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Stability and stabilization of potential feed additive enzymes in rumen fluid☆

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Abstract

Four commercial preparations of fibrolytic enzymes, from *Irpex lacteus*, *Trichoderma viride*, *Aspergillus niger*, and a mixture designed to be similar to the *I. lacteus* extract, were incubated in vitro with digesta taken from the rumen of sheep receiving a grass hay/concentrate diet, and the survival of major enzyme activities was measured. Some activities, including the β-1,4-endoglucanase and xylanase from the extract derived from *Aspergillus niger*, were stable for at least 6 h in rumen fluid. The same activities in the other extracts also retained substantial activity for several hours. β-Glucosidase and β-xylosidase activities were much more labile, most being almost completely destroyed after 1 h, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that most proteins in the extracts were digested extensively after up to 7 h of incubation. Adding bovine serum albumin (0.5 g/l) to the incubation increased the half-life of *Trichoderma viride* β-glucosidase activity from less than 0.5 h to 3 h. Proteins extracted from plant materials, particularly the soybean 7S globulin fraction, also conferred protection from proteolytic breakdown, but none was as effective as bovine serum albumin. It was concluded that the stability of most fibrolytic enzymes in rumen fluid is not likely to be a limiting factor in the use of enzymes as feed additives for ruminants; but if the enzymes are not stable, means can be found for their stabilization.

Keywords: Enzymes; Ruminants; Cellulase; Xylanase; Stability; Proteolysis

1. Introduction

Industrially produced enzymes are increasingly being used as feed additives to improve the nutritional efficiency of farm animals. Enzyme-containing supplements are widely utilized in the poultry and swine feed industries [1]. Their application in ruminant diets is still under development. Early results show promise, although many factors such as the precise nature of beneficial enzymes and the diet specificity of the response, remain to be resolved [2,3]. A potential problem, which was identified previously by Kopecny et al. [4], is that protein in the diet, particularly soluble protein, is usually degraded rapidly in the rumen [5,6]. 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.

The rate and extent of hydrolysis of individual proteins are affected by their chemical structure: their secondary and tertiary conformation govern their susceptibility to proteases [5,7]. Most commercial enzyme preparations are the product of fungal fermentation, predominantly by *Trichoderma* and *Aspergillus* species, and they consist of a mixture of hydrolytic enzymes. As some of these are used as feed additives in nonruminants, they presumably resist degradation by gastric and pancreatic proteinases and may have structures that are also resistant to rumen microbial proteases.

The objective of this study was to determine the resistance of the enzyme activities in potential feed additives to rumen microbial proteolytic activity, and to investigate

☆ A paper by Hristov et al. [24] has been published since this paper was first submitted, which also concludes that fibrolytic enzymes are stable in rumen digesta. Furthermore, Hristov et al. [23] demonstrate that xylanase survives passage to the duodenum, opening up the possibility that dietary enzyme supplements for ruminants may function postruminally, as well as in the rumen.

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means of enhancing their resistance to proteolytic breakdown.

2. Materials and methods

2.1. Enzyme preparations

The enzymes used were powdered commercial preparations from fungal extracts that contained a variety of plant polysaccharide hydrolases. The extracts were from *Irpex lacteus* (Driselase, Meito Sangyo, Tokyo, Japan), *Aspergillus niger* (Energex, Novo Nordisk, Copenhagen, Denmark), *Trichoderma viride* (Roxazyme G, Roche Products, Heanor, Derbyshire DE75 7SG, UK), and a mixed product—preparation M—containing enzymes from different sources designed to simulate the activities of Driselase (Nutec, Lichfield, Staffordshire WS13 7SE, UK).

2.2. Incubations with rumen fluid

Rumen inocula were obtained from four rumen-fistulated sheep receiving a mixed diet consisting of hay, barley, molasses, fish meal, and minerals and vitamins (500, 299.5, 100, 91, and 9.5 g/kg dry matter, respectively) fed in equal meals of 500 g at 8 a.m. and 4 p.m. Rumen fluid was removed before the morning feeding and strained through four layers of muslin. Enzyme preparations, calculated to have similar carboxymethylcellulase (CMCase) activities, were added to Hungate tubes and dissolved in 1 ml of 0.1 M anaerobic sodium phosphate buffer, pH 6.5. Then 9 ml of strained rumen fluid were added, the tubes were incubated at 39°C under O_2-free CO_2 and samples were removed periodically into microcentrifuge tubes on ice. Protein concentration in the incubation mixture ranged from 0.15 to 0.30 g/l. Particulate material was removed by centrifugation at 15 000 $\times$ *g* for 15 min. The supernatant was stored at −80°C until analyzed.

2.3. Stabilization of enzyme activity

The influence of bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) or plant proteins on enzyme stability was examined by using the *T. viride* preparation. Incubations were performed as described above, with the experimental proteins being added to the enzyme solution before addition of rumen fluid. Plant proteins used were maize zein (Sigma) and those obtained from soybean and rice flours. Soluble soybean proteins were extracted with 15 volumes (w/v) of 0.03 M Tris-HCl buffer, pH 8, containing 10 mM 2-mercaptoethanol for 1 h. The slurry was mixed thoroughly at 5-min intervals and centrifuged at 45 000 $\times$ *g* for 20 min to obtain the soluble protein fraction in the supernatant. Soluble soybean globulin fractions 7S and 11S were separated as described by Aufrère et al. [8]. Rice prolamin was isolated according to the method of Hancock et al. [9]. Purity of the protein fractions was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [10].

2.4. Enzyme assays

Endocellulase, xylanase, and amylase activities were assayed by using medium viscosity carboxymethyl cellulose (CMC), oat spelt xylan, and soluble wheat starch as substrates, respectively, and all were obtained from Sigma. Assays were carried out by adding 50 μl of sample to a tube containing 100 μl of 0.1 M sodium citrate/phosphate buffer, pH 5, and 50 μl of 2% CMC, 2% oat spelt xylan, or 1% wheat starch, respectively. The mixture was incubated at 39°C for 1 h for CMCase, 30 min for xylanase or 4 h for amylase. The reaction was stopped by adding Somogyi reagent, and reducing sugars generated were measured by the Nelson–Somogyi method [11]. Incubations were also carried out with sample in the absence of added substrate and with enzyme solutions alone plus buffer in place of rumen fluid. Each sample was incubated in duplicate and results are expressed as means of samples from four sheep.

Glycosidase activities were measured in 96-well plates. Each well contained 10 μ1 of sample, 80 μl of 0.1 M sodium citrate/phosphate buffer, pH 5, and 10 μl of 50 μM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma) or 4-methylumbelliferyl-β-D-xylopyranoside (Sigma) for β-glucosidase and β-xylosidase, respectively. Substrates were prepared by diluting 20 μl of 25 mM stock dimethylformamide solution in 10 ml of water. The plates were incubated at 39°C for 5 min for β-glucosidase or 60 min for β-xylosidase, then the reaction was stopped by adding 100 μl of 1 M glycine-NaOH buffer, pH 10.6. Release of 4-methylumbelliferone was measured fluorometrically at 365-nm excitation and 450-nm emission. Each assay was carried out in triplicate. Results are expressed as means samples from four sheep.

The proteolytic activity of strained rumen fluid was measured by using digesta taken immediately before feeding, and also using digesta taken 2 h after feeding. Proteolytic activity was measured by the rate of breakdown of casein, which had been labeled with ^{14}C by reductive methylation [7]. The results are means of duplicate incubations with each sheep.

2.5. Electrophoretic analysis of enzymic properties

Electrophoresis was performed with a Multiphor II electrophoresis system and ExcelGel SDS 8–18% precast gradient polyacrylamide gels (Pharmacia, St. Albans, Herts AL1 3AW, UK). Samples were treated with SDS under reduced (in the presence of DL-dithiothreitol) or nonreduced conditions as described by Pharmacia. Proteins were stained with Coomassie Blue or silver staining and glycoproteins were detected with a Glycoprotein Detection Kit (Sigma) based on the periodic acid-Schiff reagent method.

Table 1
Enzyme activities of rumen fluid and rumen fluid with added enzyme preparations

Enzyme preparation	Concentration of enzyme prep in rumen fluid (mg/ml)	Protein content of enzyme prep (%)	Enzyme activity (nmol/min/ml)									
			CMCase		Xylanase		β-Glucosidase		β-Xylosidase		Amylase	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Rumen fluid	0	—	10.1	5.6	3.3	1.8	1.1	0.6	0.3	0.3	1.1	0.9
RF + *I. lacteus* prep	2.0	12.1	60.2	2.4	146.7	14.5	22.1	3.5	n.d.[a]		5.8	2.1
RF + *A niger* prep	4.0	8.9	47.3	4.8	26.4	5.1	5.8	1.3	n.d.		12.9	1.4
RF + *T. viride* prep	1.0	15.4	73.8	16.8	110.4	16.3	36.1	9.8	8.6	1.7	10.5	5.8
RF + preparation M	1.0	24.8	74.6	12.6	175.4	20.6	22.3	2.8	7.4	1.3	23.9	2.3

[a] n.d., The enzyme preparations contained low β-xylosidase activities (<10% of the activity of the *A. niger* preparation), so these activities were not determined in the presence of rumen fluid.

Samples were obtained after incubation of the enzyme preparations with rumen fluid. Rumen fluid from three animals obtained as described above was pooled and incubated with the enzyme preparations under CO_2 for 7 h in a shaking water bath at 39°C. After incubation, particulate material was removed by centrifugation and the supernatant was dialyzed for 24 h against distilled water. Sample aliquots were stored at −80°C until electrophoresis. For 0 h incubations, chilled rumen fluid was added to the enzymes and kept on ice for 1 to 2 h to allow enzyme solubilization.

3. Results

3.1. Addition of enzymes to rumen fluid

The enzyme mixtures were added to rumen fluid in quantities sufficient to give a significant increase in the total extracellular CMCase activity of rumen fluid (Table 1). As a consequence, xylanase, amylase, β-glucosidase, and β-xylosidase activities also increased (Table 1). All of these enzyme activities were measured against a background of similar activities already present in rumen fluid. However, because the assays were carried out by using supernatants after centrifugation, from which microorganisms had been removed, the interference in measuring the added enzymes was not great (Table 1). Furthermore, all enzyme activities present in extracellular rumen fluid remained relatively constant throughout the incubations (not shown).

3.2. Proteinase activity of rumen fluid

The proteinase activity of strained rumen fluid was measured by using ^{14}C-labeled casein. Proteolytic activity in the four sheep was 2.10, SD 0.54 and 2.19, SD 0.57 mg casein hydrolyzed/h/ml strained rumen fluid immediately before and 2 h after feeding, respectively.

3.3. Stability of enzyme activity in rumen fluid

β-1,4-Endoglucanase activity in the *A. niger* preparation, measured by the release of reducing sugars from CMC, was particularly stable when added to rumen fluid in vitro, with no apparent decrease in activity after 6 h of incubation in rumen fluid (Fig. 1A). The other enzyme preparations had a half-life of 2 h for *T. viride* and about 4 h for *I. lacteus* and preparation M (Fig. 1A). Activity against xylan was more stable than the CMCase activities, except for *I. lacteus*, which lost 60% of its xylanase after 2 h of incubation (Fig. 1B). *A. niger* xylanase was not affected by the incubation, whereas *T. viride* and preparation M retained nearly 75% of the original activity at the end of the incubation period (Fig. 1B). Amylase was not a predominant activity in any the preparations. The highest amylase activities were detected in preparation M and *T. viride*, but they were not stable. The *A. niger* enzyme was again the most stable (Fig. 1C).

β-Glucosidase and β-xylosidase activities were unstable in all the preparations, particularly those of *T. viride* (Fig. 2). Again, *A. niger* retained activity better than other preparations for β-glucosidase. β-Xylosidase activities in *I. lacteus* and *A. niger* were too low to be detected accurately.

3.4. Stabilization of enzyme activity by adding slowly degraded proteins

As incubations of crude enzyme extracts with rumen liquor had indicated that some of the constituent enzyme activities might be unstable, the possibility was investigated that enzyme activity might be stabilized by the addition of proteins resistant to rumen degradation. When bovine serum albumin was added to rumen fluid along with *T. viride* extract, the half-life of β-glucosidase was extended from less than 0.5 h with no added protein to 3 h in the presence of 0.5 mg/ml BSA (Fig. 3A). Protection was greater at higher concentrations of BSA. Several plant proteins were extracted and tested in the same way for their effects on the rate of β-glucosidase inactivation by rumen microorganisms (Fig. 3B). The soybean 7S globulin fraction conferred the best protection, closely followed by the total soluble soybean protein fraction. Rice prolamin showed some protection, but only at a high protein concentration (1 mg/ml). The 11S soybean globulin fraction and zein were not effective in prolonging the β-glucosidase half-life.

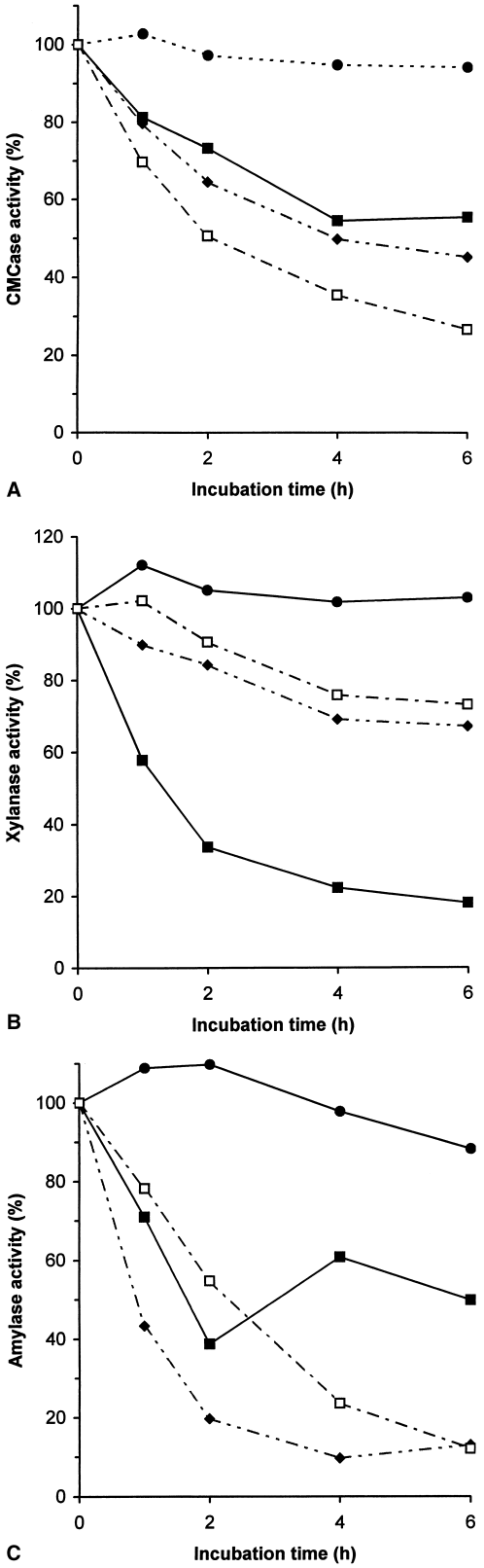

Fig. 1. Stability of (A) cellulase (CMCase), (B) xylanase, and (C) amylase activities from different enzyme preparations when incubated with rumen fluid. Activity at zero time was set at 100%. The enzyme preparations were from *I. lacteus* (■), *A. niger* (●), *T. viride* (□) and a mixture of sources, preparation M (♦).

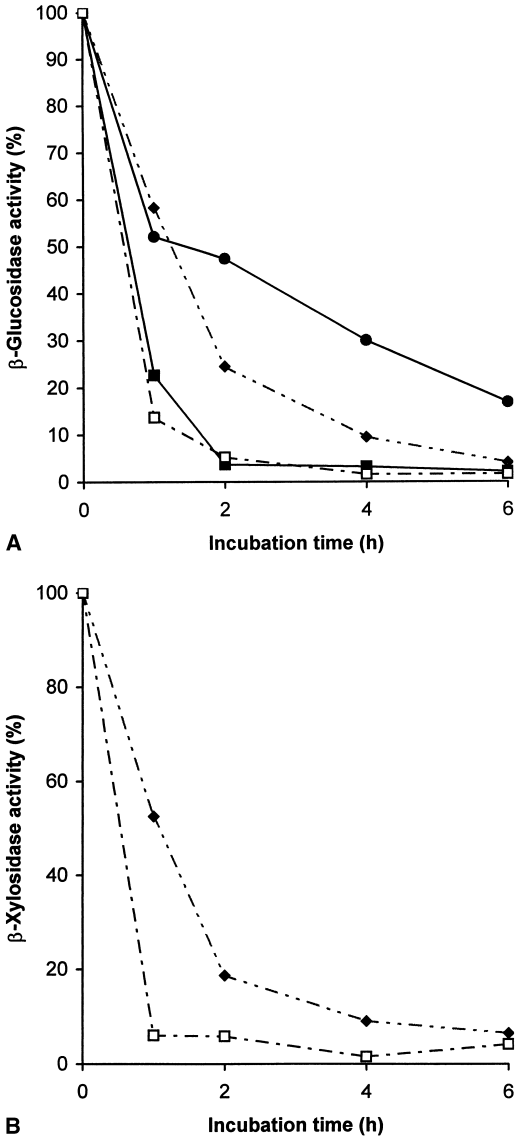

Fig. 2. Stability of (A) β-glucosidase and (B) β-xylosidase activities from different enzyme preparations when incubated with rumen fluid. Activity at zero time was set at 100%. The enzyme preparations were from *I. lacteus* (■), *A. niger* (●), *T. viride* (□), and a mixture of sources—preparation M (♦).

3.5. Relation between enzyme stability and chemical structure

The electrophoretic mobility of proteins present in the enzyme extracts was determined in SDS-PAGE, with and without reduction, and before and after incubation with rumen fluid (Fig. 4). Multiple protein bands were present in all of the extracts, particularly *I. lacteus*, and most of these bands disappeared after incubation in rumen fluid. However, certain bands appeared to survive after 7 h of incubation (Fig. 4). The bands that survived this incubation were different in the presence and absence of dithiothreitol, indicating that they were cross-linked by disulfide bonds. In

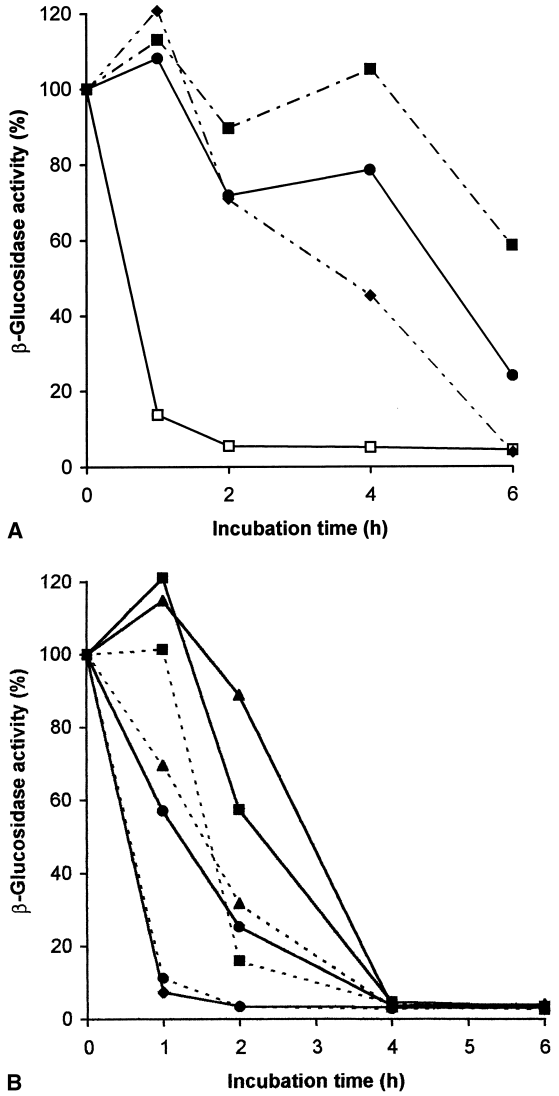

Fig. 3. Stability of β-glucosidase activity from *T. viride* (A) when incubated with rumen fluid containing different concentrations of BSA: no added BSA (□); 0.5 mg BSA/ml (♦); 1 mg BSA/ml (●), 5 mg BSA/ml (■); and (B) when incubated with rumen fluid containing plant proteins: no addition (♦), soluble soybean protein (■), soybean 7S fraction (▲), and rice prolamin (●). Dotted and continuous lines represent protein concentrations of 0.5 and 1 mg/ml respectively. Data obtained with additions of soybean 11S fraction and zein are not shown: they did not differ significantly from the activities measured in the absence of added protein. Activity at zero time was set at 100% in both graphs.

general, however, the banding patterns in the presence and absence of dithiothreitol were completely different, and it was not possible to correlate the banding patterns. An attempt was made to associate this characteristic with the presence of cystine in the protein molecule, by analysis of bands excised from the gels, but no conclusive results were found. Gels were stained for glycosylated proteins: glycosidation was a common feature in all the enzyme preparations (results not shown). However, it appeared that there was no direct relation between resistance to proteolysis and the presence of sugar moieties in proteins.

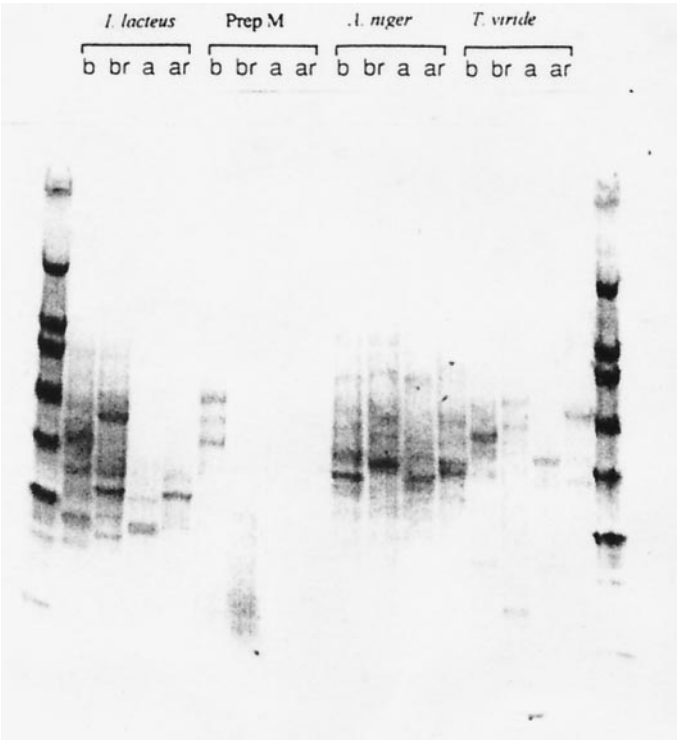

Fig. 4. SDS-PAGE of enzyme preparations before (B) and after (A) incubation with rumen fluid under reducing (r) or non-reducing conditions. Lanes at either end are molecular mass markers, containing myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The gel was stained with Coomassie Brilliant Blue G-250.

4. Discussion

The effectiveness of dietary enzymes in nonruminants is assumed to stem partly from their marked resistance to proteolytic degradation [12]. If enzymes are to be effective in diets fed to ruminants, it is reasonable to assume that similar resistance will have to be among their properties. Indications from an earlier study [4] were that cellulases from *T. viride* were not stable when incubated with rumen digesta. The rumen microbial population is generally perceived, because of the significance of rumen proteolysis in depriving the host animal of much of the protein it consumes [13], as being highly proteolytic [14,15]. In fact, in microbial terms the activity is not particularly high; it is the high microbial biomass and long residence time of protein in the digesta, which causes the high extent of breakdown of susceptible proteins [16]. Not all proteins are susceptible to rapid breakdown, depending on their solubility [5,6] and structure [6,7]. The susceptibility to ruminal breakdown of the proteins and enzyme activities present in several commercial enzyme preparations was therefore measured in order to assess whether fibrolytic enzymes were likely to be sensitive to ruminal proteolysis, and if this was likely to be an obstacle to their potential application as feed additives for ruminants.

The experiments were done by adding enzyme mixtures

to rumen fluid taken from sheep receiving a grass hay/concentrate diet. The rumen fluid was strained through muslin to remove large particulate material and therefore to aid pipetting. The rumen fluid was also taken before the morning feeding in order to minimize problems associated with soluble sugars being present in the rumen fluid after feeding, which would affect the subsequent measurement of glycanase activities. It was established that the proteolytic activity of rumen fluid immediately before and 2 h after feeding was similar, suggesting that variation in proteinase according to the time of sampling would not have a large bearing on the results; however, the straining process would remove many of the solids-associated microorganisms, and the proteolytic activity of the strained rumen fluid would inevitably be lower than whole digesta. The extent of this underestimate was not determined; however, another advantage of taking prefeeding samples would be that the solids-associated microbial population would presumably be minimal at this time.

After incubation of the enzyme preparations with the strained rumen fluid, small particulate material, principally microorganisms, was removed by centrifugation. The background cell-free activity due to rumen fluid was usually much lower than the activity of enzymes added, and it was in any case fairly stable. It should be noted that enzyme activity determinations were performed at pH 5, closer to the optimal pH of most fungal enzymes than the usual pH of rumen fluid, which is 6–6.5.

SDS-PAGE indicated that most of the proteins present in the mixtures disappeared after incubation in rumen fluid for several hours, with only a few bands remaining. The resistance of the enzyme activities tested was variable. Some of them survived for several hours, whereas others lost most of their activity within 1 h. Enzymes produced by *A. niger* appeared to be the most stable in the rumen. The *A. niger* preparation, however, was adsorbed on to a granular carrier which might have contributed to extending the enzymes' half-life. Whether enzymes in this form would be as effective as soluble enzymes in stimulating digestion would have to be established by further experimentation. Cellulases and xylanases were particularly stable in most preparations, whereas glycosidases were rapidly inactivated. All fungal species used in this work produce several cellulases and xylanases as part of their cellulolytic and xylanolytic systems [17–20]. The stability of CMCase and xylanase activities in the present 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, enzymes are usually added to the feed in a liquid form before they are given to the animal and the presence of substrate further contributes to stability [12].

Identification of protein characteristics that are associated with increased stability to proteases such as disulfide bridges and glycosylation was not particularly successful in this study, because most proteins in the extracts altered their electrophoretic mobility in the presence of dithiothreitol and most appeared to be glycosylated. The proportion of disulfide cross-links in proteins is known to be related to the rate of rumen degradation [6,7]. In the present experiments, the different banding patterns in normal compared with nondenaturing conditions indicate only that disulfide bonds were present, while giving no indication of their number, therefore it was not possible to judge how important disulfide bridges were in conferring stability. As mentioned above, many of the enzymes produced by these fungal species are glycosylated [17–21], which often protects enzymes from inactivation by temperature and proteinases [22]. However, no relation was found here between this post-translational modification and resistance to rumen proteinases. Indeed, *T. viride* β-xylosidase is glycosylated [21], yet was unstable, whereas its main xylanase is not glycosylated [23], and was much more stable.

The activity of the labile β-glucosidase of *T.viride* was extended by the addition of BSA in the incubation mixture. BSA is a soluble protein known to be highly resistant to proteolysis in the rumen [5–7]. Many plant proteins have been also shown to resist degradation in the rumen [8,9] and some of them were tested for their protective effect. It appeared that solubility was a more important factor in determining the protective effect of a protein than resistance to proteases. The insoluble maize zein, which is stable in the rumen [9], failed to protect the β-glucosidase from proteolysis. However, soybean globulin fraction S7, which is degraded rapidly in the rumen, was more effective in protecting the β-glucosidase than the more resistant S11 fraction [8]. The S11 fraction precipitates at pH 6.6 or lower and this could be the reason for its lack of protection. The use of soluble proteins, that can be part of the protein supplement, may therefore be a simple device for prolonging the half-life of labile enzymes if they are to be used as feed additives for ruminants.

It is concluded that the stability of fibrolytic enzymes in rumen fluid is unlikely to be a limiting factor in their application as feed additives for ruminants. Particular enzyme activities may, however, be more labile than others. If such an activity is a key component of the mode of action of feed-additive enzymes for ruminants, it would appear likely that enzyme activity might be protected by simple means. The emphasis should now be placed on identifying which, if any, fibrolytic activity is limiting in the rumen and on devising means of amplifying that activity. Applying enzymes as feed supplements promises to be the simplest technology for achieving such an amplification in the immediate future.

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