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Characterization of the amylolytic properties of the rumen ciliate protozoan *Eudiplodinium maggii*

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ABSTRACT

The rumen ciliates *Eudiplodinium maggii* are thought to be strongly cellulolytic. We observed, however, that they preferentially ingested starch when the sheep were fed hay and ground barley. The studies reported in this paper were undertaken in order to characterize the amylolytic activity of these protozoa. The crude enzyme preparation obtained from the bacteria-free ciliates degraded starch and dextrin at the rate of 29.5 and 19.4 μmol released glucose/mg protein/h, respectively, while the degradation rate of maltose and isomaltose was only 0.45 and 0.14 μmol released glucose/mg protein/h. The pH and temperature optimum of starch, dextrin, maltose and isomaltose hydrolysis varied in the range of 4.5-7.5 and 45-55ºC relative to substrate. Pullulan was not degraded. Four protein bands with the ability to degrade starch were identified by a zymogram technique following the electrophoretic separation of protozoal protein. The enzymes were α-amylase in nature, as they degraded starch mainly to maltose and maltotriose. Ion-exchange chromatography of a crude enzyme preparation resulted in the separation of numerous fractions which were able to degrade starch. The most amylolytic fractions were very rich in protein and also exhibited a strong ability to digest carboxymethylcellulose. Partial sequences from two genes coding for synthesis of α-mylase enzymes were identified in a cDNA library of *Eudiplodinium maggii*. The rest of the sequences were reconstructed using GeneRacer and both complete genes were sequenced and cloned. Gene *amyl 1* consisted of 1625 bp and the *amyla 2* - 1593 bp. They encoded enzymes of 505 and 431 amino acids, respectively.

KEY WORDS: rumen ciliates, starch, amylolytic enzymes, amylase genes

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INTRODUCTION

Starch is the storage polysaccharide deposited by higher plants and represents an important source of readily usable energy for herbivore mammals. It does not seem to be a significant component in the diet of wild ruminants (Langer, 1988) but can be the predominating polysaccharide in the feed of domesticated species (Whitelaw et al., 1972; Lyle et al., 1981; Towne et al., 1988; Franzolin and Dehority, 1996). Chemically, starch is a polymer of α-D-glucose bound by 1,4- and 1,6-glucosidic linkages and is readily digested and fermented in the rumen. It is well known that ruminal bacteria, as well as fungi and protozoa, are able to digest this polysaccharide (Trinci et al., 1994; Chesson and Forsberg, 1997).

Studies on the protozoal enzymes participating in starch digestion were mainly performed many years ago. They concentrated on the pH and temperature optimum of amylase, maltase and izomaltase (Coleman and Laurie, 1976; Bailey and Howard, 1963a,b; Coleman, 1969; Yarlet et al., 1981). The focus of this work was on the degradation rate of starch, amylose, maltose and isomaltose by cell-free extracts, as opposed to studies on the specific activity of purified enzymes or genes encoding their synthesis in the cells of rumen ciliates.

All the rumen ciliates studied exhibited amylolytic activity, even in species such as *Dasytricha ruminantium* which never ingest starch grains (Williams, 1986; Williams and Coleman, 1992). Moreover, it was recently reported that the strong cellulolytic ciliate *Eudiplodinium maggii* (Dehority, 1993; Michałowski, 1997) preferentially ingested starch grains in the rumen of sheep fed a hay-ground barley diet (Michałowski et al., 2003), suggesting that they could also have a significant impact on starch digestion.

Thus, the objectives of this study were to characterize the ability of *Eudiplodinium maggii* to digest starch and starch derivatives, as well as to identify and sequence genes encoding synthesis of amylases in the cells of these protozoa.

MATERIAL AND METHODS

Protozoa

Ciliates *Eudiplodinium maggii* originated from the rumen of a selectively faunated sheep fed on a hay-ground barley diet. The animal was fitted with a large rumen fistula (~10 cm) and defaunated by the washing procedure described by Michałowski et al. (1999). Protozoa used as the inoculum were isolated from the rumen of other animals and cultured *in vitro* (Michałowski et al., 1991), before they were introduced into the rumen of the ciliate-free sheep.

Preparation of ciliates free of feed particles and external bacteria

Samples of rumen content weighing about 1 kg were withdrawn from the rumen. They were diluted with 2 litre of "*caudatum*" salt solution (Coleman et al., 1972) and squeezed through a screen of pore size 260 μm. The filtrate was collected, poured into a separating funnel and allowed to stand at 40ºC for about 30 min. The protozoa forming a white pellet at the bottom of funnel were carefully collected, re-suspended in the same salt solution and sedimented again. The sedimentation procedure was repeated 2-3 times and the purified ciliates were used to prepare either a crude enzyme preparation or a cDNA library.

Preparation the ciliates free of internal bacteria

The crude enzyme preparation was prepared from protozoa free of bacteria. To kill the intracellular bacteria the purified ciliates obtained from 1 kg of rumen content (see above) were suspended in 1 l of salt solution (Hungate, 1942) supplemented with chloramphenicol, streptomycin and ampicillin. Each antibiotic was added to a final concentration of 50 μg/ml per antibiotic. The ciliates were anaerobically incubated for 18 h at 40ºC and separated by the above sedimentation technique. They were washed three times with "*caudatum*" salt solution (Coleman et al., 1972), lyophilized by a freeze-drying in a vacuum and stored at -80ºC.

Preparation of crude enzyme

Samples of lyophilized 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 4ºC and the supernatant was collected and used as a crude enzyme preparation.

Fractionation of crude enzyme preparation.

The crude enzyme preparation was fractionated by ion-exchange chromatography using a glass column (400×16 mm) packed with DEAE Sephadex A-50. The samples of crude enzyme preparation (10 ml), containing 7-8 mg protein, were applied to the top of the column and eluted initially with 0.2 M sodium potassium buffer (pH 6.0) and then a mixture of this buffer and a solution of 0.5 M sodium chloride. The concentration of sodium chloride increased with the time of elution up to 0.5 M. The elution rate was 5 ml/h and the volume of the collected fractions - approximately 1.5 ml.

Degradation of α- and β-glucose polymers

The degradation rate of starch, dextrin, pullulan, carboxymethylcellulose (CMC) and xylan was determined by quantification of reducing sugars released from the appropriate substrate following incubation with a crude enzyme preparation and/or its fractions. The 0.4% solutions of starch, dextrin and pullulan as well as 2% solutions of CMC and xylan were used as substrates. Reaction mixtures consisted of 750 µl substrate solution, 50 µl enzyme preparation and 450 µl of 0.02 M McIlvain buffer of the appropriate pH (see Figure 1). The mixtures were incubated for 1 h at 40ºC then hydrolysis was stopped by addition of 1.5 ml of salicylic acid reagent (Miller et al., 1960) and absorbance was measured at 560 nm using a Beckman DU 64 Spectrophotometer. The concentration of reducing sugars in the examined sample was calculated by comparison with the absorbance of glucose or xylose standards. The true quantity of the released sugars was calculated by subtraction of their content in blanks as well in enzyme preparations and substrates incubated separately. The quantities of released products were expressed as equivalents of simple sugar per mg protein and time unit.

Degradation of maltose and isomaltose was determined by a similar method. The reaction mixture consisted of 100 µl of a 0.1% solution of maltose or isomaltose (Sigma), 100 µl of enzyme preparation and 200 µl of 0.2 M TrisHCl buffer (pH according to values in Figure 1). The mixture was incubated for 1 h at 40ºC and released glucose was determined enzymatically using a Glucose Oxy kit (Pointe Scientific).

Identification of amylolytic enzymes

A native polyacrylamide gel electrophoresis (PAGE) of crude enzyme preparation in combination with a zymogram technique was applied to identify starch-degrading enzymes. The electrophoresis was performed using a 6% polyacrylamide gel and 0.05 and 0.005 M Tris/glycine buffer (pH 8.3) as gel and electrode buffer, repectively. The 0.2% solution of soluble starch was co-polymerized with the gel as a substrate for the identified enzymes. Ten µl samples of the crude enzyme preparation, containing about 20 µg protein, were applied to the top of the gel and separated using a constant power of 100 W in a Mini Dual Chamber (Sigma). The gels were then incubated in 0.02 M McIlvaine buffer (pH 6.0) for 15 min at 40ºC and stained with tetrazolium chloride according to Gabriel and Wang (1960). Red bands showed the location of starch degrading enzymes on the gel lanes.

Isolation of enzymes from the gel and identification of the end products of starch hydrolysis

A crude enzyme preparation was separated electrophoretically and the first two lanes of the gel were excised to allow visualization of the proteins exhibiting amylolytic activity (see above). The relative mobilities of the particular bands were precisely measured and used to determine the location of enzymes in the untreated (native) portion of gels. The appropriate slices (1 mm) were excised by hand from the gel. They were immersed in a cold (4ºC) 0.02 M sodium phosphate buffer (pH 6.0) and homogenized in a glass homogenizer while the homogenate was used to digest starch. The reaction mixture consisted of 0.1 ml homogenate or crude enzyme preparation, 0.5 ml of a 2% solution of soluble starch and 0.5 ml of 0.02 M sodium phosphate buffer (pH 6.0). This was incubated anaerobically at 40ºC for 24 h and then sampled for the identification of end products of substrate hydrolysis.

End products were identified by thin layer chromatography. Samples of reaction mixtures (15 µl) were spotted on the plates of silica gel (Silufol, Kavalier) and developed three times using a mixture of butanol-ethanol-water (5:5:1) according to Kozumi et al. (1985) while glucose, maltose, maltotriose and malto-oligosascharides were used as standards. The sugars were visualized using a mixture of diphenylamine-alanine-orthophosphoric acid-acetone as described by Stahl and Kaltenbuch (1965).

Detection of living bacteria in the ciliate cells

Ciliates which had been incubated overnight with antibiotics were centrifuged at 3000 *g* for 3 min, washed three times with a sterile *"caudatum"* salt solution (Coleman et al., 1972) and homogenized in a Potter homogenizer with a Teflon pestle. The resulting homogenate was transferred to tubes containing a liquid medium for the culture of anaerobic bacteria (Anaerobe Laboratory Manual, 1973) supplemented with soluble starch (0.5% w/v) and incubated for 24 h at 37ºC. After incubation the turbidity of the medium was measured at 600 nm using a Beckman DU 4 spectrophotometer and the presence of undigested starch was detected by adding Lugol solution. Homogenates prepared from ciliates incubated without antibiotics were introduced to the control tubes and incubated simultaneously.

Statistical analysis

Mean values and standard errors were calculated and differences between mean values were compared using the Student t-test.

Genetic studies

An *Eudiplodinium maggii* cDNA library produced during ERCULE (www.ercule.com), a project funded by the EU, was used to identify and sequence the genes encoding for α-amylase enzymes.

Screening of the cDNA library for amylase encoding genes

A phage λ ZAP Express (Invitrogen) and plasmid library were screened for the presence of complete genes encoding α-amylase. *Escherichia coli* strain JM 109 transformed with the appropriate library were grown on 0.7% agar plates containing 5 g/l yeast extract, 10 g/l casein hydrolysate, 5 g/l NaCl, 2 g/l $MgSO_4 \cdot 7H_2O$. The medium was covered by a mixture of agar and starch-azure and the dishes were incubated for 16 h at 37ºC. Plates were then screened for bacteria which had been transformed by phage with amylase genes.

Identification of amylase encoding genes using random sequencing

Previously when random sequencing of around 550 clones from a cDNA library from *Eudiplodinium maggii* was carried out (Ricard et al., 2006), two clones (A06 and G05) were found which had derived protein sequences similar to those encoding amylase genes. Sequences from these clones were used to design primers which could be used to obtain the full-length gene by using the GeneRacer technique (Invitrogen BV).

Having determined the remainder of the cDNA sequence, the complete gene was amplified from the GeneRacer library using PCR. The PCR products of complete genes were cloned using the pTrcHis2 TOPO TA Expression Kit (Invitrogen) and amplified by being transformed into *E. coli* strain TOP 10'. Colonies of the transformed bacteria were isolated, and their biomass increased before plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega).

Bioinformatical analysis

The mutational response index (Gatherer and McEwan, 1997), effective codon number (Wright, 1990), usage of AGA to encode arginine and usage of G in position 3 of codons encoding lysine, glutamic acid and glutamine were calculated as described previously (McEwan et al., 2000a).

DNA sequences were checked for the potential absence of the 'universal' polyadenylation signal, a motif which is generally absent from cDNAs isolated from rumen ciliates (McEwan et al., 2000b). Protein sequences were screened for the presence or absence of putative signal peptides (http://cbs.dtu.dk/services/SignalP/).

RESULTS AND DISCUSSION

Degradation of starch and starch derivatives by the protozoal crude enzyme preparation

Michałowski et al. (2003) observed an increase in the quantity of starch engulfed by *Eudiplodinium maggii* during the first 4 h after sheep were fed and this was followed by a continuous decrease. These changes seem to result from ingestion and intracellular digestion of dietary starch and the results presented in this report support this suggestion.

It was found here that starch, dextrin, maltose and isomaltose were digested following incubation with a crude enzyme preparation from *Eudiplodinium maggii*, while the digestion rate was related to pH and temperature. The optimum pH varied 4.5 and 7.5 depending on the digested carbohydrate (Figure 1). The pH optimum for degradation of starch and dextrin was found to be 5.0 (Table 1). No data concerning the environmental factors influencing the degradation rate of both of the carbohydrates were found in the available literature, while the pH optimum for digestion of amylose by cell-free extracts of *Entodinium caudatum, Epidinium ecaudatum, Dasytricha ruminantium* and *Isotricha* sp. varied between 4.8 and 6.5 relative to the ciliate species (Mould and Thomas, 1957, 1958; Bailey, 1958; Abou Akkada and Howard, 1960; Coleman, 1986). It was found that the degradation rate of starch at pH 3.0 and 8.0 reached around 60 % of the maximum

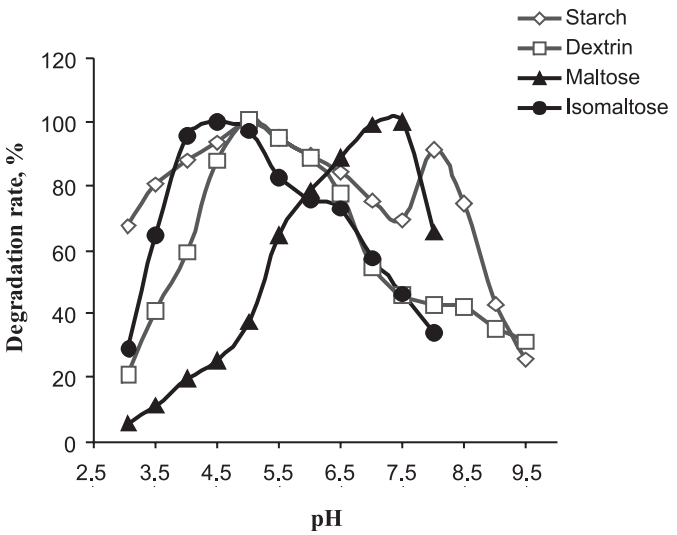

Figure 1. The effect of pH on the degradation rate of starch, dextrin, maltose and isomaltose by crude enzyme preparation obtained from the cells of *Eudiplodinium maggii*

Table 1. The degradation rate of starch, dextrin, maltose and izomaltoze (μM released glucose/mg protein/h) by crude enzyme preparation obtained from the cells of *Eudiplodinium maggii* and optimum conditions for degradation of particular substrates. Mean values ± standard deviations (n=3)

Substrate	Degradation rate	Optimum pH	Optimum temperature °C
Starch	29.5 ± 0.40	5.0	55
Dextrin	19.4 ± 2.26	5.0	45
Maltose	0.45 ± 0.01	4.5	40
Isomaltose	0.14 ± 0.02	7.5	50
Pullulan	0.0	N/A	N/A

N/A - not applicable

(Figure 1). No such phenomenon has been described by other authors with respect to starch digestion. However, function of ciliate enzymes in the basic range is not unprecedented, with the optimum pH for degradation of carboxymethylcellulose in the presence of CMCase synthesized by *Epidinium ecaudatum* being reported as 8.45 (Wereszka et al., 2004).

It was stated in this study that the optimum pH for the digestion of maltose was 4.5. This is lower than the value observed in the case of a cytosolic fraction from *Metadinium medium* which had an optimal pH of 6.0 (Naga and El Shazly, 1963). Previous data generated from crude preparations from other ciliates were also higher than we report, with values of 5.7-6.1, 6.7 and 5.5 for *Entodinium caudatum*, *Epidinium ecaudatum* and *Dasytricha ruminantium,* respectively (Bailey and Howard, 1963a). Conversely, the optimal degradation rate of isomaltose in the presence either of a cytosolic fraction or crude enzyme preparation from *Metadinium medium* and *Epidinium ecaudatum* were reported at pH 6.0 (Bailey and Howard, 1963b; Naga and El Shazly, 1963) whilst in the present study we found an optimum at a slightly higher pH, 7.0-7.5.

The temperature optimum varied between 40 and 55ºC depending on carbohydrate digested (Figure 2). The maximum rate for digestion of starch and dextrin were achieved at 55 and 45ºC, respectively. This is first time that such a figure has been reported for dextrin digestion by a rumen ciliate. However, the optimal reaction temperature for the degradation of amylose is higher than has been reported previously for other rumen ciliates. Previous values for degradation of amylose using cell-free extracts from *Epdinium ecaudatum*, *Entodinium caudatum* and *Polyplastron multivesiculatum* were 37-45, 38 and38ºC, respectively (Bailey, 1958; Abou Akkada and Howard, 1960; Abou Akkada et al., 1963).

In this work we found that the optimal temperature for degradation of maltose and isomaltose was 40 and 45-50ºC, respectively (Figure 2). We were unable to find previous reports on isomaltose breakdown in rumen ciliates, but the optimal temperature for maltose breakdown was previously described as 38 and 50ºC in

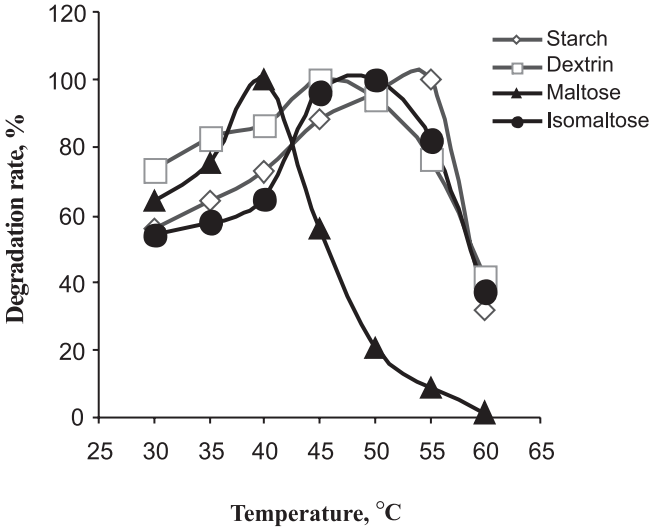

Figure 2. The effect temperature on the degradation rate of starch, dextrin, maltose and isomaltose by crude enzyme preparation obtained from the cells of *Eudiplodinium maggii*

the case of enzyme preparations from *Epidinium ecaudatum* and *Dasytricha ruminantium* (Bailey and Howard, 1963a).

The above data suggest that these enzymes when present in the rumen protozoa exhibit species-specific features when environmental factors affecting their activity are considered.

It was found that the reducing sugars were released from soluble starch at a rate equivalent to 29.5 μmol glucose/mg protein/h (Table 1). This digestion rate was comparable to the degradation rate of amylose by a cytosolic fraction extracted from *Eudiplodinium maggii* (Coleman, 1986). However, this author reported that only 1.3 μmol maltose/mg protein/h was released by the same preparation from granular rice starch. In addition to this, Naga and El Shazly (1963) reported that digestion rate of rice starch by a crude enzyme preparation of *Metadinium* (*Eudiplodinium*) *medium* did not exceed 0.22 μg released glucose/mg protein/h. Our findings support the earlier reports of Coleman (1986), suggesting that the amylolytic activity of rumen ciliates is a species-specific feature. On the other hand, however, the physical form of substrates should also be considered as a factor influencing the quantity of reducing sugars released from the digested polysaccharide.

The digestion rate of maltose was 0.45 μmol of released glucose/mg protein/h and was similar to that described by Bailey and Howard (1963a) for *Entodinium caudatum*, while crude enzymes from *Dasytricha ruminantium* and *Epidinium ecaudatum* digested this disaccharide with distinctly slower rate than *Entodinium*

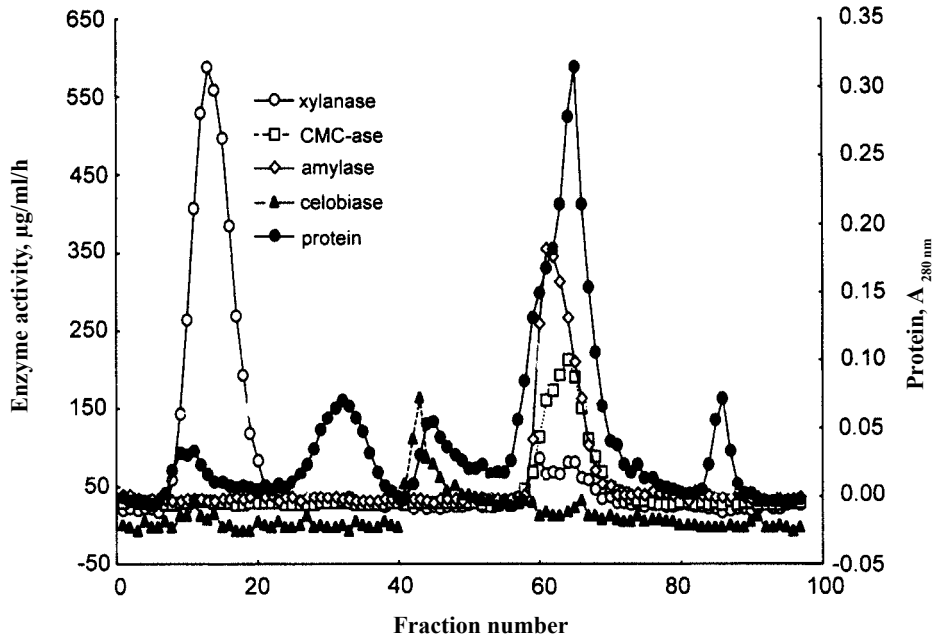

Figure 3. Distribution of protein and the activity of amylase, CMC-ase, xylanase and cellobiase over the fractions obtained following the fractionation of crude enzyme preparation by ion-exchange chromatography on DEAE sephadex A-50

caudatum. On the other hand isomaltose was digested 3 times slower than maltose and this result is in agreement with the data of Bailey and Howard (1963b). Digestion of pullulan was not detected (Table 1). This suggests that no pullulanase was synthesized by the ciliates examined in this study.

Of the four substrates used in this experimental work, starch was digested with the highest rate, followed by dextrin, while both of the disaccharides were hydrolysed 40 to 200 times slower. These results suggest that depolymerases were responsible for the high intensity of digestion of starch by *Eudiplodinium maggii*. Coleman (1986) proposed that α-amylases play the most important role in starch degradation by rumen ophryoscolecids and the results presented here seem to support this hypothesis.

To characterize the amylase enzymes synthesized by *Eudiplodinium maggii,* an attempt was undertaken to separate them from the other carbohydrate hydrolases. Fractionation of the crude enzyme preparation by ion-exchange chromatography resulted in identification of three peaks (Figure 3). Amylolytic activity was exhibited by fractions 57-69, with the most active being fraction 61. Unfortunately, the fractions with the highest amylolytic activity also had high activity of CMC-ase and some activity of xylanase. Zymographic studies revealed that quite different

enzymes were responsible for the degradation of starch and carboxymethylcellulose and that all of them were present in the examined 57-69 fractions (Bełżecki, 2004). It was, however, impossible to separate amylolytic enzymes by ion-exchange chromatography. Thus crude enzyme preparation was separated by native polyacrylamide gel electrophoresis while the starch degrading enzymes were identified and excised following visualization by a zymogram technique (Gabriel and Wang, 1969). A similar method was used earlier for a successful isolation of the fructanolytic enzymes synthesized by rumen treponemes (Kasperowicz and Michałowski, 2002). Zymographic studies resulted in identification of four protein bands able to degrade soluble starch (Figure 4). A visible difference in band intensity was observed, suggesting differences in the quantity of the reducing sugars released from the degraded substrate. This suggested either a difference in the activity or quantity of particular enzymes. To explain this question, the slices with particular enzymes were cut from the native gel and checked for their ability to degrade starch. The obtained results showed that homogenized slices of gel

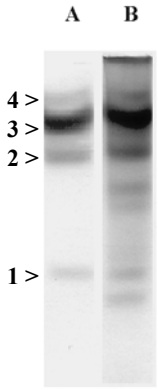

Figure 4. Zymogram of the starch degrading enzymes prepared following separation of a crude enzyme preparation by polyacrylamide gel electrophoresis. A-gel lane stained specifically with tetrasolium chloride. B-gel lane stained with Coomassie brilliant blue. 1, 2, 3, 4 - the identified amylolytic enzymes

from samples 2, 3 and 4 (see Figures 4 and 5) contained enzymes where maltose, followed by maltotriose, where the main end products detected after incubation with starch. Plaques of glucose and maltotetraose were also detectable, but only at a very low threshold level (Figure 5). On the other hand the end products of starch degradation by enzyme 1 were only just above the detection threshold. These products are similar to those detected following the incubation of starch with a crude enzyme preparation. The data obtained here are in good agreement with the findings of Satoh et al. (1993, 1997) who identified the end products of starch hydrolysis by α-amylases cloned from the rumen bacterium *Streptococcus*

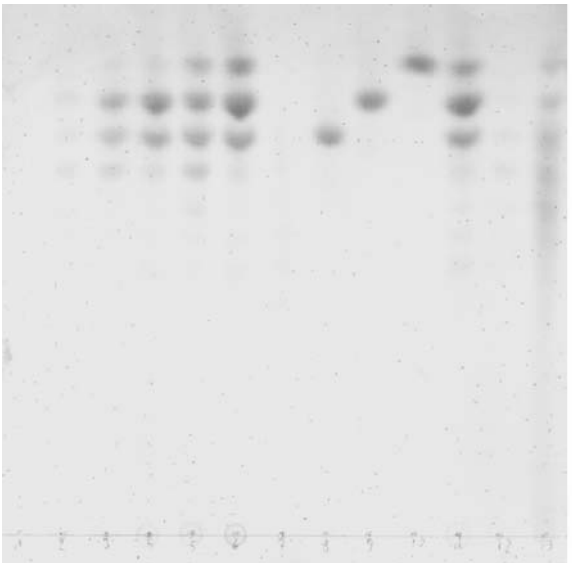

Figure 5. Thin layer chromatography of the end products of starch hydrolysis by particular enzymes isolated from the polyacrylamide gel following electrophoretic separation of crude enzyme preparation. Lanes 1 and 12 - starch; Lanes 2-5 - end products released from starch by enzymes no 1, 2, 3, 4, respectively (see Figure 4). Lanes 6 and 11 - end products of starch digestion by crude enzyme preparation. Lanes 8, 9, 11 - maltotriose, maltose and glucose, respectively. Lane 13 - maltooligosaccharides

bovis strain 148, supporting our assumption that the enzymes excised form the gel following separation of crude enzyme preparation of *Eudiplodinium maggii* were α-amylases.

Presence of the living amylolytic bacteria in ciliates incubated with antibiotics

It was found that the medium inoculated and incubated with homogenate originating from protozoa which had been incubated overnight with antibiotics remained transparent, while its turbidity measured spectrophotometrically was 0.004±0.0005. In contrast the control tubes were not transparent and their turbidity was of 0.95±0.04. Examination of the incubated material with Lugol solution showed the presence of indigested starch in the medium incubated with the homogenate from the protozoa which had been treated with antibiotics, whereas no starch was found in the control tubes. These results suggest that the incubation of the purified ciliates with antibiotics resulted in the killing of amylolytic bacteria existing in their cells. Thus they confirm the suggestion that the enzymes identified here were of protozoa origin.

Genetic studies

No complete gene encoding amylase was found during the screening of gene library of *Eudiplodinium maggii*. However, two clones (A06 and G05) of the length of about 1500 and 1000 bp (Figure 6) were identified during a random sequencing project which had high levels of similarity to the catalytic domains of α-amylases belonging to family 13 of the glucan hydrolases. The both clones contain motifs most similar to the Pfam 00128 family.

Following GeneRacer amplification and DNA sequencing, both clones were characterized as having a polyadenylated tail in the 3' untranslated region. The

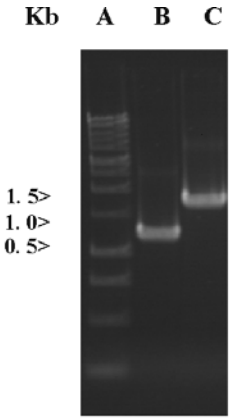

Figure 6. Length of the identified fragments of genes encoding for α-amylase in *Eudiplodinium maggii*. A- nucleotide ladder; B-the cDNA clone G05; C - the cDNA clone A06; Kb - Kilo basis

total length of the cDNA determined as the *amyl 1* gene (originally identified as clone the A06) was 1625 bp. In a similar way, cDNA *amyl 2* (reconstructed on the basis of the clone G05) was found to be 1593 bp long and interestingly appears to be the first example of a rumen ciliate not using ATG as a start codon - using ATT instead. The two cDNAs identified here are of different lengths and although both members of the same Pfam group, are most similar to different sequences in the GenBank database. They are also different in respect to signal peptides, with the shorter of the two rather surprisingly being the one which has such a signal. These sequences have been deposited in the EBI database. The two genes are most similar to different sequences in the Gene Bank database; an amylase from the *Tetrahymena thermophila* SB210 genome project (Eisen et al., 2006) and an amylase from the *Entamoeba histolytica* HM-1:IMSS genome (Loftus et al., 2005) project, respectively.

The two cDNAs identified here are of different lengths with *amyl 1* encoding a derived protein of 505 residues, and *amyl 2* encoding a derived protein of 431 residues. *Amyl 1* shares 55% identity and 70% similarity with an amylase from *Tetrahymena thermophila* SB210. Interestingly, the area of similarity included the C-terminal part of the sequence, despite the *Eududiplodinium maggii* sequence being over 130 residues shorter. No signal peptide was detected in this sequence. At the DNA level, the 3' untranslated region of the gene is relatively short and does not possess the 'universal' polyadenylation signal - features which are common to messages from rumen ciliates (McEwan et al., 2000b). *Amyl 2* shares 56% identity and 71% similarity with an amylase from *Entamoeba histolytica* HM-1: IMSS. In this case the *Eududiplodinium maggii* sequence is again shorter than the corresponding one in *E. histolytica*, however this time there is no area of similarity at the C-terminal region, despite this clone having a putative signal peptide present - supporting the idea that the clone is a complete cDNA. Interestingly, the 3' untranslated region of the gene is longer than that seen in most rumen ciliate messages, and although it possesses the 'universal' polyadenylation signal - its position is out with the normal region occupied by a functional polyadenylation signal. The genes had high mutational response index values, 0.42 and 0.45, (typically values of greater than 0.2 are considered large), low values for the effective codon number, 31.5 and 32.7 (typically values of less than 40 are considered small), high usage of AGA to encode arginine, 84 and 94%, and low usage of G in position 3 of codons encoding lysine, glutamic acid and glutamine, 3.3 and 5.5%, are in keeping with patterns observed previously (McEwan et al., 2000a).

A combination of factors also gives us confidence that we are dealing with ciliate genes, and not just bacterial contaminants. Firstly the measurements were performed using homogenetes obtained from ciliates after treatment with antibiotics, thereby reducing the likelihood that the enzymes have been carried over from bacterial contamination. Secondly, by looking at the codon usage pattern, we find that the usage pattern is similar to that seen in other genes from rumen ciliates. Most notably the high values for the mutational response index, low values for the effective codon number, high usage of AGA to encode arginine and low usage of G in position 3 of codons encoding lysine, glutamic acid and glutamine are in keeping with patterns observed previously (McEwan et al., 2000a).

We propose that the identified cDNAs are of eukaryotic origin, due to their similarity to other protozoal genes. The realization that as much as 4% of the genes in a rumen ciliate may have a bacterial origin (Ricard et al., 2006) means that where a particular phenotype is present in both eukaryotes and prokaryotes, it is worth checking the most likely origin of this gene from a rumen ciliate. It is also interesting to note that in keeping with other protozoal amylase genes, these genes appear shorter than those described in some rumen bacteria (Rumbak et al., 1991; Freer, 1993; Satoh et al., 1993, 1997). Nevertheless, the estimated mass of the two prod-

ucts from the clones described here are within the normal range (30-60 kDa), which is considered typical for microbial amylases (Rumbak et al., 1991).

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