Proteinase Activity in Rumen Ciliate Protozoa

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Azocasein-degrading proteinase activity was detected in all rumen ciliate protozoa that were examined from four entodiniomorphid and two holotrich genera. All of the activities were optimal in the range pH 4.0–5.0 and were inhibited by cysteine proteinase inhibitors, notably leupeptin. The inhibition profiles and extent of inhibition observed with the different groups of inhibitors were organism-specific. Gelatin-SDS-polyacrylamide gel electrophoresis of protozoal lysates revealed multiple forms of the proteinases in the species examined. The number of enzymes detected, their molecular masses, the level of activity and inhibitor susceptibility was genus-dependent. The proteinase profiles of the two holotrich species differed and inter-species differences were also apparent among species of the genus *Entodinium*. The characteristics and molecular size distribution of rumen bacterial proteinases were different to the protozoal proteinases. Low levels of proteinase activity, of apparently bacterial origin, were detected by gelatin-SDS-PAGE analysis of cell-free rumen liquor.

INTRODUCTION

The degradative activities of the rumen microbial population adversely affect the availability of dietary protein to the host ruminant. The proteolytic bacteria are mainly involved in the degradation of plant protein (Nugent & Mangan, 1981), whereas the rumen ciliate protozoa are thought to degrade bacterial and insoluble particulate proteins (Hino & Russell, 1987; Wallace *et al.*, 1987). The predation of the bacteria by ciliates increases the rate of bacterial protein turnover (Wallace & McPherson, 1987) and decreases the efficiency of bacterial growth in the rumen (Veira, 1986), and defaunation (i.e. elimination of protozoa from the rumen) of animals receiving low protein diets has resulted in enhanced animal productivity and wool growth (Bird & Leng, 1983, 1984). Nevertheless, despite the apparent ecological and economic importance of protozoal proteolysis, the proteolytic enzymes of the rumen ciliates have not been well characterized.

The studies undertaken to date that demonstrate protozoal proteolysis have either compared protein breakdown in faunated and ciliate-free rumen contents (Shinchi *et al.*, 1986; Wallace & McPherson, 1987; Wallace *et al.*, 1987) or have used washed suspensions of mixed populations of protozoa, that contained both holotrich and entodiniomorphid ciliates, to examine proteolysis (Onodera & Kandatsu, 1970; Forsberg *et al.*, 1984) and amino acid metabolism (Williams, 1986). Some properties of the intracellular (Brock *et al.*, 1982; Forsberg *et al.*, 1984) and extracellular (Shinchi & Kandatsu, 1980, 1981, 1983) proteinases present in mixed ciliate populations have also been described. Information on the proteinases of individual species of protozoa is, however, very limited. Proteolysis has been demonstrated in representative species from eight genera of rumen entodiniomorphid ciliates, isolated directly from the rumen or

Abbreviations: TLCK, *N* α -*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulphonyl fluoride.

grown *in vitro* (Abou Akkada & Howard, 1962; Naga & El-Shazly, 1968; Coleman, 1983), although only with *Entodinium caudatum* have the enzymes involved been studied (Abou Akkada & Howard, 1962; Coleman, 1983). Here we describe the characteristics of the proteinases present in ciliate protozoa from both holotrich and entodiniomorphid genera.

METHODS

Preparation of protozoal suspensions. The protozoal species studied were all recovered directly from ovine rumen contents that had been withdrawn 2 h after the ration had been consumed. *Dasytricha ruminantium*, *Ophryoscolex caudatus* and *Isotricha* spp. were isolated by filtration from the rumen ciliate population of two animals receiving a diet of molassed sugar beet pulp (600 g d⁻¹) and hay (300 g d⁻¹) as previously described (Williams & Yarlett, 1982). The *O. caudatus* preparation was collected on a 55 µm porosity filter after an initial pre-filtration (64 µm porosity cloth) to remove all the large and most of the intermediate sized protozoa. The protozoa retained by the 55 µm porosity filter were repeatedly resuspended in sterile salts solution D (Coleman, 1978) and allowed to stand for 2–3 min at 39 °C, resulting in the differential sedimentation of *O. caudatus* and *Isotricha* spp. Contaminating *Isotricha* spp. were lysed by incubation at 39 °C for 60 min in salts solution D containing mannose (10 mg ml⁻¹). The subsequent cell preparation was washed thoroughly with sterile salts solution on a 30 µm filter. This resulted in a single-species preparation of *O. caudatus* as described by Ogimoto & Imai (1981).

Epidinium caudatum ecaudatum/*Entodinium simplex* and *Polyplastron multivesiculatum*/*Entodinium* spp., originally isolated from rumen contents and kindly supplied as cultures by Dr G. S. Coleman, were present as co-cultures in animals receiving a daily diet of hay (500 g) and goat mix (a commercially compounded feed containing rolled barley, rolled oats, wheat feed, soya bean, flaked maize and minerals; 500 g). The co-cultures were the only protozoa present and had been introduced into, and established in, the ruminal contents of ciliate-free (defaunated) animals. The protozoa were readily separable, without any cross-contamination, by differential filtration and were collected on defined aperture textiles of the appropriate porosity following pre-filtration through a 100 µm porosity filter cloth (*Ep. caudatum ecaudatum* 30 µm; *En. simplex* 15 µm; *P. multivesiculatum* 40 µm; mixed entodinia 15 µm). The larger protozoa were washed thoroughly on a 30 µm porosity filter; *En. simplex* and the mixed *Entodinium* spp. preparations were washed five times by resuspension in sterile salts solution D and recovered by centrifugation (500 g, 1 min, room temperature). Anaerobiosis was maintained during the isolation procedures and the filter-sterilized (0.45 µm filter) salts solution was deoxygenated by autoclaving and gassing with N₂ and pre-warmed to 39 °C prior to use as previously described (Williams & Yarlett, 1982). The washed protozoal suspensions were sedimented by centrifugation (500 g, 1 min, room temperature) and resuspended in sterile deoxygenated distilled water. The contamination of the preparations by other species of protozoa was negligible (<1%) (Williams & Yarlett, 1982). All protozoal preparations were extensively washed with sterile buffered salts solution to ensure complete removal of free bacteria and also to minimize contamination of the protozoa by adherent and ingested bacteria. Since some bacteria may have been present in all the preparations, and so bacterial enzymes could have contributed to the proteinase activity detected in the protozoal preparations, control experiments on a population of mixed rumen bacteria were undertaken.

Mixed rumen bacteria were prepared from the rumen contents, withdrawn 2 h after feeding, from a sheep receiving the hay and goat mix ration. The rumen contents were initially filtered through cheesecloth to remove the large particulate material and were pre-centrifuged (1000 g, 10 min, 4 °C) to remove the protozoa and small particulate debris. The supernatant material was carefully decanted and centrifuged (38 000 g, 30 min, 4 °C) to sediment the bacteria. The supernatant was taken as the cell-free rumen liquor fraction. The bacterial cell pellet was resuspended by homogenization in sterile deoxygenated distilled water.

The samples were stored at -20 °C in screw-capped ampoules with a N₂ headspace. Prior to use, Triton X-100 was added to 0.25% (v/v), which resulted in the complete lysis of the protozoa as judged by microscopic observation. The lysates were centrifuged (10 000 g, 5 min, room temperature) and the supernatants used for the enzyme analyses.

Proteinase assays. Proteinase activity was assayed using azocasein as the substrate as previously described (Coombs, 1982). Assays were done at 39 °C in duplicate or triplicate. An increase of 1 absorbance unit was caused by the hydrolysis of 0.4 mg azocasein under the reaction conditions stated. Buffers used were 0.1 M-glycine/HCl (pH 3.0), 0.1 M-sodium acetate/acetic acid (pH 3.5–5.5) and 0.1 M-sodium phosphate (pH 6.0).

Protein concentration was determined by the method of Sedmak & Grossberg (1977) using bovine serum albumin as standard.

The effect of proteinase inhibitors was tested by the addition to cell lysates of pepstatin and leupeptin at a final concentration of 100 µg ml⁻¹ and *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulphonyl fluoride (PMSF), 1,10-phenanthroline and iodoacetic acid at a final concentration of 1 mM. The lysates were then incubated at room temperature for 1 h prior to assaying for proteinase activity at the optimum pH. Pepstatin and TPCK were dissolved in 5% (v/v) dimethylsulphoxide

(DMSO), PMSF was dissolved in ethanol and the remaining inhibitors were dissolved in water. Controls, preincubated and assayed without inhibitor but with water, ethanol or DMSO, were done as appropriate.

Gelatin-SDS-PAGE. Analysis of the proteinases of protozoal lysates was done by gelatin-SDS-PAGE (Lockwood *et al.*, 1987). The acrylamide concentration of the separating gel was 7.5% (w/v) and electrophoresis was done at 6 mA per gel (175 mm $\times$ 140 mm, 1.5 mm thick) overnight. The proteinase bands were developed in 0.1 M-sodium acetate/acetic acid buffer pH 5.0 containing 1 mM-dithiothreitol (DTT) for 4 h at 39 °C. The bands were visualized by staining with PAGE blue 83 and destaining as previously described (Lockwood *et al.*, 1987). Molecular masses of individual proteinases were determined from the mobility of protein standards. The mobility of these standards was not affected by the presence of gelatin.

The effect of inhibitors on the proteinases was tested by the addition of leupeptin and pepstatin at 20 μ g ml⁻¹ and iodoacetic acid, PMSF, and 1,10-phenanthroline at 1 mM to the incubation buffer.

Materials. Iodoacetic acid, TLCK, TPCK, 1,10-phenanthroline, DTT, azocasein, gelatin (type A: from porcine skin, approx. 300 Bloom), leupeptin and pepstatin were obtained from Sigma. PMSF was supplied by Boehringer. All other reagents were AnalaR grade and obtained from BDH. The defined aperture textiles were obtained from Henry Simon Ltd, Stockport.

RESULTS

Proteinase activity towards azocasein was detected in cell lysates of all the protozoa investigated, although at different levels. Relatively low activities were detected in *Isotricha* spp. whereas the levels were higher in the putatively closely-related *D. ruminantium*. Activities in the entodiniomorph species ranged from 2.4 μ g azocasein hydrolysed min⁻¹ (mg protein)⁻¹ in *O. caudatus* to 12.0 μ g azocasein hydrolysed min⁻¹ (mg protein)⁻¹ in *E. simplex* (Fig. 1). All of the activities were optimal at acidic pH in the range 4.0–4.5 (Fig. 1) and some were markedly stimulated by the addition of 1 mM-DTT although it had no significant effect on the activity detected in *O. caudatus* (Table 1). The proteinase activities of all species were inhibited by cysteine proteinase inhibitors, notably leupeptin, but were relatively unaffected by inhibitors of other classes of proteinase (Table 1).

Azocasein-hydrolysing activity was also detected in rumen bacteria. The activity differed, however, from that found in the protozoa in that it was maximal [6.9 μ g azocasein hydrolysed min⁻¹ (mg protein)⁻¹] at a higher pH (5.0), had considerable activity at pH 6.0 and was apparently unaffected by 1 mM-DTT (Table 1). The inhibition profile of the bacterial activity (Table 1) showed that it was less affected by inhibitors of cysteine proteinases. No activity towards azocasein was detected (limit of detection, 0.01 μ g azocasein hydrolysed min⁻¹) in the extracellular rumen liquor, although low levels of high molecular mass activity were detected by gelatin-SDS-PAGE analysis.

Analysis of the proteolytic enzymes by gelatin-SDS-PAGE revealed multiple forms of proteinase present in the lysates of each of the rumen ciliates. The proteinase band patterns obtained with the various species differed with respect to the number of enzymes, their molecular masses and the level of activity detected (Figs 2, 3 and 4).

Some similarities were apparent in the proteinases of the holotrich species. Both were found to have a number of high molecular mass proteinases and a single major low molecular mass band. However, the molecular masses of the enzymes in the two species differed appreciably; there were also distinct differences between the minor, less active bands detected (Fig. 2) and in inhibitor sensitivity. Whereas all the bands detected with *D. ruminantium* were sensitive to leupeptin, iodoacetic acid and 1,10-phenanthroline, with *Isotricha* spp. only the low molecular mass band (F) was inhibited by the cysteine proteinase inhibitors, although the high molecular mass enzymes were sensitive to 1,10-phenanthroline.

Comparison of the proteinases detected in *En. simplex* with those found in a mixed *Entodinium* population showed that the major bands apparent in the mixed population do not correlate with those detected in *En. simplex*, although there were a number of minor bands in the mixed preparation that had similar molecular masses to those of the major enzymes found in the single species (Fig. 3a, b).

There was some indication of differences in substrate specificities of some of the enzymes. Although relatively low proteinase activity towards azocasein was detected in *O. caudatus*, a number of bands of activity could be detected on the gels (Fig. 3c). In contrast, although

Fig. 1. pH profiles of the azocasein-degrading proteinase activities in rumen ciliates. (a) *Isotricha* spp.; (b) *D. ruminantium*; (c) *En. simplex*; (d) *O. caudatus*; (e) *P. multivesiculatum*; (f) *Ep. caudatum ecaudatum*. The error bars represent the SD values of the means from the number of experiments described in Table 1.

P. multivesiculatum had relatively high levels of azocasein-hydrolysing activity, only low activity bands were observed using gelatin gels (Fig. 3d).

All of the proteinase activities detected in *En. simplex* and *O. caudatus* were sensitive to the cysteine proteinase inhibitors leupeptin and iodoacetic acid and also to 1,10-phenanthroline but were unaffected by PMSF or pepstatin. The inhibitor sensitivities of the bands detected with *P. multivesiculatum* were not fully studied, although band A was shown to be sensitive to the cysteine proteinase inhibitors but not to 1,10-phenanthroline.

Controls of rumen bacteria and extracellular rumen liquor demonstrated that the proteinases detected were indeed of protozoal origin and not due to contaminating bacteria. The only proteinase bands apparent for the bacterial samples on the gelatin gels were of very high molecular mass and of relatively low activity. No bands that correlated with the lower molecular mass protozoal enzymes were detected (Fig. 4). Only very low activity bands of high molecular mass were detected in the extracellular rumen liquor, with molecular masses similar to the bacterial enzymes. There was no evidence for extracellular proteinases of protozoal origin.

Table 1. *Effect of inhibitors and DTT on the proteinase activities of rumen ciliates and bacteria*

Results are expressed as the activity towards azocasein in the presence of inhibitors or DTT as a percentage of the activity in their absence. The assays were done at pH 4.5 except for *Isoetricha* spp. (pH 4.0) and the rumen bacteria (pH 5.0). The figures are means $\pm$ SD from three independent determinations using separate samples from three (a), two (b) or a single (c) preparation of micro-organisms.

Addition	<i>Isoetricha</i> spp. ^a	<i>D. ruminantium</i> ^b	<i>En. simplex</i> ^b	<i>O. caudatus</i> ^b	<i>P. multivesiculatum</i> ^b	<i>Ep. caudatum</i> <i>ecaudatum</i> ^f	Rumen bacteria ^c
Leupeptin	8 $\pm$ 4	34 $\pm$ 4	16 $\pm$ 3	12 $\pm$ 10	3 $\pm$ 3	47 $\pm$ 5	67 $\pm$ 14
Iodoacetic acid	33 $\pm$ 6	70 $\pm$ 12	72 $\pm$ 6	41 $\pm$ 4	30 $\pm$ 8	49 $\pm$ 4	74 $\pm$ 7
TPCK	29 $\pm$ 12	95 $\pm$ 5	102 $\pm$ 20	10 $\pm$ 1	49 $\pm$ 4	87 $\pm$ 35	86 $\pm$ 12
TLCK	14 $\pm$ 8	42 $\pm$ 7	79 $\pm$ 23	22 $\pm$ 4	11 $\pm$ 5	70 $\pm$ 30	78 $\pm$ 6
PMSF	99 $\pm$ 12	113 $\pm$ 6	69 $\pm$ 14	91 $\pm$ 9	123 $\pm$ 20	106 $\pm$ 29	82 $\pm$ 13
Pepstatin	114 $\pm$ 10	123 $\pm$ 5	120 $\pm$ 3	89 $\pm$ 14	94 $\pm$ 11	100 $\pm$ 6	101 $\pm$ 2
1,10-Phenanthroline	87 $\pm$ 1	110 $\pm$ 1	106 $\pm$ 11	37 $\pm$ 4	78 $\pm$ 3	93 $\pm$ 8	86 $\pm$ 11
DTT	247 $\pm$ 56	163 $\pm$ 34	326 $\pm$ 24	112 $\pm$ 4	219 $\pm$ 51	164 $\pm$ 17	103 $\pm$ 6

Fig. 2. The proteinases of rumen holotrich ciliates detected using gelatin-SDS-PAGE. (a) *Isotricha* spp., track 1, 100 µg protein; track 2, 50 µg protein. (b) *D. ruminantium*, 25 µg protein. The positions of the molecular mass markers are indicated by the arrows; the major ciliate proteinases detected are labelled A-F.

DISCUSSION

The ability of rumen protozoa to transform both dietary and microbial proteins can markedly affect the supply of nitrogen to the host ruminant. All of the entodiniomorphid ciliates examined by Coleman (1983) were able to degrade fraction 1 leaf protein (ribulose 1,5-bisphosphate carboxylase). Different protein substrates were used in the survey reported here and their use confirmed that the entodiniomorphid protozoa *P. multivesiculatum*, *O. caudatus*, *Ep. caudatum*, *ecaudatum* and *En. simplex* possess proteolytic enzymes. This study has also demonstrated, for the first time, that proteinases are present in the rumen holotrich ciliate *D. ruminantium* and a mixed preparation of *Isotricha prostoma* and *Isotricha intestinalis*. Multiple forms of proteinase were shown to be present in both protozoal groups although the activities towards azocasein were generally higher in the entodiniomorphs. Our results concerning pH optima, inhibitor susceptibility and stimulation by DTT are, in the main, consistent with the majority of the findings of previous studies (Abou Akkada & Howard, 1962; Coleman, 1983; Shinci & Kandatsu, 1983; Forsberg *et al.*, 1984) and indicate that cysteine proteinases are present in all six genera examined, although their contribution to overall proteolysis appears to differ with species. Bacteria were included in this study simply as a control to take into account the possible contribution to proteolysis of the bacteria associated with the purified protozoa. Our results, however, confirm the many previous reports that the bacteria contain proteinases. Our inability to detect proteolytic activity in rumen liquor contrasts with the reports of others (Brock *et al.*, 1982; Kopečný & Wallace, 1982) but probably reflects the use of different substrates as we confirmed the presence of cell-free enzymes using gelatin-SDS-PAGE.

The use of gelatin-SDS-PAGE revealed marked differences between species with respect to the numbers and molecular masses of proteinases present in the protozoal lysates. This

Fig. 3. The proteinases of rumen entodiniomorph ciliates detected using gelatin-SDS-PAGE. (a) *En. simplex*, 10 µg protein. (b) *Entodinium* spp., track 1, 25 µg protein; track 2, 10 µg protein. (c) *O. caudatus*, 100 µg protein. (d) *P. multivesiculatum*, 100 µg protein. The positions of the molecular mass markers are indicated by the arrows; the major ciliate proteinases detected are labelled A-P.

Fig. 4. The proteinases of rumen bacteria and in extracellular rumen liquor detected by gelatin-SDS-PAGE. (a) Rumen bacteria, 75 µg protein. (b) Rumen liquor, 50 µl. The positions of the molecular mass markers are indicated by the arrows; the major proteinases detected are labelled A and B.

technique has also been used to detect and characterize the extracellular serine proteinases of a strain of the rumen bacterium *Butyrivibrio fibrisolvens* (Strydom *et al.*, 1986) and to facilitate the study of the proteinases present in other non-rumen protozoa (Lockwood *et al.*, 1987). Most interesting were the differences detected between the proteinase profiles of the two holotrich preparations; the individual enzymes also differed in their sensitivity to inhibitors. Thus, although the metabolic characteristics of the two genera are apparently very similar (Williams, 1986), there are clear differences between the species in both the proteinases and carbohydrases (Williams, 1986) present. Further biochemical studies may well reveal other differences.

Inter-species differences in the proteinases present within the genus *Entodinium* are also apparent. The results presented here show that a mixed preparation of *Entodinium* spp. possessed several electrophoretically distinct proteinases that were not present in *En. simplex* lysates. In both this study and that of Coleman (1983), the highest specific activities of proteinase among the rumen ciliate protozoa were measured in the *Entodinium* isolates examined. This suggests that further characterization of the proteinases present in the principal species of rumen entodinia would be justified, particularly as the protozoa from this genus are normally numerically predominant in the rumen protozoal population.

The characteristics of the protozoal proteinases are different to those described for other rumen micro-organisms. The major proteinase of the anaerobic rumen fungus *Neocallimastix frontalis* has been reported to be a high molecular mass metalloproteinase with maximal activity at pH 7.5 (Wallace & Joblin, 1985). The bacteria possess cysteine, serine, aspartic and metalloproteinases (Hazlewood & Edwards, 1981; Brock *et al.*, 1982; Kopecny & Wallace, 1982). The predominant proteolytic enzymes of the rumen bacteria are cysteine proteinases with broad pH optima that are associated with, and easily removed from, the complex surface layers of the bacteria. We confirmed that the bacteria possess proteinases that are active over a broad pH range although our finding that the activity was optimal at pH 5.0 differs a little from some previous reports (Kopecny & Wallace, 1982). This probably reflects the use of different

substrates. It is clear, however both from this and from other studies (Brock *et al.*, 1982; Kopečný & Wallace, 1982; Forsberg *et al.*, 1984) that the inhibition profiles of the bacterial and protozoal enzymes differ in various ways. Notably we found that the bacterial activity was largely unaffected by DTT and inhibitors that characteristically inhibit cysteine proteinases. As other inhibitors were also ineffective our results do not allow us to comment further on the types of proteinase present in bacteria. Gelatin-SDS-PAGE analysis has also demonstrated that whereas the protozoal lysates typically contained lower molecular mass enzymes, no corresponding bands were present in the bacterial preparation. Measurements of proteolytic activity indicate that the rumen bacterial population has more overall activity than the protozoal fraction (Brock *et al.*, 1982; Wallace *et al.*, 1987). The protozoa, however, possess a very different array of proteolytic enzymes with largely undefined substrate specificities and function. Further studies are required to establish the role and contribution to proteolysis of the proteinases of the various groups of micro-organisms in the rumen.

Carbohydrate-degrading and bacteriolytic enzymes have been detected in cell-free culture supernatants of certain rumen protozoa following incubation *in vitro* (Williams, 1986). Shintchi & Kandatsu (1980) were also able to detect proteolytic enzymes in the incubation supernatants of mixed rumen protozoa and subsequently examined the properties of the extracellular activity (Shintchi & Kandatsu, 1981; 1983). They also observed that the proteolytic activity of cell-free rumen contents from defaunated animals was lower than that from conventionally faunated ruminants (Shintchi *et al.*, 1983) and concluded that the rumen protozoa secreted proteolytic enzymes in the rumen. However, in the present work only high molecular mass proteinases were detected in cell-free rumen liquor. The molecular size distribution was similar to that of the rumen bacterial proteinases and no lower molecular mass, protozoal enzymes were observed. Thus our studies refute a role for secretory protozoal proteinases, although it is possible that cell-free enzymes may have been inactivated during processing whereas they could retain activity for some time in the rumen digesta. A contribution of such enzymes to the overall digestion in the rumen cannot yet be fully discounted.

A more detailed understanding of the rumen protozoal proteinases and their effects is therefore required if improvements in the efficiency of nitrogen utilization in the rumen are to be gained. Further information on these enzymes may allow their detrimental activities to be selectively controlled without recourse to defaunation as this procedure, at present, is not suitable for on-farm usage and does not always achieve improvements in animal productivity as the protozoa are advantageous in other metabolic areas.

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