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Binding of Extracellular Carboxymethylcellulase Activity from the Marine Shipworm Bacterium to Insoluble Cellulosic Substrates

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The binding of extracellular endoglucanase, a carboxymethylcellulase (CMCase), produced by the marine shipworm bacterium to insoluble cellulose substrates was investigated. Up to 70% of CMCase activity bound to cellulosic substrates, and less than 10% bound to noncellulosic substrates. CMCase binding to cellulose was enhanced in basal salt medium or sodium phosphate buffer containing 0.5 M NaCl. Increased cellulose particle size correlated with decreased CMCase binding. Also, cellulose treated with either 5 N NaOH or commercial cellulase reduced the CMCase binding to these surfaces. Pretreatment of CMCase preparations with 0.01% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 5 mM EDTA or ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) had little effect on binding to cellulose. While pretreatment of CMCase with trypsin, chymotrypsin, and pronase had little effect on CMCase enzymatic activity, the ability to bind to cellulose was greatly diminished by these treatments.

Wood-boring bivalves of the family Teredinidae, commonly known as marine shipworms, possess a specialized gland, known as the gland of Deshayes (27). This gland harbors a pure dense culture of a single species of bacterium which is capable of both fixing nitrogen and synthesizing enzymes for cellulose degradation (27). It has been reported that this bacterium is capable of selectively colonizing particulate cellulosic surfaces (19). Specific interactions between the bacterium and cellulosic surfaces appear to be mediated via trypsin-sensitive proteins associated with the cell surface (19).

The shipworm bacterium produces an extracellular enzyme(s) in defined culture medium which dramatically increases the reducing-sugar content from carboxymethyl cellulose (CMC) but does not solubilize sugar from particulate cellulose (12). Greene et al. (11) described multiple endoglucanase activities in the extracellular medium of shipworm bacterium cultures and have purified at least one endoglucanase (CMCase) from such culture medium. The purified enzyme degraded cellodextrins larger than cellotriose (G_3). In addition, only interior cellodextrin chain linkages were cleaved and the centermost bond of cellohexose was preferentially cleaved. Although it has been suggested that the CMCase activity of the marine shipworm bacterium binds tenaciously to insoluble cellulosic substrates (11), the binding properties of the activity have not yet been characterized. Studies of enzyme binding to insoluble natural substrates may yield important information regarding the enzymatic mechanisms involved in cellulose degradation. In this report, we describe our initial efforts to characterize the binding of marine shipworm CMCase activity to cellulose.

MATERIALS AND METHODS

Chemicals. Unless mentioned otherwise, all reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Cell culture. Bacterial cultures (Woods Hole Oceanographic Institution strain T8301), isolated from marine shipworm *Psiloteredo healdi*, were a gift from John Waterbury. Cultures (1 liter) were grown aerobically as previously described (10, 11) in 3-liter Fernbach flasks with 1% Sigmacell type 50 as the carbon source and 0.1% NH_4Cl as the nitrogen source.

Enzyme preparation. Procedures used for the enzyme preparation have been described previously (11). Briefly, four 1-liter cell-free culture supernatants were obtained by removing the cells from 10- to 12-day-old cultures by centrifugation (15,000 × *g* for 30 min). To further clarify, the supernatant was ultracentrifuged at 100,000 × *g* for 60 min. This supernatant was concentrated 10-fold by ultrafiltration (Amicon PM 30 membrane), diafiltered (an ultrafiltration technique in which microsolutes are removed from or added to a constant process volume by convective transport in a buffer through a sized hollow-fiber membrane [Amicon operating instructions I-1231]) with 6 volumes of distilled water, lyophilized, and stored at −20°C for subsequent use. A 1-mg amount of protein from this preparation contained approximately 6 U of CMCase activity (1 U of activity is defined as the number of micromoles of glucose reducing-sugar equivalents formed from CMC per minute at 37°C).

Cellulose-binding assay. Unless otherwise indicated, the lyophilized enzyme preparation was resuspended at a protein concentration of 1 mg/ml in 20 mM sodium phosphate buffer (pH 7.0). A suspension of insoluble microcrystalline cellulose (Sigmacell type 50) equilibrated with the phosphate buffer was dispensed into a minicolumn (Quick Snap Columns with bottom disk [QS-NP], 8 cm long by 1 cm wide; Isolab Inc., Akron, Ohio) to give a 1-ml bed volume. A 1-ml bed contained 250 mg of microcrystalline cellulose (dry weight) or an equivalent weight of other cellulose substrates. Columns were washed several times with fresh buffer prior to use. The enzyme preparation was loaded onto a column. The column was sealed shut and gently rocked for about 15 min at 24°C to allow adequate mixing of enzyme and microcrystalline cellulose. The cellulose in the column was then allowed to settle, and unbound enzyme activity was

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collected in the flowthrough volume. The column was then washed with 3 to 4 ml of phosphate buffer, and 1-ml fractions were collected and analyzed for CMCase activity (see below). Bound CMCase activity was determined by the following formula: % CMCase activity bound to substrate = [(total CMCase activity loaded − total unbound CMCase activity eluted off the column)/(total CMCase activity loaded)] × 100. Enzyme solutions and cellulose suspensions routinely contained 0.02% (wt/vol) sodium azide to avoid contamination. All datum points in graphs and tables represent average values of at least three experiments having <5% variability in results.

To determine the optimal temperature for CMCase binding to cellulose, enzyme solutions and substrate suspensions were individually equilibrated at the desired temperature and allowed to react at that same temperature. For pH studies, buffer solutions containing 50 mM each sodium citrate, potassium phosphate, Tris, and glycine were prepared. The pH was adjusted to the desired value with either HCl or NaOH. This procedure has been described elsewhere (19).

Analytical methods. CMCase activity was analyzed from the reducing-sugar content of 1% CMC by the method of Hoffman (13). Typically, 0.5 ml of sample in 20 mM sodium phosphate buffer (pH 7.0) was mixed with 0.5 ml of 2% CMC dissolved in 20 mM sodium phosphate buffer (pH 7.0). The reactants were allowed to incubate at 37°C for 30 min, at which time the reducing-sugar content was determined with a Technicon Autoanalyzer. Assays were standardized to known quantities of glucose.

The protein content was determined by using a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) based on the dye-binding method of Bradford (3) adapted for microtiter plates. The A_{600} of the samples was read with a Dynatech-MR5000 microtiter plate reader.

Substrate and CMCase treatments. Microcrystalline cellulose was incubated overnight with 5 N NaOH (EM Science, Gibbstown, N.J.) (see Table 2). The suspension was then neutralized with HCl and washed several times with phosphate buffer. The indicated cellulase treatment was as follows. Microcrystalline cellulose was suspended in 100 mM sodium acetate buffer (pH 5.5). Cellulase (1 mg/ml from *Trichoderma reesei* ATCC 26921) (EC 3.2.1.4) was added, and the mixture was incubated for 1 h at 25°C. The remaining particulate cellulose was collected and washed four times with sodium phosphate buffer (pH 7.0) by centrifugation. For protease treatments, either 10 μg of trypsin (EC 3.4.21.4; bovine type XI) per ml and 100 μg of α-chymotrypsin (EC 3.4.21.1) per ml or 100 μg of peptidase (porcine intestinal mucosa) per ml was added to a CMCase preparation (equivalent to 1 mg/ml protein in sodium phosphate buffer [pH 7.0]) and incubated for 1 h at 25°C. These reactions were stopped by addition of 1 mg of trypsin inhibitor per ml.

RESULTS

Preliminary experiments were conducted to determine the enzyme and substrate concentrations to be used in subsequent assays. A preparation containing CMCase activity (1 mg of protein per ml dissolved in sodium phosphate buffer [pH 7.0]) was mixed with increasing concentrations (0.1 to 1.0%, wt/vol) of CMC and incubated as described in Materials and Methods. Product formation (reducing sugars) increased linearly up to about 0.6% CMC, but at higher CMC concentrations a plateau was observed, indicating substrate saturation, and about 0.3 mg of product per ml was formed (data not shown). In another set of experiments, a constant amount of CMC (1.0%, wt/vol) was mixed with variable amounts of CMCase activity. Results indicated a linear increase in the formation of reducing sugars. Again, a maximum of 0.3 mg/ml at 1.0 mg of CMCase preparation per ml was achieved (data not shown). In addition, about 50% of the CMCase activity present in this preparation (1 mg of protein per ml) bound to 250 mg of microcrystalline cellulose (bed volume, 1 ml) (data not shown). Since perturbation of this system could result in significantly enhanced or inhibited binding, which could be accurately detected by the CMC–reducing-sugar assay, about 1.0 mg of both CMCase and CMC per ml along with 250 mg of particulate substrate were chosen for further experimentation.

TABLE 1. CMCase binding to insoluble cellulose substrates

Substrate[a]	Bound CMCase activity (%)[b]
Microcrystalline cellulose	55
Whatman no. 1 filter paper	42
Cotton	40
DEAE-cellulose	60
DEAE-acrylamide (control)	10
Microcrystalline cellulose + 0.5 M NaCl	70
Microcrystalline cellulose + basal salt medium	70
Microcrystalline cellulose (−NaCl)	52

[a] Equivalent of 250 mg of substrate was used in each case.
[b] Amount of CMCase activity bound to 250 mg of substrate.

Binding of CMCase to a variety of insoluble celluloses. In a control experiment, only 10% of CMCase activity bound to non-cellulose-containing DEAE-acrylamide (Table 1). When the preparation was individually mixed in suspension with microcrystalline cellulose, Whatman no. 1 filter paper, cotton, or DEAE-cellulose, 40 to 60% of the CMCase activity bound to these materials within 15 min (Table 1). About 20 to 30% of the cellulose-bound activity could subsequently be removed by repeatedly washing the cellulose with buffer solution (data not shown). Enhanced CMCase binding to microcrystalline cellulose, Whatman no. 1 filter paper, or DEAE-cellulose was observed in the presence of either basal salt medium or sodium phosphate buffer containing 0.5 M NaCl (Table 1; microcrystalline-cellulose results are shown only). Furthermore, very little, if any, of this activity could be removed by a variety of treatments, including washing the cellulose with sodium phosphate buffer alone or with 0.01% sodium dodecyl sulfate (SDS), 0.1% Triton-X-100 (Union Carbide Chemicals and Plastics Co. Inc.), 100 mM dithiothreitol or 2.8 M lithium chloride, and soluble CMC. Various pH values and ionic strengths were included in the protocols (data not shown).

For further experiments, only microcrystalline cellulose was used to characterize CMCase binding.

Effects of temperature and pH on CMCase binding to microcrystalline cellulose. Somewhat unexpectedly, the binding of CMCase activity to microcrystalline cellulose was more favorable at lower temperatures. A maximum of 60% CMCase activity bound to cellulose at about 8°C, and the binding decreased with increasing temperatures (Fig. 1A). CMCase binding to cellulose was stable within a fairly wide range of pH values. About 40 to 60% binding occurred within the pH range of 6 to 8 (Fig. 1B). CMCase binding to cellulose was greatly reduced below pH 5.0 and above pH 9.0 (Fig. 1B).

Binding of CMCase activity to modified microcrystalline

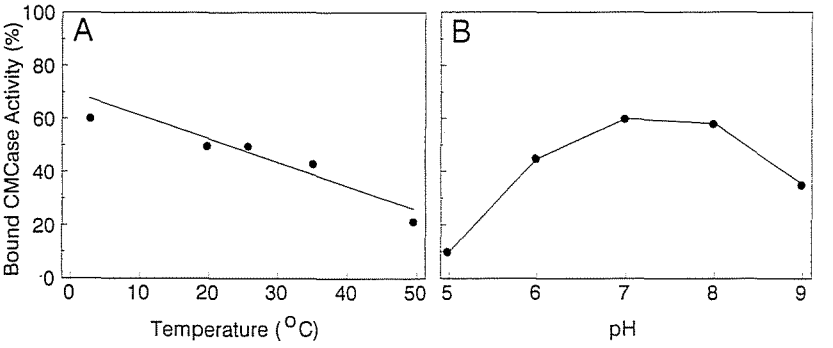

FIG. 1. Effect of temperature (A) and pH (B) on CMCase binding to cellulose.

cellulose. The amount of CMCase activity bound to microcrystalline cellulose that had been suspended in sodium phosphate buffer was very similar to the amount bound after direct addition to powdered (nonhydrated) microcrystalline cellulose (Table 2). Increased particle size of the cellulose substrate reduced the amount of bound CMCase activity (Table 2). Cellulose pretreated with 1% bovine serum albumin showed little, if any, reduction in the amount of CMCase binding to such substrate. Interestingly, only 10% of the CMCase activity bound to celluloses that had been pretreated with either 5 N NaOH to disrupt the degree of crystallinity of the cellulose or briefly with commercial cellulase to induce enzymatic modifications (Table 2).

Pretreatments of CMCase activity. CMCase preparations pretreated with either SDS, EDTA, or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) exhibited no obvious effects regarding binding to cellulose (Table 3). Pretreatment with β-mercaptoethanol, on the other hand, resulted in some reduction (<10%) in such binding. When preparations were preincubated with either trypsin, chymotrypsin, or pronase, a 35 to 55% reduction in CMCase binding to cellulose was observed (Table 3). Pretreatment of the preparation with SDS or the proteases had little effect on CMCase enzymatic activity. However, β-mercaptoethanol did reduce some CMCase enzymatic activity (data not shown). Attempts were also made to characterize the effects of 6 M urea on CMCase binding to cellulose. However, the results were inconclusive because urea interfered with the reducing-sugar assay.

CMCase binding as a function of protein content. Experiments were conducted to analyze the protein from cellulose-bound and unbound fractions of the CMCase preparation. For Fig. 2, the CMCase preparation was adjusted to protein concentrations between 0.10 and 1.0 mg/ml and mixed with a constant amount of microcrystalline cellulose. For all cases, a relatively small proportion of the total protein bound to cellulose. Relative CMCase activity in the cellulose-bound fraction reached 90% at the lowest protein concentration (0.10 mg/ml) investigated. This indicated that if cellulose-binding sites were available, all the CMCase activity would adhere, or, conversely, that none of the CMCase activity was associated with soluble substrate, such as cellodextrins. When the protein concentration of the CMCase preparation was increased from 0.10 to 0.50 mg/ml, the amount of cellulose-bound protein increased. Above this level, the amount of bound protein remained constant. Also, expectedly, as the protein concentration of the preparation increased, the relative amount of bound CMCase activity decreased and, consistent with previous observations, approximately 55% of the CMCase activity was associated with the cellulose at 1 mg of protein per ml. Preliminary SDS-polyacrylamide gel electrophoresis analysis of the cellulose-bound protein fraction indicated that at least four major and six minor polypeptides adhered to cellulose (data not shown).

DISCUSSION

Cellulosic biomass represents an abundant source of renewable energy, and prospects for utilizing it as a replacement for petroleum-based fuel appear promising. We have been studying microbial attachment to and degradation of plant-derived polymeric materials for several years (4, 9, 14–22, 25). Cellulose-degrading enzymes provide a first step toward conversion of cellulosic biomass into biofuel. There-

TABLE 2. CMCase binding to pretreated microcrystalline cellulose

Treatment[a]	Bound CMCase activity (%)[b]
Hydrated	52
Nonhydrated	55
Particle size, 50 μm	42
Particle size, 100 μm[c]	20
5 N NaOH	10
1% BSA[d]	40
Partial hydrolysis by cellulase	10

[a] See text for experimental details.
[b] Amount of CMCase activity bound to 250 mg of substrate.
[c] Sigmacell type 100.
[d] BSA, bovine serum albumin.

TABLE 3. Pretreatment of CMCase activity and its effect on binding to cellulose

Treatment[a]	Concn	Bound CMCase activity (%)[b]
No treatment		55
Buffer + 1 mM $CaCl_2$		48
SDS (0.1%)	0.1% (wt/vol)	38
β-Mercaptoethanol	5% (vol/vol)	50
EDTA	5 mM	54
EGTA	5 mM	25
Trypsin	10 μg/ml	35
Chymotrypsin	100 μg/ml	30
Pronase	100 μg/ml	

[a] See text for experimental details.
[b] Activity bound to 250 mg of microcrystalline cellulose (bed volume, 1 ml).

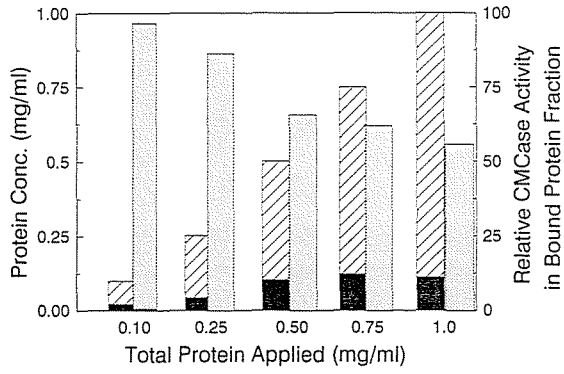

FIG. 2. Analyses of cellulose-bound (■) and unbound (▨) proteins in a CMCase preparation of variable protein concentration and CMCase activities associated with the cellulose-bound protein fractions (□).

fore, it is important to learn more about the surface interactions of such soluble enzymes with insoluble substrates and the roles they play in substrate degradation. Data presented here indicated that the extracellular CMCase activity from marine shipworm bacterium bound preferentially and tenaciously to cellulosic substrates but not to noncellulose substrate (Table 1), suggesting that the binding may be specific. The extent of adherence was more favorable at lower temperatures (Fig. 1A). In this regard, Peitersen et al. (24) have also reported that the cellulase from *Trichoderma reesei* bound favorably to filter paper at lower temperatures. The increased binding of CMCase to cellulose in the presence of salts (Table 1) is not surprising, considering the marine origin of the microorganism. Also, maximum hydrolysis of CMC by purified enzyme was observed in ionic strengths equivalent to seawater (11).

Increasing the cellulose particle size reduces the surface area-to-volume ratio, which should in turn reduce the surface concentration of CMCase-binding sites (7). Indeed, reduced protein binding was observed with larger cellulose particles (Table 2). In this regard, it has been suggested (26) that the availability of the binding sites on the cellulose is limited to the surface of pores with radii greater than that of enzyme. Other evidence indicates the intricate nature of the adhesion process, which appears to be influenced by the macroscopic structure of the substrate (23). Significant loss of CMCase binding was observed when the crystalline structure of the cellulose was chemically disrupted or when the substrate was altered enzymatically (Table 2). NaOH treatment of cellulose, which increases the surface area significantly (23), reduced CMCase binding (Table 2), indicating that other structural features of cellulose may be limiting the availability of binding sites. Lee et al. (23), who obtained similar results, suggested that these results may be explained by the known conversion of the crystalline form of native cellulose from cellulose I to a less reactive form, cellulose II. In any event, further studies are needed to define factors limiting the availability of binding sites on the cellulose. With regard to reduced binding to cellulose pretreated with *T. reesei* cellulase (Table 2), it is possible that *T. reesei* cellulase saturated the cellulose, thus inhibiting the binding. This, however, remains to be verified.

Pretreatment of CMCase preparation with proteases had little effect on the enzymatic activity, but the ability to bind to cellulose was significantly inhibited (Table 3). It is likely that CMCase possesses a protease-sensitive cellulose-binding domain which is distinct from the active site. Din et al. (8) demonstrated that the cellulase from the bacterium *Cellulomonas fimi* contains two distinct domains on the same molecule: a cell-binding domain and a catalytic domain. The fact that treatment with detergent, reducing agent, or chelating agents did not affect enzyme binding to cellulose (Table 3) indicates that the binding probably does not require ionic or disulfide linkages or the presence of divalent cations. Similar results were also obtained by Castanon and Wilke (5), who studied the adsorption and recovery of cellulases during the hydrolysis of newspaper.

Many natural biopolymers are being used to develop environmentally friendly biodegradable consumer materials. Studies pertaining to the binding of enzymes to natural solid substrates may yield valuable information on substrate biodegradation. Necessarily, binding of CMCase to cellulose is an important initial step in the degradation process. It has been suggested that the binding of cellulolytic bacteria to plant cell walls and cellulose matrices facilitates cellulose fiber degradation (1, 2, 6).

We have also studied the preferential adherence of intact shipworm bacterium to cellulosic substrates (19). Although cell-associated exoglucanase activity appears to rely on the extracellular CMCase activity to generate cellulose chain ends (11), it is interesting that whole-cell binding to cellulose does not appear to be influenced to any extent by the presence of extracellular CMCase activity (19). Perhaps future experiments will reveal a closer relationship between the two adherence processes.

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