Characterization of a Bifunctional Cellulase and Its Structural Gene

THE cel GENE OF BACILLUS SP. D04 HAS EXO- AND ENDOGLUCANASE ACTIVITY*

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Bacillus sp. D04 secreted a bifunctional cellulase that had a molecular weight of 35,000. This cellulase degraded Cm-cellulose, cellotetraose, cellopentaose, and avicel PH101. Based on the high performance liquid chromatography analysis of the degradation products, this cellulase randomly cleaved internal \( \beta \)-1,4-glycosidic bonds in cellotetraose and cellopentaose as an endoglucanase. It also hydrolyzed the aglycosidic bond in p-nitrophenyl-\( \beta \)-o-cellobioside and cleaved avicel to cellobiose as an exoglucanase. Cellulobiose competitively inhibited the p-nitrophenyl-\( \beta \)-o-cellobioside degrading activity but not Cm-cellulose degrading activity. Ten mM p-chloromercuribenzoate inhibited p-nitrophenyl-\( \beta \)-o-cellobioside degrading activity completely, but Cm-cellulose degrading activity incompletely. Cm-cellulose increased p-nitrophenyl-\( \beta \)-o-cellobioside degrading activity, and vice versa, whereas methylumbelliferyl-\( \beta \)-o-cellobiose strongly inhibited p-nitrophenyl-\( \beta \)-o-celllobioside degrading activity. The cellulase gene (cel gene), 1461 base pairs, of Bacillus sp. D04 was cloned. The nucleotide sequence of the cel gene was homologous to those of Bacillus subtilis DLG and B. subtilis BSE616. The cel gene was overexpressed in Escherichia coli, and its product was purified. The substrate specificity and substrate competition pattern of the purified recombinant cellulase were the same as those of the purified cellulase from Bacillus sp. D04. These results suggest that a single polypeptide cellulase had both endo- and exoglucanase activities and each activity exists in a separate site.

Cellulose is an unbranched glucose polymer composed of an anhydro-\( \beta \)-1,4-glucose units linked by a \( \beta \)-1,4-\( \alpha \)-glycosidic bond. Cellulolytic enzymes degrade cellulose by cleaving this glycosidic bond. Cellulases can be classified into three types: endoglucanases (1,4-\( \beta \)-D-glucan 4-glucohydrolase, EC 3.2.1.4), exoglucanases (\( \beta \)-1,4-\( \beta \)-D-glucan cellobiohydrolase), and \( \beta \)-glucosidases (\( \beta \)-o-glucosidase glucohydrolase, EC 3.2.1.21). Endoglucanases randomly hydrolyze internal \( \beta \)-1,4-\( \alpha \)-glycosidic bonds in cellulose. As a result, the polymer rapidly decreases in length, but the concentration of the reducing sugar increases slowly (1). Exoglucanases hydrolyze cellobiose by removing the cellobiose unit from the nonreducing end of cellulose; the reducing sugars are rapidly increased, but the polymer length changes little (1–3). \( \beta \)-Glucosidases cleave cellobiose and oligosaccharides to glucose (1). Therefore, crystalline cellulose is efficiently hydrolyzed by the synergistic action of all three types of cellulases.

Cellulosic substrates hydrolyzed by only one type of cellulase are categorized as follows. Acid-swollen cellulose, Cm-cellulose, cellulase azure, and trinitrophenyl Cm-cellulose are hydrolyzed by endoglucanases (1). MUC\(^1\) (4) and pNPC (5) are used as substrates for the determination of exoglucanase activity, and MUG (4) and pNPG (5) are cleaved by \( \beta \)-glucosidases. Filter paper and avicel are efficiently hydrolyzed by the synergistic action of endo- and exoglucanases, but not by either one alone (6).

Some organisms (for example, Trichoderma sp.) (6–9, 11) produce all three types of cellulases and efficiently degrade cellulose by their synergistic effect. A cellulolytic hydrolase with a considerable level of endo-, exoglucanase, and xylanase activity has been described (3, 12–14). For example, Saul reported a cellulase gene (cel B) of Caldoccum saccharolyticum with a Cm-cellulose-degrading domain in the C-terminal region and an MUC degrading domain in the N-terminal region (14). A polysaccharide hydrolase of the rumen fungus Neocallimastix patriciarum has a multifunctional catalytic domain with high endoglucanase, cellulobiohydrolase, and xylanase activities (12, 13).

Extensive recent studies on proteins (such as cellulase, protease, and amylase) secreted by Bacillus species (15) have shown that the following Bacillus species produce cellulases: Bacillus cereus (16), Bacillus licheniformis (17), Bacillus subtilis (18), and Bacillus polymyxa (19). Because these strains did not produce all three types of cellulase, they did not extensively hydrolyze crystalline cellulose. We have investigated another strain of this species, Bacillus sp. D04, having the ability to degrade crystalline cellulose. We have determined that the cellulase of Bacillus sp. D04 differed from that of other Bacillus species in several respects. In particular it has both endo- and exoglucanase activity. It degraded Cm-cellulose, cellotetraose, and cellopentaose as an endoglucanase and cleaved aglycosidic bonds in pNPC as an exoglucanase. It also cleaved avicel to cellobiose. Substrate competition assays showed that the cellulase of Bacillus sp. D04 had separate sites for endo- and exoglucanase activity.

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\( ^{1} \) The abbreviations used are: MUC, methylumbelliferyl-\( \beta \)-o-celllobiose; MUG, methylumbelliferyl-\( \beta \)-o-glycopyranoside; pNPC, p-nitrophenyl-\( \beta \)-o-celllobioside; pNPG, p-nitrophenyl-\( \beta \)-o-glycopyranoside; pCMB, p-chloromercuribenzoate; PCR, polymerase chain reaction; IPTG, isopropylthio-\( \beta \)-D-galactoside; FAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Purification of Cellulase

Bacillus sp. D04 was cultured in 2 liters of medium (containing M9 minimal salts, 0.5% glucose and 0.5% avicel) at 45 °C for 13 h. After the medium was centrifuged at 11,000 × g for 10 min, the supernatant was concentrated by ultrafiltration (10,000 nominal molecular weight cut-off membrane was used). Ten mM potassium phosphate buffer (pH 5.8) at a flow rate of 10 ml/h. The potassium phosphate buffer (pH 5.8) and eluted with a 30-ml 10–250 mM potassium phosphate salt gradient at a flow rate of 10 ml/h. The concentration of protein was measured with Bradford solution (Bio-Rad).

Cellulase Enzyme Assay

Cm-cellulase and Avicel Degrading Activity Assay—The Cm-cellulase assay consisted of 800 µl of 1% Cm-cellulose in 10 mM potassium phosphate buffer (pH 5.8) and 200 µl of diluted enzyme solution, incubated at 45 °C for 20 min. Avicel degrading activity was measured as follows: 500 µl of 10 mg/ml avicel in 10 mM potassium phosphate buffer (pH 5.8) was mixed with 500 µl of suitably diluted enzyme and then incubated for 72 h at 45 °C in a shaking incubator. The remaining avicel was removed by centrifugation, and the amount of reducing sugar was detected with 3,5-dinitrosalicylic reagent. One unit of Cm-cellulose and avicel degrading activity was defined as the amount of enzyme required for producing 1 µmol of glucose/min.

pNPC Degrading Activity Assay—The reaction mixture, consisting of 800 µl of pNPC at 1 mg/ml in 50 mM sodium acetate buffer (pH 5.8) and 200 µl of suitably diluted enzyme, was incubated at 45 °C for 20 min. The p-nitrophenol released from pNPC was detected at 420 nm after adding 1 ml of 0.5 M glycine/NaOH buffer (pH 10.4). Fluorescence measurements were made on a Toso FP-777 spectrophuorometer at 20 °C, with an excitation wavelength of 365 nm and detection at 450 nm. One unit of enzyme activity was defined as the amount of enzyme required for producing 1 µmol of 4-methylumbelliferone/min (9).

High Performance Liquid Chromatography Analysis of Degradation Products

The reaction mixture, consisting of 80 µl of oligosaccharides released from cellulotic substrates, 20 µl of 10% trichloroacetic acid, and 100 µl of 0.3% (w/v) ethanolic solution of dansyl hydrazine, was heated at 80 °C for 10 min, and then cooled (20–22). Samples were dried, dissolved in 78% acetonitrile solution, and analyzed with μ-Bondapack® NH₂ column (3.9 × 300 mm, Waters) (23). The dansyl hydrazone of oligosaccharides was detected at 254 nm. The 78% acetonitrile solution was used as an elution solvent, and the flow rate was 1.5 ml/min.

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TABLE I

| Purification steps             | Total Protein (mg) | Total volume (ml) | Total activity (units) | Specific activity (units/mg) | -Fold |
|-------------------------------|--------------------|-------------------|------------------------|-------------------------------|-------|
| 1. Cm-cellulose degrading activity |                    |                   |                        |                               |       |
| Ultrafiltration               | 35.40              | 100.0             | 122.10                 | 3.44                          | 2.1   |
| Cm-Sepharose                  | 2.44               | 120.0             | 18.00                  | 7.22                          | 37.2  |
| Hydroxylapatite              | 0.07               | 2.4               | 9.00                   | 128.00                        |       |
| 2. pNPC degrading activity    |                    |                   |                        |                               |       |
| Ultrafiltration               | 35.40              | 100.0             | 2.10                   | 0.06                          |       |
| Cm-Sepharose                  | 2.44               | 120.0             | 0.19                   | 0.08                          | 1.3   |
| Hydroxylapatite              | 0.07               | 2.4               | 0.16                   | 2.28                          | 36.0  |
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Cloning and Determination of Nucleotide Sequence of the Cellulase Gene

Chromosomal DNA from Bacillus sp. D04 was partially digested with Sau3A1 producing 3-5 kb DNA fragments. These were isolated by density gradient centrifugation in 10 to 40% sucrose using an SW 28 rotor at 20,000 rpm for 20 h at 20 °C. Fragments were ligated with the dephosphorylated BamHI site of pBluescript KS(+) and then transferred into an Escherichia coli DH5α strain. Transformants with Cm-resistance were screened on an L-agar plates containing ampicillin at 100 µg/ml. To screen MUC-degrading activity, transformants were transferred into L-agar plates containing ampicillin at 100 µg/ml and MUC at 50 µg/ml. For the determination of the nucleotide sequence of the cel gene, serial deletion of the gene was done by using Erase-a-Base kit (Promega). The sequence of cellulase gene was determined from both strands by the dideoxynucleotide chain termination method using a Sequenase kit (U.S. Biochemical Corp.).

Table II
The substrate specificity of cellulase

| Substrate   | Specific activity | Cm-cellulase | pNPC | MUC | pNP | pUG | Avicel |
|-------------|------------------|--------------|------|-----|-----|-----|--------|
| mg/ml       | units/mg         | 10            | 1    | 1   | 1   | 1   | 1      |

º Concentration indicates weight concentration of each substrate.
º The unit of each substrate is described under “Experimental Procedures.”

PCR Amplification of the cel Gene and Construction of pCO1

The cel gene in pBluescript KS(+) was amplified by PCR with 5’-CATATGAAACGGTTAACATTCT-3’ (ATG in the NdeI site of CATATG) was a start codon of the cel gene) and M13 reverse primer. Amplification was done by 30 cycles of PCR at standard reaction conditions: reaction volume, 50 µl; reaction composition, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 50 µM dNTP, 2 fmol of template, 10 pmol primer, and 2 units of Vent DNA polymerase; cycle profile, 1 min at 95 °C, 1 min at 50 °C, 1.5 min at 72 °C. The PCR products were purified and digested with HindIII. These fragments were ligated with pBluescript KS(+) that had been digested with SmaI and HindIII. This recombinant DNA was named pCO1.

Construction of pCO₂ and Transfer into E. coli BL21(DE) pLysS

The pCO1 vector was digested with EcoRV and then partially digested with NdeI to generate the 1.5-kb cel gene. The overexpression vector, pET-3a-d (Novagen, Inc.), was digested with BamHI, and then end-filling was done with a Klenow fragment. This overexpression vector was digested with NdeI and then ligated with the 1.5-kb cel gene. This recombinant plasmid was named as pCO2. The pCO2 vector was transferred into E. coli BL21(DE) pLysS by electroporation (BTX electro cell manipulator). The pCO2 vector was transferred into E. coli BL21(DE) pLysS(F’ompT dcm gal(DE) pLysS, Cm’I) by electroporation (BTX electro cell manipulator). The pCO2 vector was transferred into E. coli BL21(DE) pLysS(F’ompT hadS cloB gal(DE) pLysS, Cm’I) by electroporation (BTX electro cell manipulator). The pCO2 vector was transferred into E. coli BL21(DE) pLysS(F’ompT hadS cloB gal(DE) pLysS, Cm’I) by electroporation (BTX electro cell manipulator).

Overexpression of the Cellulase Gene

E. coli BL21(DE)pLysS with pCO2 was cultured in Luria-Bertani medium containing ampicillin at 50 µg/ml and chloramphenicol at 30 µg/ml for 12 h and then transferred into TBGM9 medium (tryptone at 10 mg/ml, NaCl at 5 mg/ml, M9 salt, 0.4% (w/v) glucose, and 1 mM MgSO₄) containing ampicillin at 50 µg/ml. To obtain high levels of transcription, these cells were grown to mid-log phase, IPTG was added to a final concentration of 0.5 mM, and growth continued for 3 h at 30 °C.

Fig. 3. HPLC analysis of degradation products. Panel A, HPLC analysis of products released from cellotetraose. The reaction mixture containing 10 µl of 10 mM potassium phosphate (pH 5.8), 40 µl of the purified cellulase (0.1 mg/ml), and 30 µl of 10 mg/ml cellotetraose were incubated for 0(a), 60(b), and 120(c) min at 45 °C. Panel B, HPLC analysis of products released from cellulose. 40 µl of 10 mg/ml cellulose was used as a substrate instead of cellobiose. These samples were incubated for 0(a), 60(b), and 120(c) min at 45 °C. Panel C, HPLC analysis of products released from Avicel. 800 µl of a 1% (w/v) avicel solution and 800 µl of the purified cellulase (0.1 mg/ml) were mixed and incubated for 72 h at 45 °C. (a) was a control that did not contain cellulase, whereas (b) contained purified cellulase. These reaction products were modified with dansyl hydrazine as described under “Experimental Procedures.” The absorbance was measured at 254 nm. The numbers 1-4 represent the dansyl hydrazones of 1. cellobiose; 2. cellotriose; 3. cellotetraose; and 4. cellopentaose.
Table III

| Reagents | Concentration | Control cellulase activity<sup>a</sup> | CM-cellulose degrading activity | pNPC degrading activity |
|----------|---------------|----------------------------------------|---------------------------------|-------------------------|
| PMSE<sup>b</sup> | 1 | 100 | 100 |
| pCMB | 10 | 100 | 100 |
| Cellulose | 10 | 33.1 | 0 |
| Ca<sup>2+</sup> | 5 | 140.9 | 110.8 |
| Zn<sup>2+</sup> | 5 | 37.4 | 48.0 |
| Mg<sup>2+</sup> | 5 | 91.5 | 113.8 |

<sup>a</sup> The control cellulase activity was measured without reagents.

<sup>b</sup> Phenylmethylsulfonyl fluoride.

Purification of Overexpressed Cellulase

The overexpressed recombinant cellulase from E. coli BL21(DE) pLysS was partially purified by ammonium sulfate fractionation as previously described (24). This partially purified cellulase was dialyzed in 20 mM sodium acetate buffer (pH 4.8) and then loaded onto Cm-Sepharose CL-6B (15 × 50 mm) equilibrated with dialysis buffer. The proteins were eluted with a 80-ml 20–500 mM sodium acetate (pH 4.8) salt gradient at a flow rate of 20 ml/h.

Activity Staining of Cellulase

The protein sample was mixed with protein loading dye and then incubated at 68°C for 1 h. These samples were loaded onto a 10% polyacrylamide gel containing 0.1% Cm-cellulose, then subjected to electrophoresis. After SDS-PAGE, one of the gels was stained with Coomassie Blue R250. Another was soaked and gently shaken in 50 mM phosphate buffer (pH 6.8) containing 25% isopropanol for 30 min. It was transferred to 50 mM phosphate buffer (pH 6.8) and shaken for 30 min. The buffer was removed, and the gel was incubated for 20 min at 37°C. This gel was stained with 1% Congo Red solution for 5 min and destained with 1 M NaCl/NaOH solution.

RESULTS

Purification of Cellulase—Because Bacillus sp. D04 secreted cellulase into medium, concentrated medium was used as starting material for enzyme purification. Many other proteins were removed by passage through the Cm-Sepharose CL-6B (Fig. 1A). The sample that eluted at 180 ml potassium phosphate from hydroxylapatite had both Cm-cellulase and pNPC degrading activities (Fig. 2). SDS-PAGE of this sample revealed only a 35,000-Da single polypeptide (Fig. 1A). The molecular weight of the native form of this cellulase, determined by gel permeation chromatography (Superoxide 12, Pharmacia Biotech Inc.), was also about 35,000. Activity staining showed that this purified protein had Cm-cellulase degrading activity (Fig. 1B). The steps in purification of this protein are given in Table I.

Substrate Specificity of the Purified Cellulase—The activity of the purified cellulase was assayed with various cellulose substrates. This cellulase degraded Cm-cellulose, pNPC, MUC, and avicel PH101 (Table II). However, the specific activity toward avicel was much lower than that of the soluble substrates. Neither MUG nor pNPG was hydrolyzed (Table II).

HPLC Analysis of Oligosaccharides from Cellulolytic Substrates—HPLC analysis showed that a single peak was detected at 280 nm as a reaction product on hydrolysis of pNPC by this cellulase (data not shown). The retention time of it was the same as that of p-nitrophenol. Therefore we identified it as p-nitrophenol. This means that enzyme cleaved only aglycosidic bond in pNPC, producing cellobiose and p-nitrophenol. The enzyme cleaved cellulotic substrates (such as cellulotetraose and cellolentaose) to glucose, cellobiose, and other oligosaccharides. Since these compounds are not detected at any wave length, we modified them with dansyl hydrazine because sugar dansyl hydrazones could be detected at 254 nm. On the basis of HPLC analysis, the purified cellulase cleaved cellulotetraose to cellobiose and cellotriose (Fig. 3A). It also produced cellobiose, cellotriose, and cellolentaose from cellobiose (Fig. 3B). These results indicate that the purified cellulase randomly cleaved internal β-1,4-glycosidic bonds in these cellulolytic substrates as an endoglucanase. Based on the above result, it would seem that the smallest substrate recognized by the endoglucanase of Bacillus sp. D04 is a cellotetraose. Both endo- and exogluconase activities were detected by using cellulotetraose and cellolentaose as substrates. But pNPC is not a substrate for endoglucanase of Bacillus sp. D04 because it is shorter than cellotetraose. Therefore only exogluconase activity was detected by using pNPC as a substrate. The enzyme also produced cellobiose from avicel as an exoglucanase (Fig. 3C).

Differential Inhibition of Cellulase Activity with Various Inhibitors—The Cm-cellulase and pNPC degrading activities of
this cellulase were differentially inhibited by several inhibitors. pCMB at 10 mM completely inhibited the pNPC degrading activity but inhibited Cm-cellulose degrading activity by 67%. In 40 mM cellobiose, Cm-cellulose degrading activity was not inhibited, but the pNPC degrading activity was inhibited by 57.8% (Table III). Cellobiose changed the $K_m$ but not the $V_{	ext{max}}$ of pNPC degrading activity as a competitive inhibitor (Fig. 4A), whereas both the $V_{	ext{max}}$ and $K_m$ of pNPC degrading activity were changed by pCMB as a mixed-type inhibitor (Fig. 4B).

Both Cm-cellulose and pNPC degrading activity required Ca$^{2+}$, but were strongly inhibited by Zn$^{2+}$. Mg$^{2+}$ slightly increased pNPC degrading activity and weakly inhibited Cm-cellulose degrading activity (Table III).

Substrate Competition—To investigate whether a purified cellulase contains each endo- and exoglucanase active site, we performed substrate competition assays. At a high ratio of Cm-cellulose (0.5%, w/v) to pNPC (0.005%, w/v), pNPC degrading activity was not inhibited, but was increased by Cm-cellulose (Fig. 5A). Cm-cellulose degrading activity was not inhibited, but was slightly increased in the presence of various concentrations of pNPC (Fig. 5B). But 60% of the pNPC degrading activity was inhibited by MUC even at a low ratio of MUC (0.01%, w/v) to pNPC (0.005%, w/v) (Fig. 5C).

Cloning and Nucleotide Sequence of the cel Gene—L-agar plates containing Cm-cellulose and trypan blue were used to clone the gene for Cm-cellulose degrading activity from the genomic library in pBluescript KS(−), which was described under “Experimental Procedures.” Cm-cellulose was stained with trypan blue, but the hydrolyzed Cm-cellulose was not. As a result, a halo formed around the colony with the Cm-cellulose degrading activity. Eleven colonies having Cm-cellulose degrading activity were obtained (data not shown). To determine the MUC degrading activity of these colonies, they were transferred onto an L-agar plate containing MUC. The colony with exoglucanase activity cleaved MUC to cellobiose and methylumbelliferone which emitted fluorescence when it was exposed to UV light. All colonies with Cm-cellulose degrading activity emitted fluorescence under the UV light after incubating for 12 h at 37 °C on L-agar plates containing MUC (data not shown). Therefore, 11 colonies had a cellulase gene with both Cm-cellulose and MUC degrading activities. The nucleotide sequence of this gene (Fig. 6) showed one open reading frame of 1461 base pairs was a possible gene encoding the cellulase. A potential promoter ($−35$ (TAGACAAT) and $−10$ (TACAAT)) and the Shine-Dalgarno sequence (ribosomal binding site) were identified in the upstream region. Based on the nucleotide sequence homology with other cellulase genes, the cellulase gene of Bacillus sp. D04 has a high homology with those of B. subtilis DLG (26) and B. subtilis BSE616 (27) (Fig. 6).

Overexpression of Recombinant Cellulase Gene and Purification of Recombinant Cellulase—E. coli BL21(DE) pLysS with pCO2 overexpressed a 55,000-Da protein after IPTG was added (Fig. 7). Activity staining showed that 55,000 and 35,000-Da proteins had Cm-cellulose degrading activity (Fig. 8B). The 35,000-Da protein with Cm-cellulose degrading activity was purified by Cm-Sepharose CL-6B chromatography (Fig. 8A).

Characteristics of Recombinant Cellulase—The purified recombinant cellulase degraded Cm-cellulose, pNPC, and...
Fig. 6. Nucleotide sequence of the cel gene and homology between cellulase genes from Bacillus subtilis. The potential promoter region (−35 (TATA-GACA)), −10 (TACAAAG) region, the Shine-Dalgarno sequence (AAGGAGG) are underlined. The stop codon is marked as ***. The nucleotide sequence of the cel gene is shown as line 1. Line 2 indicates amino acid sequence deduced from the cel gene and the underlined amino acid sequence is a typical β-glucanase signal peptide of Bacillus species. Lines 3 and 4 indicate nucleotide sequences of the cellulase genes of Bacillus subtilis BSE616 and DLG, respectively.
Bifunctional Cellulase from Bacillus sp. D04

The overexpression of cellulase gene from E. coli BL21(DE) pLysS with pCO2. Lane a showed proteins which were extracted before IPTG was added. Lanes c, d, and e showed proteins which were extracted at 1-h intervals after IPTG was added. Molecular weight markers were in lane a: 1, β-galactosidase (118,000); 2, bovine serum albumin (78,000); 3, ovalbumin (47,100); 4, carbonic anhydrase (31,400); 5, soybean trypsin inhibitor (25,000); and 6, lysozyme (18,800). The arrowhead points to overexpressed products.

Fig. 7.

Purification and activity staining of the overexpressed cellulase. Panel A, SDS-PAGE of purified overexpressed cellulase. Lane b was a resuspended ammonium sulfate pellet. Lane c was a purified cellulase by Cm-Sepharose. In Panel B, lanes d and e show the activity staining of lanes b and c in Panel A, respectively. Molecular weight markers were in lane a: 1, β-galactosidase (118,000); 2, bovine serum albumin (78,000); 3, ovalbumin (47,100); 4, carbonic anhydrase (31,400); 5, soybean trypsin inhibitor (25,000); and 6, lysozyme (18,800). The arrowheads point to zones in which Cm-cellulase was degraded by cellulase.

Avicel (Table II), but proteins extracted from E. coli DH5α strain did not degrade these cellulosic substrates. Cm-cellulase slightly increased pNPC degrading activity, and vice versa (Fig. 5, D and E). The pNPC degrading activity was strongly inhibited by MUC (Fig. 5F).

Discussion

The following results suggest that the purified 35,000-Da cellulase secreted by Bacillus sp. D04 has both endo- and exoglucanase activity. The endoglucanase of Clostridium thermocellum, Cellulomonas fimii, and other Bacillus species hydrolyze Cm-cellulose, swollen cellulose, cellotetraose, and cellopentaose, but not pNPC (1). The exoglucanase of Ruminococcus flavefaciens FD-1 (2) and Aspergillus fumigatus (4) hydrolyzed pNPC, MUC, and filter paper, but not Cm-cellulose. However, the cellulase of Bacillus sp. D04 hydrolyzed Cm-cellulose, pNPC, and MUC (Table II). Moreover, this cellulase cleaved only the aglycosidic bond in pNPC as does an exoglucanase of Trichoderma viride and Sporotrichum pulverulentum (5), and randomly cleaved internal β-1,4-glycosidic bonds in cellotetraose and cellopentaose (Fig. 3, A and B) as an endoglucanase. These results imply that cellulase of Bacillus sp. D04 has both endo- and exoglucanase activity. The presence of both activities in the purified cellulase is confirmed by the fact that this cellulase also degraded crystalline cellulose (Fig. 3C), even though the hydrolysis efficiency of avicel was less than that of soluble cellulosic substrates. Probably, this was due to the low affinity of the purified cellulase against a crystalline cellulose.

To determine whether the active site of endo- and exoglucanase are separatedly existed, we studied differential effects of compounds that specifically inhibited one type of cellulase activity. The cellulase competitively inhibited pNPC degrading activity, but did not inhibit Cm-cellulose degrading activity (Table III). However, since the K_i of cellulase was 35.4 mM (Fig. 4A), cellulase was not a strong inhibitor in pNPC degrading activity, pCMB, a thiol protease inhibitor, inhibited pNPC degrading activity completely and Cm-cellulose degrading activity incompletely (Table III). Therefore endo- and exoglucanase activities were differently inhibited by cellulose and pCMB. Xue et al. (13) showed that the polysaccharide hydrolyase from N. patriciarum has a multifunctional catalytic domain that contains endoglucanase, cellbiohydrolase, and xylanase activities. On the basis of the substrate competition assays of this enzyme, Cm-cellulose and xylan strongly inhibited hydrolysis of MUC (13). Thus, they clearly demonstrated that only one active site has three types of enzyme activities. But the substrate competition pattern of the cellulase of Bacillus sp. D04 was different from those of N. patriciarum. At a high ratio of Cm-cellulose to pNPC or vice versa, one substrate did not inhibit hydrolysis of the another substrate (Fig. 5, A and B). But as MUC and pNPC were common substrates for exoglucanase, MUC strongly inhibited pNPC degrading activity even if the ratio MUC (0.01%, w/v) to pNPC (0.005%, w/v) was low (Fig. 5C). Thus, above results imply that the purified cellulase has separate sites of endo- and exoglucanase activity.

In order to rule out the possibility that enzymatic activity of either the endo- or the exoglucanase in the purified cellulase from Bacillus sp. D04 is due to a minor contaminating protein, we overexpressed the cd gene from a PET family vector in E. coli and compared its characteristics to those of the purified cellulase from Bacillus sp. D04. We deduced amino acid sequence from the cd gene. The 29 amino acids (from Met (1) to Ala (28), Fig. 6) in the N terminus was a typical β-glucanase signal peptide of Bacillus species (28). As an estimated molecular weight based upon amino acid composition of the cd gene was about 55,000. E. coli BL21(DE) pLysS with this gene produced 55,000-Da protein with Cm-cellulose degrading activity. But a 35,000-Da protein with this activity was also detected, which is the molecular mass of the cellulase purified from Bacillus sp. D04. These results indicate that the cellulase was produced as a precursor form from the cd gene and then processed (such as elimination of signal peptide, etc.) to its mature form. The purified 35,000-Da protein with cellulase activity was used as a recombinant cellulase. The substrate specificity and competition pattern of recombinant cellulase were the same as those of a purified cellulase from Bacillus sp. D04. These results clearly eliminate the possibility that the purified cellulase from Bacillus sp. D04 might have a minor contaminating protein involved in catalyzing either the endo- or the exoglucanase activity. Therefore a single polypeptide cellulase of Bacillus sp. D04 has both two kinds of activity. To localize each endo- and exoglucanase activity site in the cellulase, we are attempting to develop mutant which has only one type of glucanase activity.

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