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SYNERGISM BETWEEN ENZYMES OF *Sclerotium rolfsii* INVOLVED IN THE SOLUBILIZATION OF CRYSTALLINE CELLULOSE*

JAI C. SADANA† AND RAJKUMAR V. PATIL
Biochemistry Division, National Chemical Laboratory, Poona 411 008 (India)
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ABSTRACT

Synergism between (1→4)-β-D-glucan cellobiohydrolase, endo-(1→4)-β-D-glucanases, and β-D-glucosidases of *Sclerotium rolfsii* for solubilization of native and amorphous celluloses is discussed. Besides synergism between cellobiohydrolase and endo-β-glucanases of *S. rolfsii*, a synergistic effect between endo-β-glucanases and β-glucosidases [which behaved rather as (1→4)-β-D-glucan glucohydrolases] was observed for solubilization of crystalline and amorphous celluloses. It seems that a cellobiohydrolase initiates the attack on crystalline cellulose and an endo-β-D-glucanase the attack on amorphous cellulose.

INTRODUCTION

The purification, biochemical characterization, and mode of action of cellulase components, namely endo-(1→4)-β-D-glucanases (EC 3.2.1.4) (endo-β-glucanase), (1→4)-β-D-glucan cellobiohydrolase (EC 3.2.1.91) (cellobiohydrolase), and β-D-glucosidases (EC 3.2.1.21) from *S. rolfsii* have been reported[1–4]. In the present paper, the mode of action of the various components of the cellulase complex from *Sclerotium rolfsii* for the breakdown of crystalline cellulose is discussed in relation to the synergism shown between mixtures of the various components.

EXPERIMENTAL

General. — All materials, methods for determination of reducing sugars, D-glucose, and total carbohydrate, and enzyme assays (cellobiohydrolase, endo-β-glucanase, β-glucosidase, and cellobiose dehydrogenase) were as described previously[1–4].

Cellulase (Avicel, cotton, or H_3PO_4-swollen cellulose-solubilizing) activity. — The assay mixture contained Avicel or cotton sliver (2 mg), or H_3PO_4-swollen cellulose (20 mg), enzyme(s) and mM NaN_3–50mM citrate buffer, pH 4.5, to give a

*N.C.L. Communication No. 3456.
†To whom all correspondence should be addressed.

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total vol. of 3 mL. After incubation at 37° for 7 days for Avicel or cotton, and 24 h for H_3PO_4-swollen cellulose, the total carbohydrate released in the supernatant fluid or the residual cellulose was determined by the anthrone–H_2SO_4 method[3].

RESULTS

Solubilization of Avicel, cotton, and H_3PO_4-swollen cellulose by cellulase components of S. rolfsii. — With *S. rolfsii* culture filtrate, the optimum pH and temperature for solubilizing Avicel or H_3PO_4-swollen cellulose were 4.5 and 37°, and that for cotton 4.5 and 45° under standard assay conditions. For the synergistic experiments reported below, the solubilization of Avicel or cotton (2 mg) in 7 days was studied at 37°, pH 4.5, as described by Wood[5]. The proportions of solubilization of cellulose calculated from the total carbohydrate solubilized and present in the supernatant fluid, and the residual cellulose (both determinations by the anthrone method[3]) were more or less similar.

A 2-mL culture filtrate of *S. rolfsii* [or the equivalent as $(NH_4)_2SO_4$ precipitate] solubilized 45 and 77% of cotton and Avicel cellulose, respectively; 1.0-, 0.6-, 0.3-, and 0.1-mL culture filtrate produced 32, 23, 15, and 9.5% solubilization of cotton, respectively, and 74, 49, 32, and 18% solubilization of Avicel, respectively. Avicel and cotton cellulose were hydrolyzed to the same extent when the hydrolysis was carried out under air or nitrogen, and under shaking or non-shaking conditions.

For synergistic studies for solubilization of Avicel or cotton cellulose, the amount of enzymes taken corresponds to that present in 0.5 mL of culture filtrate: cellobiohydrolase, 1.5 mg; endo-β-glucanases A, B, and C (mixed in equal amounts on protein basis), 1.0 mg (89.5 IU); and β-glucosidases I-IV (mixed in equal amounts on protein basis), 0.25 mg (10.5 IU). The amount of cellobiohydrolase present in the culture filtrate was determined by taking endo-β-glucanases (1 mg), β-glucosidases (0.25 mg), and various amounts of cellobiohydrolase so that the proportion of solubilization of a 2-mg sample of Avicel or cotton was equal to that obtained with 0.5 mL of culture filtrate.

Synergism between cellulase components of S. rolfsii *in solubilizing Avicel, cotton, and H_3PO_4-swollen cellulose.* — The ability of the different components of the cellulase system, acting alone or in combination, to degrade Avicel, cotton, and H_3PO_4-swollen cellulose was compared to that of the full complex and unfractionated culture filtrate (see Table I). A mixture of cellobiohydrolase and endo-β-glucanases showed a high degree of cooperation in solubilizing Avicel, cotton, and H_3PO_4-swollen cellulose, followed by a mixture of endo-β-glucanases and β-glucosidases, and least with cellobiohydrolase and β-glucosidases. Thus, β-glucosidases also act synergistically with endo-β-glucanases in solubilizing Avicel, cotton, and H_3PO_4-swollen cellulose. All the three endo-β-glucanases from *S. rolfsii* individually show synergistic effect to varying degrees with cellobiohydrolase (data not shown). When cellobiohydrolase, endo-β-glucanases, and β-glucosidases were acting in concert, the solubilization of Avicel was enhanced further from 31.7

TABLE I

SYNERGISM BETWEEN ENDO-β-GLUCANASES, CELLOBIOHYDROLASE, AND β-GLUCOSIDASES COMPONENTS OF *Sclerotium rolfsii* CELLULASE IN SOLUBILIZING AVICEL, COTTON, AND H_3PO_4-SWOLLEN CELLULOSE

Enzyme	*Avicel*[a]			*Cotton*[a]			*H_3PO_4-swollen cellulose*[b]
	Total sugar[c] *(μg)*	*Reducing sugar*[d] *(μg)*	*Solubilization*[e] *(%)*	*Total sugar*[c] *(μg)*	*Reducing sugar*[d] *(μg)*	*Solubilization*[e] *(%)*	*Solubilization*[e] *(%)*
Cellobiohydrolase (CBH)	102	50.8	4.6	56.5	28	2.6	3.3
Endo-β-glucanases[f] (Endo)	32	18.0	1.9	38.0	17.0	1.7	9.1
β-Glucosidases[g] (BG)	4.5	4.4	0.2	0	0	0	0.3
Cellobiose dehydrogenase (CBDH) + DCPIP[i]	0	0	0	[h]	[h]	[h]	[h]
CBH + BG	264	267	12.0	63.3	60.0	2.9	3.7
CBH + Endo	700	420	31.7	375	222	17	22
Endo + BG	354	252	16.0	153	111	6.9	15.7
Endo + CBDH + DCPIP	[h]	[h]	3.4[j]	[h]	[h]	[h]	[h]
CBH + Endo + CBDH + DCPIP + BG	1038	968	47	[h]	[h]	[h]	[h]
CBH + Endo + BG	1040	984	47	542	506	24.5	26.7
CBH[k] + Endo + BG	1080	1004	48.9	[h]	[h]	[h]	[h]
Culture filtrate (0.5 mL)	1060	940	48.0	554	519	25.0	[h]

[a]Standard assay as described in Experimental section: CBH (1.5 mg), Endo (1.0 mg), BG (0.25 mg), and CBDH (300 μg) + DCPIP (20 μmol). [b]Standard assay as described in Experimental section: CBH (20 μg), Endo (20 μg), and BG (20 μg) 24 h. [c]Total soluble sugar released was determined by anthrone–H_2SO_4 method. [d]Reducing end-groups were determined by Nelson–Somogyi method. [e]Solubilization was determined from total soluble sugar released in supernatant solution. [f]Endo-β-glucanases A, B, and C mixed in equal amounts on protein basis. [g]β-Glucosidase (I–IV) mixed in equal amounts on protein basis. [h]Not determined. [i]2,6-Dichlorophenol–indophenol. [j]Percent of solubilization calculated from residual cellulose. [k]CBH 3.0 mg.

TABLE II

INHIBITION OF CELLULASE ACTIVITY (CELLULOSE SOLUBILIZATION) BY CELLOBIOSE AND D-GLUCOSE AND OF SYNERGISTIC ACTION BETWEEN THE COMPONENTS OF THE CELLULASE COMPLEX OF *Sclerotium rolfsii*

Enzyme	*Inhibitor (mM)*	*Inhibition by cellobiose (%)*				*Inhibition by* D-*glucose (%)*		
		Avicel[a]	*Cotton*[a]	*H_3PO_4-swollen cellulose*[b]	*CMC*[c]	*Avicel*[a]	*H_3PO_4-swollen cellulose*[b]	*CMC*[c]
Cellobiohydrolase (CBH)	0.5	52	68	10	[d]	[d]	0	[d]
CBH	2.0	86	85	14	[d]	[d]	0	[d]
Endo-β-glucanases[e] (Endo)	0.5	36	42	6	12	[d]	[d]	0
Endo	2.0	48	56	8	18	[d]	[d]	0
CBH + Endo	0.5	45	[d]	12	[d]	18	8	[d]
CBH + Endo	2.0	78	[d]	18	[d]	30	12	[d]

[a]Standard assay as described in Experimental section: CBH (300 μg) and Endo (200 μg). [b]Standard assay as described in Experimental section: CBH (20 μg) and Endo (20 μg). [c]CMC, carboxymethylcellulose, standard assay as described previously[3] containing 0.1 μg of enzyme. [d]Not determined. [e]Endo-β-glucanase A, B, and C mixed in equal amounts on protein basis.

TABLE III

HYDROLYSIS OF H_3PO_4-SWOLLEN CELLULOSE AND AVICEL AFTER A PRETREATMENT WITH CELLOBIOHYDROLASE OR ENDO-β-GLUCANASES AND WHEN ACTING IN CONCERT

Enzyme used for[a]		*Solubilization*[b] *(%)*	
Pretreatment	*Hydrolysis*	*H_3PO_4-swollen cellulose*[c]	*Avicel*[d]
None	Cellobiohydrolase (CBH) (30 min)	0.03	[e]
None	CBH (24 h)	0.82	0.88
None	CBH (7 days)	[e]	3.35
None	CBH (14 days)	[e]	3.67
None	Endo-β-glucanases[f] (Endo) (30 min)	0.24	[e]
None	Endo (24 h)	3.80	0.42
None	Endo (7 days)	[e]	1.30
None	Endo (14 days)	[e]	1.40
CBH (30 min)	Endo (24 h)	4.10	[e]
CBH (24 h)	Endo (24 h)	4.80	[e]
CBH (24 h)	Endo (7 days)	[e]	2.90
CBH (7 days)	Endo (7 days)	[e]	6.20
Endo (30 min)	CBH (24 h)	1.90	[e]
Endo (24 h)	CBH (24 h)	8.80	[e]
Endo (24 h)	CBH (7 days)	[e]	4.20
Endo (7 days)	CBH (7 days)	[e]	5.20
	CBH + Endo (24 h)	9.0	[e]
	CBH + Endo (7 days)	[e]	10.00

[a]Figures given in parenthesis indicate the time of incubation with enzymes. [b]Solubilization was determined by measuring the total soluble sugar released in the supernatant solution with the anthrone method. [c]Standard assay as described in Experimental section: CBH (5 μg) and Endo (5 μg). [d]Standard assay as described in Experimental section: CBH 300 (μg) and Endo 200 (μg). [e]Not determined. [f]Endo-β-glucanases A, B, and C mixed in equal amounts on protein basis.

to 47% (Table I), which is about the same as obtained with 0.5 mL of unfractionated culture filtrate (48%). Addition of twice the amount of cellobiohydrolase (3 mg in place of 1.5 mg) to endo-β-glucanases (1 mg) and β-glucosidases (0.25 mg) further enhanced Avicel solubilization to 49%, *i.e.*, only marginally (Table I). When acting in concert on H_3PO_4-swollen cellulose, the three types of enzymes caused an increase of 110% in solubilization over the sum of solubilizations obtained when they were acting separately. The synergistic effects observed between the three types of enzymes with cotton as substrate were similar to that obtained with Avicel, except that the extent of the solubilizations obtained was smaller. Thus, the best solubilization of all forms of cellulose is obtained with the three types of enzymes acting in concert.

Inhibition of cellulase (cellulose-solubilization) activity by cellobiose and D-*glucose.* — The inhibition of cellulose (Avicel, cotton, and H_3PO_4-swollen cellulose) degradation by cellobiose was greater with ordered cellulose than with amorphous cellulose (Table II). D-Glucose, up to a 2mM concentration, did not inhibit cellulose degradation either by cellobiohydrolase or endo-β-glucanases,

TABLE IV

HYDROLYSIS OF AVICEL, COTTON, AND H_3PO_4-SWOLLEN CELLULOSE BY VARIOUS CONCENTRATIONS OF CELLOBIOHYDROLASE AND ENDO-β-GLUCANASES[a]

Substrate	*Enzyme concentration (μg)*	*Solubilization[b] by*	
		Cellobiohydrolase (%)	*Endo-β-glucanases[c] (%)*
Avicel	100	1.8	0 95
	200	2.9	1.3
	300	3.35	1.5
	1500	4.6	2.1
Cotton	500	1.6	1.0
	1000	2.3	1.7
H_3PO_4-swollen cellulose	10	1.6	7.5
	20	3.3	9.1
	30	4.6	10.7
	50	7.6	15.4

[a]Standard assay as described in Experimental section. [b]Percent of solubilization determined by measuring the total soluble sugar released in the supernatant solution by the anthrone method. [c]Endo-β-glucanase A, B, and C mixed in equal amounts on protein basis.

though it caused a slight inhibition when the two enzymes were acting together.

Initiation of enzymic degradation of cellulose. — In order to obtain some information on the initiation by cellobiohydrolase or an endo-β-glucanase of the attack on crystalline cellulose, the following experiments were conducted. Firstly, the effect of pretreating H_3PO_4-swollen cellulose or Avicel with either cellobiohydrolase or endo-β-glucanases prior to the addition of the alternative type of enzyme was studied. When H_3PO_4-swollen cellulose was treated with cellobiohydrolase, followed (after heating and cooling) by endo-β-glucanases, the extent of solubilization was nearly equal to that obtained from a simple summation of enzyme activity (Table III). However, when H_3PO_4-swollen cellulose was treated first with endo-β-glucanases, either for 30 min or 24 h, followed by cellobiohydrolase, the extent of solubilization was about two times greater than that anticipated from a summation of separate enzyme activities. With Avicel, on the other hand, the results were just the reverse; a greater solubilization was obtained when Avicel was treated first with cellobiohydrolase, followed (after heating and cooling) by endo-β-glucanases. Furthermore, when cellobiohydrolase and endo-β-glucanases acted in concert on Avicel or H_3PO_4-swollen cellulose, a further increase in the solubilizations was observed.

In the second experiment, Avicel and H_3PO_4-swollen cellulose were treated, with either cellobiohydrolase or endo-β-glucanases, under identical conditions at several equivalent concentrations. With Avicel (which is a crystalline cellulose), it was observed that in all cases (from 100 μg to 1.5 mg of enzymes), the solubilization obtained with cellobiohydrolase was about two times higher than that obtained with the corresponding amount of endo-β-glucanases (Table IV). However, with

H_3PO_4-swollen cellulose (which is an amorphous cellulose), the extent of solubilization was 2.0–4.6 times higher (with 10–50 μg of enzymes) with endo-β-glucanases than that obtained with the corresponding amounts of cellobiohydrolase (Table IV).

DISCUSSION

The mechanism of cellulase activity in the hydrolysis of crystalline cellulose has been one of the long standing problems, and whether a cellobiohydrolase or an endo-β-glucanase initiates the attack on crystalline cellulose is currently discussed[6–8]. Reconstitution experiments with pure enzymes from *S. rolfsii* for the solubilization of crystalline (Avicel, cotton) or amorphous (H_3PO_4-swollen) cellulose showed maximum synergism with cellobiohydrolase and endo-β-glucanases, followed by endo-β-glucanases and β-glucosidases, and least with cellobiohydrolase and β-glucosidases. In contrast, Halliwall and Griffin[9] reported that a cellobiohydrolase from *Trichoderma koningii* is synergetic only with cellobiase in hydrolyzing crystalline and less complex forms of cellulose, and no other component of the cellulase system is required. However, synergism between cellobiohydrolase and endo-β-glucanases from a number of organisms has been reported for the solubilization of Avicel and H_3PO_4-swollen cellulose[6,10–14]. Whereas all the three endo-β-glucanases from *S. rolfsii* show synergistic effect with cellobiohydrolase, Wood and McCrae[15] reported that only two of the four major endo-β-glucanases isolated from *T. koningii* show synergism with the cellobiohydrolase component. Streamer *et al.*[6] could not observe a synergistic effect between cellobiohydrolase and endo-β-glucanases of *Sporotrichum pulverulentum* with H_3PO_4-swollen cellulose, though a strong synergistic response was observed between these enzymes for solubilization of Avicel and cotton. Shepherd *et al.*[16], on the other hand, reported that the degradation of crystalline cellulose could be catalyzed independently by each of the *Thermoascus aurantiacus* cellulase components, and no synergistic effect was observed between purified endo- and exo-cellulases.

When acting in concert, endo-β-glucanases and β-glucosidases from *S. rolfsii*, also showed a synergistic effect in the solubilization of Avicel and cotton (with H_3PO_4-swollen cellulose, the synergistic effect was much smaller), the solubilization being 16.0 and 6.9% when the two enzymes acted in concert as compared to 2.1 and 1.7% (sum of the solubilizations) when acting separately (Table I). The synergistic effect observed was much greater than could be explained merely by removal of cellobiose (an inhibitor of endo-β-glucanase[3,17]) by β-glucosidases. At 0.5 and 2mM concentrations, cellobiose inhibited Avicel hydrolysis by endo-β-glucanases by 36 and 48%, respectively; and that by cellobiohydrolase by 52 and 86%, respectively (Table II). The concentrations of cellobiose used as inhibitor were much higher (25–100 times) than those that would have been produced during solubilization of Avicel or cotton. Thus, it appears that the effect is more likely due to synergism between the two enzymes, and only partially due to the removal of the

inhibitory effect of cellobiose. This is supported by the observation that addition of pure cellobiose dehydrogenase (purified from *S. rolfsii*[18]) and its electron acceptor 2,6-dichlorophenol–indophenol (instead of β-glucosidases) to endo-β-glucanases to remove cellobiose had only marginal effect on Avicel solubilization. At optimum pH and temperature of Avicel solubilization (pH 4.5 and 37°), the absolute cellobiose dehydrogenase activity of *S. rolfsii* culture filtrate was 2.6, and that of BG-III β-glucosidase [which forms about 80% of the total β-glucosidase activity of *S. rolfsii* culture filtrate[1]] 2.65 μmol of cellobiose oxidized–hydrolyzed · min^{-1} · mL^{-1} of culture filtrate. Thus, even though the absolute activities of the two enzymes for removal of cellobiose were about the same, the synergistic effect observed on addition of β-glucosidases to either endo-β-glucanases, or to endo-β-glucanases plus cellobiohydrolase was much greater than that observed by the addition of an equivalent (or even greater) amount of cellobiose dehydrogenase (Table I). This result lends further support to our concept that the synergistic effect observed with endo-β-glucanases and β-glucosidases could only be due partly to the removal of cellobiose inhibition by β-glucosidases. Synergism between endo-β-glucanases and β-glucosidases have so far not been reported for other organisms. The concept that β-glucosidases do not participate directly in cellulose hydrolysis and play a role merely to relieve the inhibition of endo-β-glucanases and cellobiohydrolase does not appear to be valid, at least for *S. rolfsii*.

The four β-glucosidases purified from *S. rolfsii* behaved rather as (1→4)-β-D-glucan glucohydrolases[2]. Apparently, these glucan glucohydrolases from *S. rolfsii* act synergistically with endo-β-glucanases as does cellobiohydrolase, though to a lesser degree. This is in contrast to the findings of Wood and McCrae[19] that endo-β-glucanases do not show any synergistic effect with glucan glucohydrolase in solubilizing crystalline cellulose; *i.e.*, in *S. rolfsii*, the enzyme cellobiohydrolase that removes cellobiose units, or the glucan glucohydrolase that removes glucose residues from the cellulose-chain ends, can cooperate with endo-β-glucanases for solubilizing crystalline cellulose.

Though both cellobiohydrolase and endo-β-glucanases by themselves are able to hydrolyze both amorphous and crystalline celluloses, the present results showed that the production of a larger number of bonds accessible to hydrolysis was essential for better action of both cellulases. The greater solubilization of Avicel by cellobiohydrolase acting alone and of H_3PO_4-swollen cellulose by endo-β-glucanases acting alone (Table IV), and the beneficial effect of pretreatment of Avicel with cellobiohydrolase, and of H_3PO_4-swollen cellulose with endo-β-glucanases prior to the addition of alternative type of enzyme (Table III), suggest that endo-β-glucanase initiates the attack on amorphous cellulose (and creates more ends for the cellobiohydrolase to act) and cellobiohydrolase initiates the attack on crystalline cellulose (thereby making the substrate more accessible to hydrolysis). The recent observation that cellobiohydrolase from *S. rolfsii* also dis-

plays an initial endo-type mode of action[20] further lends support to this concept. The initiation role of cellobiohydrolase on crystalline cellulose postulated by us is in contradiction with the reports that attribute this role to an endo-β-glucanase[6,7,10,11]. Recently, Chanzy *et al.*[8] have reported that cellobiohydrolase I from *Trichoderma reesei* displays an initial, endo-type mode of action and is responsible for the disruption of cellulose crystals without the help of endo-β-glucanases. They suggested that the initial attack on crystalline cellulose was more likely due to cellobiohydrolase I.

A quantitative duplication of the solubilization of Avicel or cotton cellulose by the unfractionated culture filtrate from *S. rolfsii* was obtained by recombining cellobiohydrolase, endo-β-glucanases, and β-glucosidases in their original proportion. Therefore, it seems highly unlikely that any other type of enzyme is required for the hydrolysis of crystalline cellulose, at least in *S. rolfsii*. Reese[21] has suggested that enzyme C_1 (which has been postulated to be responsible for disruption of hydrogen bonds[22]) and cellobiohydrolase may be two different proteins which could not be separated by the purification procedure adopted. We have not observed the non-identity of enzyme C_1 and cellobiohydrolase peaks of activity by use of Avicel to detect synergism. The data also suggest that formation of the complex of the three types of enzymes is required for the efficient utilization of crystalline cellulose. The complex probably is adsorbed on the surface of the crystallite through cellobiohydrolase owing to its high affinity for crystalline cellulose. Leatherwood[23] observed, in roll-tube cultures of *Ruminococcus albus*, a protein–protein interaction which resulted in the formation of a cellulase complex that could degrade native cellulose. Wood and McCrae[24] have suggested that crystalline cellulose is hydrolyzed only by the sequential action of the two enzymes, *i.e.*, endo-β-glucanases (C_x) initially, and cellobiohydrolase (C_1), which have formed a loose complex at the surface of the cellulose chains. The postulated "initiation" role of cellobiohydrolase described herein is in contradiction to this suggestion.

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