Endo-1, 4-β-Glucanase and Endo-1, 4-β-Xylanase of the Ciliate Epidinium ecaudatum free of Cellulolytic and Hemicellulolytic Bacteria

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ABSTRACT. Cultures of Epidinium ecaudatum were prepared without any cellulolytic and hemicellulolytic bacteria. By viscometry and reducing power measurements, endo-1, 4β-glucanase activity towards soluble derivatives of cellulose (hydroxyethylcellulose, carboxymethylcellulose) and endo-1, 4β-xylanase activity towards xylan, were demonstrated from the Epidinium ecaudatum acellular extract. β-D-glucosidase and β-D-xylosidase activities towards p-nitrophenyl derivatives were also observed. These optimum pH range was 6.0 to 7.0.——KEY WORDS: endo-1-4-β-glucanase, endo-1-4-β-xylanase, epidinium.

The structural carbohydrates of plant cell walls are degraded and utilized in ruminants by the synergistic action of their endosymbiotic microorganisms. Defaunated ruminants generally show the decreased over all fiber digestibility [19, 15, 23]. Shift of the fiber digestion from the rumen to the large intestine, gives a defaunated animal a typical “pot-bellied” appearance [14]. Ciliates are responsible for 30 to 50% of rumen microbial fiber digestion [11, 16]. This may be due to intrinsic protozoal activity, the presence of heat stable protozoal constituents, stimulating bacterial cellulolysis [25] or to an increase in the number of cellulolytic bacteria due to the presence of protozoa [18]. According to some authors [13, 9, 10], the main part of the protozoan activity is due to the associated bacteria. But recently, it has been proved that Polyplastron multivesiculatum free of extracellular and intracellular cellulolytic bacteria degrade soluble derivatives of cellulose [4]. Cell-free extracts of Epidinium ecaudatum hydrolysed three major hemicelluloses in both Lolium perenne and Trifolium pratense [3, 4] and they release also 14C from 14C cellulose [6]. There was possibility that these enzymes came from intracellular bacteria. The aim of the present study is to investigate the nature and origin of the cellulolytic and hemicellulolytic activities of Epidinium ecaudatum.

MATERIALS AND METHODS

Preparation of Epidinium ecaudatum for the cellulase and hemicellulase studies: The rumen contents from a bovine which contained Epidinium ecaudatum as the dominant rumen ciliate (85–90%) were filtered through gauze. The filtrates were incubated for 2 days in a Coleman nutritive medium [7] under N2 at 38°C (Holotrichiidae, Ophryoscolex and Ostracodinium disappeared). The bottoms of the incubation were collected and incubated for 24 hr in a nutritive medium with antibiotics under N2 at 38°C. The medium contained 1.5ml of wheat starch suspension (1.5%) for 200ml of Coleman caudatum medium [7] and filter paper. The antibiotics were aminobenzylpenicillin (250 μg/ml), carbenicillin (250 μg/ml), cephaloridin (250 μg/ml), chlorampheni-
Bovine rumen contents with *Epidinium ecaudatum* as major microorganism

\[ \rightarrow \]
Filtration on gauze

\[ \downarrow \]
Culture in Coleman nutritive medium (2d)

\[ \downarrow \]
Incubation (24hr) in nutritive medium with antibiotics under N₂ at 39°C

\[ \downarrow \]
Centrifugation (300g, 3min)

\[ \downarrow \]
Pellet with sterile mineral medium without antibiotics

\[ \downarrow \]
Preparation of cell-free extracts

\[ \downarrow \]
Centrifugation (300g, 3 min)

\[ \downarrow \]
Washing with sterile mineral solution

\[ \downarrow \]
Sonication (20 kHz/s, 3 min)

\[ \downarrow \]
Centrifugation (10000 g/30 min)

\[ \downarrow \]
Millipore filtration (0.22 μm) of supernatant

Fig. 1. Preparation of the ciliates *Epidinium ecaudatum* for cellulase and hemicellulase studies.

*Time of bacteriological tests.

Nicol (100 μg/ml) and spiramycin (50 μg/ml). After incubation, the ciliates were prepared as described in Figure 1.

Protozoa counts: Protozoa counts were made in a Fuchs Rosenthal cell counting chamber (Preciss) before preparation of cell-free extracts.

Bacteriological tests: After the nutritive medium had been changed by another one without the antibiotics, bacteriological tests were conducted on the cell-free extracts before and after sonications. We verified that these preparations contained no extracellular or intracellular cellulytic and hemicellulolytic bacteria. Bacteria were counted in RGCA medium [5] in which cellobiose and glucose were replaced by cellulose (Whatman n° 1 filter paper) for cellulytic bacteria and xylan (0.5%) for hemicellulolytic bacteria.

**Determination of protein:** Protein in cell-free extracts were determined by the colorimetric method of Lowry et al. [18].

**Viscometric assay:** Reaction mixtures contained 15ml of 0.25% hydroxyethyl cellulose solution in citrate buffer (pH5.4) and 1ml of cell-free extract filtrate of *Epidinium ecaudatum*. The mixture was incubated in an Ostwald viscometer at 39°C. Viscometry determination of enzymic activity was performed by the methods of Almin and Eriksson [1] and Nisizawa [21].

**Determination of reducing sugars:** The incubation mixtures contained [1] Carboxymethyl cellulose (CMC) (Fluka) at 0.05 and 0.1% in 0.25ml 200mM acetate buffer (pH5.4) and 0.25ml of cell-free extract filtrate. [2] 0.05ml xylan (from larchwood-Sigma) at 1%, 0.125ml 100mM citrate buffer (pH5.0, 6.0) or 100 mM phosphate-citrate buffer (pH7.0, 8.0) and 0.125ml of cell-free extract filtrate. One drop of toluene was added to prevent bacterial contamination. After 22 hr and 2 hr incubation (respectively for reaction mixtures 1 and 2) at 39°C, the reducing sugars were determined according to Nelson’s technique [20]. The specific activities are expressed as nMoles of glucose or xylose released per milligram of protein per hour.

**β-glucosidase activity:** This activity was determined by the colorimetric method. The incubation mixture contained p-nitrophenyl-β-D-glucoside or p. nitrophenyl β-D-xylopyranoside in 0.7ml 100 mM sodium citrate buffer (pH5.0, 6.0) or 100 mM phosphate-citrate buffer (pH7.0, 8.0) and 0.1ml of cell-free extract filtrate. After incubation for 4 hr at 39°C, 2ml of 0.2 M sodium borate was added and the liberated nitrophenol was estimated by its absorbance at 400nm. As for the endoglucanase and the endoxylanase activities, enzyme and substrate controls were also incubated and assayed.
Cellulase and hemicellulase of *Epidinium* 545

Table 1. Variations of the intrinsic viscosity (η) of a hydroxyethylcellulose induced by incubation with filtrate of cell-free extracts cellulolytic bacteria-free *Epidinium ecaudatum*.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>n/100</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>81.5</td>
</tr>
<tr>
<td>7</td>
<td>79.7</td>
</tr>
<tr>
<td>12</td>
<td>71.7</td>
</tr>
<tr>
<td>15</td>
<td>64.9</td>
</tr>
<tr>
<td>30</td>
<td>57.7</td>
</tr>
<tr>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>50</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 2. Cellulolytic activity in filtrate of cell-free extracts cellulolytic bacteria-free *Epidinium ecaudatum*.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>nMol of glucose/hr per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>E\textsuperscript{3}-CMC\textsuperscript{b) 1}</td>
<td>66.5</td>
</tr>
<tr>
<td>&gt; 2</td>
<td>110</td>
</tr>
</tbody>
</table>

a) Filtrate of cell free extract *Epidinium ecaudatum*, protein value (μg/ml incubation medium using bovine albumin as standard): 110
b) Carboxymethylcellulose; 1) 0.05% 2) 0.1%.

Table 3. Hemicellulolytic activity in filtrate of cell-free extract hemicellulolytic bacteria-free *Epidinium ecaudatum*.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>nMol of xylose/h per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>E\textsuperscript{3}-p NG\textsuperscript{b) 5}</td>
<td>6.0</td>
</tr>
<tr>
<td>E\textsuperscript{3}-p NX\textsuperscript{b) c}</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>227.3</td>
</tr>
<tr>
<td></td>
<td>79.5</td>
</tr>
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</table>

a) Filtrate of cell-free extract *Epidinium ecaudatum* E protein value (μg/ml incubation medium using bovine albumin as standard): 22.
b) p-nitrophenyl-β-D-glucopyranoside.
c) p-nitrophenyl-β-D-xyl pyranoside.
d) μMol of p-nitrophenol/h/per mg protein.

RESULTS

Cell-free extracts of *Epidinium ecaudatum* were prepared from a 4.4×10\textsuperscript{2} ml ciliates population.

Cellulase and xylanase activities: The viscometric tests showed a 50% decrease in the intrinsic viscosity of a 0.25% hydroxyethylcellulose solution in 35 min (Table 1). By measurements of the reducing power released by incubation of carboxymethylcellulose and xylan with cell-free extracts of *Epidinium ecaudatum* it was observed, that the hemicellulolytic activity of *Epidinium ecaudatum* was higher than the cellulolytic activity (Tables 2 and 3).

β-glucosidase and β-xiosidase activities: The optimum pH range of the activities were from 6.0 to 7.0. β-glucosidase appeared to be five higher than β-xylosidase activity with the same cell-free extract (Table 4).

DISCUSSION

With cellulose, two kinds of enzymic activities were found in cellulolytic bacteria-free *Epidinium ecaudatum*. The endo-1, 4-β-glucanase that degraded soluble derivatives of cellulose, produced rapid fall in viscosity of the substrate solution. This glucanase hydrolysed 1, 4-β-glucosidic linkages inside the chains. The β-D-glucosidase activity was also observed which hydrolysed cellbiose to glucose. With hemicellulose (xylan), other two kinds of enzymic activi-
ties were also found: an endo-1, 4-β-xylanase and a β-D-xylosidase activity that hydrolysed xylobiose to xylose. Recently, Coleman [8] and Williams and Coleman [24] determined the cellulase and hemicellulase activities of 15 species of entodiniomorphid protozoa. As Coleman prepared cell-free extracts, by sonication without removing cellulolytic bacteria, and without testing cellulolytic bacteria, he might observe the cellulolytic activity of protozoal and contaminated bacteria. The cellulolytic activity of protozoa had been studied also by transmission electron microscopy. Unlike Delbosse-Debacher et al. [9, 10], Grain and Senaud [12, 22], observed that the plant material was ingested by phagocytosis and digested only by the ciliates (Epidinium ecaudatum and Polyplostron multivesiculatum), in vacuoles edged with rough endoplasmic reticulum. Pseudopods without glyccalyx emerged from the cytosome, applied to plant fragments and bacteria ingested simultaneously were isolated in separated vacuoles. For the bacteria present on plant fibers, they were envacuolated with plant particles but did not participate to the plant cell degradation. They were segregated inside the vacuole and then removed near the cytoprote. Moreover, the cellulolytic and hemicellulolytic activities were detected from cell-free extracts of Polyplostron multivesiculatum (Bonhomme et al., 1986) and of Epidinium ecaudatum (in this paper) without cellulolytic and hemicellulolytic bacteria. Therefore it is concluded that ciliates can synthetise their own polysaccharases.

REFERENCES

CELLULASE AND HEMICELLULASE OF EPIDINUM


要 約

セルロース分解菌・ヘミセルロース分解菌不存下での有縄毛 Epidinium ecaudatum の endo-1,4-β-glucanase および endo-1,4-β-xylanase：Annie Bonhomme (LA 04138 CNRS 及 Reims 大学理学部動物学教室) と魚バケツリア研究者、ヘミセルロース分解菌の不存下で Epidinium ecaudatum を培養したところ、粘度、還元力の測定によって、無細胞抽出液中にセルロースの可溶性誘導体に対する endo-1,4-β-glucanase 活性および xylan に対する endo-1,4-β-xylanase 活性が見出された。また、p-ニトロフェニル誘導体に対する β-D-glucanase および β-D-xylosidase 活性もみとめられ、至適 ph は 6.0〜7.0であった。