Catalytic and Substrate-Binding Domains of Endoglucanase 2 from Bacteroides succinogenes

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Received 28 December 1988/Accepted 15 March 1989

Endoglucanase 2 (EG2) of the cellulolytic ruminal anaerobe Bacteroides succinogenes is a 118-kilodalton (kDa) enzyme which binds to cellulose and produces cellooligosaccharides as the end product of hydrolysis. The purified enzyme was treated with the protease trypsin in an attempt to isolate peptides which retained the ability to either hydrolyze soluble carboxymethyl cellulose or bind to insoluble cellulose. There was no loss in endoglucanase activity (carboxymethylcellulase) over a period of 2 hrs following the addition of trypsin. In comparison, there was a greater than eightfold reduction in the binding of carboxymethylcellulase activity to crystalline cellulose. A Lineweaver-Burk plot with amorphous cellulose as the substrate revealed that the trypsin-digested enzyme had an identical $V_{max}$ but a 1.9-fold-lower $K_{m}$ in comparison with the intact enzyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the trypsin-digested enzyme revealed two major peptides of 43 and 51 kDa (p43 and p51). The 43-kDa peptide was able to bind to both amorphous and crystalline cellulose, whereas p51 did not. Purified p51 had a molar activity toward carboxymethyl cellulase which was identical to that of the intact enzyme, but activity toward both amorphous and crystalline cellulose was reduced approximately twofold. Two high-titer monoclonal antibodies from mice immunized with the intact protein recognized p43 but not p51. The results are consistent with a bifunctional organization of EG2, in which the 118-kDa enzyme is composed of a 51-kDa catalytic domain and a highly antigenic 43-kDa substrate-binding domain. In terms of its domain structure and activity toward cellulose, EG2 is very similar to cellobiohydrolase II of Trichoderma reesei.

Materials and Methods

Enzyme purification. EG2 was purified as described previously (12).

Trypsin digestion of EG2. Trypsin digests typically contained 50 μl of purified EG2 (38 μg), 40 μl of deionized water, and 10 μl of 1.0 M potassium phosphate buffer, pH 7.5. Digestion was initiated by the addition of 10 μl of 1.5-mg/ml trypsin (T-8253; Sigma Chemical Co., St. Louis, Mo.) freshly prepared in 1 mM HCl. The final concentrations of trypsin and EG2 were 136 and 345 μg/ml, respectively. Incubation was for 1 h at 25°C. At this time, samples were either diluted 10-fold in cold 50 mM potassium phosphate buffer (pH 6.0) for assay of endoglucanase activity or were further treated by the addition of 20 μl of 1.5-mg/ml soybean trypsin inhibitor (Sigma T-9128) made up in 0.1 M potassium phosphate buffer (pH 7.5).

Preparation of antiserum. Purified EG2 (385 μg) was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Bio-Rad Protein II system with a gel thickness of 1.5 mm. Following electrophoresis, the gel was stained briefly in 0.1% Coomasie blue R-250–40% methanol–10% acetic acid and destained in 7% acetic acid–12% ethanol. The stained protein was excised with a scalpel, rinsed with distilled water, and lyophilized. The dried gel slice was diced into small pieces, reconstituted in a minimal volume of distilled water, and then mixed with an equal volume of Freund complete adjuvant. The emulsion was repeatedly drawn up through a 16-gauge needle and expressed through a 20-gauge needle. When the emulsion could be easily drawn up through the 20-gauge needle, a sample containing approximately 100 μg of protein was injected subcutaneously into the hindquarters of each of two New Zealand White male rabbits. Subsequent injections of 50 μg were given at intervals of 5, 12, and 27
days after the first injection. Blood was collected 1 week after the final injection.

**Purification of antibodies.** Antibodies were purified from rabbit serum by affinity chromatography. EG2 was partially purified from *B. succinogenes* culture fluid by passage through S-Sepharose and CM-Sepharose as described previously. The preparation containing 8.6 mg of total protein was dialyzed against 0.1 M NaHCO₃-0.5 M NaCl (pH 8.3) and coupled to 2 g of cyanogen bromide-activated Sepharose following the instructions of Pharmacia Fine Chemicals (Piscataway, N.J.). The gel was packed into a column and equilibrated with phosphate-buffered saline (PBS) (per liter: 8.0 g of NaCl, 0.2 g of KH₂PO₄, 2.9 g of Na₂HPO₄·12H₂O, 0.5 g of KCl) containing 0.5% Tween 80 and 0.01% thimerosal. Antisera was applied to the column, and it was then washed with PBS-Tween 80, followed by PBS alone, until protein could no longer be detected in the eluate. Retained immunoglobulins were eluted with 3 M sodium thiocyanate, and fractions of 1.0 ml were collected. Fractions containing protein were pooled and immediately dialyzed against 2 liters of PBS. The titer of the purified antibody was determined by enzyme-linked immunosorbent assay with 1 μg of purified EG2 per ml to coat the wells of a microtiter plate.

**MAb production.** For monoclonal antibody (MAb) production, two female BALB/c mice (8 to 10 weeks old; Charles River Canada Inc., St. Constant, Quebec, Ontario, Canada) were immunized subcutaneously on day 1 with 100 μg of purified EG2 emulsified in Freund incomplete adjuvant. On day 12, 50 μg was administered intravenously. Fusions were done on day 15. Spleen cells from immunized mice were fused with NS-1 murine myeloma cells by the procedure of Oi and Herzenberg (13) as modified by Lam et al. (10). Positive hybridomas were identified by enzyme-linked immunosorbent assay with 1 μg of purified EG2 per ml in carbonate buffer (per liter: 1.5 g of Na₂CO₃, 2.93 g of NaHCO₃, 1.02 g of MgCl₂, 6H₂O, pH 9.8) to coat the wells. The blocking agent was 1.5% gelatin in PBS. Goat anti-mouse F(ab')₂ conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used as the substrate. Proteinuria F(ab')₂ conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used as the detection system. Positive hybridomas were cloned by limiting dilutions. Antibody titer was defined as the highest dilution giving an A₄₀₅ of 0.1 or greater by enzyme-linked immunosorbent assay after a 1-h incubation with phosphatase substrate.

**SDS-PAGE and Western immunoblotting.** Trypsin-digested EG2 was subjected to SDS-PAGE by the method of Laemmli (9) with resolving gels containing 10% (wt/vol) acrylamide and 5% stacking gels. Samples were dissociated in Laemmli sample buffer by heating at 100°C for 10 min prior to electrophoresis. Protein Mₙ standards were obtained from Pharmacia. Proteins were stained with 0.1% Coomassie blue R in 40% (vol/vol) methanol-10% (vol/vol) acetic acid and destained in 20% (vol/vol) methanol-8% (vol/vol) acetic acid. When indicated, proteins were transferred to nitrocellulose (0.45-μm pore size; Bio-Rad) after SDS-PAGE by using the buffering system of Towbin et al. (17) and a Bio-Rad mini-Transblot apparatus. Immunoblots were developed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G plus immunoglobulin M (Bio-Rad) or goat anti-mouse F(ab')₂ (Jackson ImmunoResearch), according to the instructions of the Bio-Rad alkaline phosphatase Immunoblot assay kit.

**Other analytical methods.** Protein was assayed by the method of Bradford (2). Amorphous (acid-swollen) cellulose was prepared as described by Schellhorn and Forsberg (14), and the total carbohydrate content was determined by the method of Dubois et al. (3). Endoglucanase assays with carboxymethyl cellulose (CMC) (low viscosity; Sigma) as the substrate were conducted as described previously (12). To determine the Kₗₚ and Vₘₚ values of trypsin-digested and native EG2 for amorphous cellulose, we treated the enzyme with trypsin for 1 h as described above. After the addition of soybean trypsin inhibitor, the pH was adjusted by adding 20 μl of 1.0 M potassium phosphate buffer, pH 6.0. To this mixture was added water and 5.6 μg of amorphous cellulose per ml in 20 mM potassium phosphate buffer (pH 6.0) to give concentrations of amorphous cellulose ranging from 0.47 to 4.2 mg/ml. The samples were incubated at 37°C with constant shaking for 8.5 h, at which point the enzyme was inactivated by heating at 100°C for 15 min. After being centrifuged to remove cellulose, 50-μl samples of the supernatants were assayed for the presence of reducing sugar by the Nelson-Somogyi assay (1). The specific activity at each substrate concentration was plotted by the reciprocal method of Lineweaver and Burk (11), and the Kₗₚ and Vₘₚ values were determined from the x and y intercepts.

**Purification of catalytic domain.** Purified EG2 (1.5 mg) was digested with trypsin and applied to a column (2.5 by 59 cm) of Bio-Gel P-100 equilibrated in 50 mM potassium phosphate buffer, pH 6.5. Protein was eluted from the column with an equilibrium buffer at a flow rate of 15 ml/h, and fractions of 5 ml were collected. Endoglucanase activity was detected by the reducing sugar assay with CMC as the substrate. Active fractions were pooled and concentrated by ultrafiltration with a 10,000-molecular-weight-cutoff membrane (Amicon Corp., Lexington, Mass.). To remove cellulose-binding peptides, 350 mg of Avicel crystalline cellulose was suspended in 2.5 ml of 50 mM potassium phosphate buffer (pH 6.5) and centrifuged at 12,100 × g for 5 min. The pellet was washed twice in 2.5 ml of buffer and then suspended in 2.5 ml (0.49 mg) of the Bio-Gel P-100-purified trypsin digest. After incubation at 39°C for 10 min, the suspension was centrifuged as before and the supernatant was subjected to one more cycle of Avicel adsorption and centrifugation. The resulting supernatant contained purified catalytic domain.

**RESULTS**

Endoglucanase activity of trypsin-digested EG2. The stability of EG2 activity in the presence of trypsin was determined by taking samples immediately and 1 and 2 h after the addition of trypsin. In a control sample without trypsin, the enzyme retained 102 and 95%, respectively, of the original activity after 1 and 2 h of incubation, compared with 109 and 113% for the sample incubated in the presence of trypsin.

**Time course of proteolysis and binding of trypsic peptides to cellulose.** The time course of trypsin digestion of EG2 was determined by SDS-PAGE (Fig. 1). Initial proteolysis was very rapid, with the complete disappearance of the 118-kDa EG2 occurring as rapidly as a sample could be withdrawn following trypsin addition, mixed with SDS-PAGE reducing buffer, and heated to 100°C. At this point, there was a cluster of bands ranging from 74 to 94 kDa and two discrete peptides of 55 and 51 kDa (Fig. 1). The 51-kDa peptide (p51) increased in intensity over 1 h, while the 55-kDa peptide gradually disappeared. The decreasing abundance of this peptide correlated with the appearance of increasing amounts of the 43-kDa peptide (p43). After 1 h of proteolysis, p43 and p51 were the only predominant proteins in the trypsin digest, with trace amounts of a 94-kDa protein (p94). After incubation with either amorphous or crystalline cellulose and
containing 3.5 low-molecular-mass protein per digest after 0, EG2 and respectively. digestion after 0, EG2 pellet whereas pSi was catalytic activity was effectively removed, immediately mixed with SDS sample buffer, and heated at 100°C for 10 min prior to electrophoresis on a 10% polyacrylamide gel. As a control, 10 μl of 1 mM HCl was added in place of trypsin. Lanes 1 and 10, Pharmacia low-molecular-mass protein standards, indicated in kilodaltons; lane 2, EG2 incubated for 60 min in the absence of trypsin; lanes 3 to 9, trypsin digest after 0, 5, 10, 15, 30, 45, and 60 min of incubation, respectively. Arrowheads indicate the 43- and 51-kDa proteins, p43 and p51, respectively.

Purification of catalytic domain. To purify peptides retaining catalytic activity toward CMC, we passed 1.5 mg of trypsin-digested EG2 through a column of Bio-Gel P-100. This effectively removed proteins of greater than 51 kDa, and a total of 1.0 mg of protein was recovered, of which p51 was the major component. Two consecutive adsorptions of this preparation with Avicel crystalline cellulose resulted in the removal of all cellulose-binding peptides, leaving p51 as the only protein which could be detected by Coomassie blue staining (Fig. 3). As with amorphous cellulose (Fig. 2), the protein solubilized from the Avicel was enriched in p43 but contained very little p51.

Affinity of p51 for crystalline cellulose and catalytic activity toward soluble, amorphous, and crystalline substrates. To test for the effect of trypsin digestion on affinity of the catalytic activity for cellulose, we suspended two 10-mg aliquots of Avicel PH105 in 75 μl of either intact EG2 or Bio-Gel-purified trypsin digest which is essentially free of larger peptides that could have both catalytic and substrate-binding properties. After incubation at 39°C for 15 min, the samples were centrifuged, washed twice in buffer to remove nonbinding peptides, and resuspended in the original volume of buffer. When treated in this fashion, the Avicel which had been incubated with the intact enzyme retained 96% of the activity originally added, whereas only 11.5% of the activity from the trypsin digest was retained on the cellulose after two buffer washes (Table 1). In both cases the balance of the activity was present in the supernatant recovered from the two washes. For activity measurements on CMC, amorphous cellulose, and crystalline cellulose, the purified catalytic domain (p51) and undigested EG2 were adjusted to have equimolar protein concentrations (42 and 97 μg/ml, respectively) prior to incubation with substrate. With soluble CMC as the substrate, p51 and EG2 had identical activities on a molar basis (Table 2). However with either crystalline or amorphous cellulose as the substrate, the molar activity of undegraded EG2 was 1.9- or 2.3-fold greater, respectively, than that of p51. When reciprocal values of specific activity and amorphous cellulose concentration were plotted for undigested EG2 and the crude trypsin digest, the prepara-
TABLE 1. Endoglucanase activity of untreated intact and trypsin-digested EG2 bound to (Avicel) crystalline cellulosea

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (µg of reducing sugar/ ml per min)</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG2 control</td>
<td>90.5</td>
<td></td>
</tr>
<tr>
<td>EG2 washed Avicel</td>
<td>86.8</td>
<td>95.9</td>
</tr>
<tr>
<td>pS1 control</td>
<td>119.7</td>
<td></td>
</tr>
<tr>
<td>pS1 washed Avicel</td>
<td>13.8</td>
<td>11.5</td>
</tr>
</tbody>
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a Either 75 µl of untreated intact EG2 (190 µg/ml) or trypsin-digested EG2 purified by gel filtration (190 µg/ml) was added to 10 mg of Avicel PH105 crystalline cellulose (prewetted by suspending in buffer and centrifuging) and incubated for 15 min at 39°C, with gentle vortexing every 5 min. After centrifugation, the cellulose pellet was washed twice in 0.3 ml of 50 mM potassium phosphate buffer (pH 6.5) and finally suspended in the original volume (75 µl) of buffer. A 10-µl sample of the resulting Avicel suspension was diluted to 100 µl of 50 mM phosphate buffer (pH 6.0) and incubated with 100 µl of 2% CMC at 39°C for 30 min. Reducing sugars were then quantified.

As a control, intact or trypsin-digested EG2 was added directly to the Avicel and a 10-µl sample was assayed after 15 min of incubation without prior washing of the Avicel. Results are expressed as the percentage of this total activity.

Epitope mapping of trypsin digest. Two high-titer MAb specific for EG2 were isolated. MAB NS5a tissue culture supernatant had a titer of 104, while OIA had a titer of 103 as determined by enzyme-linked immunosorbent assay. Both MABs were determined to be of the immunoglobulin G1 subclass. To determine the epitope specificity of the MABs, a Western blot was probed with affinity-purified polyclonal antibody and both MABs (Fig. 5). As with the gel shown in Fig. 2, the blot consisted of separate lanes containing crude trypsin digest, supernatant after incubation with amorphous cellulose, and protein solubilized from the cellulose pellet. When probed with polyclonal antibody, the blot was identical to the gel shown in Fig. 2. Probing an identical blot with MAB NS5a clearly showed that there is no band corresponding to pS1. With the exception of the more abundant p43, incubation with cellulose and centrifugation removed all the peptides which were immunoreactive with NS5a, and these peptides were detected in the protein solubilized from the cellulose. The specificity of OIA was identical to that of NS5a. There was a strong reaction of the MAB with peptides running just below p43, and the same intensity of reaction was not apparent when the blot was probed with polyclonal antibody. This is probably due to the higher titer and avidity of the MAB allowing for a greater sensitivity of detection for peptides present in trace amounts. As noted in lanes 6 to 8 of Fig. 1, which was stained for protein with Coomassie blue, this same banding pattern can be observed just below p43. However, after a further 15 min of incubation (lane 9), the bands were barely discernable. Lanes 2 and 3 of Fig. 5B

FIG. 4. Lineweaver-Burk plot of undigested (●) and trypsin-treated (▲) EG2 with amorphous cellulose as the substrate. EG2 was digested with trypsin, followed by supplementation with soybean trypsin inhibitor and 1.0 M potassium phosphate buffer (pH 6.0) as described in Materials and Methods. For the undigested EG2, 1 mM HCl was added in place of trypsin. To this mixture was added water and 5.6 mg of amorphous cellulose per ml to give substrate concentrations ranging from 0.47 to 4.2 mg/ml. After incubation on a shaking platform at 37°C for 8.5 h, the enzyme was inactivated by steaming for 15 min. Residual cellulose was removed by centrifugation, and the supernatants were assayed for reducing sugar. Specific activities were determined based on total EG2 protein in each assay, regardless of whether or not the enzyme had been treated with trypsin.

FIG. 5. Determination of epitope specificity of MAB to EG2. EG2 was digested with trypsin for 60 min and incubated with amorphous cellulose such that lanes 1, 2, and 3 are identical to lanes 3, 4, and 5, respectively, of Fig. 2. After SDS-PAGE, the proteins were transferred to nitrocellulose. Separate strips containing lanes 1, 2, and 3 were blocked in 5% gelatin and incubated for 2 h with either 400-fold-diluted affinity-purified polyclonal antibody (A) or 400-fold-diluted MAb NS5a tissue culture supernatant (B). Second antibody was 1,000-times-diluted Bio-Rad goat anti-rabbit immunoglobulin G (A) or 5,000-times-diluted Jackson goat anti-mouse F(ab')2-alkaline phosphatase conjugate (B). MAB OIA gave a pattern identical to that observed for NS5a. Molecular size standards are indicated on the left in kilodaltons.

TABLE 2. Molar activities of intact EG2 and purified pS1 toward CMC, amorphous, and crystalline cellulosea

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific molar activity (µmol of reducing sugar/ mmol of protein per min)</th>
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<tbody>
<tr>
<td></td>
<td>EG2</td>
</tr>
<tr>
<td>CMC</td>
<td>649</td>
</tr>
<tr>
<td>Amorphous cellulose</td>
<td>5.6</td>
</tr>
<tr>
<td>Avicel crystalline cellulose</td>
<td>0.76</td>
</tr>
</tbody>
</table>

a Intact EG2 was diluted to 97 µg/ml (0.82 µmol/ml), such that the protein concentration was equimolar to that of purified pS1. With CMC as the substrate, 20-µl samples were diluted to 100 µl of 50 mM phosphate buffer (pH 6.0) and incubated with 100 µl of 2% CMC at 39°C for 15 min. For amorphous and crystalline cellulose, the enzyme was mixed with an equal volume of 0.6% (amorphous) or 2% (crystalline) substrate and incubated on a shaking platform at 37°C for 12.5 h. Reducing sugars were subsequently quantified by the method of Nelson and Somogyi (see reference 1). Specific molar activities were calculated by multiplication of the specific activity by the molecular weight of the protein (118 for EG2, 51 for pS1). As a control, it was shown that both EG2 and pS1 retained full activity toward CMC when incubated with buffer alone at 37°C for 12.5 h.
show that these peptides were present neither in the supernatant after incubation with cellulose nor in the proteins solubilized from the cellulose pellet. There are two possible explanations. The peptides may have had a weak binding affinity such that they bound to the cellulose but were removed during the subsequent buffer washes. Additionally, lanes 8 and 9 of Fig. 1 document the significant reduction in the intensity of these bands in going from 45 to 60 min of incubation with trypsin. During incubation with cellulose for an additional 15 min, continued proteolysis may have resulted in the complete degradation of these peptides.

**DISCUSSION**

Protease treatment has been used effectively to study the structural organization of purified cellulases. Papain was used to cleave celllobiohydrolases I and II (CBH I and II) from *Trichoderma reesei* (16, 18), while endoglucanase A (CenA) and an exoglucanase (Cex) from *Cellulomonas fimi* were treated with a protease produced by the bacterium during growth on glycerol (5). Trypsin catalyzes the hydrolysis of peptide bonds in which the carboxyl group is contributed by either a lysine or an arginine residue and was used in this study to examine the structural organization of EG2 from *B. succinogenes*.

As in previous work in which solubilization of membrane-associated endoglucanase by trypsin did not cause any reduction in activity (6), purified EG2 retained full activity against CMC after 2 h in the presence of trypsin. The complete conversion of the 118-kDa EG2 into smaller polypeptides was confirmed by SDS-PAGE (Fig. 1). The extremely rapid disappearance of the 118-kDa EG2 may be attributed to two factors. First, trypsin and EG2 were present at 136 and 345 μg/ml respectively, and considering its small size (23.8 kDa), trypsin is actually in excess of EG2 on a molar basis. Second, EG2 is a basic protein, having an isoelectric pH of 9.4 (12), and it should contain high levels of the basic amino acids lysine and arginine, which are cleavage sites for trypsin.

After 1 h of proteolysis, there were two major peptides of 43 and 51 kDa in the trypsic digest (Fig. 1). As there was no observable loss in activity toward the soluble substrate CMC, it was assumed that one or both of these peptides would contain an intact catalytic domain. When this trypsin-digested EG2 was subjected to Western blotting, an MAb reacted strongly with the p43 peptide but not at all with p51, providing proof that the smaller peptide could not have arisen from a proteolytic cleavage of the latter. Furthermore, p43 was found to retain affinity for both amorphous and crystalline cellulose, yet only 11.5% of the endoglucanase activity of the Bio-Gel-purified trypsin digest bound to crystalline cellulose compared with 96% for intact EG2. It is clearly evident that trypsin digestion of EG2 resulted in separation of the 118-kDa enzyme into a functional 51-kDa catalytic domain and a 43-kDa substrate-binding domain.

Purified p51 and intact EG2 had identical molar activity toward soluble CMC, but the molar activity of p51 toward both amorphous and crystalline cellulose was approximately twofold lower. This appears to be a direct reflection of the 1.9-fold-higher *Kₘ* of the trypsin-digested enzyme for amorphous cellulose.

Our results are similar to those from other studies in which proteases have been used to elucidate the protein architecture of cellulases from *C. fimi* (5), *Thermomonomospora fusca* (4), and *Trichoderma reesei* (16, 18). A *C. fimi* protease induced during growth on cellulose was used to treat a 48.7-kDa endoglucanase (CenA) and an 47.3-kDa exoglucanase (Cex) from this bacterium. The affinity of the parent enzyme for cellulose was contained independently in a 20-kDa N-terminal fragment of CenA (p20) and an 8-kDa C-terminal fragment of Cex (p8), while corresponding fragments of 30 (p30) and 35.4 (p35) kDa from CenA and Cex, respectively, contained catalytic domains. Further cleavage of the p30 CenA catalytic domain was not apparent, whereas minor products of 34.5 and 32.8 kDa (p33) were observed in the Cex preparation. For Cex, molar activities of p35 and p33 toward para-nitrophenyl-β-D-celllobiose were 7.2 and 8.9 kat/mol, respectively, compared with 9.0 kat/mol for intact Cex, and the *Kₘ* of p33 for para-nitrophenyl-β-D-celllobiose was 0.65 mM, compared with 0.70 mM for the intact enzyme. Clearly, p35 retains full activity toward the soluble chromophore para-nitrophenyl-β-D-celllobiose. However, no data were given relating the activity of p35 to insoluble cellulolistic substrates. The p30 catalytic fragment of CenA showed only a 20% reduction in activity toward crystalline cellulose in comparison with the intact enzyme, and it had a higher molar activity toward CMCC, amorphous cellulose, and cellulose micelles. The increase in activity was attributed to preferential renaturation of the truncated enzyme after protease treatment.

Our results are more consistent in showing the contribution of the binding domain toward the hydrolysis of insoluble cellulolytic substrates and are similar to those reported for the 65-kDa CBH I and 58-kDa CBH II of *Trichoderma reesei* (16, 18). Limited action of papain on CBH I and CBH II led to the isolation of respective core fragments of 56 and 45 kDa which retained full activity on small soluble substrates. Removal of the C-terminal substrate-binding domain of CBH I caused a sevenfold reduction in activity toward crystalline cellulose but had no effect on adsorption and activity toward amorphous cellulose. For CBH II, removal of the N-terminal substrate-binding domain caused a 40 to 50% reduction in both adsorption and activity on both crystalline and amorphous cellulose.

Light microscopic studies further revealed that cellulose incubated with CBH I maintained the same crystallinity index, whereas the crystallinity index increased upon incubation with CBH II (8). These results in conjunction with the data given for the activity and affinity of the respective core proteins for amorphous and crystalline cellulose are consistent with a mechanism of action in which CBH I interacts with both crystalline and amorphous regions while CBH II degrades only amorphous cellulose. Our results suggest that EG2 has a function in the hydrolysis of cellulose which is very similar to that of CBH II of *Trichoderma reesei*. First, EG2 has a greater affinity for binding to amorphous cellulose over crystalline cellulose (12), and its binding to crystalline cellulose is probably a reflection of the amorphous regions within the cellulose crystallite. Second, from the Lineeweaver-Burk plot, EG2 has a specific activity of 0.07 U/mg toward amorphous cellulose at a concentration of 0.4%, which is similar to 0.05 U/mg for CBH II at 1% substrate. Finally, removal of the binding domain from EG2 caused a reduction in activity of 47 and 57%, respectively, toward crystalline and amorphous cellulose, compared with a 40 to 50% reduction in activity in the CBH II core protein.

A similar but less complete study was done with a 43-kDa endoglucanase from *Thermomonomospora fusca* (4). In vivo proteolysis of the enzyme resulted in the generation of 30- and 14-kDa peptides. The 14-kDa peptide binds to cellulose and has no enzyme activity, while the 30-kDa product
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retains CMCase activity and binds to crystalline cellulose at 0°C but not 55°C.

The 118-kDa EG2 is a much larger protein than other cellulases for which functional domains have been characterized. While its 51-kDa catalytic domain is similar in size to the corresponding catalytic domains from the cellulases of Thermomonospora fusca (30 kDa), C. fimi (CenA, 30 kDa; Cex, 35 kDa), and Trichoderma reesei (CBH I, 56 kDa; CBH II, 45 kDa), its 43-kDa substrate-binding domain is disproportionately larger than those previously reported (Thermomonospora fusca, 14 kDa; C. fimi CenA, 20 kDa, and Cex, 8 kDa; Trichoderma reesei CBH I, 9 kDa, and CBH II, 13 kDa). Although 43 kDa is a fraction of the overall size of the two high-titer MAbs were specific for an epitope on the binding domain, suggesting that it is a prominent feature of the three-dimensional structure of the protein. These features make EG2 an ideal protein for further studies on the mechanism of endoglucanase interaction with cellulose. Recent X-ray scattering measurements of CBHs I and II from Trichoderma reesei indicated an unusual tadpole shape of the native enzyme, featuring an isotropic head containing the catalytic core protein and a long tail (7, 15). It will be interesting to determine whether the unusually large binding domain of EG2 results in a different configuration of the native protein. Finally, although EG2 is a basic protein and should contain an abundance of lysine and arginine residues at which trypsin cleaves, the two domains are comparatively resistant to further proteolysis. Hence, the domain structure may provide a high degree of intrinsic resistance to protease, which may be of particular significance in consideration of the ruminal environment from which B. succinogenes was isolated.

ACKNOWLEDGMENTS

We are grateful to Bozena Kristof for assistance in the preparation of polyclonal antibodies and to Billie Baughan for typing the manuscript. Appreciation is expressed to J. Lam and M. Y. C. Lam for guidance in the preparation of MAbs.

M.M. is the recipient of a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship. This research was funded by an operating grant from the Natural Sciences and Engineering Research Council of Canada.

LITERATURE CITED