APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1980–1986 Vol. 61, No. 5
0099-2240/95/$04.00+0

Expression, Purification, and Characterization of the Cellulose-Binding Domain of the Scaffoldin Subunit from the Cellulosome of *Clostridium thermocellum*

ELY MORAG,[1] AVIVA LAPIDOT,[2] DMITRY GOVORKO,[3] RAPHAEL LAMED,[3] MEIR WILCHEK,[1] EDWARD A. BAYER,[1] AND YUVAL SHOHAM[2]*

Department of Biophysics, The Weizmann Institute of Science, Rehovot,[1] Department of Food Engineering and Biotechnology, Technion-Israel Institute of Technology, Haifa,[2] and Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv,[3] Israel

Received 11 November 1994/Accepted 18 February 1995

The major cellulose-binding domain (CBD) from the cellulosome of *Clostridium thermocellum* YS was cloned and overexpressed in *Escherichia coli*. The expressed protein was purified efficiently by a modification of a novel procedure termed affinity digestion. The properties of the purified polypeptide were compared with those of a related CBD derived from a cellulosome-like complex of a similar (but mesophilic) clostridial species, *Clostridium cellulovorans*. The binding properties of the two proteins with their common substrate were found to be very similar. Despite the similarity in the amino acid sequences of the two CBDs, polyclonal antibodies raised against the CBD from *C. thermocellum* failed to interact with the protein from *C. cellulovorans*. Chemical modification of the single cysteine of the CBD had little effect on the binding to cellulose. Biotinylation of this cysteine allowed the efficient binding of avidin to cellulose, and the resultant matrix is appropriate for use as a universal affinity system.

The cellulosome was first described as a cellulose-binding, multicellulase-containing complex in the anaerobic thermophilic bacterium *Clostridium thermocellum* (22, 26). The original proposals for the existence of the cellulosome were based mainly upon biochemical and ultrastructural evidence (3, 4, 7, 25, 29). More recently, documentation for its structural organization has been obtained through cloning and sequencing data (12, 13). The organization of cellulases into a multienzyme complex is considered to be a crucial contribution to the observed synergistic action of bacterial cellulase systems (21, 26, 29, 48).

One of the most intriguing components of the cellulosome is a relatively large (ca. 210,000-Da), multifunctional, noncatalytic subunit, originally termed S1 (25, 26). This subunit was considered to be responsible for the major cellulose-binding function and appeared to organize the enzymatic (cellulolytic) components into the complex (3, 7). We have now termed this component scaffoldin (6).

To date, scaffoldins from two different clostridial species, i.e., CipA and CipB from *C. thermocellum* (18, 36) and CbpA from *Clostridium cellulovorans* (40), have been described in the literature. Like the enzymatically active cellulases from different bacteria and fungi, the noncatalytic scaffoldin also appears to be separated into a series of functional domains (19). In the two scaffoldins which have been sequenced, the majority of the domains are involved in integrating the enzymes into the cohesive complex (17, 18, 40). In both cases, a single cellulose-binding domain (CBD) is present. The CBD of *C. cellulovorans* is the first N-terminal scaffoldin domain, whereas in *C. thermocellum*, the CBD is an internal domain.

In the present study, the expressed CBD from *C. thermocellum* was isolated by a novel affinity-based procedure, and its properties were compared with those recently reported for the CBD from *C. cellulovorans.*

MATERIALS AND METHODS

Materials. Cellobiose was obtained from Sigma Chemical Co. (St. Louis, Mo.). Other cellodextrins were prepared as described earlier (33). Microcrystalline cellulose was purchased from E. Merck AG (Darmstadt, Germany). Amorphous cellulose was prepared from microcrystalline cellulose as reported previously (23). Maleimidopropionyl biocytin and biotin *N*-hydroxysuccinimide ester were prepared as described by Wilchek and Bayer (46). Sepharose-protein A was prepared by the cyanogen bromide method (47) with 2 mg of protein A (Sigma) per ml of resin. NeutraLite avidin was obtained from Belovo Chemicals (Bastogne, Belgium). All other chemicals and biochemicals were of the highest purity commercially available.

Bacterial strains and vectors. *Escherichia coli* HMS174 and BL21(DE3)pLysS and the T7 RNA polymerase expression vector pET3d were described elsewhere (41) and were obtained from Novagen (Madison, Wis.).

DNA manipulation. DNA was manipulated by standard procedures (1, 39). Competent *E. coli* cells were prepared by using calcium chloride (39).

Miscellaneous methods. Protein determinations were carried out as described by Bradford (14), using ovalbumin as a standard. Fast protein liquid chromatography (FPLC) was carried out with a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described by Morag et al. (33).

Cloning the CBD of the cellulosome subunit S1 from *C. thermocellum* YS. DNA primers with partial homology to the C and N termini of the CBD region were synthesized. The N-terminal primer was designed to contain an ATG translational start codon inside an *Nco*I restriction site (CCATGG). The translational start codon is in frame with the CBD domain and within the correct distance from the T7 polymerase promoter when cloned into the *Nco*I cloning site of pET3d. The C-terminal primer was designed to contain a stop codon (TAG) and a *Bam*HI restriction site at the end of the CBD domain. Amplification of the CBD domain via PCR was performed for 30 cycles in a 100-μl reaction mixture containing 20 ng of template DNA, 1 μg of each primer, 200 μM each deoxynucleoside triphosphate, and 10 μg of bovine serum albumin. The PCR cycle was 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. The reaction was carried out with 2 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass.) under the buffer conditions recommended by the manufacturer. The template DNA was a 908-bp *Hpa*I-*Pst*I fragment containing the CBD domain from plasmid pDP1 (36). The PCR product was separated on a 0.7% agarose gel, extracted from the gel with activated glass beads (Geneclean II kit; Bio 101, La Jolla, Calif.), and cleaved with *Nco*I and *Bam*HI. The cleaved fragment was again

* Corresponding author. Mailing address: Department of Food Engineering and Biotechnology, Technion, Haifa 32000, Israel. Phone: 972-4-293072. Fax: 972-4-320742. Electronic mail address: yshoham@techunix.technion.ac.il.

separated and extracted from an agarose gel and ligated with an *Nco*I-*Bam*HI-linearized pET3d plasmid DNA. The ligation mixture was used to transform competent *E. coli* HMS174 cells. Ampicillin-resistant colonies were isolated, and their plasmid DNA was purified and cleaved with restriction enzymes to verify the construct (designated pCBD).

Purification of CBD by conventional affinity chromatography. A 100-ml culture of *E. coli* BL21 (DE3)pLysS containing pCBD was grown on Luria-Bertani medium supplemented with chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml). The cells were cultured at 37°C to an A_{600} of 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 5 mM, and the cells were cultivated for an additional 4 h. The cells were centrifuged, washed twice with double-distilled water, sonicated on ice, and centrifuged again. The supernatant was brought to a final volume of 400 ml in 50 mM Tris-HCl buffer, pH 7 (Tris buffer), and 30 g of microcrystalline cellulose was then added. The suspension was stirred for 1 h at 23°C. After centrifugation, the pellet was washed three times with 1.5 liters of 50 mM phosphate buffer (pH 7.4) containing 1 M NaCl. In experiments designed to remove major contaminants, the cellulose resin was washed once with 100 ml of 3 M urea and washed twice again with the same phosphate buffer. The CBD was eluted from the cellulosic matrix with 100 ml of 1% triethylamine. The eluent was neutralized with 10% acetic acid, dialyzed against 10 mM Tris buffer, and stored at −20°C. In some cases, samples were concentrated by lyophilization, resuspended in distilled water, and purified further by FPLC.

Purification of CBD by affinity digestion. Supernatant containing crude CBD (10 ml), prepared as described in the previous section, was brought to pH 7 with Tris buffer (buffer final concentration, 50 mM). Amorphous cellulose (9 mg) was added, and the final volume was brought to 22 ml. The suspension was stirred for 1 h at 23°C and centrifuged. The pellet was washed twice with 200 ml of 50 mM phosphate buffer (pH 7.4) containing 1 M NaCl. The cellulose resin was washed with 50 ml of 3 M urea followed by 100 ml of 50 mM sodium acetate buffer (pH 5). The washed resin was then resuspended into 20 ml of the same buffer, and 2 mg of purified cellulosome was added. The suspension was incubated with constant stirring for 2 h at 60°C and centrifuged, and the supernatant fluids were saved. The pellet was again resuspended to 20 ml, and the digestion procedure was repeated with 1 mg of the cellulosome preparation. When most of the cellulosic substrate was solubilized (about 16 h), the mixture was centrifuged and the supernatant was pooled with the previous sample. The sample was dialyzed against 10 mM Tris buffer, concentrated by lyophilization, and applied to a Sephacryl S-300 column (1.5 by 85 cm). The column was equilibrated and eluted with 50 mM Tris buffer containing 0.05% sodium azide.

Antibody production. Polyclonal antibodies were elicited in rabbits by subcutaneous injection of the purified CBD (0.2 mg) in 2 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Booster injections were performed after 7 and 14 days, using a similar sample of antigen emulsified in incomplete adjuvant. The animals were bled on day 21, and the antisera were cleared by centrifugation (4,300 × *g*) and stored at −20°C.

Biotinylation of proteins. The purified CBD was biotinylated by two different procedures. To modify the single cysteine residue of the CBD, the procedure of Bayer et al. (11) was used, whereby maleimidopropionyl biocytin was introduced into a solution of 5 mg of CBD at a 25-fold molar excess. For multiple biotinylation of lysine groups, biotin *N*-hydroxysuccinimide ester was employed as described by Bayer and Wilchek (10), using a 30-fold molar excess of reagent to protein. Protein A was similarly modified with biotin *N*-hydroxysuccinimide ester, using a 20-fold molar excess. The extent of biotinylation was qualitatively determined on dot blots by using an avidin-complexed enzyme assay system (2).

Determination of cellulose-binding capacity and dissociation constants. Samples of purified CBD (between 1.5 and 10.5 μg) were added to microcentrifuge tubes containing 0.5 mg of microcrystalline cellulose in Tris-HCl buffer. The final volume of the assay mixture was 100 μl. The assay tubes were mixed by vertical rotation at 24°C for 1 h. The samples were then centrifuged at 12,000 × *g* in a microcentrifuge for 5 min to sediment the cellulose and adsorbed CBD. The amount of protein which remained in the supernatant fluids (free CBD) was determined colorimetrically. The amount of adsorbed CBD was calculated by subtracting the amount of free CBD from the total added to the assay tube. Data were analyzed by double-reciprocal plots of $(\text{bound CBD})^{-1}$ versus $(\text{free CBD})^{-1}$ as described previously (20). Experiments were performed in duplicate. The capacity of CBD to bind to crystalline or amorphous cellulose was determined as described above but with a constant amount of CBD (40 μg per tube) and different amounts of cellulose (0.006 to 10 mg per tube).

Cellulose binding in the presence of cellodextrins. Samples (5 μg) of the purified CBD were introduced into microcentrifuge tubes containing 0.5 mg of microcrystalline cellulose and 1.6% (wt/vol) of one of the series of cellodextrins (cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, and cellooctaose), and the volume was brought to 50 μl with 50 mM Tris buffer. In control samples cellodextrins were omitted. The samples were mixed for 1 h by vertical rotation as described above and centrifuged, and the presence of CBD in the supernatant fluids was examined by SDS-PAGE.

Estimation of capacity for binding to xylan and chitin. Samples of biotinylated CBD (6 μg) were added to microcentrifuge tubes containing 0.5 mg of xylan or chitin. The mixture was brought to a final volume of 20 μl with Tris buffer (50 mM final concentration). The assay tubes were mixed and centrifuged as described above, and the supernatant fluids were examined for biotinylated CBD by a dot blot assay (30).

Purification of immunoglobulin G from serum by using CBD on a cellulosic matrix. A sample of *S*-biotinylated CBD (5 mg) was brought to 5 ml with Tris buffer, and 100 mg of microcrystalline cellulose was added. The suspension was stirred for 1 h at 23°C and centrifuged, and the pellet was washed once with 50 ml of phosphate buffer. The pellet was resuspended in 5 ml of the same buffer, and 4 mg of NeutraLite avidin in 5 ml of buffer was added. The suspension was stirred as described above for 1 h, washed, and resuspended again, and 1 mg of protein A was added. The resin was stirred, and the slurry was introduced into a column. The column was washed successively with 10-ml volumes of phosphate buffer, 100 mM Tris-HCl buffer (pH 8), 10 mM Tris buffer (pH 8), and 100 mM glycine-HCl buffer (pH 3). The column was reequilibrated with 100 mM Tris buffer, and 2 ml of serum which contained the antitransferrin antibodies was applied. The column was washed as described above, and adsorbed material was eluted with the glycine-HCl buffer. Fractions of 0.8 ml were collected. The protein in each fraction was determined, and the contents of the fractions were analyzed by SDS-PAGE.

RESULTS

Cloning and expression of the CBD. The CBD region of the *cipB* gene product was deduced on the basis of sequence homology and a functional cellulose-binding assay (36). The domain consists of 167 amino acids. To clone and express the CBD of the *cipB* product, we designed two PCR primers that flank the region (Fig. 1). Following the amplification and cloning of the region into the T7 polymerase expression vector pET3d, the resulting plasmid, pCBD, was transformed into *E. coli* strains capable of producing T7 polymerase upon induction with IPTG. SDS-PAGE of cell extracts from induced and uninduced cultures indicated that an 18,000-Da protein was produced (data not shown).

Purification of the CBD by affinity digestion. Preliminary experiments were carried out for conventional affinity chromatography of the expressed CBD on both microcrystalline and amorphous cellulose. Indeed, it was found that the adsorption of the expressed protein was very efficient, but desorption procedures led to the loss of more than half of the adsorbed material. More importantly, the final product contained low, but persistent, levels of contaminating polypeptide bands. FPLC was employed in order to eliminate these contaminants.

Because of the inefficiency of conventional affinity chromatography, we used a modification of a relatively new procedure (termed affinity digestion). This approach has been previously proven to be effective for the isolation of intact cellulosomes (31). CBD-containing samples were adsorbed to a cellulosic matrix, which was then enzymatically degraded by the cellulosome. The resultant mixture of low-molecular-weight CBD and high-molecular-weight cellulosome in the liquid phase was facilely separated by gel filtration. In this experiment, amorphous cellulose was used because its capacity for binding the CBD is much higher than that of microcrystalline cellulose and because it can be degraded by the cellulosome more readily than microcrystalline cellulose.

Extracts of the transformed *E. coli* cells were thus allowed to interact with the high-adsorptive-capacity phosphoric acid-swollen cellulose. The cellulose resin was washed sequentially with a 1 M solution of buffered NaCl followed by 3 M urea and then hydrolyzed enzymatically upon addition of the intact cellulosome. Near-complete solubilization of the cellulosic matrix was observed visually, and the product, together with the enzyme complex, was released into the aqueous phase. Residual cellulosome was separated from the CBD by gel filtration on a Sephacryl S-300 column. Two well-separated peaks resulted (Fig. 2), with the second peak being an essentially homogeneous preparation of the low-molecular-weight CBD. The final yield of the CBD preparation was calculated to be 2.2 g/liter of cell culture.

Affinity of the CBD for cellulose. The capacity of CBD to bind to microcrystalline and amorphous cellulose is shown in

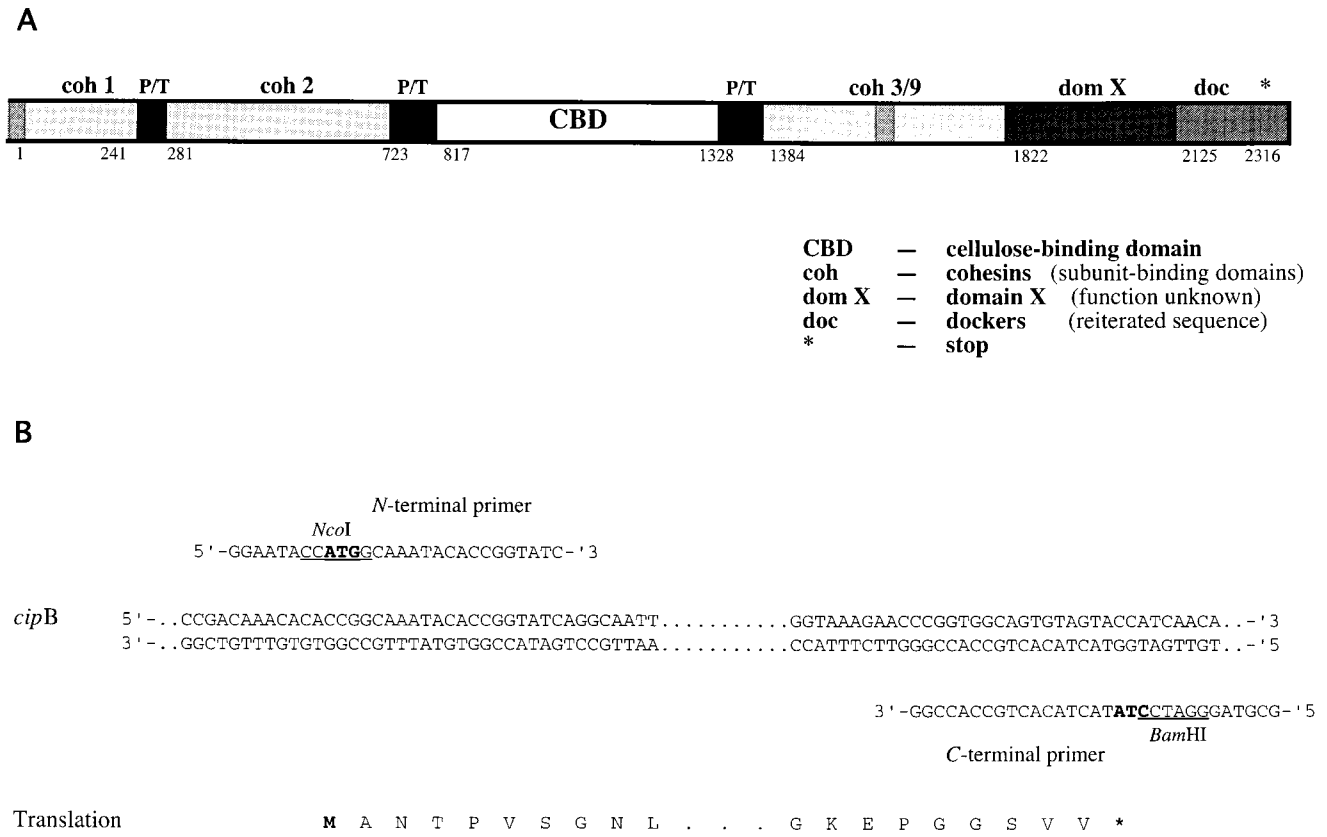

FIG. 1. Subcloning and expression of the CBD gene fragment. (A) Schematic description of the organization of the *cipB* gene from *C. thermocellum*, which harbors the CBD fragment. (B) Primers used for PCR.

Fig. 3. The binding capacity with amorphous cellulose was about 20-fold higher than that with microcrystalline cellulose. The affinity constant of the purified CBD for microcrystalline cellulose was determined by graphing a double-reciprocal plot of bound versus free CBD (Fig. 4). A linear plot resulted, indicating a single type of specific interaction with a dissociation constant of 0.4 μM and a maximum binding capacity of about 10 mg of CBD per g of microcrystalline cellulose (0.54 μmol of CBD per g of microcrystalline cellulose). These values are comparable to those reported for the CBD from *C. cellulovorans* (20). Cellobiose and soluble cello-oligodextrins failed to inhibit the binding. Interestingly, it was noted that the high affinity of the CBD for cellulose apparently led to a competitive inhibition of the hydrolysis of the substrate by the intact cellulosome (Fig. 5). The recombinant CBD also bound similarly to chitin, but it failed to bind to xylan (results not shown).

Modification of the single cysteine residue. The sequence of the CBD contains a single cysteine residue (position 62). To determine whether this residue was essential for binding activity, we modified the purified CBD by using two different sulfhydryl-specific reagents, iodoacetate and maleimidopropionyl biocytin. In both cases, the binding activity was not markedly affected (Fig. 4). Thus, biotinylation of the single cysteine of the CBD provided us a convenient tool to selectively mediate the attachment of other molecules to cellulose (see below).

Polyclonal antibodies against the recombinant CBD. The purified CBD from *C. thermocellum* was used to elicit antibodies in rabbits. Western blots (immunoblots) demonstrated selective labeling of the CBD from this organism (Fig. 6). As could be expected, the antiserum also labeled the S1 (scaffoldin) subunit, i.e., the cellulosomal subunit known to harbor the CBD in its native state. In addition, several smaller bands were weakly labeled, which apparently corresponded to degradation products of the scaffoldin subunit (32). The closely related CBD from *C. cellulovorans* failed to interact with this polyclonal anti-CBD antibody preparation.

Affinity isolation of antibodies on CBD-cellulose. The suitability of the recombinant CBD as a tool for attaching ligands to a solid phase was examined. For this purpose, the purified CBD was first biotinylated via its lone cysteine residue by using a maleimido derivative of biotin. Since this modified CBD bound tightly to cellulose, the biotinylated derivative could serve to mediate the attachment of avidin to cellulose. In turn, the remaining free biotin-binding sites of avidin could be used to bind biotinylated protein A. Thus, protein A, immobilized in this manner to cellulose, was examined as a means to isolate antibodies directly from whole serum.

In this specific example, antitransferrin immunoglobulins were isolated by the direct application of rabbit serum to the cellulosic affinity column (Fig. 7). The resultant antibodies (lane 3) appeared to be purer than those isolated on a conventional Sepharose-protein A affinity column (lane 4). The only visible contamination of the preparation appeared to be trace levels of CBD, which apparently leaked from the column. Avidin and protein A were not observed in the gel. The high-molecular-weight serum components which accompanied the isolation of antibody by the standard protein A affinity column were absent in the sample prepared by affinity chromatography on the cellulosic matrix.

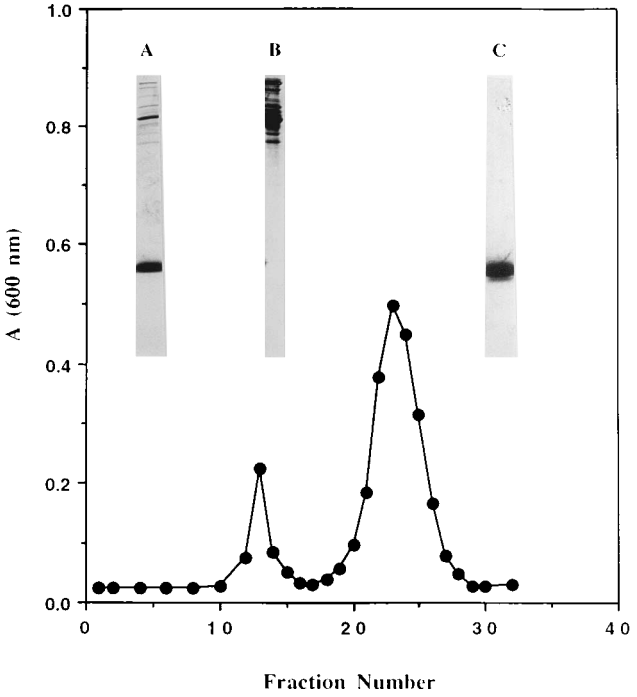

FIG. 2. Purification of the CBD by affinity digestion. The host cell bacterial sonicate was adsorbed to amorphous (phosphoric acid-treated) cellulose and washed with 3 M urea. Purified cellulosome was added in order to hydrolyze the cellulose resin with consequent release of the bound CBD. The residual cellulosome and CBD were then separated on a Sephacryl S-300 column. Lanes: A, SDS-PAGE of material applied to the gel filtration column; B, SDS-PAGE of the high-molecular-weight peak, showing the expected profile of the purified cellulosome components; C, SDS-PAGE of the low-molecular-weight peak, showing the purified CBD band.

DISCUSSION

In nature, the polypeptide chains which constitute the microbial cellulases are divided into a series of functionally defined domains (19). Interestingly, the quality of binding to the cellulose substrate is essentially separated from the quality of hydrolysis, and many cellulases thus exhibit a separate CBD and a separate catalytic domain. In some bacterial species, notably species from the genus *Clostridium*, the principal parts of the cellulolytic system are organized into a multicomponent cellulosome (16, 21, 24, 25, 40), and a central polypeptide component called scaffoldin is responsible for integrating the catalytic subunits into the cohesive enzyme complex (38, 44). The scaffoldin subunit also contains a CBD which mediates the attachment of the intact cellulosome (as well as the bacterial cell itself) to the substrate. The fact that this type of CBD is borne by a noncatalytic subunit distinguishes this system from the class of CBDs which are carried by conventional (uncomplexed) cellulolytic polypeptides.

In earlier work (36), we cloned a fragment of the gene for the cellulosomal scaffoldin from *C. thermocellum* into a suitable *E. coli* host. This fragment (termed *cipB*) exhibits a mul-

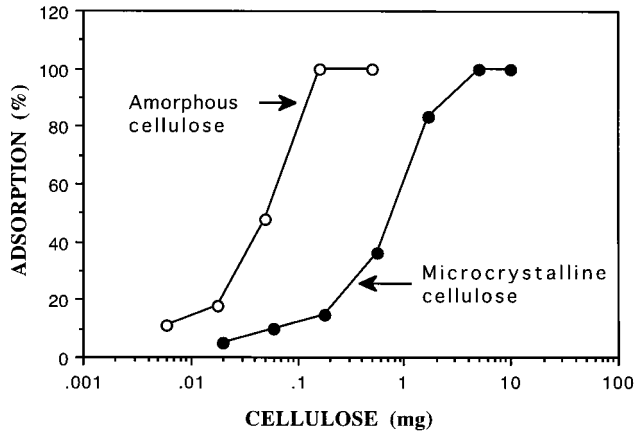

FIG. 3. Adsorption of CBD to cellulosic substrates. The capacity of CBD to bind to crystalline or amorphous cellulose was determined as described in Materials and Methods, with a constant amount of CBD (40 μg per tube) and different amounts of cellulose (0.006 to 10 mg per tube). Note the elevated level of CBD adsorption to low quantities of amorphous cellulose compared with that observed for microcrystalline cellulose.

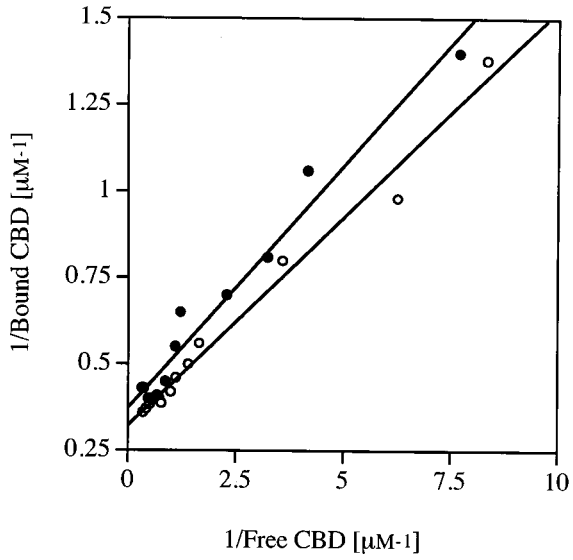

FIG. 4. Double-reciprocal plot of CBD adsorption to microcrystalline cellulose. Various amounts of CBD were added to 0.5-mg samples of cellulose. The suspension was agitated with an orbital shaker for 1 h at room temperature, and the applied and free fractions were subjected to protein analysis. The purified CBD (○) or the *S*-biotinylated derivative (●) was allowed to interact with the cellulose substrate. Following 1 h of incubation, the samples were centrifuged and total protein in the supernatant fluids was determined.

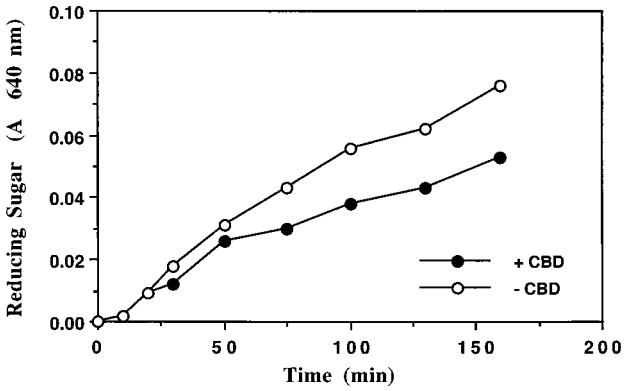

FIG. 5. Competitive effect of purified CBD on cellulosome-mediated hydrolysis of the cellulose matrix. CBD was adsorbed to amorphous cellulose, and then purified cellulosome was added. The normal degradation of cellulose by the intact cellulosome (in the absence of added CBD) is also shown.

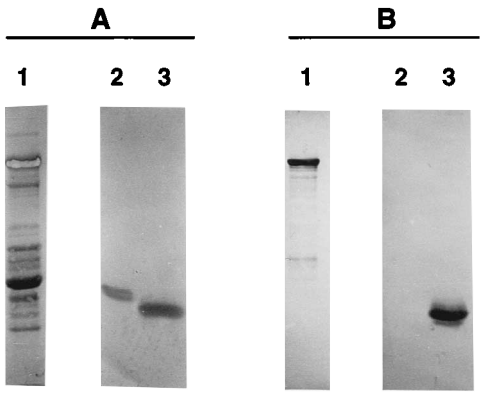

FIG. 6. Western blot analysis of the CBDs from *C. thermocellum* and *C. cellulovorans* with anti-CBD (*C. thermocellum*) antibodies. Samples were separated by SDS-PAGE and transferred electrophoretically onto nitrocelluose sheets. (A) Ponceau Red staining. (B) Labeling with anti-CBD antibodies, followed by second antibody-peroxidase conjugates. Lanes 1, samples of the purified cellulosome from *C. thermocellum*. Note the high level of immunostaining of the S1 (scaffoldin) subunit. Lanes 2, samples of the recombinant CBD from *C. cellulovorans*, separated on 6% gels. Lanes 3, samples of the recombinant CBD from *C. thermocellum*, separated on 15% gels. Note the complete lack of immunostaining of the CBD from *C. cellulovorans* by the anti-CBD antibodies from *C. thermocellum.*

tiplicity of functional domains (Fig. 1A), one of which is a CBD. In the previous work, a portion of *cipB*, which included both the putative CBD and a linker sequence, was subcloned and expressed at low product levels. The resultant protein was shown to bind strongly to cellulose, thus corroborating its function. In the current work, the CBD (lacking the linker sequence) was overexpressed.

Thus far, only one other CBD from a noncatalytic scaffoldin subunit (from *C. cellulovorans*) has been cloned and expressed (20). Its sequence is very similar to that of the CBD from *C. thermocellum* (Fig. 8), showing about 50% identity. Unlike the CBD from *C. cellulovorans*, which had to be solubilized by guanidine hydrochloride (20), the expressed CBD from *C. thermocellum* was entirely soluble upon sonication of the host cells. Moreover, the yields of CBD in this work were about 30-fold greater than that reported for the CBD from *C. cellulovorans.*

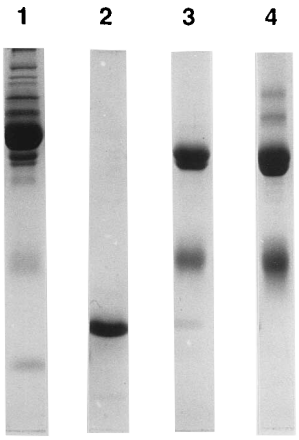

FIG. 7. Direct isolation of antibodies from serum by sequential application of *S*-biotinyl CBD, avidin, and biotinyl protein A on a cellulose support. Lanes: 1, whole serum; 2, purified CBD, 3; immunoglobulin G purified as described above; 4, immunoglobulin G purified by conventional affinity chromatography (on a CNBr-activated Sepharose-protein A column).

Despite the striking similarity in their primary structures, polyclonal antibodies raised against the CBD from *C. thermocellum* failed to interact with the protein from *C. cellulovorans*. This result indicates that the exposed epitopes are different in the two proteins, although a similar overall three-dimensional fold would have been expected. However, this result may not be entirely surprising. In this context, avidin and streptavidin are two structurally related proteins with a high degree of identity in their primary sequences and an identical folding (28), but polyclonal antibodies raised against one of the proteins fail to recognize the other.

In many respects, the CBDs from the two clostridial species appear to be quite similar. The respective affinities for the substrate and binding capacities are similar. The substrate specificities are also similar: both CBDs adsorbed to cellulosic substrates and to chitin but failed to adsorb to xylan, and cellobiose and other simple oligosaccharides do not inhibit their binding to cellulose.

It is clear that the adsorption of the CBD to cellulose is dependent on a structured arrangement of the cellulosic substrate. However, the elevated adsorption of both the recombinant CBD and the intact cellulosome with amorphous (phosphoric acid-treated) cellulose compared with that with microcrystalline cellulose would favor the amorphous rather than crystalline regions of the substrate as initial sites of binding.

One of the goals of a protein chemist is to be able to selectively modify a protein with a distinctive probe in such a way that the biological activity is maintained. For this reason, it was especially gratifying that the biotinylation of the lone cysteine residue resulted in a fully active CBD. Subsequent interaction with avidin provides a novel mode for binding other biologically active molecules to cellulosic resins in a variation of the universal avidin column (8, 9, 45). This approach also obviates the necessity to prepare fusion proteins for such purposes (27, 35, 37). In this context, we demonstrated the isolation of antibodies by using biotinylated protein A attached to such a cellulose column. The performance of this affinity column was at least as efficient as that of conventional protein A affinity chromatography.

The purification strategy used in this work exploited the selective adsorption of the CBD to a cellulosic matrix, which was subsequently solubilized by the enzymatic action of the intact cellulosome. This approach, called affinity digestion (31), was previously developed to isolate the cellulosome itself—the cellulosome simply consumed its substrate in toto and remained free in solution, in a purified state. The current work is the first in which the approach was used to purify an extraneous molecule (i.e., the recombinant CBD). Affinity digestion will eventually be even more useful for isolating hybrid constructs which contain a CBD fused to biologically active protein components (e.g., heterologous enzymes or immunochemically active molecules, etc.).

There are several advantages inherent in using affinity digestion for the isolation of such molecules. First, the use of amorphous cellulose instead of microcrystalline cellulose provides an adsorbent of much higher capacity (23), which is easier to degrade enzymatically. The "elution" of the purified material from the resin is thus more efficient. Moreover, affinity digestion obviates the need for harsh elution conditions (e.g., acid or urea, etc.), which sometimes limit conventional affinity chromatographic techniques.

The cellulosome is an intricate macromolecular complex which contains several subunits, each of which is composed of

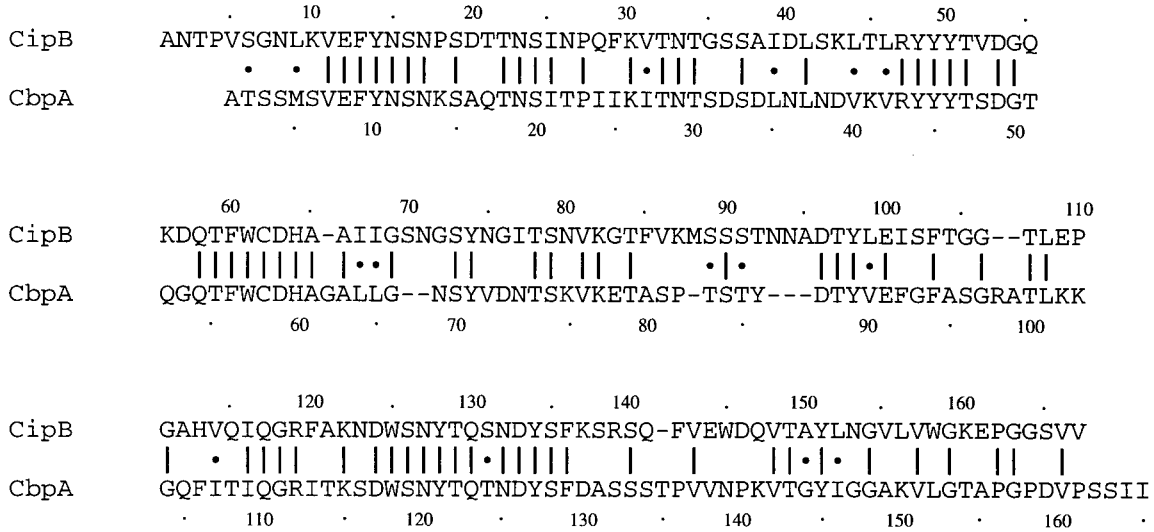

FIG. 8. Similarity between the aligned CBDs of *C. thermocellum* (CipB) and *C. cellulovorans* (CbpA). Identical residues are indicated by vertical lines. Similar residues (A and G, S and T, and V, L, I, and M) are denoted by dots.

multiple domains. The CBD described here is but one of the components of but one of its subunits. Other types of CBD are presumably contained in one or more of the catalytic subunits as well (15, 33, 34). Moreover, the CBD of the scaffoldin subunit is only one of its many domains; most of the others are involved in the integration of the cellulase components into the complex (5, 13, 42, 43). In order to understand such an elaborate biological system, it is imperative to analyze the structures and roles of the individual domains, both in an isolated state and in combination with adjacent domains. The present work is an initial step in this direction.

ACKNOWLEDGMENTS

We thank Oded Shoseyov for providing samples of the purified CBD from *C. cellulovorans* and Orit Gat for helpful comments on the manuscript.

Technical support was provided by the Technion Otto Meyerhof Biotechnology Laboratories established by the Minerva Foundation.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Short protocols in molecular biology, 2nd ed. John Wiley & Sons, New York.
2. **Bayer, E. A., H. Ben-Hur, and M. Wilchek.** 1990. Analysis of proteins and glycoproteins on blots. Methods Enzymol. **184:**415–427.
3. **Bayer, E. A., R. Kenig, and R. Lamed.** 1983. Studies on the adherence of *Clostridium thermocellum* to cellulose. J. Bacteriol. **156:**818–827.
4. **Bayer, E. A., and R. Lamed.** 1986. Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. J. Bacteriol. **167:**828–836.
5. **Bayer, E. A., and R. Lamed.** 1992. The cellulose paradox: pollutant *par excellence* and/or a reclaimable natural resource? Biodegradation **3:**171–188.
6. **Bayer, E. A., E. Morag, and R. Lamed.** 1995. The cellulosome—a treasure-trove for biotechnology. Trends Biotechnol. **12:**379–386.
7. **Bayer, E. A., E. Setter, and R. Lamed.** 1985. Organization and distribution of the cellulosome in *Clostridium thermocellum*. J. Bacteriol. **163:**552–559.
8. **Bayer, E. A., and M. Wilchek.** 1990. The application of avidin-biotin technology for affinity-based separations. J. Chromatogr. **510:**3–11.
9. **Bayer, E. A., and M. Wilchek.** 1990. Avidin column as a highly efficient and stable alternative for immobilization of ligands for affinity chromatography. J. Mol. Recog. **3:**102–107.
10. **Bayer, E. A., and M. Wilchek.** 1990. Protein biotinylation. Methods Enzymol. **184:**138–160.
11. **Bayer, E. A., M. G. Zalis, and M. Wilchek.** 1985. 3-(*N*-Maleimido-propionyl) biocytin: a versatile thiol-specific biotinylating reagent. Anal. Biochem. **149:** 529–536.
12. **Béguin, P.** 1990. Molecular biology of cellulose degradation. Annu. Rev. Microbiol. **44:**219–248.
13. **Béguin, P., and J.-P. Aubert.** 1994. The biological degradation of cellulose. FEMS Microbiol. Lett. **13:**25–58.
14. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
15. **Durrant, A. J., J. Hall, G. P. Hazlewood, and H. J. Gilbert.** 1991. The non-catalytic *C*-terminal region of endoglucanase E from *Clostridium thermocellum* contains a cellulose-binding domain. Biochem. J. **273:**289–293.
16. **Fierobe, H.-P., C. Bagnara-Tardif, C. Gaudin, F. Guerlesquin, P. Sauve, A. Belaich, and J.-P. Belaich.** 1993. Purification and characterization of endoglucanase C from *Clostridium cellulolyticum*—catalytic comparison with endoglucanase-A. Eur. J. Biochem. **217:**557–565.
17. **Fujino, T., P. Béguin, and J.-P. Aubert.** 1992. Cloning of a *Clostridium thermocellum* DNA fragment encoding polypeptides that bind the catalytic components of the cellulosome. FEMS Microbiol. Lett. **94:**165–170.
18. **Gerngross, U. T., M. P. M. Romaniec, T. Kobayashi, N. S. Huskisson, and A. L. Demain.** 1993. Sequencing of a *Clostridium thermocellum* gene (cipA) encoding the cellulosomal S_L-protein reveals an unusual degree of internal homology. Mol. Microbiol. **8:**325–334.
19. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. J. Miller, and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. **55:**303–315.
20. **Goldstein, M. A., M. Takagi, S. Hashida, O. Shoseyov, R. H. Doi, and I. H. Segel.** 1993. Characterization of the cellulose binding domain of the *Clostridium cellulovorans* cellulose-binding protein A. J. Bacteriol. **175:**5762–5768.
21. **Lamed, R., and E. A. Bayer.** 1988. The cellulosome concept: exocellular/extracellular enzyme reactor centers for efficient binding and cellulolysis, p. 101–116. *In* J.-P. Aubert, P. Beguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation. Academic Press, London.
22. **Lamed, R., and E. A. Bayer.** 1988. The cellulosome of *Clostridium thermocellum*. Adv. Appl. Microbiol. **33:**1–46.
23. **Lamed, R., R. Kenig, E. Setter, and E. A. Bayer.** 1985. The major characteristics of the cellulolytic system of *Clostridium thermocellum* coincide with those of the purified cellulosome. Enzyme Microb. Technol. **7:**37–41.
24. **Lamed, R., J. Naimark, E. Morgenstern, and E. A. Bayer.** 1987. Specialized cell surface structures in cellulolytic bacteria. J. Bacteriol. **169:**3792–3800.
25. **Lamed, R., E. Setter, and E. A. Bayer.** 1983. Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. J. Bacteriol. **156:**828–836.
26. **Lamed, R., E. Setter, R. Kenig, and E. A. Bayer.** 1983. The cellulosome—a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol. Bioeng. Symp. **13:**163–181.
27. **Le, K. D., N. R. Gilkes, D. G. Kilburn, R. C. J. Miller, J. N. Saddler, and R. A. J. Warren.** 1994. A streptavidin-cellulose-binding domain fusion protein that binds biotinylated proteins to cellulose. Enzyme Microb. Technol. **16:**496–500.
28. **Livnah, O., E. A. Bayer, M. Wilchek, and J. L. Sussman.** 1993. Three-dimensional structures of avidin and the avidin-biotin complex. Proc. Natl. Acad. Sci. USA **90:**5076–5080.

29. **Mayer, F., M. P. Coughlan, Y. Mori, and L. G. Ljungdahl.** 1987. Macromolecular organization of the cellulolytic enzyme complex of *Clostridium thermocellum* as revealed by electron microscopy. Appl. Environ. Microbiol. **53:**2785–2792.
30. **Morag, E., E. A. Bayer, and R. Lamed.** 1990. Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum* to cellulose-degrading enzymes. J. Bacteriol. **172:**6098–6105.
31. **Morag, E., E. A. Bayer, and R. Lamed.** 1992. Affinity digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*. Enzyme Microb. Technol. **14:**289–292.
32. **Morag, E., E. A. Bayer, and R. Lamed.** 1992. Unorthodox intra-subunit interactions in the cellulosome of *Clostridium thermocellum*. Appl. Biochem. Biotechnol. **33:**205–217.
33. **Morag, E., I. Halevy, E. A. Bayer, and R. Lamed.** 1991. Isolation and properties of a major cellobiohydrolase from the cellulosome of *Clostridium thermocellum*. J. Bacteriol. **173:**4155–4162.
34. **Navarro, A., M.-C. Chebrou, P. Béguin, and J.-P. Aubert.** 1991. Nucleotide sequence of the cellulase gene *cel*F of *Clostridium thermocellum*. Res. Microbiol. **142:**927–936.
35. **Ong, E., J. M. Greenwood, N. R. Gilkes, D. G. Kilburn, R. C. J. Miller, and R. A. J. Warren.** 1989. The cellulose-binding domains of cellulases: tools for biotechnology. Trends Biotechnol. **7:**239–243.
36. **Poole, D. M., E. Morag, R. Lamed, E. A. Bayer, G. P. Hazlewood, and H. J. Gilbert.** 1992. Identification of the cellulose binding domain of the cellulosome subunit S1 from *Clostridium thermocellum*. FEMS Microbiol. Lett. **99:**181–186.
37. **Ramírez, C., J. Fung, R. C. J. Miller, R. A. J. Warren, and D. G. Kilburn.** 1993. A bifunctional affinity linker to couple antibodies to cellulose. Bio/Technology **11:**1570–1573.
38. **Salamitou, S., K. Tokatlidis, P. Béguin, and J.-P. Aubert.** 1992. Involvement of separate domains of the cellulosomal protein S1 of *Clostridium thermocellum* in binding to cellulose and in anchoring of catalytic subunits to the cellulosome. FEBS Lett. **304:**89–92.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Shoseyov, O., M. Takagi, M. A. Goldstein, and R. H. Doi.** 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. Proc. Natl. Acad. Sci. USA **89:**3483–3287.
41. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
42. **Takagi, M., S. Hashida, M. A. Goldstein, and R. H. Doi.** 1993. The hydrophobic repeated domain of the *Clostridium cellulovorans* cellulose-binding protein (CbpA) has specific interactions with endoglucanases. J. Bacteriol. **175:**7119–7122.
43. **Tokatlidis, K., P. Dhurjati, and P. Béguin.** 1993. Properties conferred on *Clostridium thermocellum* endoglucanase CelC by grafting the duplicated segment of endoglucanase CelD. Protein Eng. **6:**947–952.
44. **Tokatlidis, K., S. Salamitou, P. Béguin, P. Dhurjati, and J.-P. Aubert.** 1991. Interaction of the duplicated segment carried by *Clostridium thermocellum* cellulases with cellulosome components. FEBS Lett. **291:**185–188.
45. **Wilchek, M., and E. A. Bayer (ed.).** 1990. Avidin-biotin technology. Methods Enzymol. **184:**1–746.
46. **Wilchek, M., and E. A. Bayer.** 1990. Biotin-containing reagents. Methods Enzymol. **184:**123–138.
47. **Wilchek, M., T. Miron, and J. Kohn.** 1984. Affinity chromatography. Methods Enzymol. **104:**3–55.
48. **Wu, J. H. D., W. H. Orme-Johnson, and A. L. Demain.** 1988. Two components of an extracellular protein aggregate of *Clostridium thermocellum* together degrade crystaline cellulose. Biochemistry **27:**1703–1709.