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Mode of Action and Synergism of Cellulases from *Penicillium funiculosum*†

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ABSTRACT

A 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) and 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4) were purified from the culture filtrates of *Penicillium funiculosum* by using preparative isoelectric focusing. Both the enzymes were homogeneous on polyacrylamide gel with and without sodium dodecyl sulphate. The mol wt of the cellobiohydrolase and endoglucanase were 14,400 and 25,000 respectively. The purified enzymes were free of β-glucosidase activity. Acting in isolation, the cellobiohydrolase had little capacity for solubilizing Avicel or Walseth cellulose, but showed increased rates of hydrolysis when combined with endoglucanase. Cellobiose inhibition (50%) was observed in the initial rate of the hydrolysis of Walseth cellulose. It was also observed that cellobiohydrolase initiates the attack on crystalline cellulose.

Index Entries: Cellulases; synergism; *Penicillium funiculosum*.

INTRODUCTION

It is now well established that cellulase is a multicomponent enzyme system. The crystalline cellulose is hydrolyzed by the synergistic action of different enzymes of cellulase complex (*1,2*). Previous work from our laboratory has indicated that *Penicillium funiculosum* produces an extracellular cellulase complex consisting of endoglucanase, exoglucanase, and

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β-glucosidase (*3,4*). The hydrolysis of various pure cellulose and lignocellulosic substrates using *P. funiculosum* cellulase has also been reported by us (*4,5*). The present paper describes the purification and characterization of a cellobiohydrolase and endoglucanase from culture filtrate of *P. funiculosum*. The role of these purified enzymes in the overall hydrolysis of crystalline substrate such as Avicel has also been discussed.

MATERIALS AND METHODS

Microorganism

The strain of *Penicillium funiculosum* (*3*) was obtained from the National Chemical Laboratory, Pune (National Collection of Industrial Microorganism, NCIM).

The chemicals used were of analytical grade. The sources are shown in parenthesis: sodium salt of CM-cellulose, low viscosity (Sigma C 8758, DS = 0.7, DP = 400) 3,5-dinitrosalicyclic acid and SDS molecular weight determination kits (Sigma Chemical Co., St. Louis, MO). The CM-cellulose (high viscosity Hercules, 7H35) was received as a gift from K. E. Eriksson (Sweden). Sodium dodecyl sulphate (SDS), Tris, TEMED, acrylamide were from Koch Light Laboratories (UK). UM-10 ultrafiltration membrane (Amicon Corporation Lexington, MA) and Collodion membrane (Sartorius membrane filter GmbH, Federal Republic of Germany) were also used. Purified β-D-glucosidase (B-D-glucosidase glucohydrolase EC 3.2.1.21) was a gift from B. C. Lakshmi Kantham for the author's laboratory.

Walseth cellulose was prepared according to Walseth (*6*). Cellooligosaccharides were prepared according to Miller et al. (*7*).

Enzyme Assays

Activity toward CM-cellulose and Walseth cellulose was determined by incubating 1 mL of reaction mixture containing suitably diluted enzyme, 0.5 mL CM-cellulose (1%) (C8758) and Walseth cellulose (0.3%), in acetate buffer, pH 4.8, 0.05*M* for 30 min at 50°C. The reducing sugar was determined using D-glucose as a standard (*8*). Activity toward filter paper, cotton, and D-xylan was determined according to the method of Mandels and Weber (*9*). The reaction mixture contained enzyme and substrate in 2 mL 0.05*M* acetate buffer, pH 4.8. The amount of substrate and period of incubation were respectively, 50 mg Whatman No. 1 filter paper for 1 h and 5 mg xylan for 30 min.

Viscosity reducing activity was determined using CM-cellulose (*10*).

Unit of Activity

One unit of enzyme activity was defined as that amount of enzyme that produces 1 μmol of reducing sugar in 1 min under assay conditions. Protein was determined by the method Lowry et al. (*11*).

Purification

The enzyme for purification studies was produced in a modified Reese's medium containing 2.5% cellulose powder as a carbon source (*3*). The clarified culture broth (40 mL) was concentrated by ultrafiltration with a UM-10 membrane to a residual vol of 12 mL. The concentrated broth was dialyzed against 0.05*M* acetate buffer (pH 4.8).

Preparative Isoelectric Focusing

Preparative isoelectric focusing was carried out as described by LKB Produkter AB, Stockholm, Sweden (application note 198). Isoelectric focusing was performed by using polyurethane strips in place of ultrodex. Isoelectric focusing of proteins was carried out in the LKB Multiphore apparatus with the LKB Multiphore tray (24.5×11×0.5 cm) and Ampholines in the 4–6 pH range. Ultrodex was substituted with 24 strips of foam (5×1×9.5 cm each). The strips were soaked in 7 mL of 2% (w/v). The Ampholines in water were then placed horizontally on the tray. The anodic strip was soaked in 1*M* H_3PO_4 and cathodic strip was soaked in 1 *N* NaOH. The strips were removed, squeezed, dipped in the solution containing enzyme sample, and replaced on the tray. The electrofocusing was run with a constant current of 12 mA and a constant power of 8 W for 18 h at 10°C. After termination of the run, foam strips were squeezed and the material was collected in different test tubes and assayed for the protein and activity. The pH was measured using a surface electrode.

SDS Gel Electrophoresis

Electrophoresis in slab gel of polyacrylamide containing SDS was performed by the method of Weber and Osborn (*12*) by using lysozyme (14,000 mol wt), trypsin (24,000) pepsin (34,700), ovalbumin (45,000), bovine serum albumin (68,000), and transferrin (90,000) as standard proteins. Electrophoretic mobilities of purified cellulases and reference proteins on SDS polyacrylamide were plotted vs their molecular weights.

Analytical Isoelectric Focusing

Isoelectric focusing in thin polyacrylamide gel was carried out by the method of Vesterberg (*13*). The Ampholine range used was pH 3.5–5.0.

Disk Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in 7% acrylamide gel with Tris glycine buffer (*14*).

Substrate Specificity

The enzyme (10 μg) was incubated with 10 mg of Avicel, Filter paper, Walseth, or 100 mg of cellotriose to cellopentaose in 0.05*M* acetate buffer,

pH 4.8 for 24 h. The end products were analyzed by paper chromatography (*15*).

Effect of Inhibitors

Effect of cellobiose (25 m*M*) and gluconolactone (50 m*M*) on cellobiohydrolase and endoglucanase were studied under assay conditions. Walseth cellulose and CM–cellulose were used as substrates for assaying cellobiohydrolase and endoglucanase activities respectively.

Synergism

Ten mg Avicel or Walseth cellulose was incubated with purified enzymes (40 μg each) in a total vol of 1 mL with 50 m*M* acetate buffer, pH 4.8 at 37°C for 18 h. The glucose formed was estimated according to Bergmeyer and Bernt (*16*).

Hydrolysis of Avicel after a Pretreatment with Enzymes

Avicel (10 mg) was treated for 18 h at 37°C either with cellobiohydrolase or endoglucanase (45 μg). The reaction mixture was heated at 100°C for 10 min, cooled, and incubated either with endoglucanase or cellobiohydrolase for 18 h at 37°C. Samples were estimated for glucose.

Kinetics

The Michaelis constant (K_m) was determined from Lineweaver-Burk plots by measuring the enzyme reaction rates using substrate concentration ranging from 1 to 20 mg of CM–cellulose and 10 to 75 mg of Walseth cellulose.

RESULTS

Purification

The cell-free culture filtrate (40 mL) was concentrated and dialyzed by ultrafiltration to 12 mL. The concentrated preparation (6 mL, 72 mg) was subjected to preparative isoelectric focusing over a pH range of 4–6 (Fig. 1). The eluates were analyzed for activity toward CM–cellulose (endoglucanase) and Walseth cellulose (cellobiohydrolase). Fractions 4 and 22 were dialyzed and concentrated through collodion membrane to 0.5 mL. Fraction 4 showed a high ratio of CM–cellulase to Walseth cellulase activity, whereas fraction 22 showed a high ratio of Walseth cellulase to CM cellulase. Purification of endoglucanase and cellobiohydrolase are summarized in Table 1. The enzymes were found to be homogeneous on SDS and non-SDS polyacrylamide gels (Fig. 2). The yield of cellobiohydrolase was 125 mg/L of culture broth.

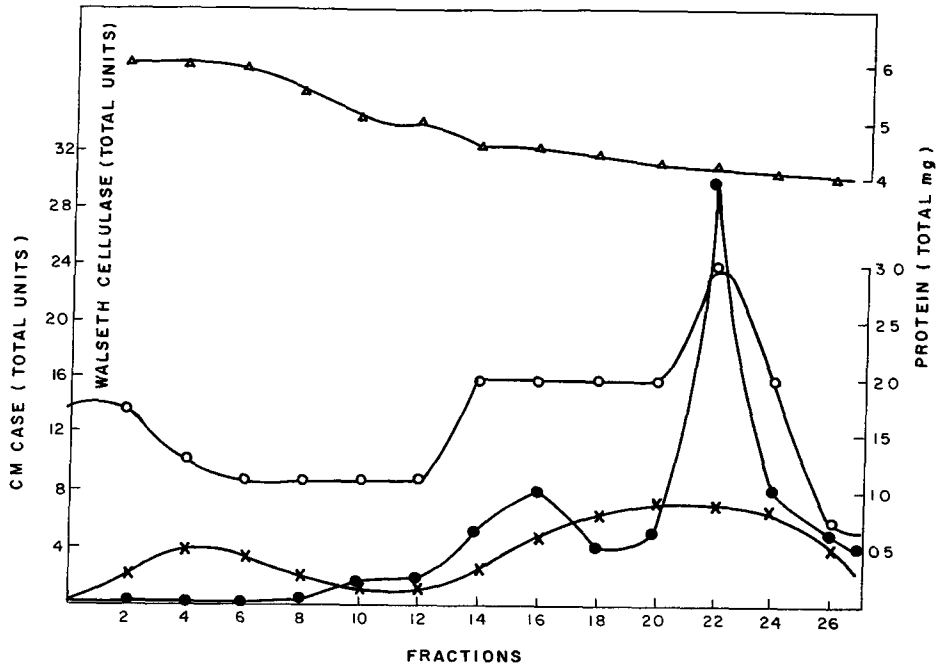

Fig. 1. Purification of cellobiohydrolase and endoglucanase using preparative isoelectric focusing, pH 4–6: (●) cellobiohydrolase; (x) endoglucanase; (△) pH; and (○) protein.

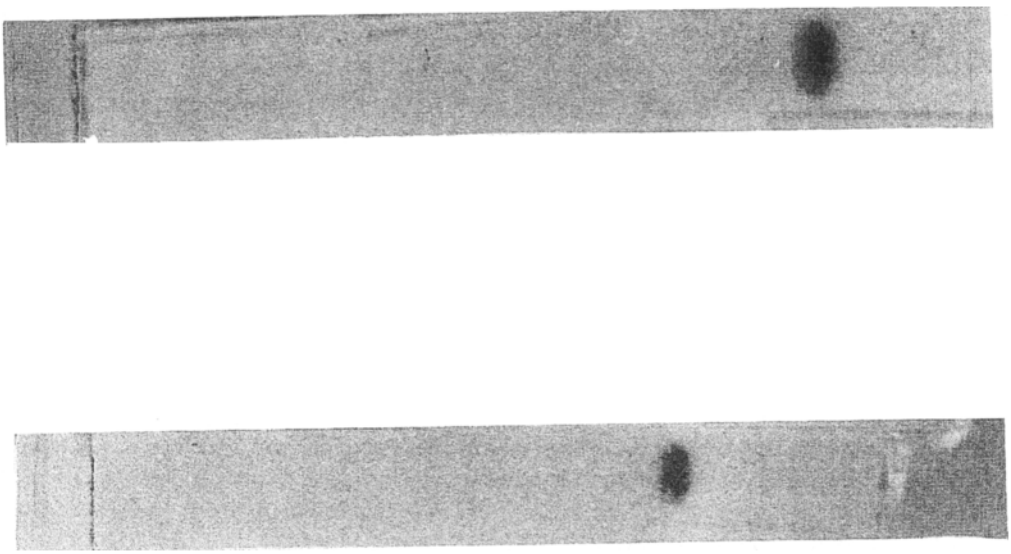

Fig. 2. SDS gel electrophoresis (Protein 100 μg was loaded and stained with Coomassie blue): (A) Cellobiohydrolase and (B) Endoglucanase.

Table 1
Purification of Endoglucanase and Cellobiohydrolase from *P. funiculosum*

	Protein, mg	Activity toward Walseth cellulose, total units	Activity toward CM cellulose, total units	Specific: Endoglucanase, U/mg	Specific: Cellobiohydrolase, U/mg	Ratio: CM cellulose / Walseth cellulose activities	Ratio: Walseth cellulose / CM-cellulose activities
Culture filtrate	100	200	300	3.0	2.0	1.5	0.7
Ultrafiltered enzyme[a]	72	180	300	4.0	2.5	1.6	0.6
Isoelectric focusing							
Cellobiohydrolase (Fraction 22)	2.4	25	5	—	10.0	—	5
Endoglucanase (Fraction 4)	0.5	—	10	20.0	—	20.0	—

[a] mL ultrafiltered dialyzed enzyme was subjected to preparative isoelectric focusing pH (4-6), current 12 mA voltage 500 V, power (constant)—8 W period of run 18 h, temperature 10°C.

The molecular weights of endoglucanase and cellobiohydrolase by SDS–PAGE were 25,000 and 14,400, respectively (Fig. 3). The enzymes showed optimum pH and temperature of 4.8 and 50°C respectively. The endoglucanase and cellobiohydrolase showed p*I* value of 5.6 and 4.2 respectively. The apparent K_m value for endoglucanase with CM-cellulose was 16 mg/mL and K_m value for cellobiohydrolase with Walseth cellulose 8 mg/mL.

Substrate Specificity

Endoglucanase showed viscosity reducing activity and catalyzed hydrolysis of carboxymethyl cellulose. It had no activity towards Avicel, Walseth cellulose, filter paper, xylan, and *p*-nitrophenyl-β-D-glucoside. Cellobiohydrolase showed high activity towards Walseth cellulose. The

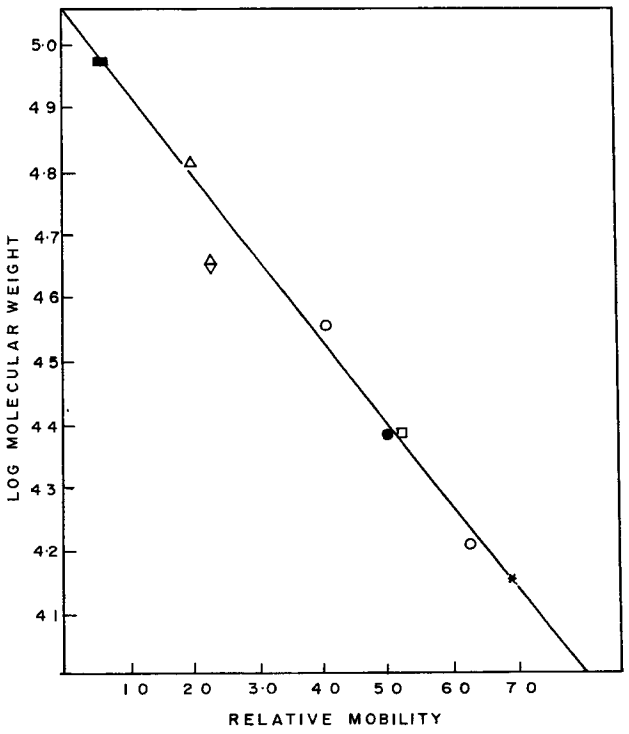

Fig. 3. Estimation of the molecular weights of the cellobiohydrolase and endoglucanase from *P. funiculosum* by SDS gel electrophoresis. Standard proteins were: (■) Transferrin; (△) Bovine serum albumin; (◈) Ovalbumin; (○) Pepsin; (●) Trypsin; (⊙) Lysozyme; (*) Cellobiohydrolase; and (□) Endoglucanase.

purified enzyme also acted on filter paper and Avicel and produced a major product as cellobiose with trace quantity of glucose as detected by paper chromatography. Cellobiohydrolase had no action on cellobiose, *p*-nitro-phenyl-β-D-glucoside and xylan. The endoglucanase hydrolyzed cellotriose to cellobiose and glucose which was identified by paper chromatography. The enzyme degraded cellotetraose and cellopentaose to a mixture of cellobiose and cellotriose with glucose. Cellobiohydrolase acted rapidly upon cellooligosaccharides (G_3–G_5) yielding mainly cellobiose. In case of cellotriose, glucose was also detected along with cellobiose (Fig. 4).

Effect of Inhibitors

The purified cellobiohydrolase showed 50% inhibition in the initial reaction velocity of the hydrolysis of Walseth cellulose using 25 m*M* cellobiose. The enzyme was not inhibited by gluconolactone (50 m*M*). Endoglucanase was inhibited by cellobiose and glucanolactone to 90 and 100% respectively.

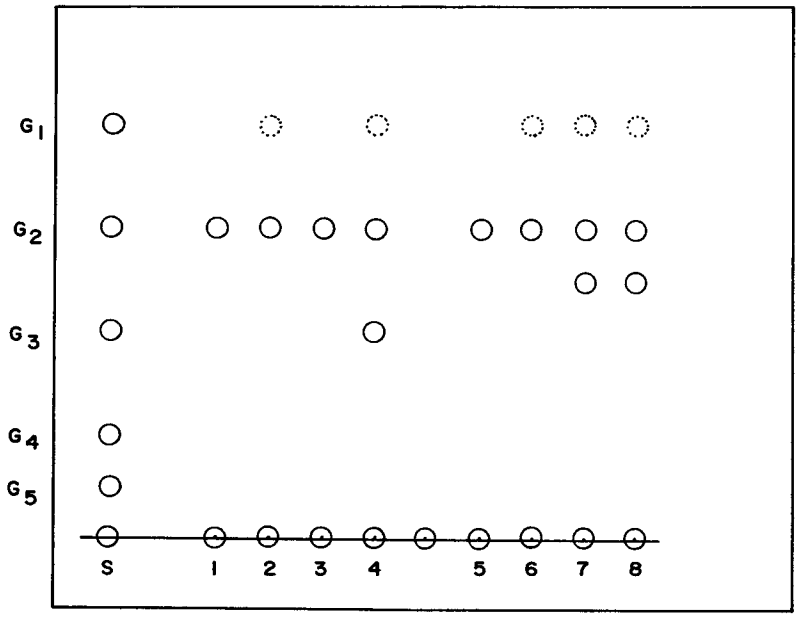

Fig. 4. Action of cellobiohydrolase and endoglucanase on cellooligosaccharides. Substrates used were cellobiose, cellotriose, cello-tetraose, and cellopentaose. Standard is denoted by S. Oligosaccharides are designated by symbol G followed by a subscript indicating degree of polymerization. Action of cellobiohydrolase on G_2(1), G_3(2), G_4(3), and G_5(4). Endoglucanase on G_2(5), G_3(6), G_4(7), G_5(8).

Synergism

The action of cellobiohydrolase when acting alone and in concert with endoglucanase and β-glucosidase on Avicel was compared (Fig. 5). It was found that there was increased hydrolysis of Avicel when cellobio-hydrolase was supplemented with endoglucanase, but maximum hydrolysis was obtained when β-glucosidase was added to the system. The purified cellobiohydrolase and endoglucanase also showed synergism in solubilization of Walseth cellulose (Table 2).

Initiation of Enzymic Degradation of Cellulose

The effect of pretreating Avicel with either cellobiohydrolase or endoglucanase prior to the addition of the alternative type of enzyme was studied. When Avicel was treated with endoglucanase, following hydrolysis with cellobiohydrolase, it had no effect in the release of glucose formation. But there was a pronounced increase in glucose formation when the substrate was first pretreated with cellobiohydrolase followed by endoglucanase (Table 3).

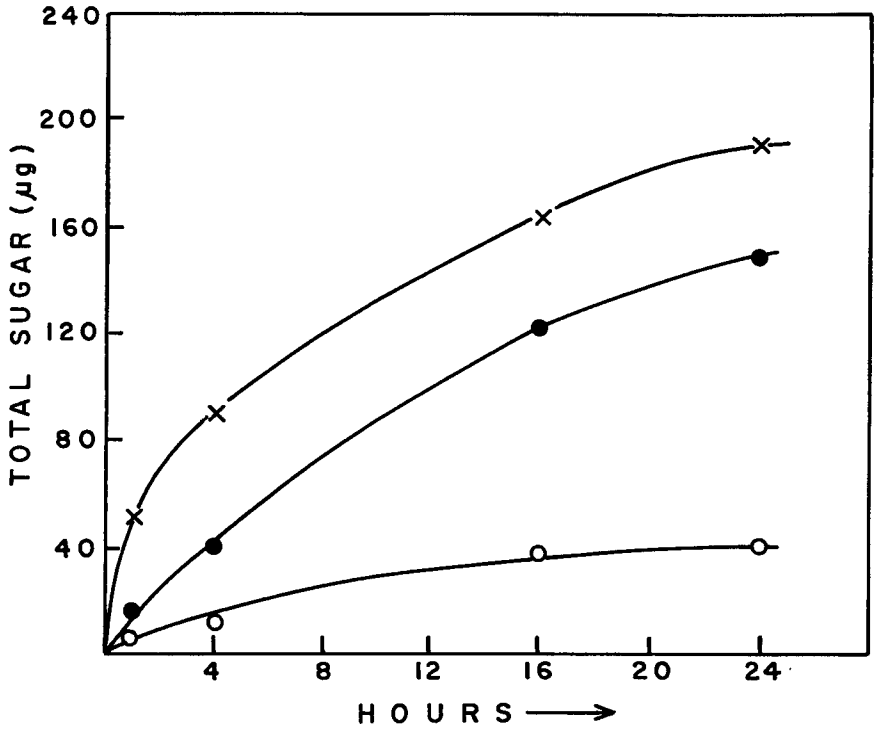

Fig. 5. Synergistic effects between the cellobiohydrolase and the other components of the cellulase complex in hydrolysizing Avicel. Glucose liberated was estimated as described in Materials and Methods: (○) Cellobiohydrolase; (●) Endoglucanase + Cellobiohydrolase; and (x) Endoglucanase + Cellobiohydrolase + B-glucosidase.

Table 2
Synergistic Hydrolysis of Walseth Cellulose by Cellobiohydrolase and Endoglucanase[a]

	Glucose released, μg
Endoglucanase	1
Cellobiohydrolase	25
Endoglucanase + cellobiohydrolase	85

[a]Ten mg of Walseth cellulose was treated with the purified enzyme (40 μg of protein) in a total vol of 1 mL with 50 m*M* acetate buffer, pH 4.8 at 37°C for 18 h.

Table 3
Hydrolysis of Avicel After a Pretreatment with Endoglucanase or Exoglucanase[a]

Enzyme used for Pretreatment	Degradation by	Glucose, μg
None	Endoglucanase	0
None	Cellobiohydrolase	24
Endoglucanase	Cellobiohydrolase	24
Cellobiohydrolase	Endoglucanase	48

[a]Avicel (10 mg) was treated for 18 h at 37°C either with cellobiohydrolase or endoglucanase (45 μg). The reaction mixture was heated, cooled, and treated with either endoglucanase or cellobiohydrolase as described in Materials and Methods.

DISCUSSION

Fractionation studies of *P. funiculosum* cellulase by preparative isoelectric focusing showed the presence of an endoglucanase and two exoglucanases. The cellobiohydrolase and an endoglucanase were purified to a state of homogeneity as judged by gel electrophoresis. The cellobiohydrolase isolated from *P. funiculosum* showed activity towards highly ordered substrates such as Avicel and dewaxed cotton fiber, but acted more rapidly on filter paper and Walseth cellulose. The enzyme acted upon cellooligosaccharides (G_3–G_5). Cellobiose was the major end product, but in the cases of G_3 and G_5 the hydrolysates showed traces of glucose that may be owing to the transferase action of the enzyme. The presence of higher cellooligosaccharides was not detected as analyzed by paper chromatography.

Cellobiohydrolase from the present studies was not inhibited by gluconolactone as observed for cellobiohydrolase from *F. solani* (*17*) and *P. funiculosum* (*1*). In contrast, the *S. pulverulentum* enzyme (*18*) was inhibited 83% by 1 μ*M* D-gluconolactone. The cellobiohydrolase was inhibited by

25 m*M* cellobiose up to 50%, whereas the cellobiohydrolase from *T. reesei* (*19*) is inhibited only up to 10–25% by higher concentrations of cellobiose.

The endoglucanase of *P. funiculosum* has no action on Walseth cellulose, xylan, and cellobiose. Few endoglucanases from cellulolytic cultures show activity toward xylan (*10,20,21*), but the absence of xylanase activity from the purified endoglucanase has also been reported (*21*). The mode of action of the endoglucanase on cellooligosaccharides was identical with those reported earlier for endoglucanase of *Sclerotium rolfsii* (*23*).

The purified cellobiohydrolase and endoglucanase were free of β-glucosidase activity. The cellobiohydrolase alone had little capacity for solubilizing highly ordered cellulose, such as Avicel, but when recombined with endoglucanase showed increased rates of hydrolysis. The supplementation of β-glucosidase from *P. funiculosum* (*24*) yielded a further increase in hydrolysis. Similar synergistic effects between the purified cellobiohydrolase and the mixture of endoglucanase and β-glucosidase for the hydrolysis of Walseth cellulose has been observed (*1,2,25*).

Present studies on initiation of enzymic degradation of cellulose suggested that cellobiohydrolase initiates the attack on substrates more accessible to hydrolysis. Streamer et al. (*2*) have shown that endo-β-glucanase initiates the attack on crystalline cellulose. Recently, Chanzy et al. (*26*) have reported that one of the cellobiohydrolases from *Trichoderma reesei* displays an initial endo-type mode of action and is responsible for the disruption of cellulose crystals. Sadana and Patil (*27*) have also reported that a cellobiohydrolase initiates the action on crystalline cellulose.

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