Chitinolytic enzymes of the rumen ciliate
Eudiplodinum maggii

R. Miltko · G. Belzecki · T. Michalowski

Abstract
The ability of rumen ciliates to digest chitin is clearly recognized. We investigated the chitinolytic system of the rumen ciliate Eudiplodinum maggii. The ciliates were grown in a selectively faunated sheep. They were isolated from the rumen and purified by sedimentation. A crude enzyme preparation was prepared following incubation of ciliates with antibiotics. This was done in order to reduce their contamination with intracellular bacteria. The activity of particular enzymes was examined by quantification of the products released from specific substrates. It was stated that the optimum conditions for the detected activities varied between 4.5 and 5.5 pH, and 45 and 55 °C. β-N-Acetylglucosaminidase was found as an enzyme of the highest activity (4.2 μmol/l released product per mg protein per h). The activities of endochitinase and exochitinase were almost two times lower than that of β-N-acetylglucosaminidase. Zymographic studies revealed the presence of two endochitinases, two exochitinases and two β-N-acetylglucosaminidases in the examined preparation.

Introduction
Rumen ophryoscolecid protozoa engulf readily the fungal zoospores which are rich in chitin (Williams and Coleman 1997; Lee et al. 2001). We already found that the ciliates Eudiplodinum maggii digest and ferment chitin (Miltko et al. 2010). However, no information is available on chitinolytic enzymes of this species of rumen protozoa. The objective of this study was to identify and characterize chitinolytic enzymes of this species of ciliates.

Materials and methods
The ciliates E. maggii were identified after Dogiel (1927). They were isolated from the natural rumen fauna of sheep. The ciliates were cultured under in vitro conditions according to Michalowski et al. (1991) and were then inoculated into the rumen of ciliates-free sheep (Michalowski et al. 1999). The ciliates living in the rumen of monofaunated sheep (see above) were used to perform the enzymic experiments. The samples of the fluid (about 1 l) were withdrawn from the rumen and the protozoa were isolated and purified by sedimentation (Michalowski 1990). The purified ciliates were incubated overnight with a mixture of antibiotics (chloramphenicol, streptomycin and ampicillin) each of
which was supplied at the final concentration of 50 μg/ml. The antibiotics were used in order to restrict the intracellular bacteria. After incubation, the ciliates were washed three times with warm (40 °C) caudatum type salt (Coleman et al. 1972). Finally, they were concentrated by the sedimentation method and stored at −80 °C. To obtain the enzyme preparation the frozen ciliates were thawed and homogenized in a glass homogenizer equipped with a Teflon pestle. The resulting homogenate was centrifuged (22,000×g, 30 min, 4 °C) and the supernatant was collected and used as a crude enzyme preparation. The activity of endochitinase was determined by quantification of reducing sugars released from carboxymethylchitin (Wirth and Wolf 1990) following its incubation with crude enzyme preparation. Reaction mixture consisted of 0.4 ml substrate, 0.4 ml enzyme preparation and 0.2 ml 0.1 mol/l citrate–phosphate buffer. The mixture was incubated for 1 h at 40 °C and the released products were measured spectrophotometrically according to Miller et al. (1960). The exochitinase and β-N-acetylglucosaminidase activities were determined by measurements of nitrophenyl released by crude enzyme preparation from 4-nitrophenyl-β-D-N-acetylchitobioside and 4-nitrophenyl-β-N-acetylglucosaminide, respectively. Reaction mixture consisted of 100 μl solution of 1 μmol/l substrate, 50 μl enzyme preparation and 150 μl 0.1 mol/l citrate–phosphate buffer. It was incubated for 1 h at 40 °C and the released product was quantified according to Yem and Wu (1976). Native polyacrylamide gel electrophoresis (PAGE) of crude enzyme preparation in combination with zymography technique was applied to identify the chitin degrading enzymes (Wirth and Wolf 1990). Carboxymethylchitin, 4-methylumbelliferyl-β-D-N-acetylchitobioside and 4-methylumbelliferyl-β-N-acetylglucosaminide were added as the specific substrates to the separating gels to identify endochitinase, exochitinase and β-N-acetylglucosaminidase, respectively.

### Results and discussion

We showed that *E. maggii* ciliates possess endochitinase, exochitinase and β-N-acetylglucosaminidase which were responsible for the breakdown of chitin. This finding supports the earlier results concerning of chitinolytic properties of *Diploplastron affine* (Belzecki et al. 2008). They showed also that the most active was β-N-acetylglucosaminidase. It was 12 times more active than the similar enzyme which was found in *E. maggii* by Williams et al. (1986). The endochitinase and exochitinase were similar in their activities (*p*>0.05) which were about two times lower than β-N-acetylglucosaminidase (Table 1).

In general, six protein bands exhibited the ability to degrade chitin or its derivatives (Fig. 1). Two of them were endochitinases, two were exochitinases, and two were β-N-acetylglucosaminidases.

![Fig. 1 Chitinolytic enzymes of the rumen ciliate *Eudiplodinium maggii* identified by the zymogram technique; protozoal proteins were separated by native PAGE.](https://example.com/fig1.png)

**Table 1** Characterization of chitinolytic enzymes of *Eudiplodinium maggii*

| Chitinolytic activity   | pH optimum | Temperature optimum (°C) | Degradation rate (μmol/l released product per mg protein per h) |
|------------------------|------------|--------------------------|---------------------------------------------------------------|
| Endochitinase          | 5.5        | 45–55                    | 1.7                                                           |
| Exochitinase           | 4.5–5.0    | 45                       | 2.0                                                           |
| β-N-Acetylglucosaminidase | 4.5       | 55                       | 4.2∗                                                          |

*Mean values (n=3)

*p* < 0.05

Acknowledgements This study was supported by Grant No. N311 046134, from the Polish Ministry of Scientific Research and Information Technology.
Belzecki G, Miltko R, Michalowski T, Šimůnek J, Kopečný J (2008) Chitinolytic activity of sheep rumen ciliate Diploplastron affine. Folia Microbiol 53:201–203
Coleman GS, Davies JI, Cash MA (1972) The cultivation of rumen ciliates Epidinium ecaudatum caudatum and Polyplastron multivesiculatum in vitro. J Gen Microbiol 73:509–521
Dogiel VA (1927) Monographie der Familie Ophryoscolecidae. Arch Protistenk 59:1–288
Lee Ss, Ha JK, 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. Can J Microbiol 47:754–760
Michalowski T (1990) The distribution of ciliates through the reticulorumen of sheep. Acta Protozool 29:213–222
Michalowski T, Harmeyer J, Belzecki G (1999) The importance of washing the omasum for successful defaunation of sheep. Anim Feed Sci 8:611–619
Michalowski T, Muszynski P, Landa I (1991) Factors influencing the growth of rumen ciliates Eudiplodinium maggii in vitro. Acta Protozool 25:419–426
Miller GL, Blum R, Glennon WE, Butron AL (1960) Measurement of carboxymethylcellulase activity. Anal Biochem 2:127–132
Miltko R, Belzecki G, Kwiatkowska E, Michalowski T (2010) The ability of the rumen protozoan Eudiplodinium maggii to utilize chitin. Folia Microbiol 55:349–351
Wirth SJ, Wolf GA (1990) Day-label substrates for the assay and detection of chitinase and lysozyme activity. J Microbiol Method 12:197–205
Williams AG, Coleman GS (1997) The rumen protozoa. In: Hobson PN, Stewart CS (eds) The rumen microbial ecosystem, 2nd edn. Blackie Academic and Professional, London, pp 73–120
Williams AG, Ellis AB, Coleman AG (1986) Subcellular distribution of polysaccharide depolymerase and glycoside enzymes in rumen ciliate protozoa. Curr Microbiol 13:139–147
Yem DW, Wu HC (1976) Purification and properties of β-N-acetylglucosaminidase from Escherichia coli. J Bacteriol 125:324–331