Journal of Animal and Feed Sciences, 24, 2015, 203–207
The Kielanowski Institute of Animal Physiology and Nutrition, PAS, Jabłonna

Chitin as a source of energy for rumen ciliates

R. Miltko[1], B. Kowalik, T. Michałowski and G. Bełżecki

The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland

KEY WORDS: rumen protozoa, chitin, degradation, fermentation, VFA

Received: 21 April 2015
Revised: 3 July 2015
Accepted: 9 September 2015

[1] Corresponding author:
e-mail: r.miltko@ifzz.pl

ABSTRACT. The objective of the present study was to examine and compare the ability of *Diploplastron affine* and *Entodinium caudatum* to digest and ferment chitin. Cultivation studies showed that enrichment of the growth medium with this polysaccharide increased the ciliate count ($P < 0.05$). After 2 h of incubation of ciliates with chitin, a statistically significant increase in the percentage of individuals containing chitin particles was observed ($P < 0.05$), followed by a continuous decrease ($P < 0.05$) in this percentage. Enzymatic studies confirmed the ability of the examined protozoa to digest chitin. The chitinolytic activity of *Diploplastron affine* was 3.9 and that of *Entodinium caudatum* was 5.9 µmol N-acetyl glucosamine/mg protein/h. The production rates of volatile fatty acids from fermented chitin were 3.9 and 0.5 pmol/ciliate cell/h for *Diploplastron affine* and *Entodinium caudatum*, respectively.

Introduction

The biocenosis of the rumen ecosystem is composed of anaerobic microorganisms. Bacteria are the most abundant at 10^{11}, followed by ciliates up to 10^6, and fungi at 10^3–10^5 per millilitre of rumen fluid (Doré and Mackie, 1997). The most common interaction between protozoa and the other groups is predation (Lee et al., 2001). However, comprehensive studies of the relationship between protozoa and fungi are limited to only a few publications (Morgavi et al., 1993, 1994a,b; Miltko et al., 2014). The predation of fungi by protozoa is a two-step process during which zoospores are engulfed and digested by ciliates. Digestion begins with the degradation of chitin, an important carbohydrate component of the fungal cell wall (Miltko et al., 2014). Chitin is composed of acetyl glucosamine residues linked by β-1,4-glucosidic bonds. This compound is structurally very similar to cellulose. In contrast to cellulose, however, information about how chitin is degraded and the potential of this carbohydrate as an energy source for protozoa is restricted to *Eudiplodinium maggii* (Miltko et al., 2010a,b, 2012) and *Diploplastron affine* (Bełżecki et al., 2008).

Taking the above into account we undertook the studies reported here. The main goal was to examine and compare the process of digesting chitin by rumen ciliates that exhibit different nutritional preferences: the amylolytic *Entodinium caudatum* (Abou Akkada and Howard, 1960) and the fibrolytic *Diploplastron affine* (Michałowski et al., 1986).

Material and methods

The isolation of rumen ciliates and maintenance of stock cultures

Protozoa were isolated from the rumen of fistulated sheep. The ciliates *Diploplastron* (also known as *Metadinium affine)* and *Entodinium caudatum* were identified according to Dogiel (1927). Cultivation was initiated by picking 20–30 individuals with features typical of the selected species and inoculating them into separate Erlenmeyer flasks (50 ml) containing 40 ml of two-week-old cultures

of ruminal bacteria growing on '*caudatum*' salt solution (Coleman et al., 1972). The initial cultures of *D. affine* were fed 15 mg of food consisting of: powdered meadow hay (60%), wheat gluten (16%) and microcrystalline cellulose (24%), whereas in the diet for *E. caudatum*, microcrystalline cellulose was replaced by barley flour. Wheat gluten (G5004) and microcrystalline cellulose (310697) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The protozoa were maintained according to the method of Michałowski et al. (1986). Some of the developed cultures were used to inoculate the *in vitro* experiments. The remaining cultures were separately transferred into the rumen of defaunated sheep (Michałowski et al., 1999). They were multiplied there and used to perform the enzymatic and fermentation experiments.

Effect of chitin on ciliate growth *in vitro*

Ciliates were cultivated in a culture medium consisting of '*caudatum*' solution (see above) and food. The food of the control cultures was the same as initial cultures and was composed of powdered meadow hay, wheat gluten and barley flour (*E. caudatum*) or the first two components and microcrystalline cellulose (*D. affin*e). The particular ingredients were supplied in a proportion of: 0.3, 0.08 and 0.06 mg · ml^{-1} culture per day, respectively. The same food was supplemented with different doses of chitin (C7170, Sigma-Aldrich, St. Louis, MO, USA) at a rate of 0.015, 0.03, 0.06 and 0.12 mg · ml^{-1} culture per day (experimental cultures). The examined protozoa belong to the family *Ophryoscolecidae*, which prefer solid food particles. The meadow hay and chitin were ground in a high-speed electric grinder. The smallest particles adhering to the lid of the grinder were collected and sieved through a filter of 10 μm pore diameter. Three experimental and three control cultures were simultaneously initiated and cultivated for 28 days according to Michałowski et al. (1986). Briefly, the cultures were fed every day, and every fourth day they were transferred to a fresh medium. A new set of flasks was filled with 20 ml of fresh '*caudatum*' solution. Each of the maintained cultures was thoroughly mixed and 20 ml-portions were quickly transferred into flasks containing fresh medium. Finally, each culture was fed, saturated with CO_2 and incubated at 40ºC. On the transfer days samples were collected for counting of the protozoa and preserved with 4% formaldehyde.

Chitin ingestion and digestion

The experiment was initiated after 24-h starvation of the 6 replications of ciliates growing on a control diet (see above). After starvation, three of them were incubated without chitin (control cultures), whereas the remaining three (experimental cultures) were fed powdered chitin (0.6 mg · ml^{-1}). All cultures were incubated for 24 h and sampled to estimate the proportion of individuals filled with chitin. The sampling was performed just before the start of incubation as well as 2, 4, 8, 12, 18 and 24 h thereafter.

Measurement of chitinolytic activity

Ciliates cultivated in the rumen of monofaunated sheep were isolated, separated, purified and incubated with chloramphenicol, streptomycin and ampicillin. The final concentration of each of the antibiotics was 50 μg · ml^{-1} (Miltko et al., 2010a). The purified protozoa were collected, lyophilized and stored at –80ºC. On the day of the experiments, the samples of protozoa were thawed and homogenized. The obtained homogenate was centrifuged at 22000 *g* for 30 min at 4ºC and the supernatant was collected and used as a crude enzyme preparation.

Chitinolytic activity was determined by the quantification of reducing sugars released during the incubation of the crude enzyme preparation with substrate. Chitin resembles cellulose in that it is an insoluble carbohydrate, thus to obtain a soluble form and homogenous suspension of the substance, chitin was modified to a colloidal form and used in enzymatic studies. Colloidal chitin was prepared according to Shimahara and Takiguchi (1988). The reaction mixture consisted of 0.4 ml of a 0.2% colloidal chitin solution, 0.4 ml of enzyme preparation and 0.2 ml of 0.1 mol · l^{-1} citric-phosphate buffer (pH 6.0). The mixture was incubated for 1 h at 40ºC and the reducing sugars were measured according to Miller et al. (1960). The enzyme preparation without substrate and the substrate without the enzyme preparation were always concomitantly incubated as controls. The optimal conditions for chitin digestion were estimated by the measurement of the degradation rate of the substrate at pH and temperature ranges of 3.0 to 8.0 and 30°C to 60°C, respectively. The measurements were repeated three times using three different crude enzyme preparations. The protein content in the crude enzyme preparation was measured with the use of a Microprotein-PR kit (611A Sigma-Aldrich, St. Louis, MO, USA) according to Fujita et al. (1983). In summary, the method is based on measuring the shift in the absorption that occurs when the pyrogallol red molybdate complex binds to the basic amino acid groups of protein molecules. Absorbance measured at 600 nm is directly proportional to the protein concentration in the sample.

Fermentation of chitin

Ciliates were isolated from the rumen of monofaunated sheep (see above) and prepared as described by Bełżecki et al. (2007). In brief, the protozoa were washed with '*caudatum*' solution and suspended in Hungate solution (1942), containing a new portion of antibiotics at the same concentration and composition as described above. The samples of protozoa (40 ml) were transferred into Erlenmeyer flasks and incubated under anaerobic conditions. Three of them were incubated without chitin (control) and three were incubated with 50 mg of chitin (experimental). The bacterial controls were obtained by filtration of the protozoa suspension through a filter with a 15 μm pore diameter. The filtrate was collected and 40 ml portions were poured into the Erlenmeyer flasks and incubated simultaneously with the cultures of ciliates in presence of the same doses of chitin. Samples for counting the protozoa and measuring the concentration of volatile fatty acids (VFA) were collected just before the start of incubation and at 3, 6, 9 and 12 h thereafter. The VFA concentration was measured according to Ziołecki and Kwiatkowska (1973). The protozoa count was determined as described by Michałowski et al. (1986).

Statistical analysis

Statistical analysis was processed by the Statistica 10.0 software package (StatSoft, Inc., 2011). The statistical significance of differences was calculated using one-way and two-way analysis of variance (ANOVA). Mean values were compared by Fisher's *least significant difference* (LSD) Test or using Student's t-test. Treatment effects were considered to be significant at $P < 0.05$.

Results

Effect of chitin on ciliate growth *in vitro*

The results of the growth experiment are presented in Table 1. Differences between the density of *D. affine* and *E. caudatum* populations were found. Calculations revealed that the number of both species of protozoa was positively correlated with the amount of chitin in the diet ($P < 0.05$). The increase in the population density of *D. affine* was 26% higher than that of *E. caudatum*.

Chitin ingestion and digestion

The ingestion of chitin by ciliates during the 24-h incubation under *in vitro* conditions is summarized in Table 2. The presented data revealed that only 13.3% of individuals of *D. affine* and 15.3% of *E. caudatum* contained food particles in the cell just before feeding. The number of such individuals of *D. affine* and *E. caudatum* increased by about three- and two-fold, respectively, during the first 2 h after the chitin supplementation ($P < 0.05$). After this period, a continuous decrease of about 50% towards the end of incubation was observed ($P < 0.05$). In the control groups (A), there was a continuous decrease of individuals containing food particles ($P < 0.05$).

Table 1. The mean concentration of ciliates cultured *in vitro* on the diet without chitin (A) and diets supplemented with chitin at a ratio of: 0.015 (B), 0.03 (C), 0.06 (D), 0.12 (E) mg · ml^{-1} per day, respectively

Protozoa	Diet					SE
	A	B	C	D	E	
Entodinium caudatum	2023^{a}	2108^{a}	2162^{ab}	2230^{ab}	2355^{b}	34.8
Diploplastron affine	1504^{a}	1674^{ab}	1851^{bc}	2042^{cd}	2149^{d}	65.9

^{a,b,c,d} values with different superscipts within a row are significantly different at $P < 0.05$; mean values (n = 3)

Table 2. Changes in the proportion of protozoa with engulfed food particles (% of total number) during 24-h incubation in control group (starved – A) and experimental group (supplemented chitin – B)

Protozoa	Group	Incubation time, h							SE
		0	2	4	8	12	18	24	
Diploplastron affine	A	16.1^{a}	16.2^{a}	14.8^{b}	14.3^{b}	14.2^{b}	11.8^{c}	9.5^{d}	0.34
	B	13.3^{a}	38.0^{b}	36.0^{b}	34.7^{b}	28.0^{c}	21.3^{d}	17.7^{d}	1.39
Entodinium caudatum	A	15.9^{a}	15.8^{a}	15.6^{a}	12.1^{b}	11.1^{b}	11.6^{b}	8.5^{c}	0.41
	B	15.3^{a}	31.3^{b}	31.1^{b}	28.0^{bc}	22.7^{c}	19.7^{cd}	15.0^{ad}	1.00

^{a,b,c,d} values with different superscipts within a row are significantly different at $P < 0.05$; mean values (n = 3)

Degradation of chitin

The degradation rate of chitin differed between the ciliate species. The crude enzyme preparation obtained from *D. affine* (1.2 mg protein · ml^{-1}) and *E. caudatum* (1.6 mg protein · ml^{-1}) digested colloidal chitin at a rate of 3.9 ± 0.31 and 5.9 ± 0.76 μmol N-acetyl glucosamine· mg^{-1} protein per h, respectively. The optimal conditions for the degradation of colloidal chitin were 45°C and pH 5.0 in the case of both species.

Fermentation of chitin

The ciliates incubated for 12 h with and without chitin released VFA into the medium (Figures 1 and 2). Incubation of *D. affine* and *E. caudatum* with chitin resulted in an increase in the VFA concentration by about 33% and 29%, respectively ($P < 0.05$). The VFA content in control cultures increased by 22% and 18% and was significantly lower than in the experimental cultures ($P < 0.05$). The calculated net production rate of VFA by a single

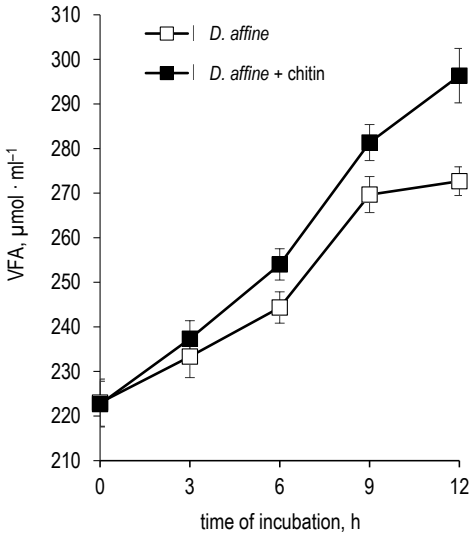

Figure 1. Changes in the concentration of volatile fatty acids (VFA) during the incubation of *Diploplastron affine* (*D. affine)* with and without chitin; mean values (n = 3)

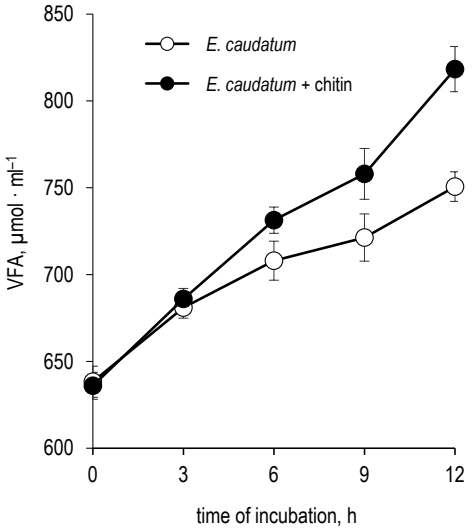

Figure 2. Changes in the concentration of volatile fatty acids (VFA) during the incubation of *Entodinium caudatum (E. caudatum)* with and without chitin; mean values (n = 3)

cell of *D. affine* and *E. caudatum* from control cultures was 3.9 ± 0.37 and 0.5 ± 0.03 pmol VFA · h^{-1}, respectively. The main products of fermentation were acetic acid (72%) followed by butyric acid (24%) and propionic acid (4%), irrespective of the ciliate species. No changes were found in the concentration of VFA in the absence of protozoa (bacterial control).

Discussion

Enrichment of the culture medium with chitin increased the population of both *D. affine* and *E. caudatum.* A similar effect of chitin was found by Miltko et al. (2010b) in relation to *E. maggii* and by Bełżecki et al. (2008), who cultivated *D. affine.* The positive effect of chitin was observed here despite the differences in nutrient preferences of the cultivated protozoa. It is assumed that *E. caudatum* uses starch to cover their requirement for carbohydrates (Abou Akkada and Howard, 1960). This is in contrast with *D. affine,* which grows well in cultures that are fed a diet devoid of starch (Michałowski et al., 1986).

The experiments on ingestion of chitin by protozoa showed an increase followed by a decrease in the number of individuals containing chitin particles. The obtained results indicate that this carbohydrate was digested inside the cell. This observation is in agreement with the data presented by Morgavi et al. (1993, 1994a,b, 1996), Bełżecki et al. (2008), and Mitlko et al. (2010a,b, 2012). The proportion of individuals engulfing chitin to the total number of protozoa was larger in the case of *D. affine* than *E. cautatum.* Although the length of *E. caudatum* is almost half that of *D. affine,* the width of the two is similar and amounts to 52 and 59 µm, respectively (Williams and Coleman, 1992). Comparison of the width of protozoa to the size of the chitin particles (below 10 µm) suggests that their diameter seems to be appropriate for both species and does not affect the engulfing rate. The higher uptake rate of chitin by *D. affine* may indicate that this protozoan is more chitinolytic than *E. caudatum*, however, the enzymatic studies do not support this suggestion. This could be because chitinolytic activity was calculated per milligram of protein in the crude enzyme preparation. The opposite effect would be obtained if this calculation were based on the number of cells from which the enzyme preparation was obtained. The optimum degradation rate of colloidal chitin for both protozoa was observed at pH 5.0 and 45°C. The same pH value was obtained by Belżecki et al. (2008), who assayed the chitinolytic activity of *D. affine*. However, the degradation rate of this carbohydrate found these authors was almost 55% higher. This may have been caused by different concentrations of protein in the crude enzyme preparation, as well as the condition of the protozoa after incubation with antibiotics. Similar results of enzymatic studies were obtained by Morgavi et al. (1994b). However, it should be noted that the cited authors worked on a mixed population of protozoa and used specific substrates to determine exochitinolytic activity. The colloidal chitin used in the present study permitted determination of total chitinolytic activity (sum of endo- and exochitinolytic activities). Accordingly, it could be expected that the obtained values would be higher than presented by Morgavi et al. (1994a). The reasons for this could be: different methods of crude enzyme preparation, lower

concentration of protein and the sensitivity of measurements.

The results of fermentation studies also confirmed that *D. affine* is more chitinolytic than *E. caudatum*. It was calculated that the net production rate of VFA by the small protozoan, *E. caudatum*, was almost 8 times lower in comparison with the larger *D. affine* when this parameter was expressed per ciliate cell. The main products of chitin fermentation were acetate, followed by butyrate and propionate. Similar proportions between the particular acids were found when cellulose (Michałowski et al., 1997), murein (Bełżecki et al., 2010) and chitin (Miltko et al., 2010b) were fermented by various species of rumen protozoa.

Conclusion

The obtained results confirm that *Diploplastron affine* can digest and utilize chitin and show that *Entodinium caudatum* also possesses this ability; their comparison indicates that *Diploplastron affine* is the more chitinolytic species.

References

Abou Akkada A.R., Howard B.H., 1960. The biochemistry of rumen protozoa. 3. The carbohydrate metabolism of *Entodinium.* Biochem. J. 76, 445–451

Bełżecki G., Miltko R., Kwiatkowska E., Michałowski T., 2010. Mureinolytic ability of the rumen ciliate *Diploplastron affine*. Folia Microbiol. Prague 55, 312–314

Bełżecki G., Miltko R., Michałowski T., Simůnek J., Kopecný J., 2008. Chitinolytic activity of the sheep rumen ciliate *Diploplastron affine*. Folia Microbiol. Prague 53, 201–203

Bełżecki G., Newbold C.J., McEwan N.R., McIntosh F.M., Michałowski T., 2007. Characterization of the amylolytic properties of the rumen ciliate protozoan *Eudiplodinium maggii*. J. Anim. Feed Sci. 16, 590–606

Coleman G.S., Davies J.I., Cash M.A., 1972. The cultivation of rumen ciliates Epidinium ecaudatum caudatum and *Polyplastron multivesiculatum in vitro*. J. Gen. Microbiol. 73, 509–521

Dogiel V.A., 1927. Monographie der Familie *Ophryoscolecidae*. Arch Protistenk. 59, 1–288

Doré J., Mackie R.I., 1997. Microbial diversity in gut ecosystem. Reprod. Nutr. Develop. 37, 15–20

Fujita Y., Mori I., Kitano S., 1983. Color reaction between pyrogallol red-molybdenum (VI) complex and protein. Bunseki Kagaku 32, E379–E386

Hungate R.E., 1942. The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. Biol. Bull. 83, 303–319

Lee S.S., Ha J.K., Cheng K.J., 2001. The effects of sequential inoculation of mixed rumen protozoa on the degradation of orchard grass cell walls by anaerobic fungus *Anaeromyces mucronatus* 543. Can. J. Microbiol. 47, 754–760

Michałowski T., 1997. Digestion and fermentation of the microcrystalline cellulose by the rumen ciliate protozoon *Eudiplodinium maggii*. Acta Protozool. 36, 181–185

Michałowski T., Harmeyer J., Bełżecki G., 1999. The importance of washing the omasum for successful defaunation of sheep. J. Anim. Feed Sci. 8, 611–619

Michałowski T., Szczepkowski P., Muszyński P., 1986. The nutritive factors affecting the growth of the rumen ciliate *Diploplastron affine in vitro*. Acta Protozool. 25, 419–426

Miller G.L., Blum R., Glennon W.E., Burton A.L., 1960. Measurement of carboxymethylcellulase activity. Anal. Biochem. 1, 127–132

Miltko R., Bełżecki G., Kasperowicz A., Michałowski T., 2010a. Isolation and purification of chitinolytic enzymes of rumen ciliates *Eudiplodinium maggii*. PCACD 15, 189–196

Miltko R., Bełżecki G., Kowalik B., Michałowski T., 2014. Can fungal zoospores be the source of energy for the rumen protozoa *Eudiplodinium maggii*? Anaerobe 29, 68–72

Miltko R., Bełżecki G., Kwiatkowska E., Michałowski T., 2010b. The ability of the rumen protozoan *Eudiplodinium maggii* to utilize chitin. Folia Microbiol. Prague 55, 349–351

Miltko R., Bełżecki G., Michałowski T., 2012. Chitinolytic enzymes of the rumen ciliate *Eudiplodinium maggii*. Folia Microbiol. Prague 57, 317–319

Morgavi D.P., Onodera R., Nagasawa T., 1993. *In vitro* metabolism of chitin and protein from ruminal fungi by ruminal protozoa. Anim. Sci. Tech. (Jpn.) 64, 584–592

Morgavi D.P, Sakurada M., Mizokami M., Tomita Y., Onodera R., 1994a. Effects of ruminal protozoa on cellulose degradation and the growth of an anaerobic ruminal fungus, *Piromyces* sp. strain OTS1, *in vitro*. Appl. Environ. Microbiol. 60, 3718–3723

Morgavi D.P., Sakurada M., Tomita Y., Onodera R., 1994b. Presence in rumen bacterial and protozoal populations of enzymes capable of degrading fungal cell walls. Microbiology 140, 631–636

Morgavi D.P., Sakurada M., Tomita Y., Onodera R., 1996. Electrophoretic forms of chitinolytic and lysozyme activities in ruminal protozoa. Curr. Microbiol. 32, 115–118

Shimahara K., Takiguchi Y., 1988. Preparation of crustacean chitin. Methods Enzymol. 161, 417–423

StatSoft, Inc., 2011. STATISTICA (data analysis software system). version 10. www.statsoft.com

Williams A.G., Coleman G.S. (Editors), 1992. The Rumen Protozoa. Brock/Springer Series in Contemporary Bioscience. Springer-Verlag, New York (USA)

Ziołecki A., Kwiatkowska E., 1973. Gas chromatography of C1 to C5 fatty acids in rumen fluid and fermentation media. J. Chromatogr. 80, 250–254