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Extracellular Endoglucanase Activity by a Novel Bacterium Isolated from Marine Shipworm

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An extracellular enzyme preparation from shipworm bacterium cultures dramatically increased reducing sugar content of carboxymethylcellulose (CMC[3]), but did not solubilize sugar from particulate cellulose. The preparation degraded cellodextrins larger than cellotriose (G_3). Only interior cellodextrin chain linkages were cleaved and the center-most bond of cellohexaose (G_6) was preferentially cleaved. Activity maxima were observed at 60°C and between pH 5.0 and 7.0. The activity was resistant to protease treatment and little loss of activity was observed after 14 d at 25°C.

Adult shipworms (wood boring bivalves of the family Teredinidae) can grow on a diet of wood alone (1). There has been much disagreement over the means by which shipworms meet their nitrogen requirements when growing on wood and on the origin of enzymatic activity necessary for cellulose digestion. Waterbury et al. (2) isolated a cellulolytic, nitrogen fixing bacterium from an

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Abbreviations used: CMC, carboxymethylcellulose; G_1, D-glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose; G_6, cellohexaose.

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outpouching of the shipworm gills and alimentary tract, commonly referred to as the gland of Deshayes. Isolates from six species of shipworm were morphologically similar and appear to represent a single bacterial species. It was postulated that nitrogen fixation by the bacterium enables the shipworm to meet nitrogen requirements when growing on a nitrogen deficient diet and that the bacterium is the primary source of cellulolytic enzymes for the shipworm.

In this study we investigated the possible involvement of extracellular enzymes from the shipworm bacterium in cellulose degradation. We report on the characterization of an enzyme preparation from cell-free medium of shipworm bacterium cultures. The results presented suggest that the predominant extracellular cellulose-degrading activity from the shipworm bacterium is endoglucolytic in nature.

MATERIALS AND METHODS

Culture and enzyme preparation. Bacterial cultures, isolated from Psiloteredo healdi shipworm, were a generous gift of Dr. John Waterbury (Woods Hole Oceanographic Institution). Shaken cultures supplemented with 0.5% alkaline peroxide treated wheat straw (see below) as a carbon source and 0.3 $g \cdot l^{-1}$ NH_4Cl were grown as previously described (3). Cultures were harvested by centrifugation at 10,000 x g for 30 min. Analysis of the supernatant for activity on carboxymethylcellulose (see below) indicated maximum enzyme production was during 10-14 days, corresponding to stationary growth phase (3). All experiments were conducted with unconcentrated medium from cultures of this age. Extracellular protein content was about 0.2 $mg \cdot ml^{-1}$, as determined by the method of Bradford (4).

Preparation of carbohydrates. Alkaline peroxide treated wheat straw was prepared by the method of Gould (5). Cellodextrins were prepared by the method of Freer and Detroy (6). [^{14}C]Cellodextrins were prepared by the method of Alexander (7), utilizing partially purified cellodextrin phosphorylase from Clostridium thermocellum (8) and [^{14}C(U)]D-glucose 1-phosphate (New England Nuclear). This method specifically labels the terminal non-reducing sugar in the cellodextrin chain.

Carbohydrate analyses. For experiments to determine total sugar solubilized from particulate cellulose, a 1 x 6 cm strip of Whatman #1 filter paper, ca. 50 mg, was added to 5 ml of cell free culture medium (equivalent to a 1% suspension of cellulose). Liquid aliquots were withdrawn and total soluble sugar content was determined utilizing phenol/sulfuric acid as described by Dubois et al. (9). This procedure is described in detail by Griffin

et al. (10). Reducing sugar content of 1% carboxymethylcellulose (Sigma) was determined by the method of Pettersson and Porath (11), except shipworm bacterium basal salts buffer (3) at pH 7.0 was substituted for sodium acetate buffer. Both above assays were standardized to known quantities of glucose. Cellodextrins were separated by HPLC with a Bio-Rad Aminex HPX-42A column and an in-line carbohydrate deashing system. The eluent was H_2O. Peaks detected by refractive index (Spectra-Physics model SP 6040 refractometer) were processed with a Hewlett-Packard model 3393A integrator and identified by comparison to retention times of authentic standards. [^{14}C]Cellodextrins were identified with an in-line Radiomatic Instruments radioactive flow detector.

RESULTS

As determined by phenol/sulfuric acid (9), cell free culture medium from the shipworm bacterium solubilized little sugar from a 1 x 6 cm strip of filter paper (Fig. 1). HPLC analysis of the reaction medium confirmed this finding (not shown). This was a somewhat unexpected result because extensive disruption of the filter paper was visible after 10 min of incubation with the extracellular preparation. However, the preparation dramatically increased the reducing sugar content of 1% carboxymethylcellulose (CMC) (Fig. 1), suggesting that the activity was endoglucolytic. No increase in CMC reducing sugar content was observed if a boiled

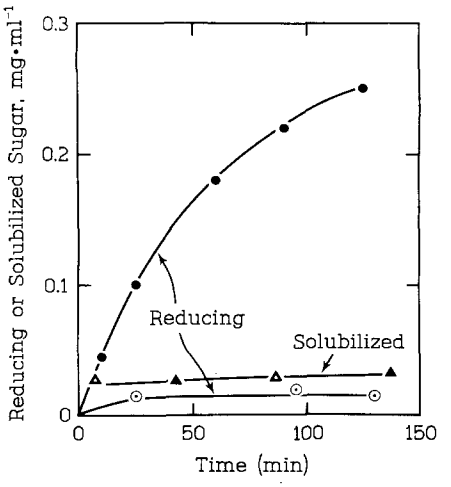


Figure 1. Effect of shipworm bacterium culture medium on CMC and particulate cellulose. Symbols: (O—●), reducing sugar content of 1% CMC; (Δ—▲), total solubilized sugar from a 1 x 6 cm strip of Whatman #1 filter paper (equivalent to a 1% suspension of cellulose). Open symbols represent preparations boiled for 10 min.

TABLE 1. Degradation products from various chain length cellodextrins

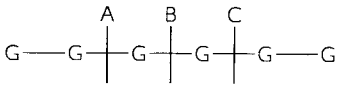
SUBSTRATE (1.5 mM)	CONCENTRATION OF PRODUCTS (mM)*											
	30 MIN INCUBATION						360 MIN INCUBATION					
	G_6	G_5	G_4	G_3	G_2	G_1	G_6	G_5	G_4	G_3	G_2	G_1
G_6	0.63	-†	0.36	0.68	0.37	-	-	-	0.09	1.02	1.69	-
G_5	-	0.49	0.08	0.81	0.78	-	-	-	-	1.44	1.49	-
G_4	-	-	0.68	-	1.42	-	-	-	-	0.19	2.53	-
G_3	-	-	-	1.44	-	-	-	-	-	1.44	0.03	-
G_2	-	-	-	-	1.44	-	-	-	-	-	1.49	-
G_1	-	-	-	-	-	1.46	-	-	-	-	-	1.44

*Cellodextrin concentrations were determined by HPLC analysis (Bio-Rad HPX-42A column). Reactions were conducted with cell-free culture medium for the indicated times at 25°C and pH 7.
†Not detectable (less than 0.03 mM).

enzyme preparation was employed. Ultracentrifugation (100,000 x g for 60 min) of the preparation removed 50-70% of the protein as an apparent membrane fraction. However, >90% of the activity on CMC remained in solution, indicating the enzyme(s) was truly soluble.

The data in Table 1 show that the enzyme preparation exhibited little apparent activity on shorter chain cellodextrins (G_1 through G_3), but readily cleaved cellodextrins of chain length G_4 or larger. Furthermore, glucose (G_1) was not a product from the cleavage of any cellodextrin. These observations suggest that the enzymatic activity was directed towards the interior glucosyl linkages of the cellodextrin chain, perhaps requiring a minimum of two glucosyl moieties adjacent to both sides of the cleavage site.

The initial degradation of G_6 (Table 1, 30 min incubation) yielded a G_4:G_3:G_2 product ratio of approximately 1:2:1. Theoretically, if the three interior bonds of G_6 were equally accessible to cleavage, this ratio should have been 1:1:1 (see Fig. 2). Similarly, in a random cleavage reaction, the end products formed from G_6 degradation should be G_3 and G_2 in a ratio of 1:3

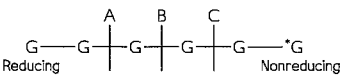


Bond Cleaved	Initial Products
A	$G_2 + G_4$
B	$G_3 + G_3$
C	$G_4 + G_2$
Overall initial products	$2G_4 + 2G_3 + 2G_2$

Figure 2. Theoretical initial product formation from random cleavage of interior G_6 glucosyl bonds.

(assuming two G_2 from G_4). However, the observed G_3:G_2 ratio was about 2:3 (Table 1, 360 min incubation). These observed ratios imply that the cleavage of interior cellodextrin bonds was directed.

Initial degradation of [^{14}C]G_6, specifically labeled in the terminal non-reducing glucose unit, yielded labeled products as indicated by the percentages in Fig. 3. Since [^{14}C]G_3 was the predominant product, the central bond of G_6 (bond B) appears to be the most susceptible to cleavage by the enzyme preparation. Between the remaining two interior bonds, a higher cleavage rate was exhibited for the bond nearest the reducing end (bond A). Interestingly, as is also shown in Fig. 3, if the observed percentages of labeled products were converted into relative reaction rates, an overall initial G_4:G_3:G_2 product ratio of



Bond Cleaved	Initial Products	Relative Reaction Rate	Relative Rate of Initial Product Formation (Initial Product × Rel. Reac. Rate)
A	G_2 + *G_4 (33%)	0.6	0.6 G_2 + 0.6 G_4
B	G_3 + *G_3 (53%)	1.0	1.0 G_3 + 1.0 G_3
C	G_4 + *G_2 (14%)	0.3	0.3 G_4 + 0.3 G_2
	Overall initial products		0.9 G_4 + 2.0 G_3 + 0.9 G_2

Figure 3. Products formed from initial degradation of [^{14}C]G_6, labeled in the terminal non-reducing glucose moiety. *G represents labeled sugar. Parenthetic values represent percent of total product observed after 5 min incubation at 25°C and pH 7.0. The relative reaction rate was calculated by normalization to percent *G_3 formed. Initial G_6 concentration was 1.5 mM. Specific activity was 1.6 mCi·mmole^{-1}.

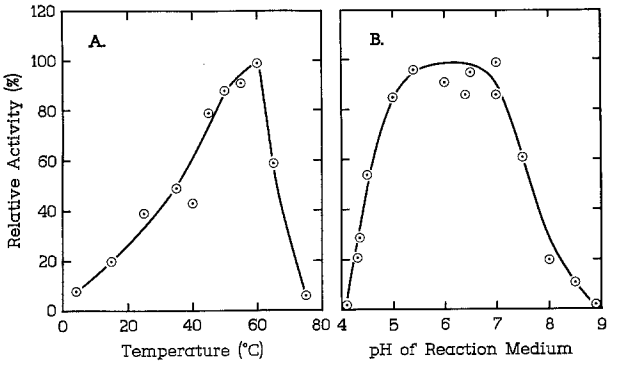


Figure 4. Effect of temperature and pH on activity. Relative activity was determined as loss of G_4 after 20 min. Initial concentration of G_4 was 1.5 mM.

0.9:2.0:0.9 was derived. This is in good agreement with the ratio observed for the data in Table 1.

It is possible that some of the $[^{14}C]G_2$ observed in Fig. 3 was derived from further degradation of accumulated $[^{14}C]G_4$ product. However, this amount should be insignificant since during the time course of this experiment <15% of the original $[^{14}C]G_6$ was degraded. Furthermore, in an analogous experiment using $[^{14}C]G_5$ as initial substrate (which does not yield G_4, see Table 1), a ratio of $[^{14}C]G_3$:$[^{14}C]G_2$ was obtained which was similar to that obtained from $[^{14}C]G_6$ (not shown).

G_4 degradation was utilized to characterize some of the physiological properties of the enzymatic activity. Maximum activity was achieved at 60°C, while activity was absent at 75°C (Fig. 4A). A rather broad activity maximum was observed from pH 5.0 to 7.0, as well as the presence of substantial activity from pH 4.3 to 8.0 (Fig. 4B). Table 2 illustrates that the activity was resistant to protease treatment and that the preparation was stable at room temperature, retaining 77% of original activity after 2 weeks. Activity was successfully reconstituted after lyophilization and the enzyme(s) can be stored indefinitely in this manner (Table 2).

TABLE 2. Stability of activity

TREATMENT	RELATIVE RATE OF G_4 DEGRADATION*
None	1.00
Protease†	
Trypsin	1.05
Chymotrypsin	0.88
Pronase	0.87
Storage	
7 d (25°C)	0.83
14 d (25°C)	0.77
14 d (4°C)	0.85
Lyophilization and reconstitution	0.93

*Degradation was determined as loss of G_4 (1.5 mM) after 30 min incubation at 25°C and pH 7.
†Culture medium preincubated with 0.03 mg·ml^{-1} of indicated protease for 60 min at 25°C.

DISCUSSION

An accepted model developed from studies of fungal enzymes depicts a complete cellulase system as being comprised of three major components (12). The components are endo-1,4-β-glucanase (CMCase), exo-1,4-β-glucanase (cellobiohydrolase) and β-glucosidase (cellobiase). The endoglucanase is thought to attack particulate cellulose by cleaving β(1,4) glucosyl linkages in the cellulose chain creating new chain ends which then become substrates for the exo-enzymes.

Several variations in the above model have been reported (13). Often in bacteria the primary extracellular activity observed is endoglucolytic (14-17). This also appears to be the case for the shipworm bacterium since the enzyme preparation described here did not solubilize significant amounts of sugar from filter paper, but did increase the reducing sugar content of CMC (Fig. 1).

Furthermore, the enzymatic activity cleaved only interior cellodextrin chain linkages (Table 1) and exhibited the highest activity on the center-most bond in cellohexaose (Fig. 3). At optimal temperature (60°C) and pH (7.0) the specific activity of the preparation, as determined by G_4 degradation, approached 1 $\mu mole \cdot min^{-1} \cdot mg\ protein^{-1}$. However, pending purification of the enzyme(s), it is difficult to compare this specific activity with other endoglucolytic proteins.

In a previous study (3), shipworm bacterial cultures growing on a carbon source of particulate cellulose were found to accumulate significant quantities of organic acids, but accumulated little soluble sugar. Other evidence suggested that the cultures could excise and transport glucosyl moieties from the ends of cellodextrin chains, indicative of cell-bound exoglucanase activity. The endoglucanase activity described in this communication is likely to be an important part of the developing model describing the symbiotic relationship between this bacterium and its host shipworm. The endoglucanase(s) generates cellulose chain ends necessary for excision of glucosyl units by the bacterial exoglucanase(s). Excised glucose becomes the energy force for metabolic processes of the bacterium. The end products of some of these processes are excreted organic acids, which, in turn, could provide carbon and energy for the shipworm. Validation of such a model should be aided by isolation and further characterization of the cellulolytic enzymes.

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REFERENCES

1. Gallager, S. M., Turner, R. D., and Berg, C. J. (1981) Mar. Biol. Ecol. 52, 63-69.

2. Waterbury, J. B., Calloway, C. B., and Turner, R. D. (1983) Science 221, 1401-1403.
3. Greene, R. V., and Freer, S. N. (1986) Appl. Environ. Microbiol. 52, 982-986.
4. Bradford, M. (1976) Anal. Biochem. 72, 284-354.
5. Gould, J. M. (1984) Biotechnol. Bioeng. 26, 46-52.
6. Freer, S. N., and Detroy, R. W. (1982) Biotechnol. Bioeng. 26, 453-458.
7. Alexander, J. K. (1972) Methods Enzymol. 28, 948-953.
8. Sheth, K., and Alexander, J. K. (1967) Biochim. Biophys. Acta 148, 808-810.
9. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Anal. Chem. 193, 265-275.
10. Griffin, H., Dintzis, F. R., Krull, L., and Baker, F. L. (1984) Biotechnol. Bioeng. 26, 296-300.
11. Pettersson, G., and Porath, J. (1966) Methods Enzymol. 8, 603-607.
12. Ryne, D. D. Y., and Mandels, M. (1980) Enzyme Microb. Technol. 2, 91-102.
13. Halliwell, G. (1979) Prog. Ind. Microbiol. 15, 1-60.
14. Yoshikawa, T., Suzuki, H., and Nisizawa, K. (1974) J. Biochem. 75, 531-540.
15. Petre, J., Longin, R., and Millet, J. (1981) Biochemie 63, 629-639.
16. Groleau, D., and Forsberg, C. W. (1983) Can. J. Microbiol. 29, 504-517.
17. Boyer, M. H., Chambost, J. P., Mangan, M., and Cattaneo, J. (1984) J. Biotechnol. 1, 229-239.