utilized by the rumen microorganisms to synthesize microbial proteins.

Exogenous enzymes added to ruminant feeds are complex proteins that have to be resistant to proteolysis in order to effectively alter ruminal digestion and digestion rates. The speed and extent to which proteins are broken down depends upon such factors as the surface area available for microbial attack, the physical consistency and chemical nature of the protein, and the protective action of other constituents. Susceptibility to breakdown is thus a characteristic of the protein itself and should be capable of being quantified. Claims have been made that the solubility of the protein is correlated with ease of breakdown but this hypothesis does not survive critical examination. For example, casein, which is readily degraded in the rumen, is not readily soluble while albumin, which is resistant to breakdown, is readily soluble (McDonald *et al.*, 1995).

In a study done by Hristov *et al.* (1998a) two commercial preparations of polysaccharide-degrading enzymes were tested *in vitro* and *in vivo* for rumen stability. *In vitro,* the incubation media consisted of rumen inoculum and nutrient solution containing the exogenous enzymes (CMC-ase, xylanase, β-glucanase and amylase). Supernatants collected at 0, 1, 2, 4, and 6 h were analysed for substrate degrading activities against CMC, xylan and starch. *In vivo*, the exogenous enzymes were directly introduced into the rumen of a dairy cow fed a forage/concentrate diet. Ruminal and duodenal samples were collected at 0, 1.5, 3, 6, 9, 12 and 15 h after the enzymes were introduced into the rumen and analysed for substrate degrading activities, as for the *in vivo* experiment. Results from the *in vitro* experiment indicated that the decline in CMC-ase activity within the 6 h incubation was not significant. For all treatments, the xylanase activity remained constant between 1 and 6 h incubation. The amylase activity declined steadily to an average of 63 % of the original activity at 1 h. These results indicate that CMC-ase and xylanase activities in the two enzyme systems could be resistant to microbial degradation in the rumen.

When applied directly to the rumen of a dairy cow both enzyme systems increased CMC-ase, xylanase and β-glucanase activities of the rumen fluid (Hristov *et al.*, 1998a) and this increased fibrolytic activity was also reflected in the lowered viscosity of the fluid fraction (Hristov *et al.*, 1998b). The average concentration of reducing sugars in the rumen fluid was higher when the enzymes were introduced, indicating that supplemental enzymes were active in the rumen in degrading structural carbohydrates to reducing sugars (Hristov *et al.*, 1998a). In another study done by the Hristov *et al.* (1998b) exogenous polysaccharide degrading enzymes (EPDE) were supplied through the feed or continuously infused into the abomasum. Again, it was observed that the enzyme treatment increased the reducing sugar concentration and lowered the NDF content compared with untreated TMR as a result of enzymatic solubilisation of the plant fibres.

The increases in fibrolytic activities in rumen fluid, due to exogenous enzyme supplementation, do not necessarily implement enhanced ruminal digestion of fibrous feeds. The fermentation substrate in the rumen is by far more complex than the purified substrates used to test for enzyme activities (Hristov *et al.*, 1998a). EPDE primarily degraded the portion of plant structural carbohydrates that were susceptible to microbial degradation in the rumen and did not solubilise the less available recalcitrant structures to any great extent. Although the soluble fraction of the DM in the EPDE treated feed was increased, the effective degradability of the DM was not affected, and total tract digestibility of DM, NDF and CP were not improved relative to the control. These results suggest that the fraction of DM solubilised by the EPDE was the most readily digestible portion of the degradable DM (Hristov *et al.*, 1998b).

The results from this experiment demonstrate that fibrolytic enzymes supplemented through the feed can be resistant to proteolysis in the rumen and have the potential to by-pass the rumen and remain active in the small intestine. Therefore their impact on the degradation of polysaccharides in the rumen could be significant. The xylanase portion of the enzyme system retained activity in the duodenal digesta, indicating that a substantial portion of this exogenous enzyme escaped the reticulo-rumen and abomasum and could affect the utilization of nutrients in the small intestine of ruminants (Hristov *et*

al., 1998a,b). A high resistance of xylanase to proteolysis has previously been found (Fontes *et al.*, 1995). The latter author also reported that only one cellulase was resistant to proteolytic attack, but, when substrate was provided, all tested cellulases were resistant to proteolysis. Gorbacheva & Rodionova (1977) suggested the involvement of glycosylation in proteolytic stability of EPDE, although, as pointed out by Fontes *et al.* (1995), non-glycosylated enzymes were also found to resist proteolysis.

Morgavi *et al.* (2000) found that feed additive enzymes produced by *Aspergillus niger* was the most stable in the rumen. Cellulases and xylanases were particularly stable in most of the preparations investigated, whereas glycosidases were rapidly inactivated. The stability of CMCase and xylanase activities in these experiments suggest that, if their activity is limiting the rate of fermentation in the rumen, they might be stable for long enough to accelerate digestion. Furthermore, when enzymes are added to the feed in the liquid form before they are given to the animal, the presence of the substrate further contributes to stability (Fontes *et al.*, 1995, cited by Morgavi *et al.*, 2000).

Inactivation of EPDE in the abomasum was affected by acidity, but both low pH and high pepsin concentrations in the abomasum seem to be critical factors in preventing EPDE reaching the lower digestive tract in ruminants (Hristov *et al.*, 1998b). Morgavi *et al.* (2000) reported that stability varies between commercial products and that the enzymes are likely to be more affected by the host gastrointestinal proteases and pH in the abomasum and intestines than by ruminal proteases.

Glycosylation and characteristics of enzymes that contribute to their stability:

Glycosylation is one of the major naturally occurring modifications of the covalent structure of proteins (Tanner & Lehle, 1987). Most secretory proteins become glycosylated as soon as the growing polypeptide chains enter the endoplasmic reticulum, before the final native-like folded state is reached. There are two different types of protein glycosylation: O-glycosylation at the hydroxyl groups of serine and threonine residues and N-glycosylation at aspargine residues in the consensus sequence of Asn-X-Ser/Thr (Wang *et al.*, 1996). Although much is known about the structure and

biosynthesis of oligosaccharides in glycoproteins, the central question of how glycosylation contributes to the glycoprotein structure and function is not entirely clear.

In eukaryotes, glycosylation has numerous roles. Some researchers believe that glycosylation of proteins protects them against proteolysis, as the addition of sugars to the protein molecule increases stability (Wang *et al.*, 1996; Annison, 1997; Rayon *et al.*, 1998), the catalytic activity (Helenius, 1994), the thickening properties, and thermostability of enzymes (Canton & Mulvihill, 1983; Wang *et al.*, 1996). Glycosylation is also important for solubility of the protein and results showed that fully deglycosylated phosphatase aggregates and becomes insoluble (Barbaric *et al.*, 1984; Mizunaga & Noguchi, 1982). However, none of the effects were constantly observed for all glycoproteins (Wang *et al.*, 1996). In addition, the rate and extent of hydrolysis of individual proteins are affected by their chemical structure: their secondary and tertiary configuration governs their susceptibility to proteases.

Chesson (1993) found that the extensive glycosylation of fungal enzymes protects them against proteolytic attack in monogastric animals and Rayon *et al.* (1998) stated that, in addition to the prevention of proteolytic degradation, glycosylation is important for the correct folding activity of the protein in eukaryotes. Results indicate that the proportion of disulfide cross-links in proteins is related to the rate of rumen degradation (Broderick *et al.*, 1991, cited by Morgavi *et al.*, 2001). Many enzymes produced by fungal species are glycosylated, which often protects enzymes from inactivation by temperature and proteases (Morgavi *et al.*, 2000).

Langsford *et al.* (1987) compared glycosylated cellulases from *Cellulomonas fimi* with their non-glycosylated counterparts synthesized in *Escherichia coli* from recombinant DNA. Glycosylation of the enzymes did not significantly affect their kinetic properties, or their stability toward heat and pH. However, the glycosylated enzymes were protected from attack by *C. fimi* protease when bound to cellulose, while the non-glycosylated enzymes yielded active, truncated products with greatly reduced affinity for cellulose.

Bovine pancreatic ribonuclease B occurs naturally as a mixture of five glycoforms in which the same polypeptide sequence is associated with a series of oligomannose sugars attached at the N-glycosylation site. Rudd *et al.* (1994) compared the amide proton exchange rates for individual glycoforms of RNase B and unglycosylated RNase A and showed that while the three dimensional structure was unaffected, glycosylation decreased dynamic fluctuations throughout the molecule. Consistent with the overall decrease in flexibility, and with the possibility that all of the sugars attached to the RNase B may afford steric protection to susceptible sites, was the finding that each of the glycoforms tested showed increased resistance to proteases compared with the unglycosylated protein.

Bernard *et al.* (1983) compared the sensitivity of unglycosylated RNase A to trypsin and chymotrypsin with three glycosylated species of RNase B that differed with respect to the size of the carbohydrate chain. Fully glycosylated RNase B was found to be 6-10 times more resistant to trypsin digestion than unglycosylated RNase A. RNase B' and RNase B'', with the intermediate chain sizes, were 3.0- and 1.3-fold more resistant to trypsin digestion than RNase A, respectively. With chymotrypsin, however, differences in rates of digestion were much less marked. Also, carboxypeptidase, free of carbohydrate, is degraded proteolytically three times faster than the glycosylated protein (Chu & Maley, 1982) and, in the case of acid phosphatase, a 60 % deglycosylated protein is hydrolyzed about 20 – fold more rapidly with pepsin than the native form (Barbaric *et al.*, 1984). However, to what extent such differences between glycosylated and unglycosylated forms are biologically relevant remains unknown.

There is evidence that even very slight alterations in the sugars that decorate the exterior of a protein can cause remarkable changes in the protein properties (Rudd *et al.*, 1994). In addition, the protective effect is inversely proportional to the distance between the unique site of carbohydrate attachment and the initial or primary cleavage sites for trypsin or chymotrypsin. These results are consistent with the view that the size of the oligosaccharide chain and its proximity to the primary or rate-limiting cleavage site are important for protecting the carbohydrate against proteolytic degradation (Olden *et al.*,

1985). Wang *et al.* (1996) also observed that the destabilization effect of deglycosylation seems to depend on the carbohydrate content, i.e., the maximum effect was observed for the most heavily glycosylated protein, irrespective of the types (N-linked or O-linked) or patterns (mono- or multi-branched) of the covalently attached carbohydrate chains.

How glycan moieties of glycoproteins protect against proteolysis is not known. Most obviously, the physical removal of bulky oligosaccharides could expose new protease cleavage sites directly or indirectly by inducing conformational alterations in the protein structure (Olden *et al.*, 1985). Despite this striking ability of carbohydrates to influence protein properties, systematic studies of the effects of protein glycosylation have been limited. This is due to the natural occurrence of microheterogeneous mixtures of glycoforms: glycoprotein forms that possess the peptide backbone, but differ in both nature and site of glycosylation (Davis *et al.*, 2000).

Objective:

The aim of this study was to determine whether glycosylation infers protection on a specific exogenous fibrolytic enzyme, xylanase, against proteolytic degradation under conditions similar to the rumen.

Chapter 2
General Materials and Methods:

Enzyme assays:

Protease activity: Protease activities in rumen fluid, culture supernatants and commercial proteolytic preparations were measured, using a modification of the colorimetric technique described by Brock *et al.* (1982). Duplicate tubes were used for enzyme samples, enzyme blanks and substrate blank. Identical samples (250 µl) were added to each of two microcentrifuge tubes of which only one contained 250 µl of a solution of 2% azocasein (A-2765, Sigma Chemical Co.) in 0.1 M sodium phosphate buffer pH 6.8, while the other tube served as an enzyme blank. To the substrate blank tube, 250 µl of 0.1M phosphate buffer was added instead of the enzyme preparation. After the enzyme preparations were added, tubes were incubated for 2 h at 39 °C. Following the incubation, 500 µl of 10% trichloroacetic acid was added to each tube to stop the reaction and to precipitate the undegraded protein. At this time, 250 µl of the azocasein solution was added to the blank tubes in each pair. The tubes were centrifuged at 12000 rpm for 5 min at room temperature. Soluble protein degradation products were measured by adding equal volumes of supernatant and 1N NaOH in a microcuvette. Absorbance (440 nm) was measured on a Hitachi UV/Vis spectrophotometer using 0.1 M phosphate buffer as blank. Activity was expressed as mg or µg of azocasein degraded per ml per h. Net absorbance was calculated by subtracting the absorbance measured in the enzyme blank tube from that of the tube containing the enzyme. The specific relationship between absorbance and protein content was determined from a series of dilutions (0.200 – 0.025 mg/ml) of the azocasein stock solution. These values were used to calculate the relative amount of protein degraded by each enzyme preparation during a 2 h incubation period.

Xylanase activity: Xylanase in partially purified and commercial enzyme preparations were determined by measuring reducing sugars released from xylan with dinitrosalicylic acid. Xylanase was assayed using 1% (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 & Poutanen (1989). Xylan

substrate (1.8 ml) was equilibrated in a water bath at 50 °C for 5 min in 16 x 125 mm glass tubes. Duplicate tubes were used for enzyme samples, enzyme blanks and substrate blank. Appropriate 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. Dinitrosalicylic acid (3.0 ml) was added to each test tube after exactly 5 min to stop the reaction. At this time, the enzyme dilution was added to the blank test tube and buffer to the substrate blank. The dinitrosalicylic acid (DNS) contained the following (per liter): 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 min, 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 min.

Protein: Protein concentration was determined by the method described by Bradford (1976). Protein sample or standard (250 µl) was 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 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.

Electrophoresis: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) in a Mini-PROTEAN 3 Cell (165-3301, Bio-Rad laboratories). Samples were dissolved in SDS sample buffer containing 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.5 M Tris-HCl buffer (pH 6.8) and heated in a boiling-water bath for 2 min prior to loading on the gel. The stacking and separating gels (Gibco BRL) consisted of 3.9% and 10% or 12.5% polyacrylamide, respectively, and were poured between glass plates with 0.75 mm spacers. After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (15528-011, Gibco BRL). The molecular weight standards were from the low-molecular-weight standard mixtures (M-5630, Sigma Chemical Co.) and consisted of serum albumin (66,000 Da), fumarase (48,500 Da), carbonic anhydrase (29,000 Da), β-lactoglobulin (18,400 Da), and α-lactalbumin (14,200 Da). Except when indicated otherwise, these were the conditions used for all electrophoresis.

Chapter 3

Purification of a xylanase from *Trichoderma longibrachiatum*:

Introduction:

Gel filtration is performed using porous beads as the chromatographic support. A glass column packed with such beads 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. The 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). Chen *et al.* (1997) purified a xylanase from *Trichoderma longibrachiatum* by subjecting the culture supernatant to a four-step purification scheme involving ultrafiltration, ammonium sulfate precipitation and cation exchange and gel filtration chromatographies. They produced a homogenous purified protein that migrated as a single sharp band (Mr 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. Royer & Nakas (1991) also purified two xylanases from the fungus, *Trichoderma longibrachiatum*. Their results indicated that the fungus generated a highly active xylanase enzyme of approximately 20 kDa and a less active enzyme of approximately 30 kDa. The aim of the experiment in this chapter was to produce and characterise a purified, concentrated xylanase from a crude fibrolytic enzyme preparation obtained from *Trichoderma longibrachiatum*.

Materials and methods:

Gel filtration chromatography: A portion (4.5 ml) of a 4% (w/v) crude xylanase solution in 0.1 M sodium citrate was applied to a 120 x 0.9 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 crude xylanase was a commercial enzyme preparation originating from the fermentation of *Trichoderma longibrachiatum* and was supplied by Alltech, Inc. The Bio-Gel P-30 gel beads were allowed to hydrate for 12 h at 20 °C and

half of the supernatant decanted. The solution containing the beads was then degassed by vacuum for 5-10 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 rate of 8 ml/h, and 3 ml fractions were collected with an automatic fraction collector and analysed for protein by absorbance at 280 nm. All the fractions with absorbance were analysed for xylanase activity and protein concentration by the Bradford method. 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 pool A and pool B. The xylanase activity and protein concentration of each of the two pools were subsequently determined. Molecular mass determination was by SDS-PAGE at acrylamide concentrations of 15%.

Ammonium sulfate precipitation: Protein in Pools A and B were precipitated by the ammonium sulfate method as described by Englard & Seifter (1990). High purity ammonium sulfate was used and the amount needed to bring the volume of the starting material to 80 – 85% saturation was determined. The ammonium sulfate was added in increments while maintaining a neutral pH and temperature of 4 °C. Each addition of salt was made only after the previously added amount was completely dissolved. After all the salt was added, the mixture was stirred for another 30 min to allow equilibration of the solvent and protein. The mixture was then centrifuged at 10,000 g for 10 min and the supernatant fluid decanted. The xylanase activity and protein concentration were then determined.

Dialysis: Ammonium sulfate from the previous step was removed from the partially purified protein by placing the protein solution in a Spectra/Por semi-permeable dialysis membrane with a molecular weight cut-off of 500 Da (131 048, Spectrum Medical Industries, Inc.). The membrane containing the protein solution was placed in a vessel and dialyzed against a 100 fold excess 0.05 M sodium citrate buffer, pH 5.3 for 6 h at 4 °C. The buffer was replaced every 2 hours. After dialysis the protein solution were analysed for xylanase activity and protein concentration and stored at –80 °C.

Results:

The xylanase solution (4%) was subjected to purification involving gel filtration chromatography, ammonium sulfate precipitation and dialysis. Results on the purification of the xylanase are presented in Table 3.1. Gel filtration of the xylanase produced by the fermentation of *Trichoderma longibrachiatum* resulted in two peaks of xylanase activity, denoted pool A and pool B (figure 3.1). Electrophoresis with low molecular mass standards suggested relative molecular masses of approximately 60 kDa – 30 kDa for pool B, which eluted with the excluded volume and 20 kDa and 10 kDa for pool A, which accessed the internal volume of the column (figure 3.2). The purification procedure resulted in the removal of high molecular mass proteins, but purification was only partial as the enzyme migrated as two distinct bands (Mr 20 kDa and 10 kDa).

The lower molecular mass enzyme (pool A) contained over 150-fold the total xylanase units of the higher molecular mass enzyme (pool B) after the crude xylanase were subjected to the purification scheme. The purification procedure gave about 3.0-fold overall purification, but before the ammonium sulfate salt was removed by dialysis, the purification factor was 6.10-fold. The specific activity of the partially purified enzyme on birchwood xylan (1%) at 50 °C was 24984.57 U/mg protein.

Table 3.1. Purification of a xylanase from *Trichoderma longibrachiatum*

Fraction	Step	Total protein (µg)	Total activity[a] (U)	Activity (U/ml)	Specific activity[a] (U/µg)	Purification (fold)	Yield (%)
	Xylanase solution	4938.8	41551.2	41551.2	8.4	1.00	100
Pool A	Gel filtration	2481.6	27666.0	2305.5	11.1	1.33	66.6
	Precipitation	325.5	16717.0	8798.4	51.4	6.10	40.2
	Dialysis	136.1	3400.4	871.9	25.0	2.97	8.2
Pool B	Gel filtration	1844.8	256.8	32.1	0.1	0.02	0.6
	Precipitation	1341.0	123.8	103.2	0.1	0.01	0.3
	Dialysis	232.0	22.6	11.3	0.1	0.01	0.1

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

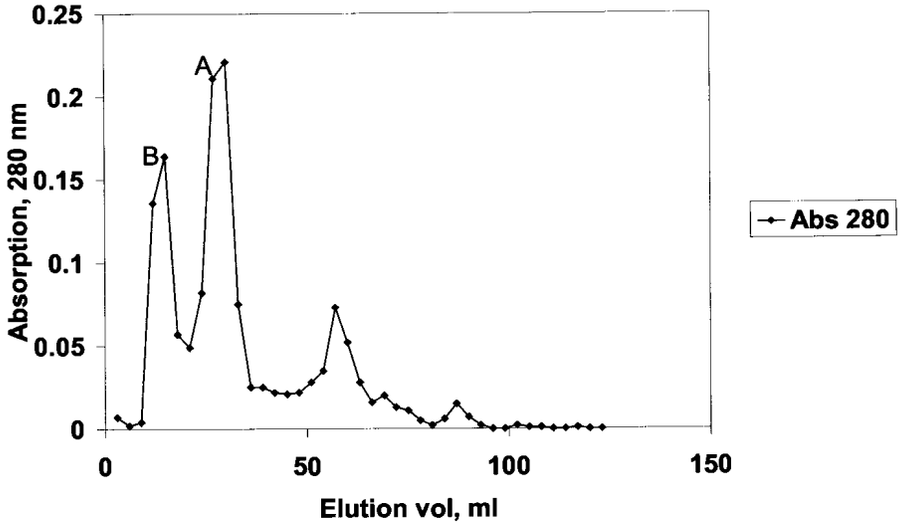

Figure 3.1. Protein concentration determination by absorbance at 280 nm. Separation of xylanase by gel filtration chromatography on Bio-Gel P-30 polyacrylamide gel.

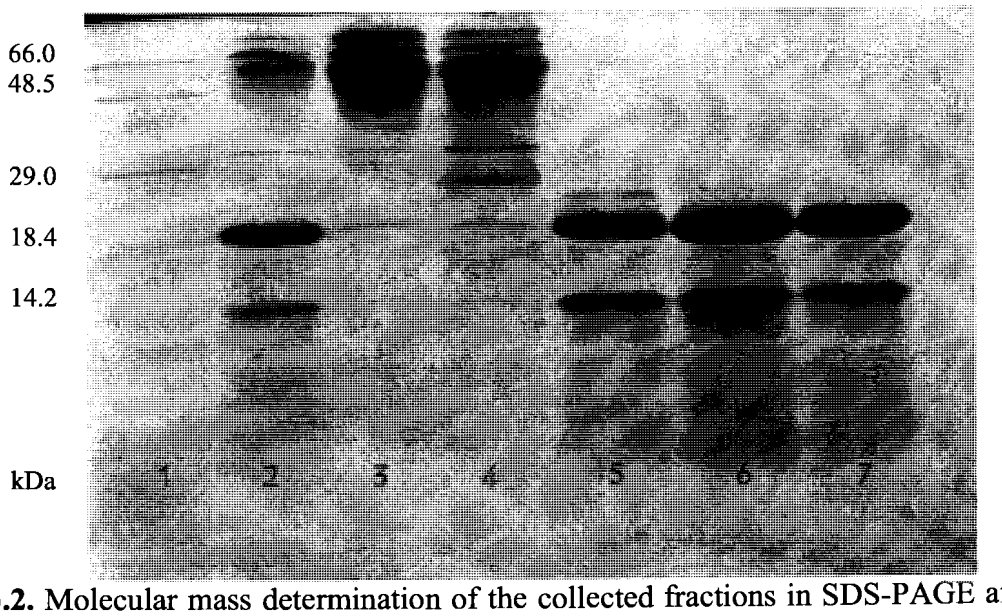

Figure 3.2. Molecular mass determination of the collected fractions in SDS-PAGE after GFC. The gel contained 15% acrylamide and proteins were stained with Coomassie brilliant blue. Lane 1, low molecular mass standards; lane 2, crude xylanase (4%); lane 3-4, unpooled fractions from pool B; lane 5-7, unpooled fractions from pool A

Discussion:

Although there was a loss in both xylanase activity (approximately 90%) and protein, pool A was partially purified about 3-fold and was used for further studies on glycosylation and xylanase stability. With gel filtration chromatography, it is difficult to separate proteins that do not differ more than 2-fold in molecular mass, hence the elution of a 20 kDa and 10 kDa heterogeneous protein together in pool A, which contained the xylanase enzyme. The enzyme complex with the 20 and 10 kDa proteins was the major contributor to the extracellular xylanase and is similar to results obtained by Royer & Nakas (1991). They also found that in purifying two endoxylanases from the culture medium of *Trichoderma longibrachiatum,* the enzyme with a molecular mass of 21.5 kDa had a specific activity of 510 U/mg protein, suggesting that it is the major contributor to the high levels of extracellular xylanase activity. A less active enzyme of approximately 30 kDa was also observed.

Gel filtration alone was effective in purifying crude xylanase as more than 60% of the xylanase activity was retained. Precipitation did not result in a major loss of activity

either, but when the ammonium sulfate salt was removed by dialysis, almost 90% of the original total xylanase activity was lost. There is an indication that the conditions during dialysis were detrimental to recovery of protein and xylanase activity. The purification procedure gave approximately 8.2% recovery of activity and the specific activity of the partially purified xylanase on birchwood xylan at 50°C was 24984.6 U/mg protein.

Chapter 4

Glycoprotein detection and deglycosylation of a xylanase produced by *Trichoderma longibrachiatum*

Introduction:

Glycosylation is one of the major naturally and most complex occurring modifications of the covalent structure of proteins. Most secretory proteins become glycosylated as soon as the growing polypeptide chains enter the endoplasmic reticulum, before the final native-like folded state is reached (Wang *et al.*, 1996). There are two different types of protein glycosylation: O-glycosylation at hydroxyl groups of serine and threonine residues and N-glycosylation at asparagine residues. Studies on the native-glycosylated, carbohydrate-depleted and recombinant non-glycosylated proteins revealed such effects as stabilization of protein conformation (Walsh *et al.*, 1990), protection from proteolysis (Bernard *et al.*, 1983; Rudd *et al.*, 1994), enhancement in nascent polypeptide solubility (Paul *et al.*, 1986). In addition, glycosylation plays a key role in determining the expression, folding, thermal and catalytic activity of enzymes (Helenius, 1994).

Fungal fibrolytic enzymes are extracellular and therefore glycosylation is important for their expression; the secretion and stability of cellulases and xylanases from fungi such as *Aspergillus* has been related to the extent of enzyme glycosylation (Calza, 1991). It is the aim of this chapter to prove that a xylanase produced by *Trichoderma longibrachiatum* is glycosylated and to deglycosylate the xylanase while maintaining enzymatic activity. It is important to retain enzyme activity of the deglycosylated xylanase in order to compare its stability to that of the naturally glycosylated xylanase against proteolytic degradation.

Materials and methods:

Glycoprotein detection: Many procedures exist for the detection of glycoproteins, such as the Periodic acid-schiff base method, Thymol-sulfuric acid staining and Lectin techniques. The Thymol-sulfuric acid staining technique was used at first to identify glycoproteins. To detect nanogram quantities of glycoproteins, the Lectin technique was used in this study in addition to the less sensitive Thymol-sulfuric acid method.

Thymol–Sulfuric Acid staining: Because this method is approximately 2-fold more sensitive than the Periodic acid-schiff base method, this was the technique of choice at first to identify all glycoproteins present and was performed according to the method described by (Gander, 1984). Glycoprotein separations were carried out in 12.5% SDS-PAGE. After electrophoresis, the gel was placed in a glass-baking dish and fixed by submerging in 25% 2-propanol/10% acetic acid. The gel was then rocked gently for 2 h at room temperature and shrinked in this solvent as it dehydrated. The solvent was drained and the process repeated. The gel was stored overnight in this solution in order to insure that SDS and Tris-glycine was leached from the gel. The gel was then immersed in the above solution containing 0.2% (v/v) thymol (5-methyl-2-isopropylphenol, T-0501, Sigma Chemical Co.) for 90 min with gentle rocking. After draining this solution, the gel was immersed in 80% sulfuric acid/20% (v/v) ethanol and rocked until the gel clarified and glycoproteins appeared as pink/red bands. The color faded within hours. The gel was soaked overnight next in 10% acetic acid/10% (v/v) methanol and counterstained with Coomassie blue to detect proteins.

Fluorescein Isothiocyanate-labeled lectin method: While the above technique is specific and direct, it requires relatively large (microgram) quantities of glycoprotein. For the detection of nanogram quantities, indirect lectin techniques are available. The Fluorescein isothiocyanate-labeled lectin procedure has specificity toward the carbohydrate region of glycoproteins to a degree dictated by the specific lectin used. Fluorescein-labeled concanavalin A reacts with numerous glycoproteins because many contain residues, such as D-mannopyranosyl, for which concanavalin A has an affinity (Gander, 1984). Glycoprotein separations were carried out on 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). The SDS was removed by washing the gel for 20 h in methanol-acetic acid-water (10:3:27) and in 5% acetic acid. The fixed gel was then washed for 4 h during each of four changes of 0.1 M NaCl-0.05 M Tris-HCl pH 7.0 containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. The concentration of the FITC labeled Concanavalin A type IV, from *Canavalia ensiformis* (C-7642, Sigma Chemical Co.) was adjusted to 1 mg/ml. The fixed and washed gels were then treated with the FITC labeled Concanavalin A for 12 h at 25 °C, followed by destaining in the same buffer for 2 days. The stained bands remaining are noted as being visible for up to 1 month.

Deglycosylation:

Two methods of deglycosylation were used. Chemical deglycosylation was used at first in an attempt to identify all glycoproteins present in the enzyme preparation. In addition, more specific and non-denaturing enzymatic deglycosylation was used to produce a deglycosylated protein with xylanase activity.

Trifluoromethanesulfonic acid method (TFMS): Chemical deglycosylation was performed according to a method described by Edge et al., 1981. Anisole (1 ml) and 2 ml of TFMS (T-1394, Sigma Chemical Co.) were mixed in a glass tube with a Teflon-lined screw cap and cooled to 0 °C. Crude xylanase (20 mg) was dissolved in 1 ml of the above solution in a 4 ml glass vial and bubbled with nitrogen for 30 seconds followed by magnetic stirring for 15 min. The deglycosylated protein was freed of reagents and low molecular weight sugars by ether precipitation and washing. A 50-fold excess of diethyl ether containing 10% (v/v) of n-hexane was used and the mixture was allowed to stand at –40 °C for 1 h. If no precipitate formed within 20 min, a drop of pyridine was added to help initiate protein co-precipitation. The protein was then pelleted by centrifugation at 2000 rpm for 5 min., followed by a second ether wash and recentrifugation. The resultant pellet was resuspended in ice-cold 95% ethanol, recentrifuged, and freed of remaining solvents by vacuum drying. The protein obtained by this method was compared with its untreated control for retention of original xylanase activity and relative molecular weights determined on 10 % SDS-PAGE .

Enzymatic deglycosylation: Partially purified xylanase was enzymatically deglycosylated with one of two endo-N-glycosidases, Peptide-N-glycosidase F (PNGase F, 170-6883, Bio-Rad Laboratories) or Endo-β-N-acetylglucosaminidase H (Endo H, from *Streptomyces griseus,* E-6878, Sigma Chemical Co.). Only the non-denaturing protocol was used to deglycosylate the xylanase because it is important to retain enzyme activity for further studies on the stability of the xylanase against various protease sources, where enzyme activity is used as measure of stability over a 24 h period

PNGase F:

Non-denaturing protocol: Glycoproteins were deglycosylated according to the procedure in the PNGase F instruction manual from Bio-Rad. Equal volumes of partially purified xylanase (containing 871.90 U/ml or 24.98 U/ µg protein) and 100 mM sodium phosphate reaction buffer pH 7.5 were mixed to a total volume of 400 µl. One 200 µl aliquot was incubated with 10 µl PNGase F (2.5 U/ml). A second 200 µl aliquot was incubated with 10 µl citrate buffer and served as the control. Both aliquots were incubated at 37 °C for 24 h and were then stored at –80 °C until used. One unit (U) of PNGase F is defined as the amount of enzyme required to catalyze the release of N-linked oligosaccharides from 60 µmoles of denatured ribonuclease B in 1 h at 37 °C, pH 7.5.

Bovine fetuin was used as a positive control. The treated and untreated glycoproteins were run in separate lanes in SDS-PAGE and visualization was by coomassie brilliant blue R-250 staining. Deglycosylated proteins will exhibit an increase in mobility due to the reduction in molecular weight.

Endo-β-N-acetylglucosaminidase H: A modification of the non-denaturing protocol described in the Bio-Rad instruction manual for PNGase F was used. Equal volumes of partially purified xylanase solution (containing 871.90 U/ml or 24.98 U/ µg protein) and 100 mM sodium phosphate buffer, pH 5.0 was mixed together in a microcentrifuge tube. To 800 µl of this mixture 0.1 U Endo H (resuspended in 50 µl of the phosphate buffer, pH 5.0.) was added. Another 800 µl aliquot was incubated with 50 µl phosphate buffer and served as the control. The mixtures were then incubated at 37 °C for 24 h and stored at –80 °C. The treated and untreated glycoproteins were run in separate lanes in SDS-PAGE and stained with coomassie brilliant blue R-250 to observe mobility changes. One unit of Endo H will hydrolyze 1.0 µmole of N-Acetyl-(^{14}C)Asn(GlcNAc)$_2$-(Man)$_5$ per min at pH 5.0 at 37 °C.

Results:

Glycoprotein detection:

Thymol-sulfuric acid staining of proteins, obtained after purification of xylanase from the previous step, separated in 12.5% SDS-PAGE resulted in the formation of a red band for the high molecular mass proteins (Mr 60 kDa) as presented in Figure 4.1A. With this staining technique, no bands were visible for the low molecular mass proteins (Mr 20 kDa and 10 kDa). Figure 4.1B indicates the low and high molecular mass proteins after purification when stained with Coomassie brilliant blue R-250. When these high- and low molecular mass proteins were subjected to the lectin technique for the detection of glycoproteins, faint yellow bands were detected for both the higher and lower molecular mass proteins (figure 4.2).

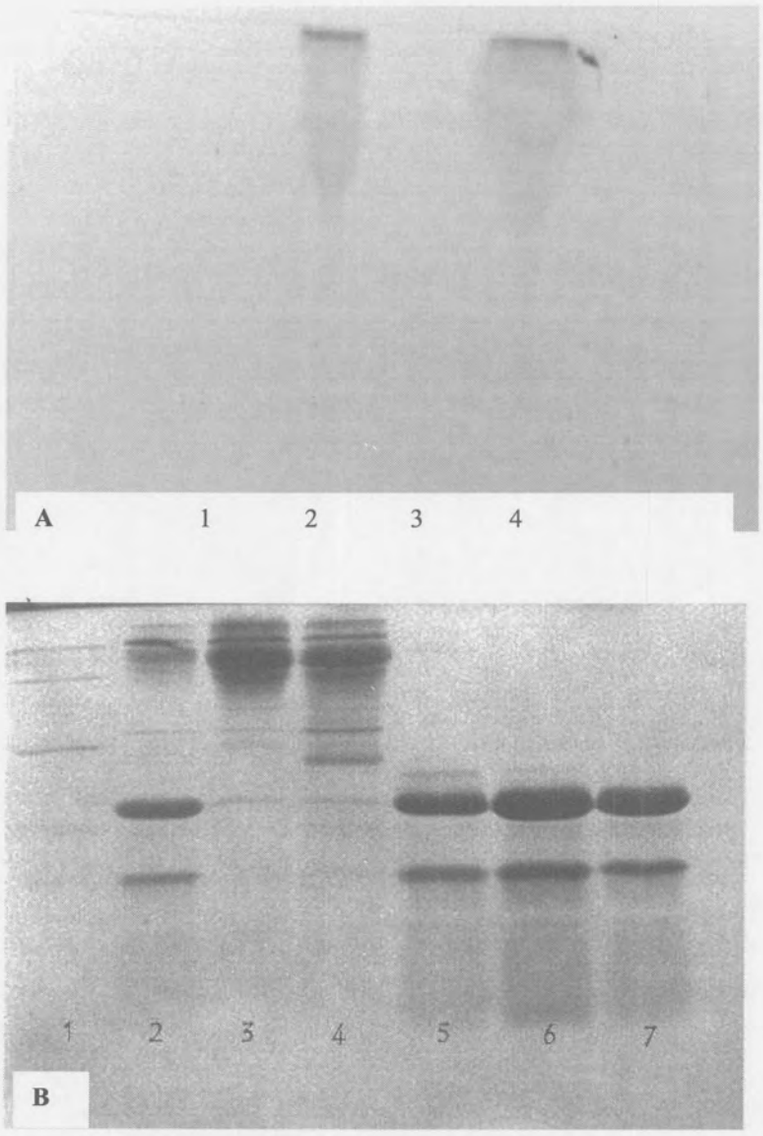

Figure 4.1. **A**: Thymol sulfuric acid staining of glycoproteins. Lane 1, negative control; lane 2, high molecular mass proteins (60 kDa); lane 3, low molecular mass proteins (20 & 10 kDa); lane 4, positive control. **B**: Coomassie blue stained proteins indicating low and high molecular mass proteins after purification. Lane 1, low molecular mass standards; lane 2, crude xylanase; lane 3-4, high molecular mass proteins (60 kDa); lane 5-7, low molecular mass proteins (20 & 10 kDa)

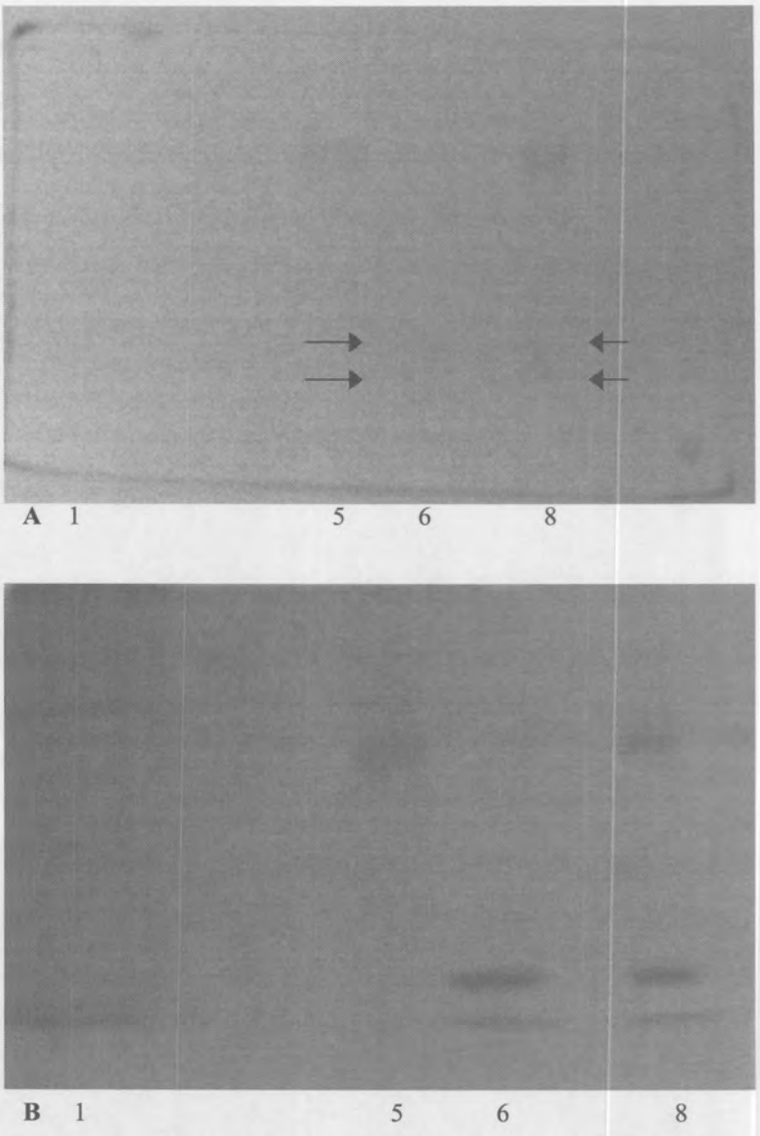

Figure 4.2. **A**: Glycoprotein detection by FITC labeled Concanavalin A method.
B: Coomassie brilliant blue counterstain of the proteins. Lane 1, molecular mass standards; lane 5, high molecular mass proteins (60 kDa); lane 6, low molecular mass proteins (20 & 10 kDa); lane 8, crude xylanase. Arrows indicate faint yellow bands of the low molecular mass proteins

Enzyme activity recovery:

Results on enzyme activity recovery after deglycosylation are presented in Table 4.1. The recovery, as a percentage of the original enzyme activity of the xylanase after enzymatic deglycosylation with PNGase F and Endo H was 93 and 98 %, respectively.

As pointed out earlier, the recovery of activity was important for subsequent work to be done on enzyme stability, as the activity of the deglycosylated xylanase would be compared with that of the naturally glycosylated xylanase against proteolytic degradation. Enzyme activity at five different time points during a 24 h incubation period of the glycosylated or deglycosylated xylanases with various protease sources would be used as an indication of the stability of the enzymes. When crude xylanase was deglycosylated with TFMS, all the original enzyme activity was lost (table 4.1). Therefore, this procedure cannot be used to produce deglycosylated xylanase for the purposes of testing enzyme activity and stability. It appears that the protein was severely denatured during this harsh acid treatment because when the protein was separated in 12.5% SDS-PAGE, it appeared as a smear across the lane. Crude xylanase was deglycosylated with TFMS to determine if this chemical method of deglycosylation could be a viable alternative to expensive enzymatic methods.

Table 4.1. Enzyme activity recovery after deglycosylation

Method	Xylanase activity (U/ml)		
	Control	Deglycosylated	Recovery of original xylanase activity (%)
Chemical[a]			
TFMS	4919.8	ND	ND
Enzymatic[a]			
PNGase F	495.4	462.0	93%
Endo H	495.4	485.5	98%

[a]Chemical deglycosylation was performed using a 2% crude xylanase solution while enzymatic deglycosylation was performed with the partially purified xylanase

Efficiency of deglycosylation:

The efficiency of deglycosylation was tested with electrophoresis. During the deglycosylation procedure with PNGase F, the GlcNAc-Asn linkage of the N-linked oligosaccarides is cleaved. Therefore a mobility shift in protein bands would be expected

as the molecular weight decreases during deglycosylation. No mobility shifts could, however, be detected after the proteins were separated in 15 % SDS-PAGE (figure 4.3) or in 7.5% - 15% gradient gels (result not shown).

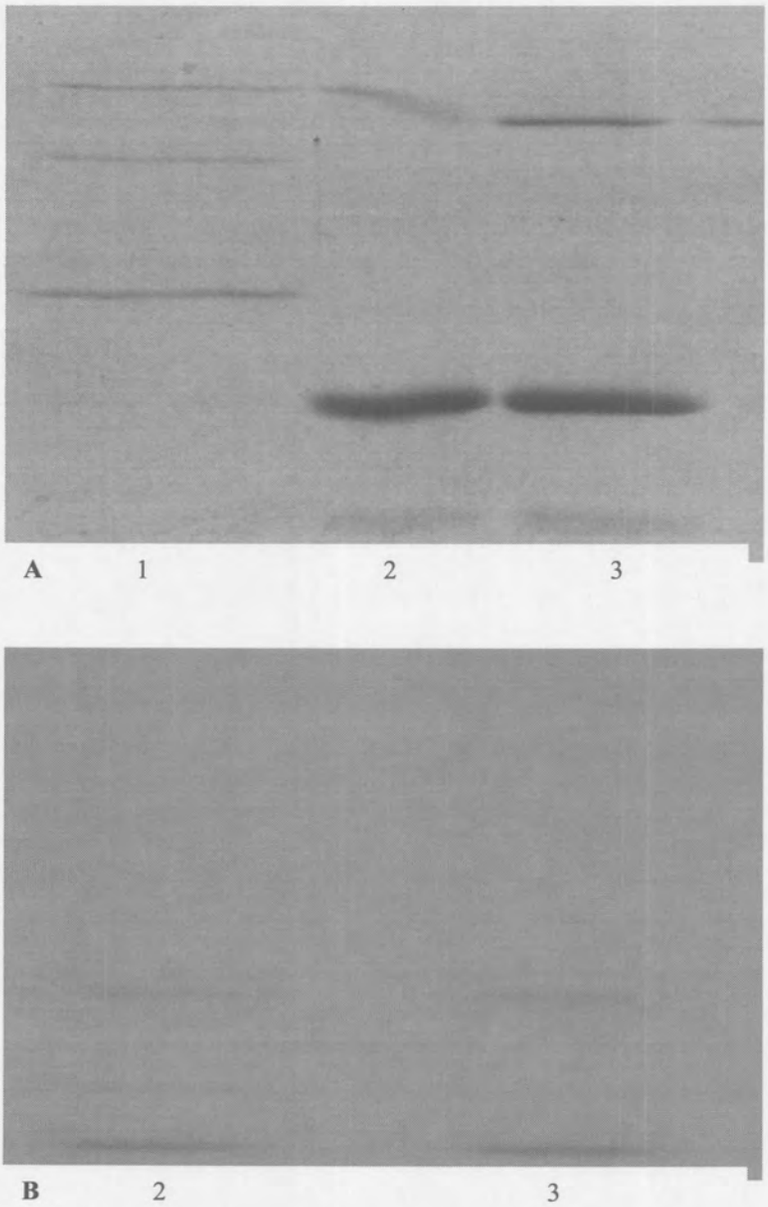

Figure 4.3. Coomassie blue stained proteins for assessing the extent of deglycosylation. Lane 1, low molecular mass standards; lane 2, control xylanase; lane 3, enzymatically deglycosylated xylanase (**A:** PNGase F deglycosylation and **B**, Endo H deglycosylation).

Discussion:

When glycoproteins were detected by the thymol-sulfuric acid method only the high molecular mass proteins (Mr 60 kDa) were stained pink/red. When glycoprotein detection was by the FITC-labelled Concanavalin A method, both the high and low molecular mass proteins stained and appeared as faint yellow bands. These results are indications that the low molecular mass proteins (Mr 20 and 10 kDa) contained only small quantities of glycoproteins because the lectin technique requires only nanogram quantities of glycoproteins while the thymol sulfuric acid method is only sensitive for microgram quantities (Gander, 1984).

After PNGase F- or Endo H deglycosylation of the partially purified xylanase, no mobility shifts were detected when the proteins were separated according to molecular mass in SDS-PAGE. Endo-N-glycosidases cleaves the GlcNAc-Asn linkage of N-linked oligosaccharides in glycoproteins and therefore an increase in mobility of the protein after deglycosylation in SDS-PAGE would be expected (Wang *et al.*, 1996). This is due to the reduction in molecular weight when the oligosaccharides are released by the deglycosylation procedure. Because only nanogram quantities of glycoproteins are present in the partially purified xylanase, this mobility shift might not be visible with separation of the proteins in SDS-PAGE and more precise methods need to be tested. More than 90 % of the enzyme activity was retained after enzymatic deglycosylation while no activity was detected after TFMS treatment. The retention of activity is important for further studies on the stability of naturally glycosylated and deglycosylated xylanase, because activity at five different time points during a 24 h incubation period with various protease sources would be used as an indication of the enzyme stability. Therefore, the chemical deglycosylation method cannot be used to produce deglycosylated xylanase and only enzymatically deglycosylated xylanases, produced using the non-denaturing protocol, would be used for further studies on the effect of glycosylation on the stability of exogenous enzymes.

In conclusion, results indicated that the partially purified low molecular mass xylanase contains glycoproteins, and due to only nanogram quantities of glycoproteins present, it is

not surprising that no mobility shifts could be detected in SDS-PAGE after deglycosylation. More sensitive techniques need to be tested to determine the exact extent of deglycosylation. The recovery of the original enzyme activity after enzymatically deglycosylating the xylanase was very high, and the stability of this deglycosylated xylanase would be compared with that of the naturally glycosylated xylanase in further studies.

Chapter 5

Stability of a partially purified xylanase form *Trichoderma longibrachiatum* in rumen fluid and *Prevotella ruminicola* culture supernatant.

Introduction:

Xylan, a major component of plant cell wall hemicellulose, is composed of a backbone of β-1,4-linked xylose units. For complete hydrolysis of xylan, many xylanolytic microorganisms often synthesize multiple groups of xylanolytic enzymes for cooperative action (Ratanakhanokchai *et al.*, 1999). Both endoxylanase and β-xylosidase are the major enzymes involved in the hydrolysis of xylan, however, a complete degradation requires the synergistic action of esterases to remove the acetyl substituents from the β-1,4-linked D-xylose backbone of xylan (Wong *et al.*, 1988 cited by Chen *et al.*, 1997). Many organisms are known to produce different xylanases; the nature of the enzymes varies between different organisms. Among them, the enzymes from fungi such as *Trichoderma* spp, deserve the most attention (Wong *et al.*, 1997) and the extensive glycosylation of these fungal enzymes might assist in protecting them against proteolytic attack in monogastric animals (Chesson, 1993). Industrially produced enzymes are increasingly being used as feed additives to improve the nutritional efficiency of farm animals. Although the application of enzyme-containing supplements is still under development in ruminant diets, they are widely utilized in the poultry and swine feed industries. A potential problem is that unprotected feed enzyme additives, particularly soluble protein, are usually degraded rapidly in the rumen (Kopency *et al.*, 1987 cited by Morgavi *et al.*, 2000). Thus, if dietary enzymes are to be effective as modifiers of rumen fermentation, the enzymes must resist proteolysis by rumen microorganisms for a time sufficiently long to affect digestion.

Recently, 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 cited by Morgavi *et al.*, 2001). Previously Chu *et al.* (1978) in studying Endo H treated enzymes, have shown that although the carbohydrate did not influence the conformation of the polypeptide backbone, its presence considerably enhanced protein stability toward heat and resistance to

proteolysis. Tanner & Lehle (1987) also observed that the less glycosylated enzymes were more susceptible to degradation by proteinases, to heat and to other denaturing conditions. The hypothesis that glycosylation is important in protecting enzymes against proteolytic degradation will be examined in this chapter.

Materials and methods:

Stability trials: Stability trials were performed using rumen fluid or *Prevotella ruminicola* culture supernatant as protease sources to estimate xylanase activity during *in vitro* incubations with the naturally glycosylated or enzymatically deglycosylated xylanase.

General xylanase stability methods: Various protease sources were incubated at 37 °C with a partially purified xylanase in its native form (control) or enzymatically deglycosylated with PNGase F or Endo H. Samples were collected in microcentrifuge tubes at 0, 3, 6, 9, and 24 h of incubation and stored at –80 °C until assayed. Before the xylanase activity, protease activity and protein concentration were determined, the samples were centrifuged at 13,000 rpm for 5 min to remove all particles. Xylanase activity is expressed as U/ml or as a specific activity, U/ μg protein.

In study 1 and 3, *Prevotella ruminicola* strain GA33 culture supernatant was used as protease source. The objective with these two studies was to compare the stability of control and PNGase F- or Endo H deglycosylated xylanase against proteolytic degradation when the protease originated from an organism with high proteolytic capabilities. In study 2 and 4, the objective was to compare the stability of the control and PNGase F- or Endo H deglycosylated xylanase when subjected to *in vitro* rumen conditions.

Study 1: Triplicate tubes containing 8.0 ml *Prevotella ruminicola* GA33 culture supernatant were incubated with 34 μl native xylanase (495.4 U/ml) or 42 μl PNGase F-deglycosylated xylanase (462.0 U/ml) and incubated at 37 °C for 24 h. Different amounts of control and deglycosylated xylanase were used in an attempt to have equal xylanase activities at 0 h.

Study 2: Control xylanase (33 µl of 495.4 U/ml) or PNGase F-deglycosylated xylanase (35 µl of 462.0 U/ml) were added to triplicate anaerobic tubes containing 9.0 ml strained rumen fluid mixture and incubated at 37 °C for 24 h.

Study 3: Control xylanase (35 µl of 495.4 U/ml) or Endo H-deglycosylated xylanase (35 µl of 485.5 U/ml) were added to triplicate tubes containing 8.0 ml *Prevotella ruminicola* GA33 culture supernatant and incubated at 37 °C for 24 h.

Study 4: Control xylanase (35 µl of 495.4 U/ml) or Endo H-deglycosylated xylanase (35 µl of 485.5 U/ml) were added to triplicate anaerobic tubes containing 9 ml of the rumen fluid mixture and incubated at 37 °C for 24 h.

Protease sources:

Strained rumen fluid: Rumen fluid was collected from a steer fitted with a rumen fistula at 09:00 and strained through four layers of cheesecloth. The fluid was centrifuged at 500 rpm for 10 min to remove large particles and 3 parts supernatant was mixed with 2 parts McDougall's artificial saliva. The McDougall's artificial saliva contained the following (per liter): 9.8 g sodium bicarbonate, 9.3 g sodium phosphate, 0.47 g sodium chloride, 0.57 g potassium chloride, 0.04 g calcium chloride (anhydrous), and 0.06 g magnesium chloride (anhydrous). The rumen fluid solution (9 ml) was then transferred to Hungate tubes, fitted with butyl rubber stoppers and kept at 37 °C. Anaerobic conditions were maintained at all times.

Culture supernatant: *Prevotella ruminicola* GA33 was obtained from Dr. K. Dawson's culture collection (University of Kentucky, Lexington, KY, USA). Cultures were started by transferring the organism, stored in rumen fluid-glucose-cellobiose-agar (RGCA) slants, to RGC liquid media. After overnight growth at 37 °C in this medium, the organism was transferred to anaerobic Hungate tubes containing Medium A and grown a further 24 h at 37 °C. The culture was maintained by frequent transfers to fresh medium A. Gram stains were performed to assure that the cultures were free from contamination. After overnight growth in anaerobic serum bottles, the stoppers were removed and the culture centrifuged twice at 3000 rpm for 10 min. The supernatant was removed, treated with 4 ppm lactoside to inhibit microbial growth and used as the protease source. The composition of broth medium A was as follow (per liter), 0.5 g

glucose, 0.5 g cellobiose, 0.5 g soluble starch, 5 g trypticase peptone, 50 ml mineral solution, 10 ml vitamin solution, 10 ml hemin solution, 10 ml volatile fatty acid solution, 10 ml ferrous sulfate, 10 ml methionine solution, 10 ml ammonium sulfate solution, 19 ml 10% sodium hydroxide, 1 ml resazurin solution, 800 ml distilled water, 50 ml sodium carbonate solution, and 20 ml cysteine solution. The composition of the rumen fluid-glucose-cellobiose medium was as follow (per liter): 0.5 g glucose, 0.5 g cellobiose, 0.5 g soluble starch, 5 g trypticase peptone, 75 ml mineral solution #1, 75 ml mineral solution #2, 400 ml clarified rumen fluid, 1 ml resazurin solution, 380 ml distilled water, 50 ml sodium carbonate solution, and 20 ml cysteine solution. All ingredients except the carbonate and cysteine solutions were mixed together and boiled in a round bottom flask. The medium was cooled under CO_2 and the carbonate and cysteine solutions added. Appropriate volumes were transferred anaerobically to either hungate tubes or anaerobic serum bottles, capped with butyl rubber stoppers and sterilized in an autoclave at 120 °C for 20 min.

Results:

Protease activity:

The protease activities of the *Prevotella ruminicola* supernatants were low for both experiments (studies 1 and 3). On average, protease activity was 0.046 mg azocasein degraded per ml per h and decreased after 9 h of incubation to 0.026 mg azocasein degraded per ml per h. Protease activity was also low in the rumen fluid used as a protease source in studies 2 and 4. Protease activity differed between batches of rumen fluid inocula used. The protease activity was less than 0.0125 mg azocasein degraded per ml per h for study 2 and 0.024 mg azocasein degraded per ml per h for study 4.

Xylanase stability:

In study 1, the xylanase activity, expressed as U/ml, was greater in the deglycosylated samples than in the control samples at the beginning of the incubation (0h). This indicates that more units of the deglycosylated xylanase were added to the culture supernatant. Therefore, xylanase activities cannot be compared directly when activity is expressed as either U/ml or as specific activity, U/mg protein. To allow for comparison

of control and deglycosylated samples, xylanase activity is expressed as a percentage by setting activity at 0 h to 100%.

Results on the xylanase stability in *P.ruminicola* culture supernatant are presented in Table 5.1 and Table 5.3. When control and PNGase F deglycosylated xylanase samples were incubated with *P. ruminicola* supernatant (study 1), there was no significant difference in percent xylanase activity remaining after incubation for 0, 3, 6 and 9 hours (table 5.1). However, xylanase activity of the deglycosylated enzyme was lower ($P<0.01$) than the control after incubation for 24 hours. There was no difference in the rate of decrease in activity between control and PNGase F deglycosylated xylanase during the initial 6 h of incubation, as reflected by the slopes of the lines. The rate of decrease was numerically greater for the deglycosylated sample than for the control sample (2.38 vs. 1.64 %/h) between 6 and 24 h incubation times (figure 5.1).

Results on the xylanase stability in rumen fluid are presented by Table 5.2 and Table 5.4. The control enzyme retained significantly more activity than the PNGase F deglycosylated sample ($P<0.05$) at 3 and 6 h hours of incubation (table 5.2). After 6 hours of incubation, no significant differences could be detected. The xylanase activity, when incubated with rumen fluid, decreased at a rate of 4.2% per hour and 3.5% per hour for the control and PNGase F-deglycosylated enzymes (figure 5.2), respectively. However, during the initial 6 hours of incubation the rate of decrease was 10 fold higher for the deglycosylated sample (6.24%/h vs. 0.65%/h). The contribution of rumen fluid to the total xylanase activity was insignificant and stayed relatively constant throughout the incubations (figure 5.3).

Table 5.1. Enzyme activities of native and PNGase F-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time,h	Control Xylanase	Deglyc Xylanase	SEM pool
	U/ml		
0	2.51	2.97	0.14
3	2.49	2.98	0.08
6	2.42	2.92	0.10
9	2.35	2.52	0.05
24	1.70	1.58	0.02
	%		
0	100.00	100.00	
3	99.00	100.61	3.07
6	96.29	98.70	4.60
9	93.35	85.39	4.43
24	67.51	53.58	2.32
Slope	-1.42	-2.10	

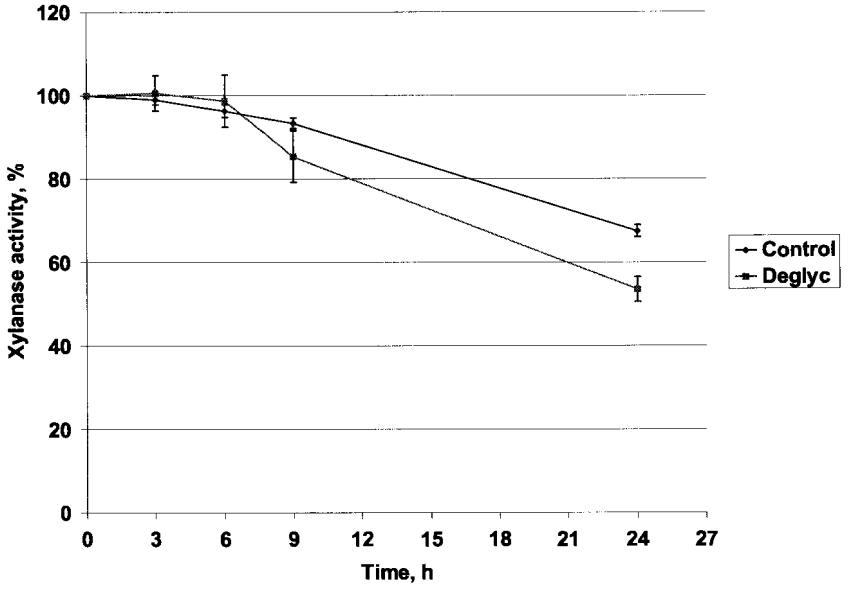

Figure 5.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Table 5.2. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time, h	Control Xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	1.67	1.71	0.07
3	1.80	1.51	0.02
6	1.59	1.09	0.09
9	1.22	1.24	0.11
24	0.13	0.24	0.03
		%	
0	100.00	100.00	
3	108.12	90.73	3.79
6	96.10	62.52	5.37
9	73.35	73.04	7.28
24	7.61	13.76	2.36
Slope	-4.27	-3.50	

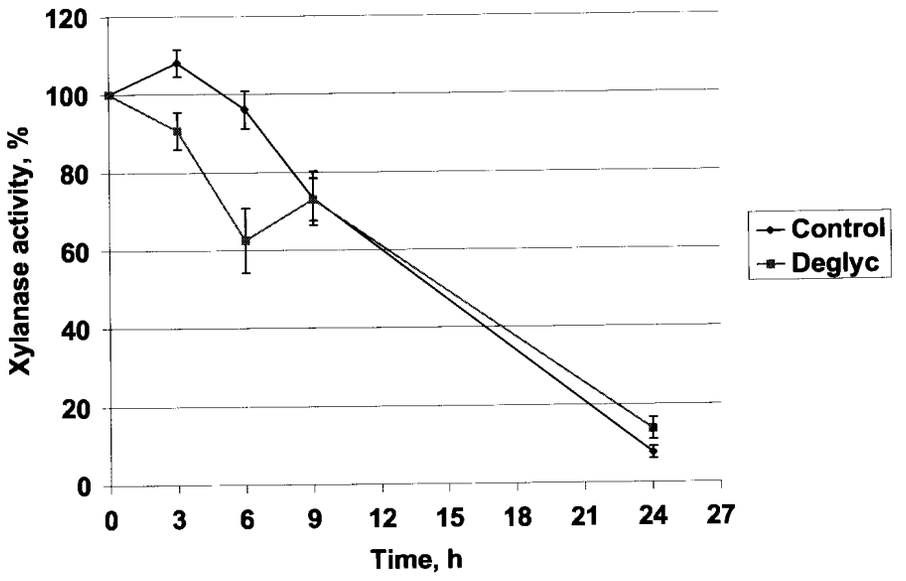

Figure 5.2. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

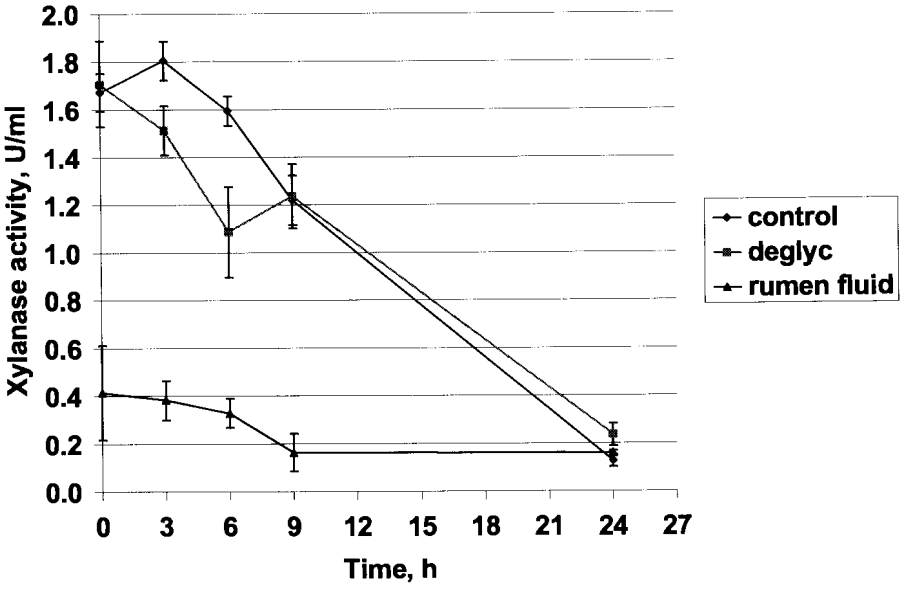

Figure 5.3. Contribution of rumen fluid to the total xylanase activity over a 24 h incubation period. Error bars represent SEM of three replicates

Results on the xylanase stability in *P.ruminicola* culture supernatant and rumen fluid when Endo H was used as deglycosylating agent are presented in Table 5.3 and Table 5.4. When the experiments were performed using Endo H deglycosylated xylanase, the xylanase activity decreased by 25% and 22% over the 24 h incubation period with *P. ruminicola* supernatant for the control and Endo H deglycosylated xylanase, respectively (table 5.3). There was no significant difference in activity at any given time point between the control and deglycosylated xylanase and the rate of decrease between the two samples did not differ (figure 5.4). In the presence of rumen fluid, the xylanase activity decreased by about 40% within the first 6 hours of incubation for both the control and Endo H deglycosylated xylanases (table 5.4) and less than 10% of the original activity was retained after the 24 h incubation period. No differences were detected in activity or the rate of decrease between the control or Endo H deglycosylated xylanase (figure 5.5).

Table 5.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with *P. ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time, h	Control xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	4.82	4.69	0.07
3	4.45	4.63	0.04
6	4.02	4.07	0.08
9	4.18	4.07	0.11
24	3.62	3.66	0.08
		%	
0	100.00	100.00	
3	92.23	98.68	1.80
6	83.35	86.79	1.59
9	86.58	86.72	1.23
24	75.13	78.03	2.65
Slope	-0.90	-0.89	

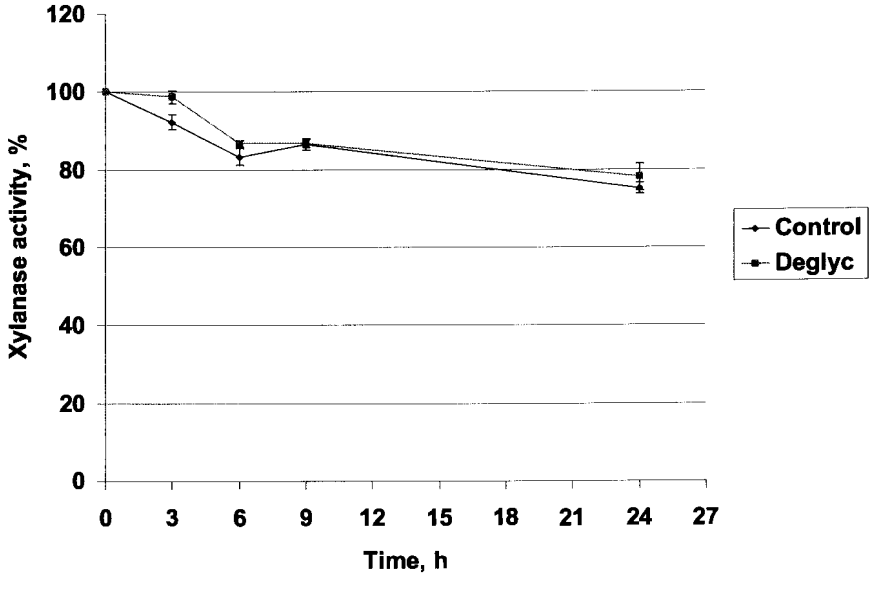

Figure 5.4. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with *P.ruminicola* supernatant at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Table 5.4. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time, h	Control Xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	4.14	4.26	0.11
3	3.42	3.43	0.09
6	2.59	2.52	0.06
9	2.18	2.24	0.10
24	0.35	0.39	0.06
		%	
0	100.00	100.00	
3	83.22	80.56	3.72
6	62.57	59.21	1.89
9	52.94	52.62	3.10
24	8.29	8.92	1.24
Slope	-3.66	-3.57	

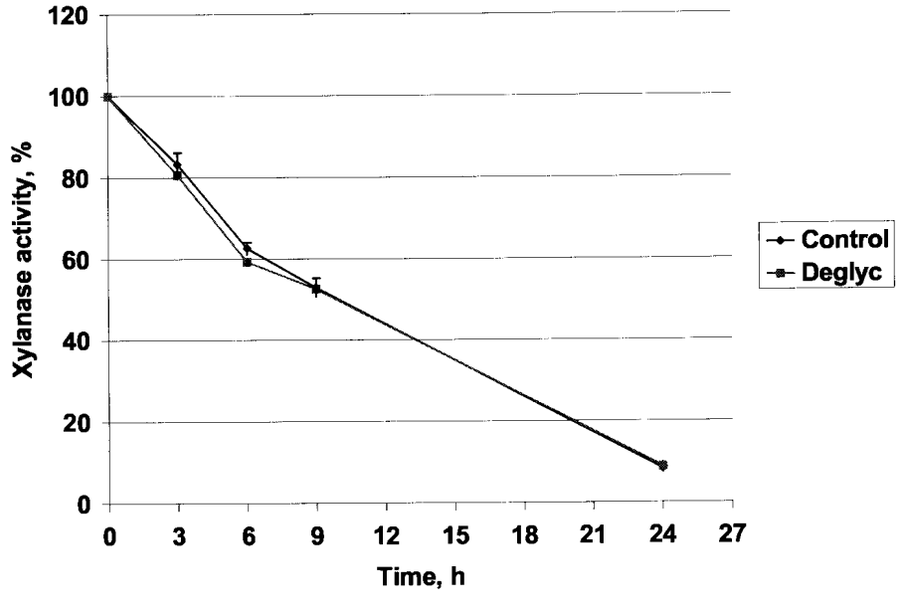

Figure 5.5. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with rumen fluid at 37 °C over a 24 h period. Xylanase activities expressed as percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Discussion:

For exogenous dietary enzymes to be effective as modifiers of rumen fermentation, they have to resist proteolysis by rumen microorganisms for a time sufficiently long to affect digestion. 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.*, 1998a). Morgavi *et al.* (2001) found that cellulase and cellulose 1,4-β-cellobiosidase were the least stable, xylanase activity decreased by 30% after 6 h of incubation with rumen fluid and β-glucanase was not affected. Results from the experiments in the current study done with a partially purified xylanase from *Trichoderma longibrachiatum* showed that this enzyme was remarkably stable in rumen fluid and *P. ruminicola* supernatant before 6 h of incubation. However, there was considerable variation between and within batches of rumen fluid. In study 2 (rumen fluid), the control enzyme retained almost all of its original activity up to 6 h of incubation, while in study 4 (rumen fluid) it had already lost about 40% of its activity at 6 h. Morgavi *et al.* (2001) found that enzymes were less stable in rumen fluid taken before feeding and proteolytic activity of rumen fluid differed between animals used. The rumen fluid for study 2 and 4 were obtained from the same donor animal, but the steer was not on a standard diet and this might explain differences observed between batches of rumen fluid. Xylanase incubated with *P. ruminicola* supernatant, an organism isolated from the rumen and known for its high proteolytic activity, retained more (>60% after 24 h incubation) of its original activity than when incubated in rumen fluid (<10% after 24 h incubation) even though the protease activity of the rumen fluid was similar to that of the *P. ruminicola* culture supernatant. The rumen microbial population is generally perceived as being highly proteolytic (Orskov, 1982; Tamminga, 1979 cited by Morgavi *et al.*, 2000). In fact, in microbial terms the activity is not particularly high; it is the high microbial biomass that causes the high extent of breakdown of susceptible proteins (Wallace & Brammall, 1985). When the culture supernatant was used as the protease source, the proteases present were produced by only one organism, while there are many protease-producing organisms present in the rumen.

The extensive glycosylation of fungal enzymes is thought to protect them against proteolytic attack in monogastric animals (Chesson, 1993). In *P. ruminicola* culture supernatant, the drop in activity for both the control and deglycosylated xylanase took place at late stages of incubation (>6h) and may not have an impact in the rumen if the enzyme effects are assumed to be important shortly after ingestion. PNGase F deglycosylated xylanase was less stable than the control enzyme only after incubation for 24 h ($P<0.01$) in culture supernatant. The drop in activity for the PNGase F deglycosylated xylanase in rumen fluid occurred earlier during the incubation period than for the control xylanase ($P<0.05$), but this effect disappeared after 6 h. It appears that the time at which the effect of glycosylation on the stability of the partially purified xylanase is observed, depends on the severity of the protease source with which it is incubated. In rumen fluid, containing protease enzymes produced by numerous microorganisms, the effect was observed much earlier than when the protease source was *P. ruminicola*, in which the protease enzymes are produced by only one organism. Because the deglycosylated xylanases lost activity earlier than the control xylanases, it is concluded that glycosylation infers some protection on the enzymes against proteolytic degradation even though the extent of glycosylation was found to be small (Chapter 4). Wang *et al.* (1996) found that the destabilization effect of deglycosylation depends on the carbohydrate content, i.e., the maximum effect was observed for the most heavily glycosylated protein, irrespective of the types (N-linked or O-linked) or patterns (mono- or multi-branched) of the covalently attached carbohydrate bands. There are indications that even very slight alterations in the sugar that decorates the exterior of a protein can cause remarkable changes in protein properties. For example, different forms of pancreatic ribonuclease B displayed a fourfold variation in hydrolytic activity although only differing in the nature of the glycans attached to the enzyme (Rudd *et al.*, 1994). Carboxypeptidase, free of carbohydrate, is degraded three times faster than the glycosylated protein (Chu & Maley, 1982) and, in the case of acid phosphatase; a 60% deglycosylated protein is hydrolyzed about 20-fold more rapidly with pepsin than the native form (Barbaric *et al.*, 1984).

No difference in stability, as reflected by the xylanase activity and the rate of decrease thereof, could be detected for the Endo H deglycosylated xylanases when incubated with either *P. ruminicola* supernatant or rumen fluid. PNGase F is a potent endo-glycosidase, cleaving most common mammalian N-linked high mannose, hybrid-, and complex-type glycans at the N-glycosidic bond (Mellors & Sutherland, 1994, cited by Wang *et al.*, 1996) and has specificity towards all asparagine-linked oligosaccharides. In addition, Endo H had also proven to be a versatile reagent for the hydrolysis of aspargine-linked high mannose oligosaccharides (Trimble & Maley, 1984), but it retains a single residue of GlcNAc attached to the asparagine. Results from the experiments conducted on the stability of control and PNGase F- or Endo H-deglycosylated xylanase indicates that Endo H deglycosylation of partially purified xylanase may not have been as efficient as PNGase-F deglycosylation because no differences in stability could be detected when the enzymes were subjected to proteolytic degradation.

Chapter 6

Stability of a partially purified xylanase from *Trichoderma longibrachiatum* in a commercial proteolytic solution and buffer

Introduction:

In this chapter, a commercial protease source was used to test the stability of the partially purified xylanase and compare it to the stability of PNGase F- or Endo H deglycosylated xylanase, even though it has been found that commercial proteolytic solutions are poor substitutes to rumen fluid (Luchini *et al.*, 1996). The objective of this study was, however, to determine the stability of native and deglycosylated xylanase under high proteolytic conditions, since, in the previous chapter, the proteolytic activity of the rumen fluid and culture supernatant was found to be low. The xylanases were, in addition to the commercial protease source, also incubated with a solution containing no protein degradative capabilities in order to establish whether any activity was lost over a 24 h period when no proteases were present.

Materials and methods:

Study 1: Control xylanase (40 µl of 495.4 U/ml) and PNGase F-deglycosylated xylanase (40 µl of 462.0 U/ml) were added to triplicate tubes containing 7.0 ml of the commercial proteolytic solution and incubated at 37 °C for 24 h.

Study 2: Control xylanase (35 µl of 495.4 U/ml) and Endo H-deglycosylated xylanase (35 µl of 485.5 U/ml) were added to triplicate tubes containing 7.0 ml of the commercial proteolytic solution and incubated at 37 °C for 24 h.

Study 3: Control xylanase (35 µl of 495.4 U/ml) and Endo H-deglycosylated xylanase (35 µl of 485.5 U/ml) were added to triplicate tubes containing 7.0 ml of 0.05 M sodium citrate buffer pH 5.3 and incubated at 37 °C for 24 h. This served as a control to the protease sources.

Protease source: A commercial proteolytic preparation from *Bacillus subtilis*, YL-NL "AMANO" (Amano Pharmaceutical Co., Ltd., Nagoya, Japan) was diluted in 0.05 M potassium phosphate buffer pH 7.2 to contain a final cell lytic activity of 10 units/ml. YL-NL "AMANO" is a cell lytic enzyme preparation produced by *Bacillus*

subtilis fermentation. *Bacillus subtilis* is historically used for fermented food production in Japan and is considered a safe organism. YL-NL "AMANO" has a cell lytic activity of not less than 350 units/ml.

Enzyme assays: The xylanase and protease activity of each sample collected at 0, 3, 6, 9, and 24 h of incubation was determined according to the methods described in chapter 2.

Results:

Results on the xylanase stability when incubated with the commercial protease from *Bacillus subtilis* are presented in Table 6.1 and Table 6.2. The proteolytic activity of the commercial enzyme solution was 1.009 mg azocasein degraded per ml per hour and was at least 20 fold and 40 fold higher than the *P. ruminicola* supernatant and strained rumen fluid, respectively. When the control and PNGase F- or Endo H-deglycosylated xylanases were incubated with this commercial enzyme preparation, both the control and deglycosylated xylanases lost more than 60 % of its original activity within the first 3 hours and retained less than 10% xylanase activity after 9 hour of incubation. The activity of the PNGase F deglycosylated xylanase was numerically smaller than the control xylanase at 3, 6 and 9 hours of incubation, but decreased at a similar rate (table 6.1). Endo H-deglycosylated xylanase lost its activity at the same rate as its control (table 6.2).

The xylanase activity was extremely variable over time when the control and deglycosylated enzymes were incubated with citrate buffer, but after 24 hours of incubation, both treatments had retained its original activity and results are presented in Table 6.3.

Table 6.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time, h	Control Xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	2.63	2.32	0.06
3	1.18	0.90	0.05
6	0.65	0.51	0.04
9	0.22	-0.26	0.48
24	0.37	0.40	0.06
		%	
0	100.00	100.00	
3	45.01	38.97	2.32
6	24.75	21.98	1.76
9	8.23	0.00	4.33
24	14.21	17.36	2.42
Slope	-2.62	-2.38	

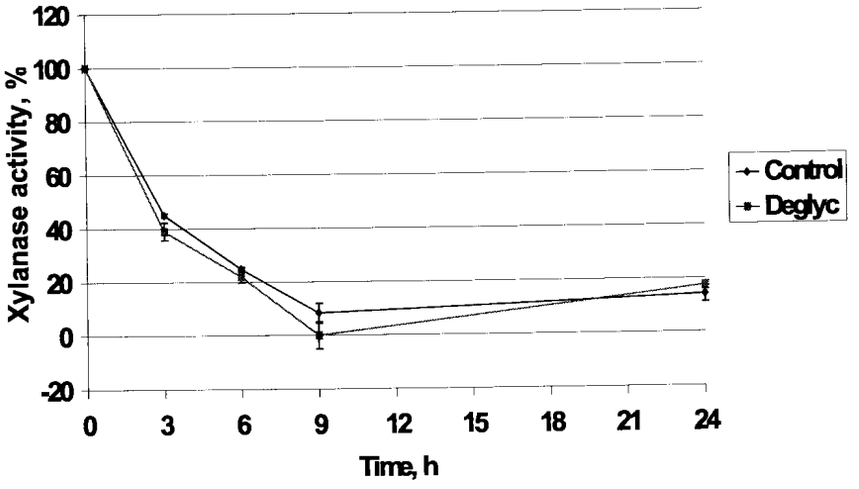

Figure 6.1. Enzyme activities of control and PNGase F-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Table 6.2. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time,h	Control Xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	3.65	3.40	0.18
3	1.18	0.96	0.14
6	0.47	0.62	0.11
9	0.37	0.43	0.09
24	0.39	0.41	0.08
		%	
0	100.00	100.00	
3	32.59	28.33	4.30
6	12.82	18.13	2.74
9	10.04	12.63	2.41
24	10.67	12.25	2.61
Slope	-2.50	-2.40	

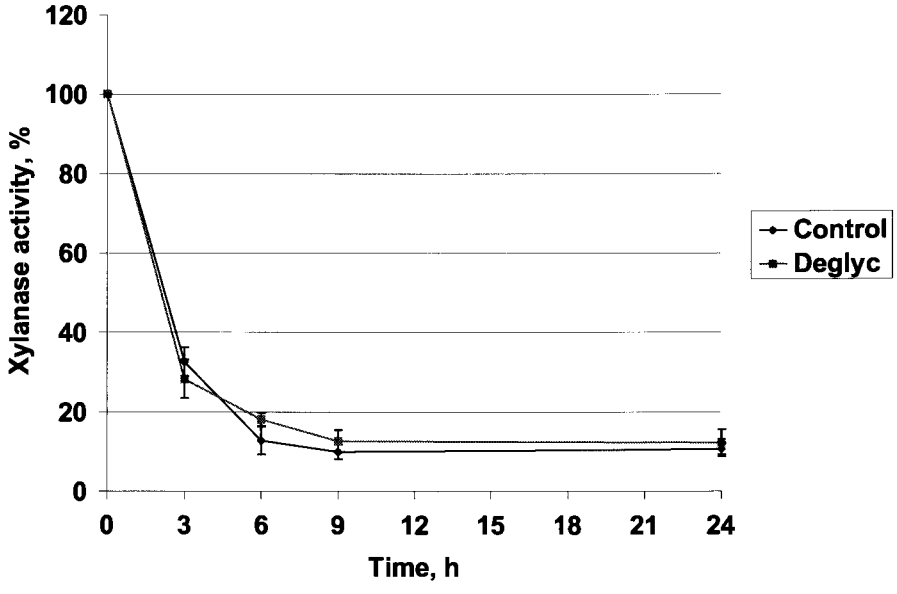

Figure 6.2. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with a commercial protease solution at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Table 6.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with citrate buffer at 37 °C over a 24 h period. Xylanase activities expressed as U/ml or as a percentage with activity at 0 h set at 100%. Values are the average of three replicates

Time,h	Control Xylanase	Deglyc Xylanase	SEM pool
		U/ml	
0	3.07	3.40	0.22
3	2.94	2.73	0.25
6	2.79	2.62	0.25
9	2.24	2.95	0.41
24	3.25	3.60	0.07
		%	
0	100.00	100.00	
3	95.57	80.24	4.88
6	91.40	78.04	10.23
9	73.76	86.84	13.27
24	106.33	106.82	5.20
Slope	0.37	0.74	

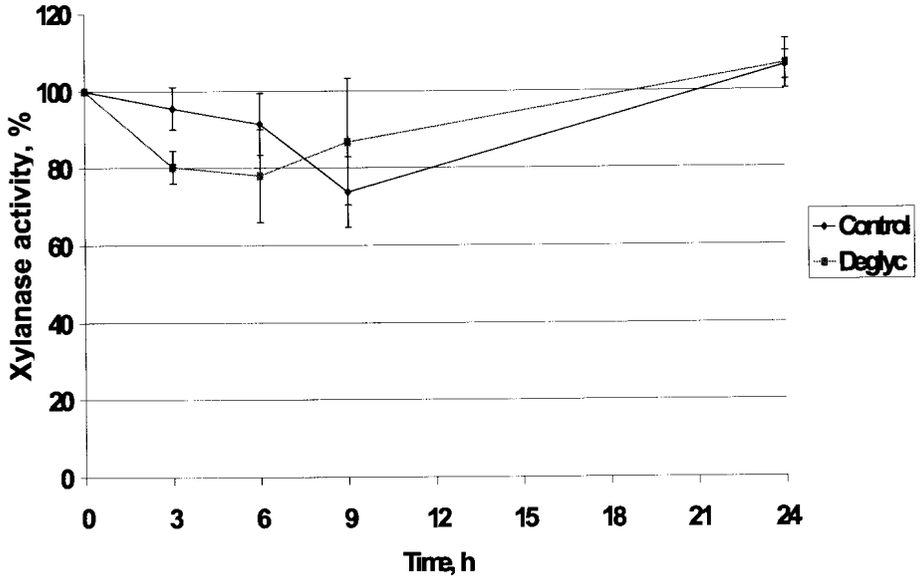

Figure 6.3. Enzyme activities of control and Endo H-deglycosylated xylanase incubated with citrate buffer at 37 °C over a 24 h period. Xylanase activities expressed as a percentage with activity at 0 h set at 100%. Error bars represent the SEM of three replicates

Discussion:

When the stability of the partially purified xylanase and deglycosylated xylanase was tested against *P.ruminicola* supernatant or rumen fluid, the enzymes retained more than 80% and 50% of its original activity after 6 hours of incubation, respectively. This high stability could be explained by the relatively low protease activities in both protease sources (0.024 mg azocasein degraded per ml per h) and an experiment was designed in which the stability of the xylanase was tested under very high proteolytic conditions (1.009 mg azocasein degraded per ml per hour). When the protease activity was increased by 20 to 40 fold by using a commercial protease source, xylanase activity was lost rapidly within the initial 3 hours of incubation. Furthermore, up to 80% of the original xylanase activity was lost within the first 6 hours of incubation. The proteolytic activity of the commercial protease was, however, so high that the stability of the glycosylated and deglycosylated xylanases observed in this experiment cannot be compared with earlier work.

Luchini *et al.* (1996) found that commercial enzyme preparations cannot mimic the protein degradative activity of strained rumen fluid. However, when Amano was used as a protease source, it was calculated to have a very high final proteolytic activity that was not comparable to either rumen fluid or *P. ruminicola* culture supernatant. This might explain why the xylanase activity was destroyed rapidly when incubated in the commercial protease solution in the experiments of the current study. The proteolytic activity of the enzyme mixture used by Luchini *et al.* (1996) was much lower and they found that even when the proteolytic activities of rumen fluid and the enzyme mixtures were similar, rumen fluid degraded the proteins two to six times faster.

There was no significant difference in stability between the control and deglycosylated xylanases and it is concluded that glycosylation can only infer protection on the enzymes when the proteolytic action of the solution is not excessively high; such is the case with rumen fluid and *P. ruminicola* supernatant.

Chapter 7

General discussion and summary:

Partially purified xylanase, containing proteins with molecular masses of 20- and 10 kDa was found to contain more than 65% of the original crude enzyme extract's xylanase activity. Furthermore, this partially purified xylanase was found to be glycosylated, although glycoproteins were only present in nanogram quantities. To evaluate the effect of glycosylation on the stability of this xylanase against proteolytic degradation under conditions similar to the rumen, the xylanase was enzymatically deglycosylated with one of two endo-N-glycosidases: PNGase F or Endo H. The efficiency of deglycosylation was tested by observing mobility shifts in SDS-PAGE or by staining protein bands with lectin specific for glycoproteins, before and after deglycosylation. Both these approaches had limited application, because the molecular weight of the xylanase was low and contained only nanogram quantities of glycoproteins. Alternatives to the destruction or elimination of the sugar moiety by the use of reagents such as glycosidases exist. They include the prevention of the attachment of the sugar to the protein during synthesis using inhibitors (Olden *et al.*, 1985) such as tunicamycin (Tanner & Lehle, 1987). Another approach is the isolation of cell mutants with defects in the biosynthesis of various intermediate glycan structures (Olden *et al.*, 1985) or cloning *T. longibrachiatum* into bacteria, such as *E. coli*, because it is known that bacteria produce non-glycosylated enzymes. With all these approaches, one faces the problem that glycosylation is important for the secretion of enzymes (Helenius, 1994). Therefore, the enzymes would have to be extracted from the cells of the organism to use them as exogenous enzyme supplements.

By incubating and comparing the stability of the enzymatically deglycosylated or original glycosylated xylanase with one of three protease sources: strained ruminal fluid, *Prevotella ruminicola* culture supernatant or "Amano", a commercial protease source from *Bacillus subtilis*, the resistance of the exogenous fibrolytic enzyme against proteolytic degradation was tested. It was observed that at relatively low protease activities, such as the strained ruminal fluid or the culture supernatant, the glycosylated

xylanase retained activity longer during a 24h incubation period than the enzymatically deglycosylated xylanase. Therefore, the glycosylated xylanase was more stable against proteolytic inactivation than the deglycosylated xylanase. Depending on the protease source, the stabilizing effect of glycosylation was observed early or late during the incubation period. For example, when the *P.ruminicola* culture supernatant was the protease source, the effect of glycosylation was observed only after 9h of the incubation period. The same effect of glycosylation on the stability of the exogenous xylanase was observed when rumen fluid was used as the protease source, although it was now observed between 3 and 6h of the incubation period and disappeared thereafter. It was concluded that the glycosylation effect was observed earlier in rumen fluid, even though rumen fluid and culture supernatant had similar protease activities, because rumen fluid contains numerous protease enzymes while, when culture supernatant was used, the protease enzymes were produced only by *Prevotella ruminicola*. It has to be pointed out that the proteolytic activity of the ruminal fluid might have been higher, had the donor animal received a standard concentrate diet.

Stability of the deglycosylated- or glycosylated xylanase was also tested with the commercial proteolytic solution, containing a very high protease activity. Under these conditions, no difference could be detected between the resistance of the deglycosylated or glycosylated enzymes to proteolytic degradation and both enzymes lost more than 90% of its original activity within the first 9h of incubation.

In terms of the objectives of this study it was concluded that glycosylation is important in inferring stability on exogenous enzymes, thereby protecting them against proteolytic inactivation under conditions similar to that of the rumen. This leads to the implication that glycosylated exogenous enzymes are stable in the rumen for a sufficiently long time to exert their effects and alter ruminal digestion.

References:

Annison, G. 1997. The use of enzymes in ruminant diets. In: Biotechnology in the feed industry, Proceedings of the 13 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics. U.K. p115.

Annison, G., Hughes, R.J. & Chocht, M., 1996. Effects of enzyme supplementation on the nutritive value of dehulled lupins. Br. Poul. Sci. 37: 157-172.

Bailey, M.J. & Poutanen, K., 1989. Production of xylanolytic enzymes by strains of Aspergillus. Appl.Microbiol.Biotechnol. 30: 5-10.

Barbaric, S., Mrsa, V., Ries, B. & Mildner, P., 1984. Role of the carbohydrate part of yeast acid phosphatase. Arch.Biochem.Biophys. 234(2): 567-575.

Beauchemin, K.A., Jones, S.D.M., Rode, L.M. & Sewalt, V.J.H., 1997. Effects of fibrolytic enzymes in corn or barley diets on performance and carcass characteristics of feedlot cattle. Can. J. Anim. Nutr. 77: 645-653.

Bernard, B.A., Newton, S.A. & Olden, K., 1983. Effect of size and location of the oligosaccharide chain on protease degradation of bovine pancreatic ribonuclease. J. Biol. Chem. 258 (20): 12198-12202.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brock, F.M, Forsberg, C.W. & Buchanan-Smith, J.G., 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl. Environ. Microbiol. 44 (3): 561-569.

Broderick, G.A., Wallace, R.J. & Orskov, E.R., 1991. Control of rate and extent of protein degradation. In: T.Tsuda, Y.Sasaki & R.Kawashime (eds.). Physiological aspects of digestion and metabolism in ruminants. PP 541-582. Academic press, San Diego, CA.

Calza, R.E., 1991. Cellulases from *Neocallimastix frontalis* EB188 synthesized in the presence of protein glycosylation inhibitors: measurement of protein molecular weights and isoelectric focusing values. Appl. Microbiol. Biotechnol. 35: 748-752.

Canton, M.C. & Mulvihill, D.M., 1983. Physico-chemical aspects of dehydrated protein rich milks. PP 339-353. Proceedings of the IDF symposium. Helsingor, Denmark.

Chen, C., Chen, J. & Lin, T., 1997. Purification and characterization of a xylanase from Trichoderma longibrachiatum for xylooligosaccharide production. Enzyme Microb. Technol. 21: 91-96.

Chesson, A., 1993. Feed enzymes. Anim. Feed Sci. Technol. 45: 65-79.

Chu, F.K., Trimble, R.B. & Maley, F., 1978. The effect of carbohydrate depletion on the properties of yeast external invertase. J. Biol. Chem. 253(24): 8691-8693.

Chu, F.K. & Maley, F., 1982. Stabilization of the structure and activity of yeast carboxypeptidase Y by its high-mannose oligosaccharide chains. Arch. Biochem. Biophys. 214: 134-139.

Davis, B.G., Maughan, M.A.T, Green, M.P., Ullman, A. & Jones, J.B., 2000. Glycomethanethiosulfonates: powerful reagents for protein glycosylation. Tetrahedron Asymmetry 11 (2000) 245-262.

Dawson, K.A. & Tricarico, J.M., 1999. The use of exogenous fibrolytic enzymes to enhance microbial activities in the rumen and performance of ruminant animals. In:

Biotechnology in the feed industry, Proceedings of the 15 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics., U.K. p303.

Edge, A.S.B., Faltynek, C.R., Hof, L., Reichert Jr. L.E. & Weber, P., 1981 Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal. Biochem. 118: 131-137.

Englard, S. & Seifter, S., 1990. Precipitation techniques. Methods Enzymol. 182: 285-300.

Feng, P., Hunt, C.W., Pritchard, G.T. & Julien, W.E., 1996. Effect of enzyme preparations on *in situ* and *in vitro* degradation and *in vivo* digestive characteristics of mature cool season grass forage in beef steers. J. Anim. Sci. 74: 1349-1357.

Fontes, C.M.G.A., Hall , J., Hirst, B.H., Hazlewood, G.P. & Gilbert, H.J., 1995. The resistance of cellulases and xylanases to proteolytic inactivation. Appl. Microbiol. Biotechnol. 43:52-57.

Fredeen, A.H. & McQueen, R.E., 1993. Effect of enzyme additives on quality of lucerne/grass silage and dairy cow performance. Can. J. Anim. Sci. 73: 581-591.

Gander, J.E., 1984. Gel protein stains: Glycoproteins. Methods Enzymol. 104: 447-451.

Gorbacheva, I.V. & Rodionova, N.A., 1977. Studies on xylan degrading enzymes. I. Purification and characterization of endo-1,4-B-xylanase from *Aspergillus niger* str. 14. Biochim. Biophys. Acta. 484:79-93.

Hatfield, R.D., Ralph, J. & Grabber, J.H., 1999. Cell wall structural foundations: Molecular basis for improving forage digestibilities. Crop Sci. 39: 27-37.

Helenius, A., 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. Mol. Biol. Cell 5: 253-265.

Howes, D., Tricarico, J.M., Dawson, K. & Karnezos, P., 1998. Fibrozyme, the first protected enzyme for ruminants: improving fibre digestion and animal performance. In: Biotechnology in the feed industry, Proceedings of the 14 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics., U.K. p393.

Hristov, A.N., McAllister, T.A. & Cheng, K.J., 1998a. Stability of exogenous polysaccharide-degrading enzymes in the rumen. Anim. Feed Sci. Technol. 76: 161-168.

Hristov, A.N., McAllister, T.A. & Cheng, K.J., 1998b. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzymes on rumen fermentation and nutrient digestibility. J. Anim. Sci. 76: 3146-3156.

Hristov, A.N., McAllister, T.A. & Cheng, K.J., 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: Effects on nutrient in cattle fed a barley grain diet. J. Anim. Sci. 78: 477-487.

Inborr, J. & Bedford, M.R., 1994. Stability of feed enzymes to steam pelleting during feed processing. Anim. Feed Sci. Technol. 46: 179-196.

Johnston, J.D., 2000. Fibrozyme and *in vitro* NDF response: moving from theory to practical commercial reality. In: Biotechnology in the feed industry, Proceedings of the 16 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics., U.K. p487.

Kopency, J., Marounek, M. & Holub, K., 1987. Testing the suitability of the addition of *Trichoderma viride* cellulases to feed rations for ruminants. Zivoc Vyroba, 32: 587-592.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680-685.

Langsford, M.L., Gilkes, N.R., Singh, B., Moser, B., Miller jr., R.C., Warren, R.A.J. & Kilburn, D.G., 1987. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. FEBS Lett. 225 (1,2): 163-167.

Leatherwood, J.M., Mochrie, R.D. & Thomas, W.E., 1960. Some effects of a supplementary cellulase preparation on feed utilization by ruminants. J. Dairy Sci. 1460-1464.

Lee, H.S., Sen, L.C., Clifford, A.D., Whitaker, J.R. & Feeney, R.E., 1979. Preparation and nutritional properties of caseinates covalently modified with sugars. Reductive alkylation of lysines with glucose, fructose or lactose. J. Agric. Food Chem. 27: 1094-1098.

Le Meste, M., Colas, B, Blond, G. & Simatos, D., 1989. Influence of glycosylation on the hydration properties of caseinates. J. Dairy Res. 56: 479-486.

Luchini, N.D., Broderick, G.A. & Combs, D.K., 1996. Characterization of the proteolytic activity of commercial proteases and strained ruminal fluid. J. Anim. Sci. 74: 685-692.

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D. & Morgan, C.A. (eds.), 1995. Animal nutrition, fifth edition. Longman, Singapore

McGilliard, M.L. & Stallings, C.C., 1998. Increase in milk yield of commercial dairy herds fed a microbial and enzyme supplement. J. Dairy Sci. 81: 1353-1357.

Mellors, A. & Sutherland, D.R., 1994. Tools to cleave glycoproteins. Trends Biotechnol. 12: 15-18.

Mizunaga, T. & Noguchi, T., 1982. The role of core-oligosaccharides in formation of an active acid phosphatase and its secretion by yeast protoplasts *Saccharomyces cerevisiae*. J. Biochem. 91: 191-200.

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

Morgavi, D.P., Newbold, C.J, Beever, D.E. & Wallace, R.J., 2000. Stability and stabilization of potential feed additive enzymes in rumen fluid. Enzyme Microb. Technol. 26: 171-177.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., McAllister, T.A Iwaasa, A.D.,Wang, Y. & Yang, W.Z., 2001. Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. J. Anim. Sci. 79: 1621-1630.

Olden, K., Bernard, B.A., Humphries, M.J., Yeo, Tet-Kin, Yeo, Kiang-Teck, White, S.L., Newton, S.A., Bauer, H.C. & Parent, J.B., 1985. Function of glycoprotein glycans. TIBS 78-82

Orskov, E.R., 1982. Protein nutrition in ruminants. New York: Academic press. 160 pp.

Paul, G., Lottspeich, F. & Weiland, F., 1986. Asparaginyl-N-acetylgalactosaminidase. Linkage unit of halobacterial glycosaminoglycan. J. Biol. Chem. 261: 1020-1024.

Pen, J., Verwoerd, T.C., van Paridon, P.A., Beudeker, R.F., van den Elzen, P.J.M., Geerse, K., van der Klis, J.D., Versteegh, H.A.J., van Ooyen, J.J. & Hoekema, A., 1993. Phytase-containing transgenic seeds as a novel feed additive improved phosphorous utilization. Bio/Technol. 11: 811-814.

PNGase F Instruction manual. Catalogue number 170-6883. Bio-Rad Laboratories, Hercules, CA.

Ratanakhanokchai, K., Kyu, K.L. & Tanticharoen, M., 1999. Purification and properties of a xylan–binding endoxylanase from alkaliphilic Bacillus sp. Strain K-1. Appl. Environ. Microbiol. 65(2): 694-697.

Rayon, C., Lerouge, P. & Faye, L., 1998. The protein N-glycosylation in plants. J. Exp. Bot. 49: 1463-1472.

Royer, J.C. & Nakas, J.P., 1991. Purification and characterization of two xylanases from *Trichoderma longibrachiatum*. Eur. J. Biochem. 202: 521-529.

Rudd, P.M., Joao, H.C., Coghill, E., Fiten, P., Saunders, M.R., Opdenakker, G. & Dwek, R.A., 1994. Glycoforms modify the dynamic stability and functional activity of an enzyme. Biochemistry 33: 17-22.

Shah, N, Mahoney, R.R. & Pellett, P.L., 1986. Effect of guar gum, lignin and pectin on proteolytic enzyme levels in the gastrointestinal tract of the rat: a time-based study. J. Nutr. 116(5): 786-794.

Stellwagen, E., 1990. Gel filtration. Methods Enzymol. 182: 317-328.

Tamminga, S., 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49: 1615-1630.

Tanner, W & Lehle, L., 1987. Protein glycosylation in yeast. Biochim. Biophys. Acta. 906: 81-99.

Trimble, R.B. & Maley, F., 1984. Optimizing hydrolysis of N-linked high-mannose oligosaccharides by Endo-B-N-acetylglucosaminidase H. Anal. Biochem. 141: 515-522.

Varel, V.H., Kreikemeier, K.K., Jung, H.J.G. & Hatfield, R.D., 1993. *In vitro* simulation of forage fibre degradation by ruminal microorganisms with *Aspergillus oryzae* fermentation extract. Appl. Environ. Microbiol. 59:3171-3176.

Wallace, R.J. & Brammall, M.L., 1985. The role of different species of bacteria in the hydrolysis of protein in the rumen. J. Gen. Microbiol. 131: 821-832.

Walsh, G., 1995. In-feed assay of enzymes by radial enzyme diffusion - recent developments and application to analysis in pelleted feed. In: Biotechnology in the feed industry, Proceedings of the 11 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics., U.K. p331.

Wang, C., Eufemi, M., Turano, C. & Giartosio, A., 1996. Influence of carbohydrate moiety on the stability of glycoproteins. Biochemistry 35 (23): 7299-7307.

Wong, K.K.Y, Tan, L.U.L. & Saddler, J.N., 1988. Multiplicity of B-1,4-xylanase in microorganisms: Functions and applications. Microbiol. Rev. 52: 305-317.

Yang, W.Z., Beauchemin, K.A. & Rode, L.M., 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. J. Dairy Sci. 82:391-403.

Zinn, R.A. & Salinas, J., 1999. Influence of Fibrozyme on digestive function and growth performance of feedlot steers fed a 78% concentrate growing diet. In: Biotechnology in the feed industry, Proceedings of the 15 th Annual Symposium. (T.P. Lyons and K.A. Jacques, eds.). Nottingham University Press, Nottingham, Leics., U.K. p313.