In vitro Metabolism of Chitin and Protein from Ruminal Fungi by Ruminal Protozoa

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Abstract The predation of ruminal anaerobic fungi by ruminal protozoa was studied using an *in vitro* system. Two fungi *Neocallimastix* sp. and *Piromyces* sp., were isolated from a goat. Mixed rumen ciliate protozoa obtained from goats and suspended in MB9 buffer solution were incubated anaerobically with each freeze-dried fungus at 39°C for up to 24 h. The total amount of chitin in cultures, and glucosamine and amino acids released in the medium were monitored. The total amount of chitin through the incubation period tended to decrease in the case of *Piromyces* sp. and in the case of *Neocallimastix* sp. it decreased a little, though not significantly in both cases. However, the glucosamine released in the medium after 24 h of incubation had a significant increment ($P < 0.05$) in both cases. The rise of glucosamine in the medium suggests that rumen protozoa have chitinolytic activity. The fact that free amino acids liberated by protozoa in the medium increased ($P < 0.01$) in the presence of fungus is a confirmation of their action in the ruminal turnover of fungal protein.

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Key words : ruminal protozoa, ruminal fungi, chitin metabolism, protein metabolism

Information on the interrelationship between fungal and protozoal populations in the rumen is limited. ORPIN²³⁾ reported that individual zoospores of fungi can be occasionally engulfed by rumen ciliate protozoa. Furthermore, it is known that defaunation leads to a higher fungal population, although this increase is not always significant^{16,26,28)}. One reason for this could be individual differences between animals¹⁶⁾ and the different methodologies used for measuring the fungal population *in vivo*²⁸⁾. NEWBOLD and HILLMAN¹⁶⁾ demonstrated that the mechanism by which defaunation increases the fungal population was due to a reduction in the turnover of fungal protein in the rumen, but this predatory activity is much less apparent with fungi than with ruminal bacteria.

Chitin, a common component in the cell wall of rumen fungi, is a polysaccharide consisting predominantly of *N*-acetyl-D-glucosamine, and is extremely resistant to degradation by micro-organisms. It may be that this component limits fungal engulfment and fungal protein turnover in the rumen. Until now no organism capable of chitin degradation has been isolated from the rumen²¹⁾. If chitin is not digested in the rumen, its synthesis must result in a net loss of energy to the host animal²⁴⁾. On the other hand, since chitin may be undegradable in the rumen, GULATI *et al.*⁹⁾ postulated that passage of the rumen fungi to the small intestine might improve the net yield of microbial protein because fungi contain good quality^{4,15)} and highly digestible protein⁹⁾.

The aim of this study was to investigate the predatory activity of ruminal protozoa on anaerobic fungi with particular emphasis on chitin metabolism and fungal protein quality.

Materials and Methods

Isolation and maintenance of ruminal fungi :

Anaerobic rumen fungi were obtained from a ruminally fistulated goat (Japanese Saanen) fed a ration consisting of alfalfa (*Medicago sativa*) haycubes (23 g dry matter/kg body-weight^{0.75}) and concentrated feed (Chubu-Shiryo, Japan ; 8 g dry matter/kg body-weight^{0.75}) in two equal portions given at 09 : 00 and 17 : 00 h.

Ruminal contents were collected 1-2 h after the morning feeding and strained through four layers of surgical gauze.

Fungi were isolated using the roll tube method described by JOBLIN¹⁴. After 3-4 days, one colony was picked up with a Pasteur pipette and transferred to a broth medium. This alternate subculturing in roll tubes and broth medium was repeated three times to ensure the purity of the isolated colonies.

The medium used for isolation and maintenance of the anaerobic fungi was based on Medium 10 of CALDWELL and BRYANT² plus the addition of an antibiotic solution (chloramphenicol sodium succinate, ampicillin sodium salt, and streptomycin sulfate, each at 0.1 mg/ml medium). The medium composition had the following modifications : 1) xylan (0.05% w/v) was included in addition to the other carbon sources⁸ ; 2) the amount of L-cysteine monohydrochloride-monohydrate and sodium sulfide was increased from 0.025 to 0.0375% (w/v) ; 3) the volatile fatty acid mixture was prepared with sodium salts (except for DL- α -methylbutyric acid).

Aseptic anaerobic techniques, methods of medium preparation and sterilization used have been described elsewhere^{2,11,12}. The antibiotic solution was filter-sterilized with a 0.20 μ m pore diameter pyrogen free filter membrane

(Dismic-25 cs, Toyo Roshi Kaisha, Ltd). The gas used was O₂-free CO₂. The incubation temperature was 39°C.

For maintenance, test tubes sealed with butyl rubber stoppers containing 10 ml medium were used. Every 4-5 days the microorganisms were transferred to new media using a 5% (v/v) inoculum from the previous culture.

Growth of ruminal fungi : Two monocentric strains were isolated, one resembled *Neocallimastix* sp. with an endogenous sporangial development and production of polyflagellate zoospores^{1,20}, and the other resembled *Piromyces* sp. bearing monoflagellate zoospores and a vegetative stage possessing a highly branched rhizoid²². The sporangial development was exogenous and endogenous¹¹.

The medium used for growth was the same as described above, but the glucose concentration was increased to 5 g/l⁸. Erlenmeyer flasks (0.5 or 1 l) sealed with butyl rubber stoppers, were employed as culture vessels; the volume of medium was half the capacity of the flask.

A 10% (v/v) inoculum was used with an incubation time of 3 days for *Neocallimastix* sp. and 4 days for *Piromyces* sp. After this period, the culture was collected with filter paper (Toyo Roshi Co., Ltd. No. 5 A with an approximate particle retention size of 7 μ m), washed twice with deionized water (50 ml), autoclaved at 121°C for 20 min and freeze-dried. These dried fungi were slightly ground and allowed to pass through a 1 mm diameter sieve. After this treatment cell wall integrity was checked by light microscopy.

Ruminal protozoal suspension : Mixed ruminal ciliate protozoa were obtained from ruminally fistulated goats fed in the same way as described above for the isolation of ruminal fungi. Ruminal contents were taken before the morning feeding and strained through four layers of surgical gauze. Samples of strained rumen fluid were then poured into a separating funnel that had been gassed with O₂-free 95%

N₂-5% CO₂. The funnel and contents were maintained at 39°C for 30-60 min to allow fine feed particles to float. The lower portion was then anaerobically centrifuged (120 × *g*, 1 min). The pellet (mainly protozoa) was washed five times with MB9 buffer solution¹⁸⁾ and re-suspended in MB9 buffer solution at about 4% (v/v) to produce a protozoal suspension. To this suspension was added chloramphenicol sodium succinate, ampicillin sodium salt, and streptomycin sulfate, each at 0.1 mg/ml suspension.

Before incubation, in order to determine the protozoal numbers, 0.5 ml of this suspension was collected and mixed with 4.5 ml of MFS (methylgreen-formalin-salt) solution¹⁷⁾. For total nitrogen measurement 1 ml samples (in triplicate) were used.

Incubation and sample treatments : Preliminary studies tested the effects of varying the concentration of dried fungi to determine a suitable ratio to the protozoa in the incubation. The effect of MB9 buffer solution plus antibiotics on the liberation of amino acids from fungi into the medium during the incubation period was also examined.

The incubations were carried out in triplicate, and each fungal strain was tested on 3 different days using protozoal isolates from different animals.

Samples (3 ml) of protozoal suspensions in test tubes (total 12 tubes) were incubated anaerobically with 12 mg of freeze-dried fungi at 39°C for up to 24 h. Control incubations consisted of protozoa alone, and fungi alone in MB9 buffer solution plus antibiotics. In the case of the fungi control 10 mg were used.

During the incubation period, samples (0.6 ml) were removed from 3 tubes and were mixed with equal volumes of 6% (w/v) sulfosalicylic acid in microcentrifuge tubes, centrifuged (16,000 × *g*, 15 min) and the supernatants were analyzed for their amino acid contents. Protozoal activity was halted by adding 0.5 ml conc. HCl to each incubation

tube. The contents were centrifuged (25,000 × *g*, 10 min, 4°C), the supernatant was assayed for glucosamine, while the pellet was washed with 5 ml deionized water, centrifuged again and assayed for chitin.

To hydrolyze fungal cells, weighed samples (5 mg) were added to 2.5 ml 6 *M* hydrochloric acid containing 0.02% (v/v) mercaptoethanol and 0.25% (w/v) phenol⁵⁾; these were heated at 110°C for 24 h in evacuated hydrolysis tubes. The hydrolyzed contents were filtered and dried by rotary evaporation, and the residue was washed 3 times with deionized water to remove the HCl. Sodium citrate buffer solution (pH 2.2) was used to dissolve these samples before amino acid analysis.

Analytical procedures : Free amino acids in the incubation media and fungal hydrolysates were analyzed by ion-exchange chromatography using an automatic high speed amino acid analyzer with a 150 × 4.6 mm column of 7 μm sulphonated polystyrene beads (Atto-Gel SC-71, Atto Corporation).

Glucosamine and chitin were determined by the method described by CHEN and JOHNSON³⁾. Rumen protozoa were counted with the aid of a Fuchs-Rosenthal hemacytometer. Total nitrogen concentrations were measured by the micro-Kjeldhal method.

Statistical evaluation of data was performed by least-squares analysis of variance¹⁰⁾.

Results

The protozoal populations used in this work were composed predominantly (98%) of small entodiniomorphid protozoa. The mean (±SE) total nitrogen content of protozoal suspensions was 0.729 ± 0.042 mg/ml (1.5 × 10⁵ protozoa/ml).

Chitin equivalent was detected in all control incubations. When *Piromyces* and *Neocallimastix* were incubated alone, concentrations (±SE) were 0.959 ± 0.017 and 1.220 ± 0.010 mg/10 mg freeze dried fungi respectively. With *Neocallimastix* the concentrations were con-

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Table 1. Concentrations of chitin in acid hydrolyzed pellets after incubation of mixed ruminal protozoa alone, with *Piromyces* sp., and with *Neocallimastix* sp.

Incubation time (h)	Protozoa alone (n = 15)	Protozoa plus <i>Piromyces</i> ^a (n = 9)	Protozoa plus <i>Neocallimastix</i> ^a (n = 9)
0	0.106 ± 0.014	1.286 ± 0.143	1.416 ± 0.166
6	0.092 ± 0.010*	1.155 ± 0.225**	1.420 ± 0.153
12	0.079 ± 0.015	1.109 ± 0.161	1.383 ± 0.136
24	0.053 ± 0.012	1.011 ± 0.117	1.346 ± 0.106

Values are expressed as mg chitin eq./3 ml incubation (mean ± SD).

^a 12 mg of freeze dried fungi.

*number of determinations = n-1, **n-3.

Table 2. Concentrations of free glucosamine in supernatants after incubation of mixed ruminal protozoa alone, with *Piromyces* sp., and with *Neocallimastix* sp.

Incubation time (h)	Protozoa alone (n = 15)	Protozoa plus <i>Piromyces</i> (n = 9)	Protozoa plus <i>Neocallimastix</i> (n = 9)
0	9.231 ^a ± 2.388*	16.938 ^a ± 4.341	21.054 ^{ab} ± 1.587
6	10.773 ^a ± 2.445	19.296 ^a ± 2.094	19.260 ^a ± 1.947
12	14.259 ^a ± 1.905	21.354 ^{ab} ± 2.295	26.832 ^{ab} ± 3.516
24	17.058 ^a ± 1.302	33.870 ^b ± 1.179	32.985 ^b ± 4.761

^a Values are expressed as µg glucosamine hydrochloride/3 ml incubation (mean ± SD). Means with different superscript letters in the same column are significantly different (P < 0.05).

* Number of determinations = n-1.

stant throughout the incubation period, but with *Piromyces* the concentrations actually increased by 12% over the 24 h period. Table 1 shows that when mixed ruminal protozoa were incubated alone, small concentrations of chitin eq. were detected; these decreased by 50% after 24 h incubation. This table also shows that when protozoa and fungi were incubated together the microbial pellet contained decreasing amounts of chitin eq.; a 21% decrease with *Piromyces*, but only 5% with *Neocallimastix* over the 24 h incubation period.

When the supernatants were analyzed, the fungal control incubations contained constant concentrations (±SE) of free glucosamine throughout the whole incubation period; they were 4.97 ± 0.29 µg/ml for *Piromyces* and 5.61 ± 0.16 µg/ml for *Neocallimastix*. When protozoa were incubated alone concentrations of

glucosamine increased throughout the incubation period, but when protozoa were incubated with fungi the amount of glucosamine in the supernatants increased significantly (P < 0.05) after 24 h of incubation (Table 2).

The concentrations of amino acids released into the media when fungi were incubated alone in MB9 buffer solution plus antibiotics were minimal during the 24 h incubation period and the values obtained were negligible (less than 2 % at 24 h) when compared with those where protozoa were present.

All free amino acids in the medium increased significantly (P < 0.01) when protozoa were incubated with *Piromyces*. In the case of *Neocallimastix* threonine, serine, proline, glycine, valine, leucine, tyrosine, and arginine (P < 0.01) and methionine and phenylalanine (P < 0.05) showed significant increases. Only dead fungi

Fig. 1. Total free amino acid in the medium when protozoa were incubated with rumen fungi. (a) ●, protozoa with *Piromyces* sp; ▲, protozoa alone; ○, (●)-(▲). (b) ●, protozoa with *Neocallimastix* sp; ▲, protozoa alone; ○, (●)-(▲).

were used to assure that any change in the compounds investigated was produced by protozoa.

Total free amino acid values, when *Piromyces* sp. was included in the medium at 6, 12, and 24 h of incubation, were 157%, 72%, and 44% larger than their corresponding protozoal control; in the case of *Neocallimastix* they were 56%, 38%, and 15% larger (Fig. 1). These results are in accordance with the changes in chitin concentrations which were more accentuated in *Piromyces*.

The cell walls, after sample preparation, were undamaged when checked by light microscopy. We are aware that the use of rather large particles of freeze dried fungi could lead to an underestimation of protozoal ability to digest chitin, but they were prepared in that way to keep cell-wall integrity.

Discussion

Neocallimastix sp. contained more chitin as a percentage of dry weight than *Piromyces* sp. and this difference between the genera was also found by other authors^{6,15,21}. This trend as well as the total chitin content, however, is variable depending on strains²⁵, age, growth rate, environmental conditions⁷, and analytical methods. In the case of *Piromyces* sp. the concentrations of chitin in the pellet increased, though not significantly, after 24 h of incubation of the fungus in MB9 buffer solution used as a control, although the reason is unclear.

The chitin detected in protozoal samples (Table 1) could have originated from a) bacterial cell wall material, inside the protozoal body or free in the medium (a small number of bacteria may remain in the washed protozoal suspension), which released *N*-acetylglucosa-

mine after acid hydrolysis of the sample⁶⁾, b) fungi ingested by protozoa in the rumen before digesta collection, and/or c) as a normal constituent of protozoa body. The first two hypothesis are supported by the fact that after incubation chitin in protozoal samples decreased by 50%, followed by an increment in glucosamine liberated into the medium.

Glucosamine liberated in the medium increased ($P < 0.05$) throughout the incubation period in protozoal suspension with each of the fungal isolates. Calculating the amount of glucosamine that was liberated from chitin, it was found that the values detected were much smaller (10 to 20%) than expected. Incubation of *N*-acetyl-D-glucosamine with supernatant preparations of homogenated protozoa showed, after 60 min, a decrease of nearly 2 μ mol (0.442 mg) *N*-acetyl-D-glucosamine/mg protein (unpublished observations). This explain the small amount of glucosamine detected, and thus the energy required for chitin synthesis can be recycled and transformed into an available source for the host animal.

The complete hydrolysis of chitin to free *N*-acetyl-D-glucosamine is performed by a chitinolytic system, consisting of two hydrolases, the action of which is consecutive. In a first step chitinase (EC 3.2.1.14) hydrolyzes the polymers of *N*-acetyl glucosamine, and then *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) hydrolyzes diacetylchitobiose and also triacetylchitotriose¹³⁾. There are reports describing *N*-acetyl- β -D-glucosaminidase activity in rumen protozoa using as substrate a *p*-nitrophenyl derivative of *N*-acetyl-D-glucosamine^{29,30)}, but chitinase activity was not examined by these authors. The degradation of whole fungal cells, used as substrate, give evidence that rumen protozoa possess a chitinolytic system with both chitinase and *N*-acetyl- β -D-glucosaminidase activities.

Protozoal suspensions liberate endogenous amino acids into the medium when they are incubated *in vitro*¹⁹⁾. The increased amount of

amino acids liberated into the medium after incubation of protozoa in the presence of rumen fungi is a confirmation of their action in the ruminal turnover of fungal protein, as described by NEWBOLD and HILLMAN¹⁶⁾.

If the total amount of free endogenous amino acids produced by protozoa alone are subtracted from those of the free amino acids produced by protozoal metabolism in the presence of fungi, it can be seen that the amino acids liberated increased linearly throughout the incubation period, indicating a steady protozoal activity (Fig. 1).

Chitin, like cellulose in plant cells, is synthesized by elaboration of microfibrils that give structural support and acts as a defensive barrier against external attacks²⁷⁾, the larger amount of this polysaccharide in *Neocallimastix* sp. could lead to more protection against protozoal attacks than *Piromyces* sp. could.

The amino acid compositions of *Piromyces communis*¹⁵⁾ and *Neocallimastix* sp.^{4,15)} has been reported previously. Though it is not the object of this report, the amino acid composition in fungal protein was analyzed to observe if there were some changes in the relation of free amino acids in the medium when protozoa were incubated alone and with fungi. Analyzing the relative pattern of emergence of amino acids when protozoa were incubated alone, we found that proline, glutamic acid, glycine, alanine, lysine and aspartic acid were most abundant. This group accounted for about 60% of the total. The same amino acids were also dominant in protozoal incubations in the presence of fungi. This can explained as follow : the most abundant amino acid in fungal protein was aspartic acid, which is easily metabolized by protozoa. Other amino acids rich in fungi were glutamic acid, lysine, alanine, glycine, and valine, and all these amino acids, except for valine, were also found to be abundant in endogenous protozoal amino acids. When protozoa were incubated with fungi, however, the

relative pattern of emergence of some amino acids such as threonine, serine, arginine, and isoleucine (usually low when protozoa were incubated alone) rose. Thus the amino acids liberated into the medium in the presence of fungi respond not only to the increased liberation of protozoal endogenous amino acids, but also to the amino acids from fungal protein after being hydrolyzed by protozoal proteases.

These results suggest that ruminal protozoa are capable of degrading chitin present in fungal species isolated from the rumen of goats. In addition fungal protein seems in part to be used by protozoa to build their own components and in part liberated in the medium. Experiments are currently in progress to confirm this action and assess its importance in the rumen microbial ecosystem.

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In vitro におけるルーメンプロトゾアによるルーメン真菌の
キチンおよびタンパク質代謝

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In vitro の系によりルーメンプロトゾアによる嫌気性ルーメン真菌の捕食を検討した。山羊から2種類の真菌, *Neocallimastix* sp. と *Piromyces* sp. を単離した。山羊から採取した混合ルーメンプロトゾアを MB9 緩衝液に懸濁し, これに凍結乾燥真菌を加えて, 嫌気的条件下で 39°C, 24h までインキュベートした。そして, 培養液中の総キチン量と培養液中に遊離したグルコサミンおよびアミノ酸を経時的に測定した。*Piromyces* の場合は有意ではなかったけれどもインキュベーション中に総キチン量が減少した。また, *Neocallimastix* ではほんの少し減少する傾向が見られた。24h 後に培地中に遊離したグルコサミンは両者とも有意に増加した ($P < 0.05$)。したがって, ルーメンプロトゾアはキチン分解活性を持つと考えられた。この点は初めての証明である。プロトゾアが培地中に放出する遊離アミノ酸は真菌の存在により増加した ($P < 0.01$) ので, ルーメンプロトゾアは真菌タンパク質を代謝していると推察された。

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