ISOLATION AND PURIFICATION OF CHITINOLYTIC ENZYMES OF RUMEN CILIATES *Eudiplodinium maggii*

Renata Miltko, Grzegorz Bełżecki, Anna Kasperowicz, Tadeusz Michałowski

The Kielanowski Institute of Animal Physiology and Nutrition
Polish Academy of Sciences. Jablonna
ul. Instytucka 3, 05-110 Jablonna, Poland
E-mail: r.miltko@ifzz.pan.pl

Abstract
Results of the earlier studies suggested an involvement of ciliates Eudiplodinium maggii in the digestion and metabolism of chitin in the rumen. In the presented paper we described the results on the preliminary identification and characterization of chitinolytic enzymes of this ciliate as well as the method of their purification. The protozoal crude enzyme preparation was used as source of enzymes, whereas the molecular filtration on Sephadex G-150 (single step method) or separation of protein by precipitation with ammonium sulfate followed by molecular filtration (two step method) were applied to purify the identified enzymes. The identification studies resulted in the detection of endochitinase, exochitinase and N-acetyl-D-glucosaminidase. The highest activity of identified enzymes were obtained in 4.0 - 4.5 pH and at 45 - 50 °C. Results of the comparative study on purification procedures showed that the single-step method enabled us to obtain enzymes of higher purity and higher activity than the two-step purification method.

Key words: *chitinase, rumen ciliate protozoa, purification.*

1. Introduction

Chitin is the second most abundant organic compound of the earth and its annual production is estimated to be 10^{10}-10^{11} tons. Chemically chitin is a linear homopolymer of *N*-acetyl-β-D-glucosamine linked by the 1,4 glicosidic bounds. The single linear chains are strong associated by the hydrogen linkages which resulted in formation of fibrils. The fibrils ensure the protective and structural function of chitin in organisms synthesizing such polysaccharides i.e. insects, crustacean or fungi.

Chitin is resistant to the enzymatic decomposition. However, the microorganisms exhibiting the ability to degrade this polysaccharide have been found in different ecosystems. One of them is the ecosystem which have been formed in the largest chamber of the ruminant's stomach i.e. in the rumen. The rumen ecosystem is composed of bacteria, fungi and protozoa. Rumen fungi synthesize chitin which contributes up to 8 % dry matter of rumen content (Orpin, 1975).

The enzymatic degradation of chitin is catalyzed by an enzymatic system consisting hydrolases of an endo and exo-mode of action. The endochitinase (EC 3.2.1.14) catalyses the first step of chitin degradation and cleaves it to *N*-acetyl-D-glucosamine oligomers. The oligomers can be attacked by two types of enzymes which release monosaccharide as end products of the substrate degradation. One of the enzymes has not been well characterized yet but it can be distinguished from endochitinase (Reguera and Leschine, 2003). The second one is the *N*-acetyl-β glucosaminidase (EC 3.2.1.30) which releases the terminal *N*-acetyl-D-glucosamine from diacetylchitobiose and from the oligomers composed of *N*-acetyl-D-glucosamine. The literature data show that the protozoa inhabiting the rumen are able to digest chitin present in fungal cell wall, and possess chitinolytic enzymes (Bełżecki et al., 2008; Morgavi et al., 1994). However, no satisfactory method for purification enzymes of rumen ciliate origin has been described to date.

The aim of our study was to develop the method enabling purification of chitinolytic enzymes synthesized by one of the most common species of the rumen ciliated protozoa *Eudiplodinium maggii.*

2. Material and methods

2.1. Protozoa

The ciliates identified after Dogiel (1927) were isolated from the natural rumen fauna of sheep. The population of *Eudiplodinium maggii* was initiated by picking the typical individuals and inoculation them to the vessel containing a developed population of rumen bacteria which were able to survive in *"caudatum"* salt solution. This solution consisting of in g/l: K_2HPO_4 - 6.3, KH_2PO_4 - 5.0, $CaCl_2 \times 7\ H_2O$ - 0.09, $MgSO_4 \times 6\ H_2O$ - 0.09, CH_3COONa - 0.75, NaCl - 0.65 and water. It is the most often used salt solution to maintain the rumen ciliates *in vitro* (Coleman et al., 1972). About 50 cells of protozoa were inoculated to each culture vessel and were cultured by a routine method (Michałowski, 1979). The developed cultures of protozoa were then transferred to the continuous culture system Michalowski (1979) and the cultivation was continued for over one year.

Protozoa growing in the continuous culture system were used as an inoculum to develop the population of *Eudiplodinium maggii* in the rumen of ciliate free sheep. Such a procedure was necessary to get satisfactory number of ciliates for enzymatic studies. The ciliate free sheep was obtained by elimination of its natural rumen fauna by the washing procedure described by Michalowski et al., (1999).

2.2, Preparation of ciliates free of food particles and external bacteria

Samples of rumen content weighing of about 1 kg were withdrawn from the rumen. They were diluted with 2 liter of "*caudatum*" salt solution (Coleman et al., 1972) and squeezed through a screen of pore the size 260 μm. The filtrate was collected, poured into a separatory funnel and allowed to stand for about 30 min. at 40 °C. The protozoa forming a white pellet at the bottom of the funnel were resuspended in about 200 ml of the same salt solution and sedimented again. The sedimentation procedure was repeated 2 - 3 times and the purified protozoa were subjected to the procedure of elimination of the intracellular bacteria.

2.3. Preparation of suspension of bacteria free ciliates

To kill the intracellular bacteria the purified ciliates (see above) were suspended in 1 L of salt solution of Hungate (1942) supplemented with chloramphenicol, streptomycin and ampicillin. The final concentration of each antibiotics was 50 μg/ml. The ciliates were anaerobically incubated over night at 40 °C and separated by the above sedimentation technique. They were washed with "*caudatum*" salt solution (Coleman et al., 1972), and stored at - 80 °C as untreated material or were lyophilized prior to storage.

2.4. Preparation of crude enzyme

Samples of ciliates were thawed and homogenized in a glass homogenizer equipped with a Teflon pestle. The resulting homogenate was centrifuged at 22000 *g* for 30 min at 4^0C and the supernatant was collected and used as a crude enzyme preparation.

2.5. Protein precipitation

The protein was precipitated from the crude enzyme preparation using ammonium sulfate which was added up to 60 % saturation and then the preparation was centrifuged for 20 min at 4 °C and 20,000 g. The supernatant was discarded whereas the sediment was collected, resolved in a cold distilled water (4 °C) and dialyzed overnight against the same water at 4 °C. The dialyzed protein solution was collected and stored at - 80 °C.

2.6. Gel filtration

The protein solution and crude enzyme preparation was applied on Sephadex G-150 column (0.8 cm × 40 cm) equilibrated and eluted with 0.05 M TRIS/HCl buffer (pH 7.4). The elution was carried out at a flow rate 6.4 cm^3/cm/h whereas the fractions 2.0 ml in volume were collected and examined for the chitinolytic activity.

2.7. Determination of chitinolytic activity

The total chitinolytic activity was determined by the quantification of reducing sugars released from colloidal chitin during 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 M citric-phosphate buffer (pH 6.0). The mixture was incubated for 1 h at 40 °C and reducing sugars were measured according to Miller et al., (1960).

The endochitinase activity was determined by the same method with an use of carboxymethylchitin as substrate. However, the buffer of pH ranged between 3 and 8 and the incubation temperature - between 30 and 60 °C were used when the optimal conditions for enzyme activities were determined.

The exochitinase and N-acetyl-β-glucosaminidase activities were determined by quantitative measurements of p-nitrophenyl released from p-nitrophenyl N,N-diacetyl-D-chitobioside or p-nitrophenyl-β-N-acetyl-glucosaminide incubated with enzyme preparation, respectively. Reaction mixture consisting 100 µl substrate, 50 µl enzyme preparation and 150 µl ml 0.1 M citric-phosphate buffer (pH 6.0). The mixture was incubated for 1 h at 40 °C and the p-nitrophenyl was measured according to Yem and Wu (1976). The determinations of optimal condition for enzyme activities were performed as described previously.

2.8. Protein assay

Protein content was determined with the urine protein kit (Sentinel 17275), using a bovine serum albumin solution as a standard. However, during purification of enzyme by molecular filtration the relative protein concentration was estimated by measurements of absorbance at 280 nm.

2.9. Chemicals and substrates

Sephadex G 150, p-Nitrophenyl *N,N*-diacetyl-D-chitobioside and p-nitrophenyl-β-N-acetyl-glucosaminide and other chemicals were supplied by Sigma Aldrich Co. Carboxymethylchtin (CM chitin) was prepared according to Wirth and Walh (1990) and colloidal chitin according to Takeshi et al., (1999).

3. Results and discussion

3.1. Chitinolytic activity characterization

The examination of crude enzyme preparation of the rumen protozoan *Eudiplodinium maggii* reveled presence of enzymes actively degraded colloidal chitin. Using specific substrates three types of activities was found there i.e. endochitinase, exochitinase and N-acetyl-β-glucosaminidase (***Table 1***).

Table 1. *Optimal conditions for degradation of chitin and its derivatives being the specific substrates for measurements of the activity of particular enzymes present in crude enzyme preparation of rumen ciliates Eudiplodinium maggii.*

Substrate	pH optimum	Temperature optimum
Colloidal chitin	5	50
Carboxymethylchitin	5.5	50
p-nitrophenyl- N,N-diacetyl-D-chitobioside	5	45
p-nitrophenyl-β-N-acetyl-glucosaminide	4.5	55

The optimum pH for the highest degradation rate of the specific substrates used to measure the activities of particular enzymes varied between 4.5 - 5. Thus it was similar to this detected in the case of *Diploplastron affine* (Bełżecki et al., 2008). The highest activity of enzymes was observed when temperature of reaction varied between 45 - 50 °C. This findings are in good accordance with the data obtained by Morgavi et al., (1994) and Bełżecki et al., (2008) and concerned to the chitinyolytic enzymes of two other species of rumen ciliates i.e. *Entodinium caudatum* and *Diploplastron affine*.

The obtained results showed that endochitinase, exochitinases and β-N-acetyloglucosamidase were presumably involved in the digestion of chitin by *Eudiplodinium maggii*. The same type of enzymes were found earlier when rumen ciliate *Diploplastron affine* (Bełżecki et al., 2008), rumen bacteria (Kopecný and Hodrová, 2000) and rumen fungi were examined (Novotná et al., 2008; Sakurada et al., 1998). The results of fractionation of crude enzyme preparation by a gel filtration method are presented on ***Figure 1***. The overlapping peaks of three different activities i.e. endochitinase, exochitinase and N-acetyl-β-glucosaminidase were identified. It was also stated that fraction no. 20 was the most active against CM-chitin, while fraction no. 21 exhibited the highest activity of exochitinase and *N*-acetyl-D-glucosaminidase.

3.2. Effect of lyophilization

Crude enzyme preparation obtained from *Eudiplodinium maggii* was either only frozen and kept at -80C or lyophilized and then stored at - 80 °C .The effect of the treatment on the activity of enzyme in the crude enzyme preparation is presented in ***Table 2***.

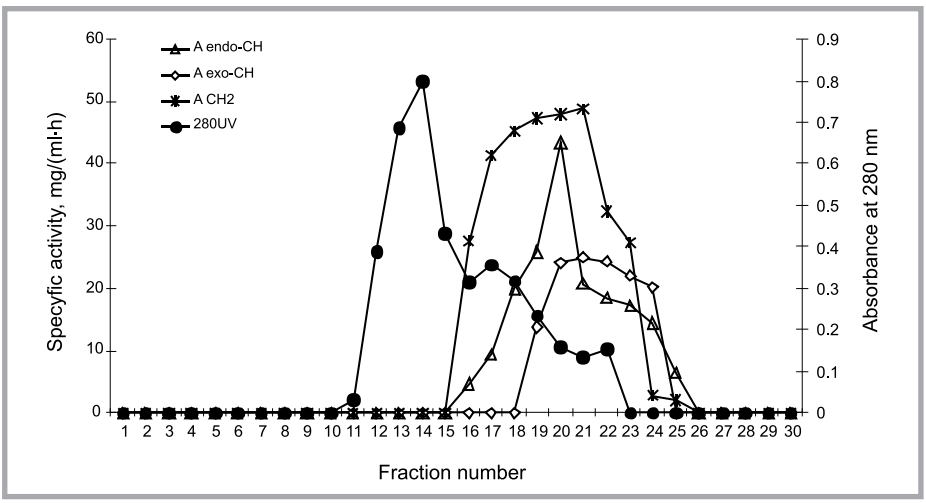

Figure 1. *Distribution pattern of protein and the endochitinase exochitinase and N-acetyl-β-glucosamidase activities over the fractions obtained following the gel filtration of crude enzyme preparation of Eudiplodinium maggii on Sephadex G-150.*

The results presented in ***Table 2*** showed that the most active was the untreated crude enzyme preparation kept frozen at - 80 °C. No differences were only found between the endochitinases activity of the fresh and the frozen crude enzyme preparation. The lyophilization of this preparation prior to storage resulted in the significant decreasing of activities of all three enzymes. The decrease was especially visible in comparison to the activities of enzymes present in the untreated crude enzyme preparation kept frozen. On the basis of these findings we used only the untreated and stored at - 80 °C preparation to continue the study.

3.3. Purification of enzymes

To purify enzymes we applied the gel filtration of crude enzyme preparation or separation of protein by precipitation with ammonium sulfate at 60 % saturation followed by gel filtration. The results of purification of enzymes by the two methods are presented in ***Tables 3, 4*** and ***5***.

A 70 fold purification of endochitinase and 48% recovery of its activity was found when enzyme was purified by gel filtration (***Table 3***). The purification degree was visibly lower when the protozoal protein was precipitated and then filtrated on Sephadex G-150. Similar relationship was found for exochitinase and N-acetyl-β-glucosaminidase (***Table 4*** and ***5)***.

As we have shown above the molecular filtration of crude enzyme preparation was more effective method for purification of enzymes involved in the chitin digestion by rumen ciliate *Eudiplodinium maggii* comparing the method composed of precipitation of protein with ammonium sulfate followed gel filtration. We suppose that the drop in the activity of enzymes purified by the later method could result from too long time of dialysis which fol-

Table 2. *Effect of treatment on the activity (μM product/mg protein/h) of chitinolytic enzymes present in crude enzyme preparation of rumen ciliates Eudiplodinium maggii.*

Treatment	Chitinolytic activity (μm product/mg protein/h)		
	Endochitinase	Exochitinase	β-N-acetyloglucosamidases
Fresh	1.70	2.00	4.20
Lyophilized	0.58	1.17	1.40
Frozen	1.52	10.32	19.46

Table. 3. *Comparison of the activity of endochitinase in the untreated crude enzyme preparation and after purification by two different methods; Specific activity - μM released product/mg protein/h. Total activity - μM released product/ protein content of the examined sample/h.*

Purification method	Total protein	Specific activity	Total activity	Yield, %	Purification folds
Untreated preparation	5.46	0.58	3.16	100	1
Gel filtration	0.04	40.89	1.53	48	70.5
Untreated preparation	2.90	1.56	4.52	100	1
Precipitation +gel filtration	0.03	43.93	1.49	33	28

Table 4. *Comparison of the activity of exochitinase in the untreated crude enzyme preparation and after purification by two different methods; Specific activity - μM released product/mg protein/h. Total activity - μM released product/ protein content of the examined sample/h.*

Step	Total protein	Specific activity	Total activity	Yield, %	Purification folds
Crude extract	5.46	1.17	6.38	100	1
Sephadex	0.04	81.22	3.45	54	69
Crude extract	2.90	3.69	10.70	100	1
Precipitation +Sephadex	0.04	143.00	5.15	48	39

Table 5. *Comparison of the activity of N-acetyl-β-glucosamidase in the untreated crude enzyme preparation and after purification by two different methods; Specific activity - μM released product/mg protein/h. Total activity - μM released product/ protein content of the examined sample/h.*

Step	Total protein	Specific activity	Total activity	Yield, %	Purification folds
Crude extract	5.46	1.40	7.64	100	1
Sephadex	0.04	152.51	6.48	85	109
Crude extract	2.90	3.14	9.11	100	1
Precipitation +Sephadex	0.04	171.05	6.16	68	54

lowed the protein precipitation. However, to explain this speculation it would be necessary to purify enzymes from the same preparation using simultaneously the both methods.

4. Conclusion

Our study showed that molecular filtration is a satisfactory method for purification of chitinolytic enzymes of the rumen ciliate *Eudiplodinium maggii.* It is possible that separation of protein by precipitation with ammonium sulfate or/and dialysis of precipitate against water affects negatively the purified enzymes.

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6. References

1. ***Bełżecki G., Miltko R., Michałowski T., Šimůnek J., Kopečný J.****; (2008) Chitinolytic activity of the rumen ciliates Diploplastron affine. Folia Microbiol. 53, pp. 201-203.*
2. ***Coleman G. S., Davies J. I., Cash M. A.****; (1972) The cultivation of rumen ciliates Epidinium ecaudatum and Polyplastron multivesiculatum in vitro. J. Gen. Microbiol. 75, pp. 509-521.*
3. ***Dogiel V. A.****; (1927) Monographie der Familie Ophryoscolecidae. Arch. Protistenk. 59, pp. 1-288.*
4. ***Hungate R. E.****; (1942) The culture of Eudiplpdinium neglectum with experiments on the digestion of cellulose. Biol. Bull. 83, pp. 303-319.*
5. ***Kopecný J., Hodrová B.****; (2000) Chitinolytic enzymes produced by ovine rumen bacteria. Folia Microbiol., 45, pp. 465-468.*

6. **Michałowski T., Harmeyer J., Bełżecki G**.; (1999) The importance of washing the omasum for successful defaunation of sheep. J. Anim. Feed. Sci. 8, pp. 611-619.
7. **Michałowski T.**; (1979) A simple system for continuous culture of rumen ciliates. Bull. Pol. Acad. Sci. Biol. 27, pp. 581-583.
8. **Miller G. L., Blum R., Glennon W. E., Butron A. L.**; (1960) Measurement of carboxymethylcellulase activity. Anal. Biochem. 2, pp. 127-132.
9. **Morgavi D. P., Sakurada M., Tomita Y., Onodera R.**; (1994) Presence in rumen bacteria and protozoal populations of enzymes capable of degrading fungal cell walls. Microbiology 140, pp. 631-636.
10. **Novotná Z, Fliegerová K, Simůnek J.**; (2008) Characterization of chitinases of polycentric anaerobic rumen fungi. Folia Microbiol. 53, pp. 241-245.
11. **Orpin C. G.**; (1975) Studies on the rumen flagellate Neocalimastix frontalis. J. Gen. Microbiol. 84, pp. 395-398.
12. **Reguera G. Leschine S. B.**; (2003) Biochemical and genetic characterization of ChiA, the major enzyme component for the solubilization of chitin by Cellulomonas uda. Arch Microbiol. 180, pp. 434-443.
13. **Sakurada M.; Morgavi D. P.; Ushirone N., Komatani K., Tomita Y., Onodera R.**; (1998) Purification and characteristics of membrane-bound chitinase of anaerobic ruminal fungus Piromyces communis OTS1. Current Microbiology. 37, pp. 60-63.
14. **Takeshi T., Shinsuke F., Shingo N., Toshiaki F., Masahiro T., Tadayuki I.**; (1999) A Unique Chitinase with Dual Active Sites and Triple Substrate Binding Sites from the Hyperthermophilic Archaeon Pyrococcus kodakaraensis KOD1. Appl. Environ Microbiol. 63, pp. 5338-5344.
15. **Wirth S. J., Walh G. A.**; (1990) Day-label substrates for the assay and detection of chitinase and lysozyme activity. J. Microbial Method. 12, pp. 197-205.
16. **Yem D. W., Wu H. C.**; (1976) Purification and properties of β-N-Acetyloglucosoamidase from Escherichia coli. J. Bacteriol. 125, pp. 324-331.