Presence in rumen bacterial and protozoal populations of enzymes capable of degrading fungal cell walls

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Ruminal bacteria and protozoa, and cell-free rumen fluid, were tested for the presence of enzymes involved in the degradation of the fungal cell wall. Protozoal homogenate obtained by ultrasonication showed chitinase (EC 3.2.1.14) and N-acetyl-β-glucosaminidase (EC 3.2.1.52) activities when assayed with fluorogenic 4-methylumbelliferyl substrates. The chitinase activity was predominantly of the ‘exo’-type. Lysozyme (EC 3.2.1.17) and 1,3-β-glucanase (EC 3.2.1.39) activities were also present in this fraction. All these activities, except lysozyme activity, were recovered mainly in the supernatant fraction of the homogenate (approximately 85% of the total activity). Lysozyme showed the same amount of activity in the precipitate and supernatant fractions. Bacterial homogenates had N-acetyl-β-glucosaminidase activity in both supernatant and precipitate fractions. The specific activity was one-third that of the protozoa. Bacteria able to grow in a medium with chitin as the sole carbon source were recognized and counted. Cell-free rumen fluid was unable to degrade any of the substrates tested.

Keywords: rumen bacteria, rumen protozoa, chitinase, N-acetyl-β-glucosaminidase, fungal cell wall degradation.

INTRODUCTION

The anaerobic fungi that inhabit the gastrointestinal tract of ruminants and other herbivores possess chitin in their cell walls (Orpin, 1977). Apart from chitin, other polysaccharides like glucans, especially 1,3-β-glucan, and protein–polysaccharide complexes are the main components of most fungal cell walls (Farkas, 1990). These cell wall polymers are cross-linked, giving structural support and protection against external attack. Chitin, in particular, is resistant to degradation by micro-organisms. Partial digestion of chitinous material in the bovine rumen has been reported, implying a chitinolytic rumen flora (Patton & Chandler, 1975). It is not known if rumen bacteria can degrade chitin or other components of the fungal cell wall. Rumenal protozoa engulf ruminal fungi with a subsequent increase of turnover of fungal protein in the rumen, and this usually leads to smaller fungal counts in faunated animals compared to defaunated ones (Newbold & Hillman, 1990; Orpin, 1984; Romulo et al., 1989; Ushida et al., 1989). Degradation of fungal protein by rumen protozoa can be accomplished only when the cell wall is broken, but the ability of protozoa to degrade protein from whole fungal cells has been observed, together with the possibility that chitin might be degraded (Morgavi et al., 1993).

The objective of the present study was to investigate the presence of bacterial and protozoal enzymes able to degrade the fungal cell wall in the rumen.

METHODS

Chemicals. 4-Methylumbelliferyl (4-MU) substrates and Micrococcus lyodeikticus lyophilized cells were from Sigma, laminarin and N,N-dimethylformamide were from Nacalai Tesque, and Coomassie brilliant blue G-250 was from Fluka. Streptomyces antibioticus chitinase was purchased from Calbiochem and chicken egg white (CEW) lysozyme was obtained from Wako Pure Chemical Industries. All other reagents were of analytical grade or the best grade available.

Preparation of cell-free rumen fluid, bacterial and protozoal fractions. Ruminally fistulated goats (Japanese native breed,
female, 35–40 kg) fed a ration consisting of alfalfa (*Medicago sativa*) hay cubes (23 g dry matter/kg body-weight) and concentrated feed (17% protein, 72% TDN, α-Dairy Mix, Chubu-Shiroyo, Japan; 8 g dry matter/kg body-weight in two equal portions given at 09:00 and 17:00 h were used as donor animals for ruminal contents. Ruminal contents were collected before the morning feeding and strained through four layers of surgical gauze. Samples of strained rumen fluid (100 ml) were poured into a separating funnel and incubated at 39 °C for up to 60 min to allow fine feed particles to float. Most of the lower portion was then anaerobically collected and centrifuged at 550 g for 5 min, to separate the protozoa.

The supernatant was carefully decanted and kept for further processing. The pellet (mainly protozoa) was washed ten times with MB9 buffer solution (Onodera & Henderson, 1980), in order to remove bacteria as completely as possible, and resuspended in 50 ml MB9 buffer solution plus a mixture of antibiotics (chloramphenicol sodium succinate, ampicillin sodium salt, and streptomycin sulfate, each at a concentration of 0.1 mg per ml suspension).

The protozoal suspension was incubated at 39 °C for 4 h in order to reduce the bacterial population (Onodera et al., 1977). After incubation the suspension was centrifuged (550 g, 5 min), the pellet washed three times with MB9 buffer solution to remove the antibiotics, and the volume made up to 50 ml. From this suspension samples were taken for enumeration of viable bacteria. It was then centrifuged, washed twice with 0.1 M sodium phosphate buffer (pH 6.0) and resuspended in 20 ml of the same buffer, with all the procedures being performed aerobically from this step. This protozoal suspension when observed microscopically had almost no contamination with feed particles. Then it was ultrasonicated (Kaijo Denki Co., ultrasonic disintegrator model TA-4280) at 4 °C for 3 min for 12 min (6 × 2 min), checked microscopically to confirm that all protozoal cells were broken, and centrifuged at 30,000 g for 30 min at 4 °C. The supernatant was used as a protozoal supernatant fraction (Ps). The pellet was resuspended in 20 ml buffer to form a protozoal pellet fraction (Pp).

The supernatant from the first centrifugation mentioned above (rumen fluid without protozoa) was centrifuged again at 550 g for 5 min, and the supernatant was then centrifuged at 27,000 g for 30 min at 4 °C. The supernatant was decanted to obtain the cell-free rumen fluid (CFRF) fraction. The pellet was resuspended in 0.1 M sodium phosphate buffer, centrifuged (27,000 g for 15 min), resuspended in 20 ml buffer, and ultrasonicated at 4 °C (3.5 A for 10 min; 5 × 2 min). The suspension was then treated in the same way as protozoa to form the bacterial supernatant (Bs) and pellet (Bp) fractions.

These five fractions were used for the determination of chitinase (EC 3.2.1.14), N-acetyl-β-glucosaminidase (EC 3.2.1.52), lysozyme (EC 3.2.1.17) and 1,3-β-glucanase (EC 3.2.1.39) activities.

**Enumeration of rumen micro-organisms.** Ciliate protozoa were determined microscopically with the aid of a Fuchs–Rosenthal haemocytometer in MFS (methylgreen/formalin/salt) fixed preparations of strained rumen fluid (Onodera et al., 1977).

For enumeration of viable bacteria and fungi, strained rumen fluid was collected aseptically in a sterilized test tube and serially diluted (10-fold) in anaerobic medium 10 of Caldwell & Bryant (1966) without carbohydrates. The roll tube method (Hungate, 1969), three tubes per dilution, was used to grow bacteria in normal medium 10 with 1.5% (w/v) agar. Tubes where the number of colonies formed was between 30 and 200 were used to estimate the total viable bacteria. Viable bacteria in the protozoal fraction, after incubation with antibiotics, were estimated in the same way.

A modified medium 10 was devised for enumeration of bacteria able to degrade chitin in roll tubes. The medium contained 1% (w/v) colloidal chitin prepared by the method of Shimahara & Takiguchi (1988), but not other carbohydrates. Degradation of colloidal chitin produced circular clear zones around colonies. The number of bacteria possessing this activity was estimated in strained rumen fluid and in the protozoal fraction using the method described above for total viable bacteria.

Anaerobic rumen fungi were enumerated using the thallus-forming units (TFU) method (Theodorou et al., 1990). Tubes (five replicates per dilution) containing 9 ml sterile medium 10 (without carbon source) with about 100 mg of autoclaved alfalfa (*Medicago sativa*) hay, milled to pass through a 1 mm mesh sieve, were inoculated with 1 ml diluted strained rumen fluid. Before inoculation, a filter-sterilized anaerobic solution of chloramphenicol sodium succinate, ampicillin sodium salt and streptomycin sulfate was added to attain a final concentration of 0.1 mg ml⁻¹ of each antibiotic.

**Enzyme assays.** 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-GlcNAC) for the determination of N-acetyl-β-D-glucosaminidase activity, and 4-methylumbelliferyl N,N',N''-triacetylchitotriosiose (4-MU-GlcNAC₃) for chitinase activity, were used as substrates. The reaction was started by the addition of 60 µl enzyme solution to 240 µl prewarmed substrate in citrate/phosphate buffer (pH 5.0). The final mixture contained 20 mM substrate and 50 mM buffer in a final volume of 300 µl. It was incubated at 39 °C for 15 min and terminated by the addition of 25 ml 1 M glycine/NaOH buffer (pH 10.6) (McCreath & Gooday, 1992). The amount of 4-MU liberated was measured with a Shimadzu RF-5000 spectrofluorometer (360 nm excitation and 450 nm emission). Substrate and enzyme blank preparations, replacing the substrate and enzyme, respectively, with water, were run through the procedure to make corrections for background fluorescence. In order to relate fluorescence output to the concentration of product released a calibration curve was constructed using 4-MU sodium salts. To determine the optimum pH of enzyme activity, different buffers were used (pH 3 to 9) at 50 mM concentrations.

Laminarin was used as a substrate for measuring 1,3-β-glucanase activity. The reaction mixture consisted of 0.25 ml 2% (w/v) laminarin dissolved in distilled water, 0.5 ml 50 mM citrate/phosphate buffer (pH 5.0), and 0.25 ml enzyme solution; incubations were carried out at 39 °C for 1 or 2 h. Reducing sugars liberated were determined by a modification of the Nelson–Somogyi colorimetric assay (Somogyi, 1952; Sprio, 1966). In some cases the neocuproine method of Dygert et al. (1965) was used for comparison. For each enzyme preparation measured, an enzyme blank, replacing the substrate with water, was carried through the procedure in addition to substrate and reagent blank preparations. One milliunit (mU) was defined as the amount of enzyme which liberates 1 nmol glucose equivalents min⁻¹.

Lysozyme activity was measured as the rate of lysis of *Micrococcus lyodekikicus* cell walls. The reaction, a decrease in A₅₇₀, was monitored at intervals using a Shimadzu UV-200S spectrophotometer at 39 °C. The reaction mixture contained, in a volume of 3 ml, 0.2 ml enzyme solution, 0.75 ml 100 mM sodium phosphate buffer (pH 6.0), and *M. lyodekikicus* cells suspended in water and adjusted to give an initial A₅₇₀ of 0.8. CEW lysozyme was used as a positive control. A decrease in A₅₇₀ of 0.001 min⁻¹ was defined as a unit.
Table 1. Specific activities of fungal cell wall degrading enzymes in protozoal (Pp and Ps), bacterial (Bp and Bs) and cell-free rumen fluid (CFRF) fractions obtained from goats

Mean values (±SD) of duplicate preparations from three goats are presented, except for 1,3-β-glucanase activity measured with the Neocuproine method, where values are mean from two goats. Figures with the same superscript letter in the same column are not significantly different (P > 0.05).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N-Acetyl-β-glucosaminidase (pkat mg⁻¹)</th>
<th>Chitinase (pkat mg⁻¹)</th>
<th>1,3-β-Glucanase (mU mg⁻¹)*</th>
<th>Lysozyme (U mg⁻¹)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monosaccharide</td>
<td>Disaccharide 4-MU</td>
<td>Trisaccharide 4-MU</td>
<td>Nelson-Somogyi method</td>
</tr>
<tr>
<td>Pp</td>
<td>257.4 ± 65.5*</td>
<td>333.4 ± 95.1*</td>
<td>224.9 ± 102.7*</td>
<td>38.8 ± 45.4*</td>
</tr>
<tr>
<td>Ps</td>
<td>1686.5 ± 243.6*</td>
<td>2873.0 ± 635.9*</td>
<td>1198.3 ± 378.5*</td>
<td>86.0 ± 72.9*</td>
</tr>
<tr>
<td>Bp</td>
<td>294.7 ± 130.6*</td>
<td>ND</td>
<td>ND</td>
<td>10.4 ± 9.0*</td>
</tr>
<tr>
<td>Bs</td>
<td>310.7 ± 80.4*</td>
<td>ND</td>
<td>ND</td>
<td>3.3 ± 2.1*</td>
</tr>
<tr>
<td>CFRF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>11.5 ± 11.1*</td>
</tr>
</tbody>
</table>

ND, Not detected.

* One milliunit represents 1 nmol glucose equivalent liberated min⁻¹.
† One unit represents a decrease in absorbance of 0.001 min⁻¹ calculated from initial rates.

RESULTS

Enzyme activities

Table 1 shows the fungal cell wall degrading enzyme activities of the five fractions when assayed on 4-MU substrates, laminarin and M. lysodeikticus cell suspension. The Ps fraction had catalytic action on the three 4-MU substrates used. Some activity was also present in the Pp fraction. About 85% of the total protozoal activity was recovered in the Ps fraction, the specific activity [pkat (mg protein)⁻¹] being more than 90% of that of the Pp fraction.

N-Acetyl-β-glucosaminidase activity was also detected in both bacterial fractions (Bs and Bp), which degraded 4-MU-GlcNAc, but did not act on the other 4-MU substrates. The CFRF fraction did not degrade any 4-MU substrate.

A commercial preparation of S. antibioticus chitinase used as a positive control was most active on 4-MU-GlcNAc, followed by 4-MU-(GlcNAc)₂; the fluorescence released from 4-MU-(GlcNAc)₃ was approximately a quarter of that from 4-MU-GlcNAc (data not shown). On the other hand, as much as 10 μg of CEW lysozyme did not release fluorescence from any of the 4-MU substrates under the conditions of the assay (data not shown).

The optimum pH for the activity of the Ps fraction on 4-MU-GlcNAc was 4.8; 50% of its activity remained at pH 6.2 and 4.0 (Fig. 1a). The same fraction assayed on 4-MU-(GlcNAc)₂ showed a wider range of optimum pH, with peaks at pH 5.6 in acetate buffer and at pH 6 in succinate buffer, 50% of its activity remaining at pH 3.5 and 7.5 (Fig. 1b). In time-course assay experiments the reaction was linear in 60 min incubations provided that there was no substrate limitation. Enzyme activities remained constant over several months at −20°C, but
when preparations were stored at 4 °C activities were lost after 2–3 d, probably due to inactivation by proteases (Nagasawa et al., 1989).

1,3-β-Glucanase activity was measured as the amount of reducing sugars liberated from laminarin. Corrections were made for reducing sugars present in the sample, which were high in CFRF and Pp fractions. Bacterial and CFRF fractions showed negligible activity, while protozoal fractions, especially the Ps, degraded the substrate (Table 1). The activity of the Ps fraction with the neocuproine method was approximately 30% greater than with the Nelson-Somogyi method. However, the amount of reducing sugars liberated from laminarin by the Pp fraction as detected by this method was half that detected by the Nelson-Somogyi method.

Lysozyme activity was detected in both protozoal fractions (Table 1). After 2 h incubation the Ps fraction caused a 50% reduction in the absorbance of the M. lysodeikticus cell suspension, while the Pp fraction caused a 58% reduction (Fig. 2). Absorbance decreased almost linearly up to 30 min, and at 120 min the reaction rate was one-third of the initial one. The other fractions did not have any activity. As controls, CEW lysozyme cleared about 90% of the cell suspension, whereas the crude S. antibioticus chinase (1 unit per tube) did not have activity under the conditions assayed (data not shown). The protozoal activity was not detectable when assayed at pH 5:0, and was small at pH 5:5 using 50 mM sodium acetate buffer. Good activity was detected with 50 mM sodium phosphate buffer at pH 6:0 to 7:0.

**Rumen micro-organisms**

The objective of counting micro-organisms was to check the presence of a normal microbiota in the ruminal samples used for enzyme assays. The number of microorganisms was within normal ranges, though total viable bacteria counts were high (Ogimoto & Imai, 1981; Theodorou et al., 1990). Protozoal counts were 1.69 ± 0.43 × 10^6 protozoa per ml rumen fluid of an A-type population (Edlae, 1962), composed predominantly (98%) of small entodiniomorphids. The number of fungi was 1.1 × 10^6 TFU ml^-1 with 95% confidence limits of 0.4 × 10^6 and 3.0 × 10^6 TFU ml^-1, lower and upper respectively. The total viable bacterial population in rumen fluid was 6.18 ± 2.6 × 10^12 bacteria ml^-1, while counts of viable bacteria remaining in the total protozoa collected from 100 ml rumen fluid after incubation with antibiotics were 2.39 ± 2.84 × 10^6 bacteria ml^-1. Incubation with diluted rumen fluid in the modified agar Medium 10 with colloidal chitin as the sole carbon source gave evidence of the presence of bacteria able to utilize this compound. Clear zones appeared after 4 or 5 d incubation. Bacteria with the ability to degrade chitin in rumen fluid were present at 2.97 ± 2.08 × 10^7 ml^-1, but the presence of micro-organisms possessing this capacity was not detected in the protozoal preparation after incubation with antibiotics.

**DISCUSSION**

Enzyme assays that use fluorogenic substrates with 4-methylumbelliferone as aglycone are very sensitive (O'Brien & Colwell, 1987) and allow detection of low levels of chitinolytic activity (McCreaeth & Gooday, 1992). They have been compared favourably with other methods for measuring chinase (Hood, 1991; O'Brien & Colwell, 1987).

N-Acetyl-β-glucosaminidase was assayed on individual ruminal bacteria and protozoa by Williams et al. (1984, 1986) using p-nitrophenyl-N-acetylglucosaminide as a substrate. *Entodinium* spp. and *Diplodiplastron affinis* had this activity. Among bacteria *Bacteroides fragilis* had a high activity, several times higher than the most active protozoa ranked at that time, though this bacterium is not dominant in the rumen. In the present experiments, where the activities of the whole bacterial and protozoal populations were compared, the abundance of entodiniomorphid protozoa could have caused the specific activity of the protozoa to be more than twice that of the bacteria.

4-MU substrates have been proposed as a tool to distinguish between endo- and exochitinase activities (Robbins et al., 1988). According to this proposal, our protozoal crude enzyme preparations exhibited predominantly an 'exo' mode of action for chinase activity, producing 4-methylumbelliferone from 4-MU-(GlcNAc)_2 2-4 times faster than from 4-MU-(GlcNAc)_3. The crude chinase from *S. antibioticus*, on the other hand, showed an 'endo' type of action.

The differences in optimum pH between 4-MU-GlcNAc and 4-MU-(GlcNAc)_2 for the Ps fraction suggest that the enzymes responsible for this action are not the same. These two activities can be separated by size-exclusion chromatography, 4-MU-(GlcNAc)_2-degrading enzymes being eluted first, followed by 4-MU-GlcNAc-degrading...
enzymes (unpublished data). The greater sensitivity of the neocuproine method for the determination of reducing sugars compared with the Nelson–Somogyi method (Dygert et al., 1965) is probably the cause of the difference in the 1,3-β-glucanase activities in protozoal fractions. The Pp fraction had less activity due to the correction for reducing sugars present in the sample, while in bacterial and CFRF fractions no activity was detected by this method. Williams & Strachan (1984) also reported more degradation of laminarin from protozoa than from bacteria.

Lysozyme is a glycosidase which splits the (1,4-β)-N-acetylmuramyl-N-acetylgalosamine linkage present in the bacterial cell walls (Ghuysen et al., 1966), and this enzyme could have an important role in the complete hydrolysis of ruminal bacteria engulfed by protozoa. Lysozyme can also act slowly as an endohydrolase (Gooday, 1990a); hydrolases possessing both chitinase and lysozyme activities have mainly been found in plants (Majeau et al., 1990). M. lysoideikticus cell suspensions were cleared in a similar way by both protozoal fractions (Pp and Ps); it was not discernible whether this activity was caused by more than one enzyme or by a single enzyme, probably membrane bound and incompletely liberated with the ultrasonic treatment. The finding of lysozyme activity in the Pp fraction without a corresponding high chitinase activity suggests that different enzymes are responsible for these actions, at least in the Pp fraction. Although 4-MU-(GlcNAc)₃ has been proposed as a substrate for lysozyme (Delmotte et al., 1975), CEW lysozyme did not degrade this substrate under the conditions of the assay. If protozoal lysozyme acts in the same way, clearing of M. lysoideikticus and degradation of chitosinous substrates in the Ps fraction could be due to different enzymes. The different pH values at which the activities are displayed support this proposition.

The methodology used to fractionate rumen microbiorganisms ignores the bacteria attached to rumen solids; enzymes digesting fungal cell walls might be more active in this population, as reported for structural polysaccharidases (Williams & Strachan, 1984; Williams et al., 1989). However, enzymes present in rumen fluid or particle-associated microbial populations are the same and only the concentration may vary (Williams et al., 1989).

Fungal zoospores were included in the protozoal fraction, at a protozoa:fungal zoospores ratio of about 150:1. The number of zoospores might decrease during the centrifugation-washing procedures due to their smaller size compared with protozoa, and also due to protozoal predation during the incubation with antibiotics. When zoospores from the anaerobic fungus Pirromyes sp. were ultrasonicated and treated in the same way as for protozoal samples, no N-acetyl-β-glucosaminidase or chitinase activities were detected. We therefore considered that the enzyme activities found originated mainly from protozoa and that fungal enzyme contamination was too small to change the pattern or amount of activity detected. Enzymes able to degrade fungal cell walls in the rumen were detected mainly in the protozoal population. This is concordant with their predatory activity against anaerobic rumen fungi. Related to this predatory activity, it must be noted that protozoa are the main cause of bacterial degradation in the rumen, and enzymes like lysozyme and N-acetyl-β-glucosaminidase play a fundamental role in cleaving the bacterial cell wall peptidoglycan (Ling, 1990). Although some bacteria were detected to be capable of degrading chitin, the rumen bacterial population did not show any chitinase activity, and their N-acetyl-β-glucosaminidase activity was lower than that of protozoa. The way in which these enzymes affect fungal counts, and the secondary effects on food degradation, remain to be elucidated to understand their role in the ruminal ecosystem.

Chitinases from protozoa may have a nutritional or pathogenic role (Gooday, 1990b). Chitinases from ruminal protozoa certainly contribute to exposing fungal material, like protein, to enzymic digestion (Morgavi et al., 1993), and they might also provide nutrients directly from chitin to protozoa.

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