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Ability of the Rumen Ciliate *Epidinium ecaudatum* to Digest and Use Crystalline Cellulose and Xylan for *in vitro* Growth

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Summary. The rumen ciliate protozoan *Epidinium ecaudatum* was isolated from rumen fluid of sheep and either grown *in vitro* or inoculated into the rumen of the ciliate-free sheep. Population density of ciliates *in vitro* was about 320 cells/ml when culture salt solution was supplemented with hay (0.6 mg/ml/d) and wheat gluten (0.15 mg/mg/d). Addition of the microcrystalline cellulose (0.25 mg/ml/d) to the control diet increased the ciliate numbers to about 440 cells/ml ($P < 0.01$). Conversely, oat spelt xylan decreased the concentration of protozoa to 250 cells/ml ($P < 0.05$). Ciliates readily ingested and digested cellulose particles while xylan particles were only sporadically engulfed. Only glucose was released from the microcrystalline cellulose and cellobiose during incubation of the both substrates with protozoal protein; the release rate was 0.19 and 14.9 μM/mg protein/h, respectively. Carboxymethylcellulose and xylan were degraded at 17.5 and 66.6 μM reducing sugars released from substrate/mg protein/ml. Degradation rate of microcrystalline cellulose, carboxymethylcellulose, cellobiose and xylan was the highest at pH 6.5, 5.5, 6.0 and 6.5, respectively. Non-denaturating polyacrylamide gel electrophoresis combined with CMC-ase and xylanase zymogram revealed the presence of three protein bands active against CMC and two protein bands degrading xylan. Thin layer chromatography showed a presence of only oligosaccharides in the end products released from CMC and xylan by enzymes isolated from gel slices. Neither glucose nor xylose were found there. Conversely only glucose was detected by TLC following incubation of microcrystalline cellulose with solution of protozoal protein. The same preparation released mainly xylose from xylan but different oligosaccharides were also present.

Key words: crystalline cellulose, digestion, β-endoglucanase, β-endoxylanase, β-glucosidase, *Epidinium ecaudatum*, xylan.

INTRODUCTION

The ciliate protozoan *Epidinium ecaudatum* belongs to the most common species of ciliates inhabiting the rumen of domestic ruminants. It is considered to participate in the degradation of plant structural polysaccharides (Dehority 1993). However, there is some disagreement regarding the contribution of this ciliate to the digestion of plant cell wall polysaccharides. Coleman (1985) showed that the ability of *Epidinium ecaudatum* to degrade microcrystalline cellulose was higher than activity of *Eudiplodinium maggii* and Williams and Coleman (1985) have stated that xylanolytic activity of *Epidinium ecaudatum* is comparable to that extracted from the cells of *Polyplastron multivasiculatum* and *Eudiplodinium maggii*. On the other hand there are also evidence showing that the xylan and especially microcrystalline cellulose digesting and fermenting ca-

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pacities of *Epidinium* isolated from the rumen are substantially lower than these of *Eudiplodinium maggii* and *Polyplastron multivesiculatum* (Jouany and Martin 1997). Due to above disagreement we decided to undertaken the study presented in this report. The objectives of our experiments were: (a) to compare the population size of ciliates grown *in vitro* in the medium supplemented with either cellulose or xylan; (b) to study the ingestion and digestion of the microcrystalline cellulose and xylan by cultured protozoa; (c) to identify and characterize some of fibrolytic enzymes produced by ciliates of the genus *Epidinium*.

MATERIAL AND METHODS

Protozoa

Ciliates *Epidinium ecaudatum* identified according to Dogiel (1927) were isolated from the rumen of sheep fed a hay-concentrate diet and kept *in vitro* as a one species population as described elsewhere (Michałowski 1995). Ciliates from *in vitro* cultures were inoculated into the rumen of defaunated sheep which was used as a source of protozoa for enzymatic assays. The sheep were defaunated by evacuation and heating of the reticulo-rumen content and washing of the rumen and reticulum walls as described by Michałowski *et al*. (1999).

Cultivation experiments

Ciliates were cultured in "*caudatum*" type salt solution (Coleman *et al*. 1972) composed of (g/l): K_2HPO_4-6.3; KH_2PO_4-5.0; CH_3COONa-0.75; NaCl-0.65; $MgSO_4 \cdot 7H_2O$-0.09; $CaCl_2 \cdot 6H_2O$-0.09. The control diet consisted of powdered hay (0.6 mg/ml/d) and wheat gluten (0.15 mg/ml/d). Experimental diets contained the same quantities of hay and wheat gluten and either microcrystalline cellulose (Sigmacell No. 20; Sigma, S-3504), or xylan from oat spelts (Sigma, X-0627) in the proportion of 0.25 mg/ml/d). Methods of initiation of the cultures and cultivation of protozoa were the same as described earlier (Michałowski 1995). Ciliates were fed every day and diluted with fresh "*caudatum*" medium (1:1, v/v) every fourth day. Three cultures were always run simultaneously on each diet for 4 weeks. The volume of each culture was 40 ml. Protozoa were counted on dilution days.

Meadow hay was used to feed the protozoa. It was ground in a high speed grinder and the smallest particles which settled on the lid of the grinder were collected and used as food components.

Cellulose and xylan ingestion

Ciliates cultured *in vitro* and fed control diet were starved for 24 h, and then fed with microcrystalline cellulose or xylan (0.5 mg/ml) and sampled to estimate the proportion of individuals with engulfed polysaccharides. The samples were taken just before feeding and at 1, 2, 4, 8, 12, 24 and 36 h thereafter. The experiment was repeated three times.

Enzyme preparation

Ciliates cultured in the rumen of sheep inoculated only with *Epidinium ecaudatum* were used. The protozoa were separated from food debris and external bacteria by repeated sedimentation according to Michałowski (1990). The sample of rumen content (about 1 kg) was diluted with warm *"caudatum"* salt solution in the proportion of 1:2 (w/v) and squeezed through a screen of the pore size of 0.5 mm. The liquid part was collected, poured into the separatry funnels and allowed to stand at 40°C for 30 min. The sedimented protozoa were collected, suspended in *"caudatum"* solution, poured into centrifuge tubes of 100 ml in volume and allowed to stand at 40°C for 3-5 min. During this time the ciliates sedimented forming a white layer at the bottom of the tubes while the plant debris collected at the top. Both the plant particles and supernatant were removed by suction while the protozoa were suspended as described above and sedimented again. The procedure was repeated 5-6 times while purity of ciliate was examined microscopically. Finally well purified protozoa were suspended in *"caudatum"* salt solution and incubated overnight in the presence of chloramphenicol, streptomycin and ampicillin (each at concentration of 100 µg/ml) to eliminate the intracellular bacteria. On the next day the bacteria free ciliates were washed three times with "*caudatum*" salt solution (Coleman *et al.* 1972) and stored at -20°C or disrupted immediately using a glass homogenizer equipped with a teflon pestle. Homogenate was centrifuged at 20000 x g at 4°C for 20 min to remove the non disrupted cells and the supernatant fraction was collected and used either as a crude enzyme preparation or for protein precipitation. Protein was precipitated using ammonium sulphate at 80 % saturation. Precipitated protein was dissolved in small volume (5-10 ml) of cold (4°C) distilled water, dialyzed against the same water and lyophilized and stored at -20°C. Protein in both the crude enzyme preparation and in lyophilizate was measured using Microprotein-PR™ reagent (Sigma 611-A).

Enzyme assays

Degradation of the microcrystalline cellulose was assayed by quantitative determination of glucose released from this polysaccharide following incubation with protozoal protein precipitated from the supernatant fraction (see above). Samples of substrate (5 mg) were added to tubes containing 1000 µl solution of protein at concentration of about 200 µg/ml., and incubated anaerobically at 40°C for 72 h. Protein was dissolved in 0.1 M McIlvaine buffer of different pH. Both the protein alone and substrate without protein were dissolved in the same buffers and incubated simultaneously as controles. Similar controles were applied in examinations of the other activities (see below). Measurements were performed just before the start of incubation as well as after 6, 12, 24, 48 and 72 h. Released glucose was measured enzymatically with the glucose oxidase-peroxidase coupled reactions using the Glucose (Trinder) reagent (Sigma No 315). The same method was used to measure cellobiose hydrolysis. Incubation mixture consisting 1 ml of protein solution and 5 mg of cellobiose (Sigma C 7252) was incubated for 4 h while released glucose was measured just before the commencement of incubation as well as at 1, 2, 3 and 4 h thereafter. Glucose was used as a standard. Carboxymethylcellulase (CMC-ase) activity was determined by measurement of reducing sugars released from carboxymethylcellulose (Sigma C 5678) following incubation of substrate with crude enzyme preparation. Incubation mixtures consisted of 750 µl

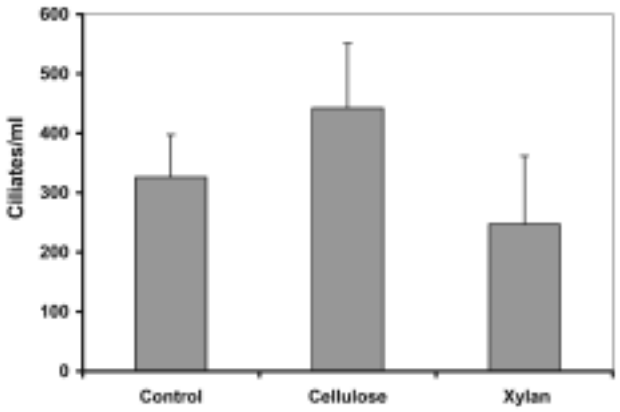

Fig. 1. The number of *Epidinium ecaudatum* in the cultures fed hay and wheat gluten (Control) or hay and wheat gluten supplemented either with microcrystalline cellulose (Cellulose) or xylan (Xylan). Hay was given at the rate of 0.6 mg and wheat gluten - 0.15 mg/ml/d. The both cellulose and xylan were supplemented at the rate of 0.25 mg/ml/d. Mean values ± S.D., n = 24

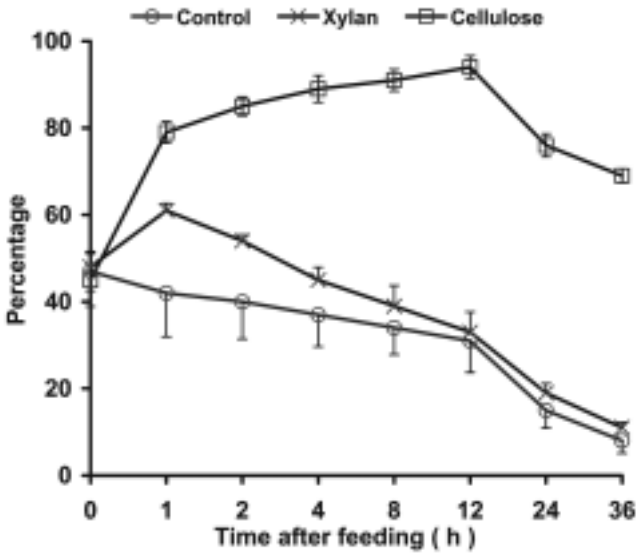

Fig. 2. Percentage of ciliates containing food particles in endoplasmic sacs at different time after giving either microcrystalline cellulose or xylan to protozoa. Control cultures were starved during the sampling period. Mean values ± S.D., n = 3

Table 1. Degradation rate of different carbohydrates (μM glucose or xylose released from appropriate substrate/mg protein/h) and pH optimum for particular activities

Substrate	Degradation rate	pH
Crystalline cellulose *	0.19 ± 0.02	6.5
Carboxymethylcellulose **	17.5 ± 3.46	5.5
Cellobiose *	14.9 ± 4.36	6.0
Xylan **	66.6 ± 0.87	6.5

* incubated with partially purified protein
** incubated with crude enzyme preparation

of 2 % sodium carboxymethylcellulose dissolved in 0.2 M McIlvaine buffer of different pH and 500 μl of crude enzyme preparation. The mixture was incubated for 1 h at 40°C and reducing sugars were determined using dinitrosalicylic reagent according to Miller *et al*. (1960) and Groleau and Forsberg (1981). Xylan hydrolysis was examined by the same method but incubation mixtures composed of 50 μl of crude enzyme preparation, 750 μl of xylan solution and 450 μl of 0.2 McIlvain buffer of different pH. Glucose and xylose standard curves were used for calculation the quantity of released sugars, respectively. Birch wood xylan (Sigma, X-4252) was used in preparation of xylan solution. The samples of substrate weighing 0.5 g were suspended in 25 ml of appropriate buffer and warmed to about 70°C for 15 min. The mixture was allowed to stand at ambient temperature and then centrifuged for 10 min at 10000 x g. Pellet was discarded while supernatant was used as substrate for determination described above.

Enzyme identification

Native polyacrylamide gel electrophoresis (NPAGE) of protozoal protein, combined with CMC-ase and xylanase zymogram technique was used for enzyme localization and identification according to Michałowski (1997). Electrophoresis was performed in Tris/glycine buffer (5 mM) at pH 8.3. A 7 % polyacrylamide gel was used. Carboxymethylcellulose or xylan were copolymerized with polyacrylamide at the final concentration of 0.1 % and used as substrates for identified enzymes. Ten times concentrated electrode buffer (50 mM) was used as gel buffer to prevent the substrate breakdown during protein migration. Electrophoresis was performed at a constant voltage of 94 V in a minidual unit (Sigma) and was followed by incubation of the gel in 0.1 M McIlvaine buffer of pH 5.5 (CMC-ase zymogram) or 6.5 (xylanase zymogram) for 15 min at 40°C. Reducing sugars released in the gel from the copolymerized CMC or xylan by the identified enzymes were visualized using 2,3,5 triphenyltetrazolium chloride solution (0.2 %) in 0.5 N NaOH according to Gabriel and Wang (1969).

End product identification

End products of CMC, xylan and microcrystalline cellulose hydrolysis were identified by thin layer chromatography (TLC). The CMC-ase and xylanase enzymes were isolated from the gels following electrophoretic separation of protozoal protein and localization of enzymes on the gel lanes. Appropriate gel slices were excised and immersed in a small volume (1-2 ml) of 0.1 M MacIlveine buffer (pH 6.0) and homogenized using glass homogenizer. CMC or xylan samples were added to homogenate to the final concentration of 0.1 % and the mixtures were incubated anaerobically at 40°C for 24 h. End products released from microcrystalline cellulose and xylan following incubation with protozoal protein were also identified. The samples of either cellulose or xylan (5 mg) were added to tubes containing protozoal protein (about 200 μg) suspended in 1 ml of McIlvaine buffer of appropriate pH and incubated anaerobically either for 24 (xylan) or 72 h (microcrystalline cellulose) at continuous agitation. Both the protein solution alone and substrates dissolved in the buffer were incubated simultaneously with experimental samples

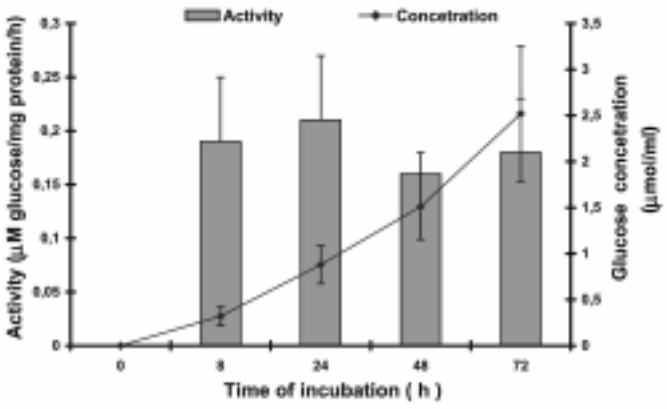

Fig. 3. Changes in cellulolytic activity and concentration of glucose released from microcrystalline cellulose during incubation with *Epididinium ecaudatum* protein. Mean values ± S.D., n = 3

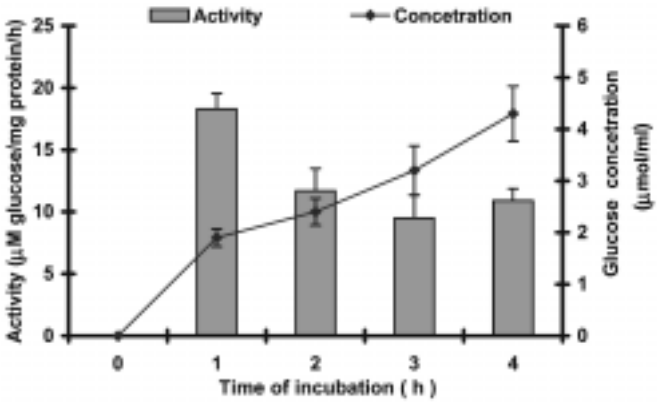

Fig. 4. Changes in enzyme activity and concentration of glucose released from cellobiose during incubation with *Epidinium ecaudatum* protein. Mean values ± S.D., n = 3

and used as control. Samples of appropriate digests were spotted in the volume of 20 µl on silica plates (Silufol, Avalier, Czechoslovakia) and developed 3 (cellulase products) or 4 fold (xylanase products) using a mixture of butanol, ethanol, water (5:5:5, v/v) or butanol, ethanol water (5:5:3, v/v), respectively. The carbohydrates were visualized using a mixture consisted of diphenylaniline (4 g), aniline (4 ml), phosphoric acid (20 ml) and acetone (200 ml).

Bacterial test

Ciliates incubated with and without antibiotics (see above) were separately disrupted in glass homogenizer equipped with a teflon pestle and homogenate was used in examination for presence of intracellular cellulolytic bacteria according to the method described earlier (Michałowski 1997). Samples of homogenate were inoculated to the tubes filled with liquid medium for cultivation of cellulolytic bacteria (Anaerobe Laboratory Manual 1973) and strips of Whatman No. 1 paper was immersed aseptically in each tube. The initiated cultures were incubated for at least 6 weeks at 40°C. The appearance of the strips was observed every day.

Chemicals

All chemicals were of analytical purity. They were supplied by Sigma Chemical Co. Wheat gluten was prepared according to Klein (1933) and Pace (1955).

Statistical analysis

Student·s t-test was used to compare the differences between mean values according to Ruszczyc (1970).

RESULTS

Ciliate growth

The population density of *Epidinium ecaudatum* cultured *in vitro* varied from about 120 to almost 600 cells/ml in relation to day and food composition. Mean number of protozoa in the cultures fed hay and wheat gluten (control diet) was 320 individuals/ml (Fig. 1). Microcrystalline cellulose supplement increased the population density by about 38 % ($P < 0.01$). Addition of xylan to the control diet resulted in decrease of the number of ciliates by about 44 % ($P < 0.05$). Microscopical examination of ciliates showed that cellulose particles were engulfed by numerous individuals.

Cellulose and xylan engulfment

Addition of the microcrystalline cellulose to the suspension of ciliates resulted in the rapid increase in the number of cells containing this polysaccharide in endoplasm. The proportion of such cells in the suspension of ciliates increased up to 12 h after feeding and was followed by continuous decrease (Fig. 2). Protozoa engulfed large quantities of cellulose and majority of them were completely filled with this polysaccharide. Xylan particles were engulfed to a lesser extent than these of cellulose. Cells containing food particles in the endoplasmic sacs increased by about 12 % during the first hour after xylan was introducing to the ciliate suspension ($P < 0.05$) and was followed by continuous decrease during the next 35 h like the control protozoa which were starved all time. There were difficulties in distinguishing the xylan and hay containing ciliates from these with only hay particles in endoplasmic sacs. Due to this the true number of the xylan engulfing protozoa could not be determined. We observed only that majority of ciliates were almost or completely empty starting from 4 h after xylan feeding.

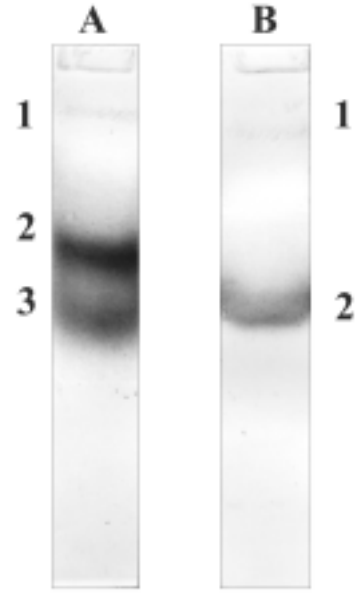

Fig. 5. Non-denaturating polyacrylamide gel electrophoresis of *Epidinium ecaudatum* protein combined with β-endoglucanase (CMC-ase) and xylanase zymogram. Lane A - three CMC-ase activities: Lane B two β-endoxylanase activities. All activities were visualized by staining with 2,3,5 triphenyltetrasolium chloride

Fig. 6. TLC of the end products of CMC and microcrystalline cellulose hydrolysis catalyzed with *Epidinium ecaudatum* enzymes. Lanes 1-5 - the standards of glucose, cellobiose, cellotriose, cellotetraose and cellopentaose, respectively. Lanes 6-8 - the products released from CMC by three β-endoglucanases obtained from the slices excised from the gel following electrophoretic separation of *Epidinium ecaudatum* protein (see Fig. 5, lane A). Lane 9 - end product of the microcrystalline cellulose hydrolysis with enzymes present in protein precipitated from the crude enzyme preparation (crude extract)

Enzyme activities

Incubation of microcrystalline cellulose, CMC, cellobiose and xylan with protozoal enzyme preparation resulted in release of reducing products. Mean degradation rate of different carbohydrates and pH optimum of particular activities are presented in Table 1. Microcrystalline cellulose was degraded at the lowest rate of all carbohydrate tested. Incubation of this polysaccharide with protozoal protein resulted in a continuous release of glucose into the medium. Degradation rate of this substrate calculated from the changes in the product concentration was constant during the incubation period (Fig. 3). No increase was found in glucose concentration in the control tubes.

Cellobiose was degraded with the rate by about 80 times higher than microcrystalline cellulose. The rate of glucose release from cellobiose during the first hour of incubation was higher ($P < 0.05$) than during the next 3 h period (Fig. 4).

Carboxymethylcellulose was degraded with the rate of about 4 times slower than xylan. This last polysaccharide was hydrolyzed with the highest rate of all carbohydrate tested ($P < 0.01$).

Enzyme separation and identification

Non-denaturating polyacrylamide gel electrophoresis combined with the CMC-ase and xylanase zymograms revealed the presence of three protein bands active against carboxymethylcellulose and two bands degrading xylan (Fig. 5).

End product identification

Oligosaccharides of the molecular mass similar to cellohexaose and disaccharides were the main products of carboxymethylcellulose hydrolysis catalyzed by all three enzymes obtained from the gel slices. Glucose was not identified there. It was, however, the only product identified following incubation of microcrystalline cellulose with the suspension of protozoal protein (Fig. 6).

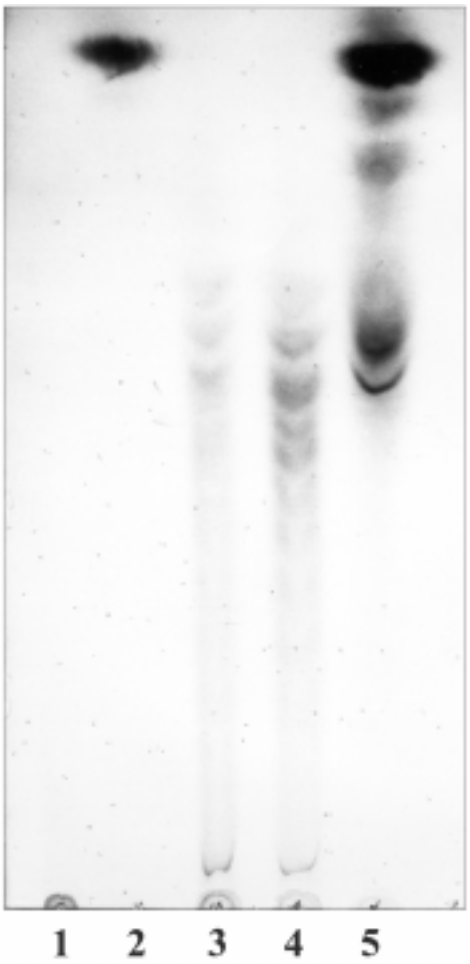

Fig. 7. TLC of the end products of xylan hydrolysis catalyzed with *Epidinium ecaudatum* enzymes. Lane 1 - xylan sample. Lane 2 - the standard of xylose. Lanes 3 and 4 - the products released from xylan by two β-endoxylanase enzymes obtained from the slices excised from the gel following electrophoretic separation of *Epidinium ecaudatum* protein (see Fig. 5, lane B). Lane 5 - end products of the xylan hydrolysis with protein precipitated from the crude enzyme preparation (crude extract)

Only oligosaccharides were found as end products of xylan hydrolysis catalyzed by the enzymes separated from the gels. Neither xylose nor xylobiose were identified there. Xylose, and perhaps xylobiose as well as longer oligosaccharides were present in the digests when xylan was incubated with the suspension of protozoal protein (Fig. 7).

Bacterial test

Intact strips of Whatman No. 1 paper were present for a period longer than 6 weeks in all tubes inoculated with homogenate obtained from the ciliates incubated overnight with antibiotics prior to homogenization. The strips disappeared, however, within 5-7 days when antibiotics were omitted. This shows that the alive cellulolytic bacteria were present only in these last tubes.

DISCUSSION

Ciliates *Epidinium ecaudatum* grew well *in vitro* in "*caudatum*" salt solution (Coleman *et al*. 1972) supplemented with powdered hay and wheat gluten. This shows that the culture medium satisfied the environmental and nutritional requirements of protozoa. It was found that microcrystalline cellulose supplemented to the control diet increased the ciliate number. Thus the reaction of *Epidinium ecaudatum* upon the changes in food composition resembled response of *Anoplodinium denticulatum, Diploplastron affine* and *Eudiplodinium maggii* stated earlier (Michałowski *et al.* 1986, 1989, 1991). One of the possible explanation of this finding seems to be the increase in energy content of the ration caused by cellulose supplement. This suggestion is confirmed by the both microscopical observation and enzymatic studies. First of them revealed that *Epidinium ecaudatum* engulfed readily and digested cellulose particles inside the cells while the second showed that glucose released from cellulose could be an important source of energy for these ciliates. On the other hand, however, it can not be also precluded that cellulose supplement improved some of environmental conditions making them more favorable for cultured ciliates. Such an effect was observed earlier in the case of non cellulolytic *Entodinium exiguum* (Michałowski *et al.* 1985). Growth of cellulolytic bacteria and more stable pH should be taken into account there. Conversely to microcrystalline cellulose the particles of oat spelt xylan were only sporadically engulfed by ciliates *Epidinium ecaudatum*. Explanation of such a behavior of ciliates towards xylan is not simple but it shows that this structural polysaccharide can not be considered as an important source of the utilizable energy for cultured protozoa. It is possible that ciliates from the same species which were examined by Jouany and Martin (1997) exhibited similar behavior towards xylan and this resulted in very low fermenting capacity in spite of high activity of the xylanolytic enzymes what was observed in our study. It is also noteworthy to point out that numerous species of rumen ciliates synthesize hemicellulolytic enzymes but their ability to ferment pentoses are not well known (Williams and Coleman

1992). Irrespective of causes influencing xylan utilization by rumen protozoa this polysaccharide diminished *Epidinium ecaudatum* numbers in our study. We suppose that xylan added to the culture medium could affect the bacterial growth and/or some environmental properties which became unfavorable for ciliates. However, further investigation are necessary to explain this reaction.

Both the microscopical observations and enzymatic studies showed that *Epidinium ecaudatum* was capable of digesting the microcrystalline cellulose. It is well known that cellulose degradation is a complex reaction catalyzed with array of enzymes of which the presence of three β-endoglucanases and β-glucosidase in *Epidinium ecaudatum* protein has been confirmed in our study. According to Wood (1992) and Chesson and Forsberg (1997) β-endoglucanases digest amorphous cellulose to oligosaccharides while β-glucosidases attack specifically cellobiose. We found, however, that only glucose was released from microcrystalline cellulose (Sigmacell 20) during incubation with the *Epidinium ecaudatum* protein. Thus the obtained results suggest that cellulolytic enzymes other than β-endoglucanases and β-D-glucosidase were also present in the preparation. One of them could be β-exoglucanase. Glucose was released from "Sigmacell 20" with the rate of about 0.19 μM/mg protein/h. This suggests that cellulolytic activity of *Epidinium ecaudatum* is not lower when compare to that of *Bacteroides* (*Fibrobacter*) *succinogenes* (Groleau and Forsberg 1981).

Xylanase exhibited the highest activity of the all fibrolytic enzymes tested. End products released from xylan with the enzymes extracted from the gel slices confirmed presence of two β-endoxylanases which released oligosaccharides from substrate. End products of the xylan hydrolysis with crude enzyme preparation suggests, however, that enzyme(s) releasing pentoses were also present in examined material.

Both the cellulolytic and xylanolytic activities were still present in examined preparation in spite of successful elimination of bacteria by incubation of ciliates with antibiotics. This finding supports the hypothesis that *Epidinium ecaudatum* is capable of synthesizing enzymes involved in degradation of the β-D-glucose and β-D-xylose polymers from plant cell walls. A successful cloning of gene encoding for β-endoglucanase from *Epidinium ecaudatum* (Sellinger *et al.* 1996) confirms partially this hypothesis. Genes encoding for xylanase were cloned from *Polyplastron multivesiculatum* (Sellinger *et al.* 1996, Wallace *et al.* 1999, Devillard *et al.* 2000) but not from *Epidinium ecaudatum*. Thus further studies seem to be necessary.

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