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Production and Characteristics of Avicel-Digesting and Non-Avicel-Digesting Cellobiohydrolases from *Aspergillus ficum*

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Two immunologically related cellobiohydrolases, cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II), were purified from *Aspergillus ficum*. The Avicel-adsorbable CBH I (molecular weight, 128,000) digested Avicel, cotton, and cellulose powder to cellobiose, but the Avicel-unadsorbable CBH II (molecular weight, 50,000) could not digest those substrates. Both enzymes hydrolyzed insoluble cellooligosaccharides ($\overline{DP}$ 25) to cellobiose. High-pressure liquid chromatographic analysis of soluble cellooligosaccharide hydrolysates revealed that both enzymes split off strictly cellobiose units from the nonreducing end of the cellulose chain with an exowise mechanism. CBH I showed glucosyltransferase activity, but CBH II did not. The *N*-bromosuccinimide-oxidized CBH I was completely inactive but retained the ability to adsorb to Avicel. This suggested that CBH I has separate sites for binding to cellulose and for catalyzing cleavage of glycosidic linkages. Cellobiohydrolases were of two types, CBH I and CBH II. The former can adsorb to and digest Avicel, while the latter can do neither.

The mechanism of enzymatic hydrolysis of cellulose has been discussed by many research workers (1, 3, 6, 14). However, the mode of action of cellulolytic enzymes, especially on native cellulose, has not been completely clarified. We had reported in a previous paper (18) that endoglucanase was divided into two types, endoglucanase I (EG I) and endoglucanase II (EG II), with the ability to adsorb to and disintegrate cellulosic substrates. EG I could be adsorbed onto cellulosic substrates and rapidly disintegrated the inner part of cellulose fibrils, and EG II was unable to adsorb to and disintegrate microcrystalline cellulose.

The multiplicity of cellobiohydrolases and their properties were reported by Fagerstam and Pettersson (5), Tilbeurgh and Pettersson (15), and Wood et al. (20–22). Cellobiohydrolase was considered to split off cellobiose units from the nonreducing end of the cellulose chain. However, as a small amount of glucose and cellotriose were also liberated from cellotetraose and cellohexaose with cellobiohydrolase, several studies (11, 17) concluded that this enzyme could hydrolyze cellulose with a less-random mechanism.

Here we tried to isolate two types of cellobiohydrolase and to elucidate their mode of action on cellulosic substrates. This paper describes the purification and properties of both Avicel-adsorbable, Avicel-digesting CBH I and Avicel-unadsorbable, non-Avicel-digesting CBH II from a strain of *Aspergillus ficum*.

MATERIALS AND METHODS

Microorganism. *A. ficum* IFO 4034 was used throughout this study and was obtained from the Institute For Fermentation, Osaka, Japan.

Preparation of crude cellulase. *A. ficum* was cultured in a 2-liter Erlenmeyer flask containing solid wheat bran medium (wheat bran, 80 g; pulp floc, 20 g; tap water, 100 ml) at 30°C for 4 days. Enzymes were extracted by addition of 600 ml of tap water and left to stand at 4°C for 5 h. The filtrate was collected and designated as crude cellulase.

Chemicals. Avicel (microcrystalline cellulose), CM (carboxymethyl)-cellulose ($\overline{DS}$. 0.41), pulp floc, and filter paper were supplied by the companies described in the previous paper (8). Soluble cellooligosaccharides were obtained from Seikagaku Kogyo Co., Tokyo, Japan. Insoluble cellooligosaccharide was prepared from pulp floc by the method of Murao et al. (13). *N*-Bromosuccinimide was purchased from Wako Pure Chemical Industries Co., Osaka, Japan.

Enzyme assay. Cellobiohydrolase activity was assayed in a reaction mixture containing 50 mg of Avicel (or insoluble cellooligosaccharide) in 1 ml of 0.1 M acetate buffer (pH 5.0) and 1 ml of enzyme solution after 2 h of incubation at 50°C. Endoglucanase activity was measured by incubating 1 ml of 1% CM-cellulose in 0.1 M acetate buffer (pH 5.0) and 1 ml of enzyme solution at 50°C for 30 min. Reducing sugar formed was determined by the dinitrosalicylic acid method (10). One unit of cellobiohydrolase or endoglucanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar from the substrate per min.

β-Glucosidase activity was measured by incubating 1 ml of 1% cellobiose in 0.1 M acetate buffer (pH 5.0) and 1 ml of enzyme solution at 50°C for 30 min. Glucose formed was determined by glucose oxidase. One unit of β-glucosidase activity was defined as the amount of enzyme releasing 2 μmol of glucose from the substrate per min.

Purification of enzymes. All purification steps were carried out at 4°C.

(i) Step 1. The culture extract was concentrated by precipitation with ammonium sulfate at a 60% (wt/vol) concentration and kept overnight. The resulting precipitate was collected by filtration and dissolved in a small volume of deionized water. The supernatant was dialyzed by PVA-hollow fiber (Kuraray Co., Osaka, Japan). The enzyme solution was then adjusted to pH 3.0 with 1 N hydrochloric acid and allowed to stand for 24 h. The precipitate was removed by centrifugation at 13,000 × *g* for 10 min. The supernatant was collected, lyophilized, and applied to a Sephadex G-50 column (4.5 by 103 cm). Those fractions that showed cellobiohydrolase activity were combined and lyophilized.

(ii) Step 2. The lyophilized samples from step 1 were

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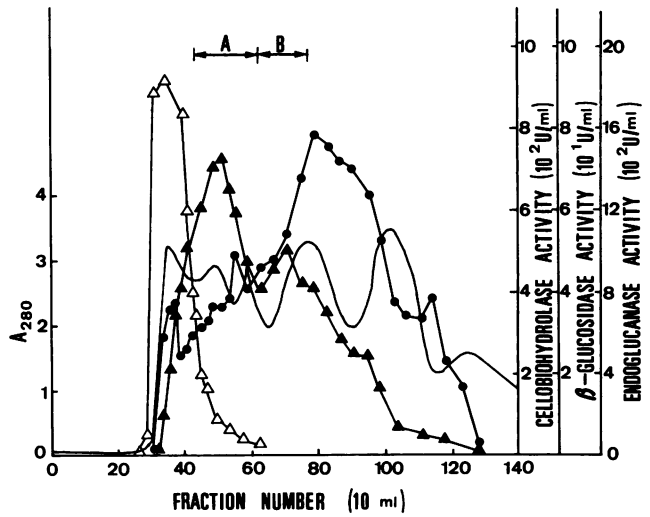

FIG. 1. Sephadex G-50 column chromatography of the cellulase from *A. ficum*. Symbols: ———, A_{280}; ▲, cellobiohydrolase activity, with Avicel as the substrate; ●, endoglucanase activity; △, β-glucosidase activity. Experimental details are described in the text.

applied to a DEAE-Sephadex A-50 column (2.5 by 103 cm) previously buffered (pH 6.8) and eluted with 500 ml of 0.05 M phosphate buffer (pH 6.8) and 500 ml of a linear gradient from 0.05 to 1 M phosphate buffer (pH 6.8). The flow rate was 5 ml/20 min. Fractions that contained cellobiohydrolase activity were combined, lyophilized, and desalted.

(iii) Step 3. The lyophilized samples from step 2 were further applied to a Sephacryl S-300 column (2.5 by 100 cm). Filtration was carried out with 0.1 M acetate buffer (containing 0.5 M sodium chloride [pH 6.0]) at a rate of 5 ml/22 min.

Hydrolysis of cellulosic substrates. The rate of hydrolysis by enzyme was calculated as a percentage of the total sugars released by acid treatment (12).

Immunological procedure. Antiserum for a purified CBH I preparation was prepared by immunizing a rabbit with three intradermal injections of a mixture consisting of 1 mg of CBH I and 1 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) at weekly intervals. On week 4, 1 ml of phosphate-buffered saline containing 1 mg of CBH I was given to the animal by intravenous injection. The rabbit was bled after 1 week, and the serum was collected after centrifugation and stored at −20°C. The double immunodiffusion technique was carried out according to Williams and Chase (19).

HPLC analysis. The products of enzymic hydrolysis of soluble cellooligosaccharides were determined by high-performance liquid chromatographic (HPLC) analysis. Before applying the sugar mixtures to an HPLC column (6 by 250 mm, YMC-pack PA-03, Yamamura Chemical Laboratory Co., Kyoto, Japan), the protein in the mixtures was removed by filtration with collodion bags (Sartorius Co., Federal Republic of Germany). The mixture (20 μl) was injected onto the column using acetonitrile-water mixtures as eluting solvents (60%, vol/vol). A flow rate of 0.5 ml/min was maintained. The sugars were determined using a differential refractometer (Shimadzu Seisakusyo Co., Kyoto, Japan).

Chemical modification of CBH I by *N*-bromosuccinimide. The purified CBH I was dissolved in 0.02 M acetate buffer (pH 4.0), containing various quantities of *N*-bromosuccinimide, and then incubated at 27°C for 5 min. The residual activity and protein concentration in the supernatant fluid before and after adsorption onto cellulosic substrates were determined.

General analytical procedures. Protein was determined by the method of Lowry et al. (9) with crystalline serum albumin as the standard. Disc gel electrophoresis in a 7.5% polyacrylamide gel at pH 8.3 in Tris-glycine buffer was carried out by the method of Davis (4). Molecular weights were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18).

RESULTS

Purification of CBH I and CBH II. The Sephadex G-50 chromatographic pattern is shown in Fig. 1. Fraction A showed the activity toward Avicel, CM-cellulose, and insoluble cellooligosaccharide, and fraction B showed higher activity toward insoluble cellooligosaccharide. Fractions A and B were collected separately, lyophilized, and then applied to a DEAE-Sephadex A-50 column. The DEAE-Sephadex A-50 chromatographic pattern of fraction A from Sephadex G-50 is shown in Fig. 2. Fractions 130 through 148

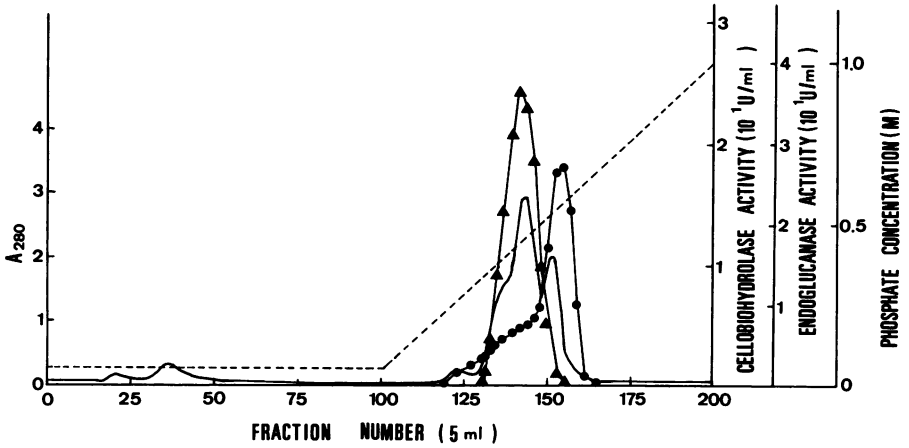

FIG. 2. DEAE-Sephadex A-50 column chromatography of fraction A from Sephadex G-50. Symbols: ———, A_{280}; ▲, cellobiohydrolase activity, with Avicel as the substrate; ●, endoglucanase activity; ——, phosphate concentration. Experimental details are described in the text.

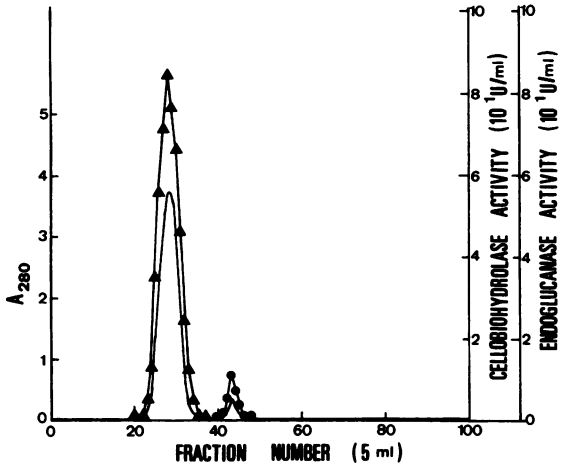

FIG. 3. Sephacryl S-300 column chromatography of CBH I after DEAE-Sephadex A-50 column chromatography. Symbols ———, A_{280}; ▲, cellobiohydrolase activity, with Avicel as the substrate; ●, endoglucanase activity. Experimental details are described in the text.

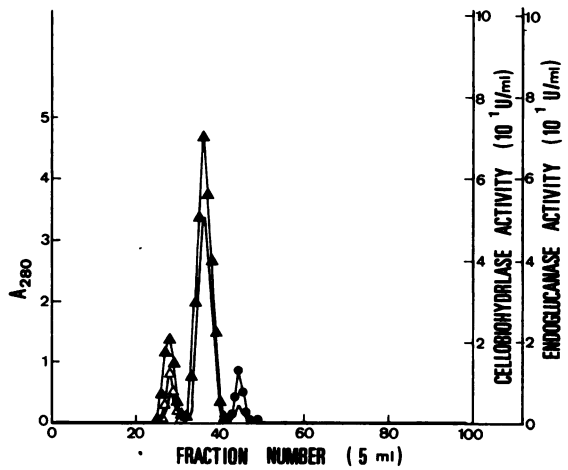

FIG. 5. Sephacryl S-300 column chromatography of CBH II after DEAE-Sephadex A-50 column chromatography. Symbols: ———, A_{280}; △, cellobiohydrolase activity, with Avicel as the substrate; ▲, cellobiohydrolase activity, with insoluble cellooligosaccharides as the substrate; ●, endoglucanase activity. Experimental details are described in the text.

that contained higher activity toward Avicel were combined, lyophilized, and then further purified by Sephacryl S-300 column chromatography (Fig. 3). The Sephacryl S-300 chromatographic pattern showed two peaks. One (fractions 41 through 47) was CM-cellulase. The other (fractions 23 through 33) that showed cellobiohydrolase activity was collected and desalted. The lyophilized sample was designated as purified CBH I. The fraction B from Sephadex G-50 was also applied to a DEAE-Sephadex A-50 column (Fig. 4). Fractions 145 through 153 exhibited activity toward insoluble cellooligosaccharide. These fractions were combined, lyophilized, and then applied to a Sephacryl S-300 column for further purification. The Sephacryl S-300 chromatographic pattern showed three peaks (Fig. 5). The first and third peaks were CBH I and CM-cellulase, respectively. The fractions (33 through 40) in the second peak were combined and desalted. The lyophilized enzyme was designated as purified CBH II and kept in an evacuated desiccator at 4°C. Recovery and specific activity of two types of cellobiohydrolase are summarized in Table 1.

Homogeneity of CBH I and CBH II. The purified CBH I was homogeneous on disc gel electrophoresis, and the purified CBH II also showed a single protein band on disc gel electrophoresis (Fig. 6). Both CBH I and CBH II also showed a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunodiffusion pattern of two types of cellobiohydrolase. Anti-CBH I serum taken out from the treated rabbit was subjected to a cross-reaction test for CBH I and CBH II, using the Ouchterlony double diffusion test on a 1% Noble agar plate. A clear precipitate band was observed between anti-CBH I serum and CBH I, and anti-CBH I serum and CBH II for 15 h after the addition of the antiserum (Fig. 7). CBH I and CBH II exhibited a pattern of identity, as shown by their smooth precipitin line formation.

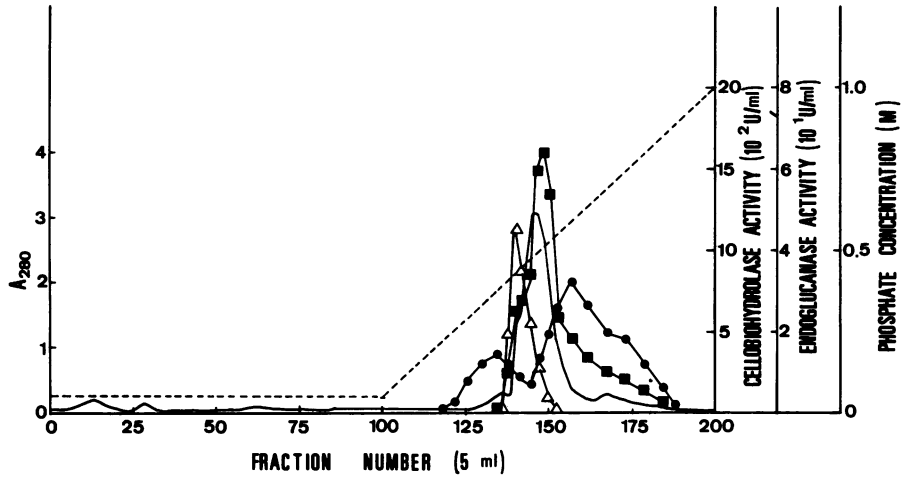

FIG. 4. DEAE-Sephadex A-50 column chromatography of fraction B from Sephadex G-50. Symbols: ———, A_{280}; △, cellobiohydrolase activity, with Avicel as the substrate; ■, cellobiohydrolase activity, with insoluble cellooligosaccharides as the substrate; ●, endoglucanase activity; — — —, phosphate concentration. Experimental details are described in the text.

TABLE 1. Purification of CBH I and CBH II from *A. ficum*

Step	Total protein (mg)		Total activity (U)		Sp act (U/mg)		Yield (%)	
	CBH I	CBH II	CBH I	CBH II	CBH I	CBH II	CBH I	CBH II
Culture extract	21,550[a]		237.05		0.011		100	
Ammonium sulfate precipitation	8,860		221.50		0.025		93.44	
Sephadex G-50	580	490	17.98	13.23	0.031	0.027	7.58	5.58
DEAE-Sephadex A-50	185	149	12.03	7.15	0.065	0.048	5.07	3.02
Sephacryl S-300	75	59	11.33	6.73	0.151	0.114	4.78	2.84

[a] Values between CBH I and CBH II columns represent cellobiohydrolase that has not yet been purified to CBH I or CBH II.

Properties of two types of cellobiohydrolase. (i) Molecular weight. The molecular weight of CBH I was estimated to be 128,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and that of CBH II was estimated to be 50,000.

(ii) Thermostability and pH stability. Portions of enzyme solutions, prepared in 0.1 M acetate buffer (pH 5.0), were kept for 10 min at various temperatures. CBH I and CBH II were stable at 55 and 60°C. CBH I retained 40% of original activity after heating at 65°C, but about 60% residual activity of CBH II was retained at 70°C. CBH I was stable at pH 2.5 to 6.0, and CBH II was stable at pH 2.0 to 7.0 after standing at 4°C for 24 h at various pHs.

(iii) Adsorbability of CBH I and CBH II to Avicel. The prepared CBH I solution (2 mg of protein/ml) in 5 ml of 0.1 M acetate buffer (pH 5.0) was applied to 1 g of Avicel, followed by standing at 4°C for 10 min. After centrifugation, cellobiohydrolase activity in the supernatant fluid was assayed. Ninety-six percent of CBH I activity was adsorbed, but the prepared CBH II solution (2 mg of protein per ml) could not be adsorbed to Avicel at the same condition.

(iv) Actions on cellulosic substrates. Hydrolysis curves of CBH I and CBH II on cellulosic substrates are shown in Fig. 8. Avicel was hydrolyzed to the extent of 26% by CBH I in 168 h of incubation at 50°C, but CBH II showed no activity toward Avicel. CBH II hydrolyzed insoluble cellooligosaccharide to the extent of 5.6% in 168 h at 50°C. Both CBH I and CBH II had little capacity to hydrolyze CM-cellulose.

(v) Carbohydrate content. Each enzyme (1 mg of protein) was dissolved in 10 ml of deionized water. To 1 ml of the enzyme solution was added 1 ml of the phenol reagent and 5 ml of concentrated sulfuric acid. The carbohydrate contents, calculated from A_{490} by using mannose as the standard, were determined to be 10.7 and 8.2% in CBH I and CBH II, respectively.

(vi) Hydrolysis products from cellulosic substrates. The reaction products from Avicel and insoluble cellooligosaccharide by CBH I and CBH II after 24 h of incubation were identified by HPLC analysis. The hydrolysis products were cellobiose and traces of glucose. Both CBH I and CBH II could attack cellotriose, cellotetraose, cellopentaose, and cellohexaose. The intermediary product, cellotetraose, was found in the hydrolysis of cellotriose by CBH I at 2 and 5 min of incubation (Fig. 9) but not by CBH II (Fig. 10). The intermediary product, cellotetraose, was hydrolyzed to cellobiose on prolonged incubation. The molar ratios of cellotriose hydrolysates (glucose-cellobiose) with CBH I and CBH II in 30 min of incubation were 1:2.6 and 1:1 (Fig. 9 and 10). When the substrate, cellotriose, was reduced with sodium borohydride, the intermediary, cellotetraose, could not be found in the hydrolysates with CBH I at 2 and 5 min of incubation (Fig. 11). The molar ratio of the products (glucose-cellobiose) was 1:1 at 30 min of incubation (Fig. 10). HPLC analyses of products released from cellohexaose with both enzymes are shown in Fig. 12 and 13. Both enzymes released cellobiose as the only product during incubation with cellohexaose.

(vii) Effect of *N*-bromosuccinimide on CBH I. *N*-bromosuccinimide at a concentration of 50 μmol/ml completely destroyed the cellulase activity of CBH I, while having no effect on its ability to adsorb to the substrate (Table 2).

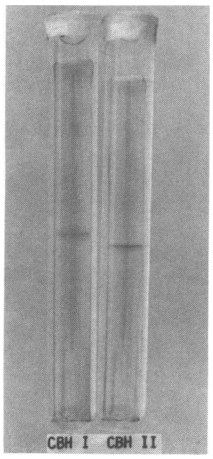

FIG. 6. Disc gel electrophoretic patterns of CBH I and CBH II. This was done on a polyacrylamide (7.5%) gel column, pH 8.3. About 10 μg of enzyme preparations was used, and 2 mA per column (0.5 by 8 cm) was applied for 120 min. Staining was done with 0.005% Coomassie brilliant blue R-250.

DISCUSSION

Wood and McCrae (20) reported that the highly purified C_1 component of *Trichoderma koningii* cellulase was a β-1,4-glucan cellobiohydrolase. This enzyme readily attacked phosphoric acid-swollen cellulose and cotton, producing cellobiose and a little glucose, but had little capacity to

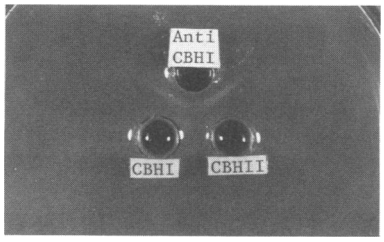

FIG. 7. Double immunodiffusion pattern demonstrating the antigenic relationships between CBH I and CBH II. Experimental details are described in the text.

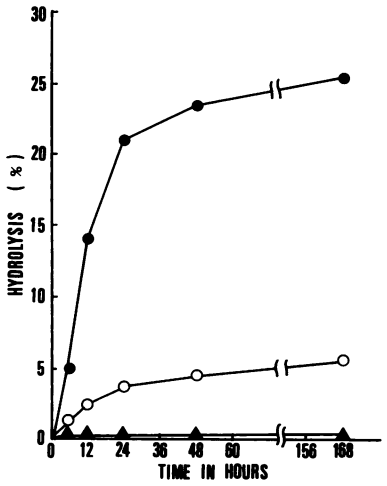

FIG. 8. Hydrolysis of cellulosic substrates by CBH I and CBH II. Symbols: ●, CBH I on Avicel; ○, CBH II on insoluble cellooligosaccharides; ▲, CBH II on Avicel. The reaction mixture, containing 50 mg of substrate in 1 ml of buffer and 1 ml of an enzyme solution (0.5 mg/ml), was incubated at 50°C in a stationary state.

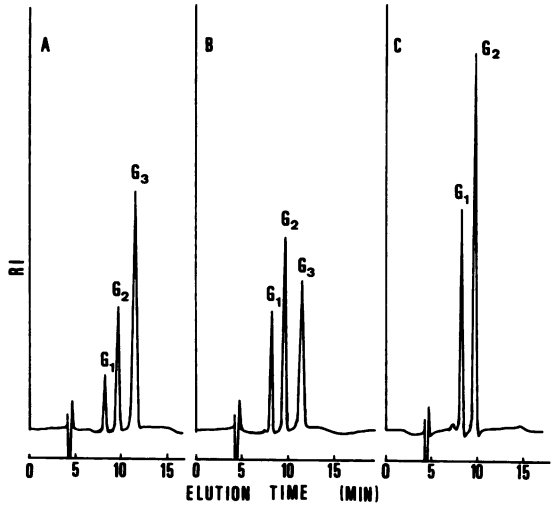

FIG. 10. HPLC analysis of products released from cellotriose (0.5%) by CBH II (0.1 mg/ml). Incubation took place at 50°C for 2 (A), 5 (B), and 30 (C) min. G_1, glucose; G_2, cellobiose; G_3, cellotriose. Experimental details are described in the text.

hydrolyze CM-cellulose. Cellotetraose and cellohexaose were similarly hydrolyzed almost exclusively to cellobiose and traces of glucose and cellotriose. Other workers reported later (18, 19) that two immunologically unrelated cellobiohydrolases I and II, isolated from the extracellular cellulase system elaborated by *Penicillum pinophilum*, acted in a synergistic manner to solubilize the microcrystalline cellulose. They, therefore, suggested that the cellobiohydrolases may be two stereospecific enzymes concerned with the hydrolysis of two different configurations of nonreducing end groups that would exist in cellulose.

According to the studies of Pettersson and his collaborators (1, 2, 5, 15, 16), *Trichoderma reesei* forms two immunologically different cellobiohydrolases, CBH I and CBH II. Both cellobiohydrolases showed activity toward Avicel and soluble cellodextrins, predominantly yielding cellobiose as the reaction product. 4-Methylumbelliferyl β-D-glycosides derived from cellobiose and 4-methylumbelliferyl-4-*o*-(β-D-galactopyranosyl)-β-D-glucopyranoside were substrates for CBH I but were not hydrolyzed by CBH II. Limited proteolysis of CBH I (molecular weight, 65,000) by papain yielded a core protein (molecular weight, 56,000) that is fully active against small, soluble cellodextrins. Activity against Avicel was, however, completely lost, and concomitantly decreased adsorption onto Avicel was observed.

The present paper proposes the existence of two types of cellobiohydrolase, CBH I (molecular weight, 128,000) and CBH II (molecular weight, 50,000) in the culture of *A. ficum*. CBH I could be adsorbed onto Avicel and digested Avicel to form cellobiose. CBH II was unable to adsorb to and digest Avicel but hydrolyzed insoluble cellooligosaccharide ($\overline{DP}$ 25) derived from pulp floc to split off cellobiose. CBH I and

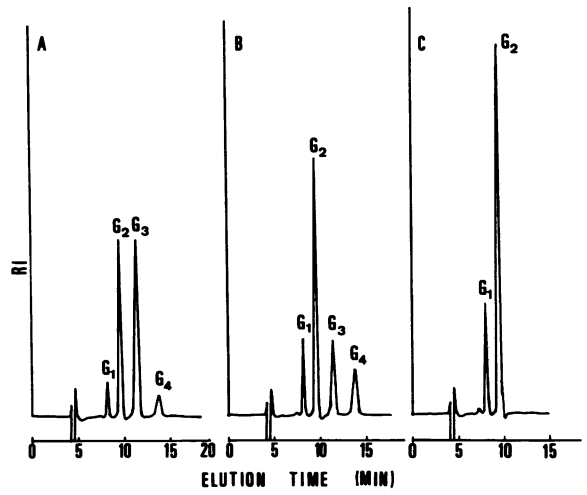

FIG. 9. HPLC analysis of products released from cellotriose (0.5%) by CBH I (0.1 mg/ml). Incubation took place at 50°C for 2 (A), 5 (B), and 30 (C) min. G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose. Experimental details are described in the text.

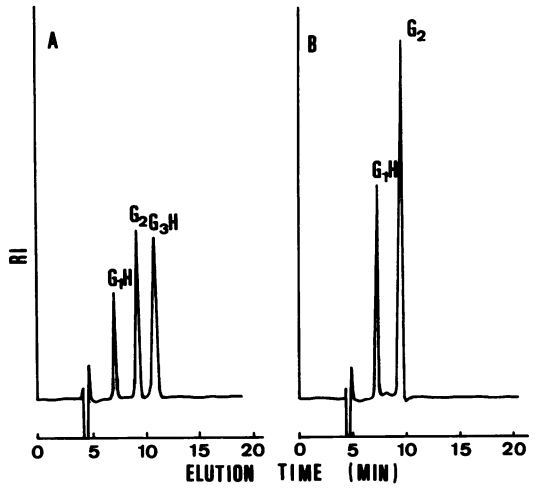

FIG. 11. HPLC analysis of products released from reduced cellotriose (0.5%) by CBH I (0.1 mg/ml). Incubation took place at 50°C for 5 (A) and 30 (B) min. G_1H, sorbitol; G_2, cellobiose; G_3H, reduced cellotriose. Experimental details are described in the text.

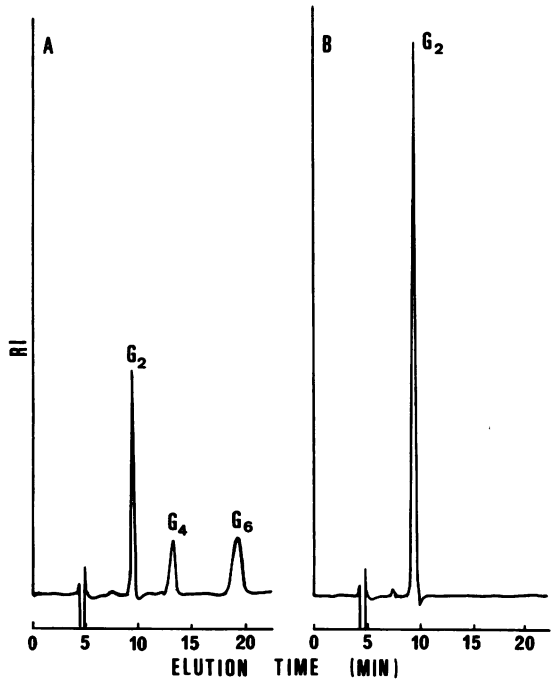

FIG. 12. HPLC analysis of products released from cellohexaose (0.5%) by CBH I (0.1 mg/ml). Incubation took place at 50°C for 5 (A) and 30 (B) min. G_2, cellobiose; G_4, cellotetraose; G_6, cellohexaose. Experimental details are described in the text.

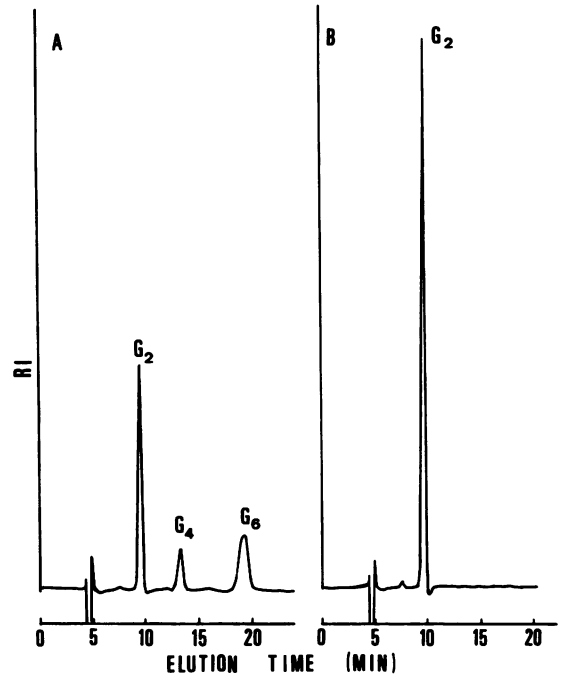

FIG. 13. HPLC analysis of products released from cellohexaose (0.5%) by CBH II (0.1 mg/ml). Incubation took place at 50°C for 5 (A) and 30 (B) min. G_2, cellobiose; G_4, cellotetraose; G_6, cellohexaose. Experimental details are described in the text.

TABLE 2. Effect of *N*-bromosuccinimide on CBH I

N-bromosuccinimide concn (μmol/ml)	Residual activity (%)	Protein concn (mg/ml)		% of protein adsorbed in 10 min
		Before adsorption to Avicel	After adsorption to Avicel	
0	100	1.19	0.06	94.96
10	71.33	–[a]	–	–
20	58.15	–	–	–
30	16.31	–	–	–
40	1.27	–	–	–
50	0.00	1.08	0.08	92.59

[a] –, Not measured.

CBH II exhibited an immunological pattern of identity by their smooth precipitin line. This indicated that the two types of cellobiohydrolase had a set of identical determinant groups with respect to the antiserum of CBH I. CBH I of *A. ficum* is the same or same kind of enzyme as CBH I of *T. reesei* and *P. pinophilum*, but CBH II is different from CBH II of *T. reesei* described above and *P. pinophilum* and seemed to be similar to papain-digested CBH I (molecular weight, 56,000) of *T. reesei*.

From HPLC analysis, the intermediary product, cellotetraose, was observed in the hydrolysis of cellotriose by CBH I but not by CBH II. It was shown that CBH I showed glucosyltransferase activity. When the substrate, cellotriose, was reduced with sodium borohydride, the intermediary product could not be detected, because CBH I split off a cellobiose unit from the nonreducing end of cellotriose and released cellobiose and a reduced glucose, sorbitol. Thus, CBH I appears to require the reducing end for glucosyltransfer. Moreover, both CBH I and CBH II released cellobiose as the sole product from cellotetraose and cellohexaose. We thus confirmed that both CBH I and CBH II split off cellobiose units from the nonreducing end of the cellulose chain with the strictly exosplitting mechanism.

The oxidation of CBH I by *N*-bromosuccinimide resulted in the complete loss of cellulase activity. This indicated that tryptophan is involved in the active site of the enzyme. However, the loss of cellulase activity of the *N*-bromosuccinimide-oxidized CBH I did not prevent the adsorption onto cellulose. It is suggested that CBH I has an affinity site whereby the enzyme characteristically adsorbs to insoluble cellulose in a manner similar to that of glucoamylase I from *Aspergillus awamori* var. *kawachi* to starch (7). Tilbeurgh et al. (16) proposed that CBH I of *T. reesei* was a bifunctional organization; one domain acts as a binding site for insoluble cellulose and the other contains the active (hydrolytic) site. The binding site could be removed with papain. Therefore, CBH I from *A. ficum* could have the affinity site and the active site as CBH I of *T. reesei*.

As previously reported (8), two types of endoglucanase, EG I and EG II, were purified from *Humicola grisea* var. *thermoidea* mutants. EG I could be adsorbed onto Avicel and rapidly disintegrated the inner part of cellulose fibrils, and EG II was unable to adsorb to and disintegrate Avicel. Both EG I and CBH I could hydrolyze native cellulose with their higher affinity for the insoluble substrates. EG II and CBH II could not directly attack native cellulose but would play an auxiliary role in hydrolyzing native cellulose. The formation of multiple types of cellobiohydrolase and the structural relationship between CBH I and CBH II will be reported later.

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