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The role of cellulase concentration in determining the degree of synergism in the hydrolysis of microcrystalline cellulose

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Microcrystalline cellulose (10 mg of Avicel/ml) was hydrolysed to glucose by different concentrations of the purified cellulase components endoglucanase (EG) II and cellobiohydrolases (CBH) I and II, alone and in combination with each other, in the presence of excess β-glucosidase. At a concentration of 360 μg/ml (160 μg of EG II/ml, 100 μg of CBH I/ml and 100 μg of CBH II/ml) the degree of synergism among them was negligible. As the concentration of cellulase decreased, the degree of synergism increased, reaching an optimum at 20 μg/ml (5 μg of EG II/ml, 10 μg of CBH I/ml and 5 μg of CBH II/ml). There was no apparent relationship between the ratio of the components and the degree of synergism. The latter is probably due, though it could not be proved, to the level of saturation of the substrate with each component. Inhibition of Avicel hydrolysis was observed when the substrate was incubated with saturating and non-saturating concentrations of a mixture of EG II and CBH I respectively. A similar result was also observed with a combination of EG I and EG II.

INTRODUCTION

The enzymic solubilization of cellulose to glucose requires the action of three enzymes, namely cellobiohydrolase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). When acting alone on a crystalline cellulosic substrate such as cotton, these components are unable to hydrolyse this substrate to any great extent, but in combination they co-operate to effect its solubilization (Wood & McCrae, 1979). Solubilization of other microcrystalline cellulosic substrates such as Avicel by the cellobiohydrolase (CBH) and endoglucanase (EG) components acting alone can occur, but when they act in combination the resulting glucose production is greater than the sum of that produced by the components acting alone (Woodward *et al.*, 1988). This phenomenon, known as synergism, can be explained by the action of the EG component on cellulose (which catalyses the hydrolysis of the glycosidic bonds along the length of the cellulose chain), resulting in the formation of new cellulose chain ends upon which the CBH component then acts, releasing cellobiose.

Two cellobiohydrolases (CBH I and CBH II), both produced by the fungi *Trichoderma reesei* and *Penicillium pinophilum*, have been purified to homogeneity and act synergistically on microcrystalline cellulosic substrates such as Avicel with large numbers of end groups (Fägerstam & Pettersson, 1980; Wood & McCrae, 1986). This type of synergism is difficult to explain, unlike the synergism between the CBH and EG components, but Wood & McCrae (1986) have postulated that CBH I and CBH II may be stereospecific enzymes attacking the two sterically different non-reducing end groups that occur in the cellulose chain.

Few studies have addressed the conditions under which synergism between the CBH and EG components occurs. Recently Woodward *et al.* (1988) measured the maximum amount of CBH I, CBH II, EG I and EG II protein that would bind to a given quantity of Avicel. They found that synergism among these components was greatest when the substrate was not being saturated with enzyme components during hydrolysis. This raised the possibility that the enzymic hydrolysis of cellulose could be more efficient in terms of enzyme requirement if the precise conditions under which synergism is greatest are understood.

The present study investigated the relationship between the concentration of CBH I, CBH II and EG II and the degree of synergism obtained among these components when acting on Avicel. An optimum concentration of these components appeared to exist for maximum synergistic effect when they were added simultaneously to a reaction mixture containing Avicel. Synergism between cellulase components also appears to be independent of their ratio in a reaction mixture and dependent only on their concentration.

EXPERIMENTAL

Materials

Purified samples of CBH I, CBH II, EG I and EG II from a proprietary strain of *Trichoderma reesei* were generously provided by Dr. Sharon Shoemaker of Genencor, San Francisco, CA, U.S.A. Practical-grade (type I) cellulase from *Aspergillus niger* was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Microcrystalline cellulose (Avicel, PH-105) was a gift from FMC Corporation, Philadelphia, PA, U.S.A.

Preparation of β-glucosidase

Cellulase from *Asp. niger* was fractionated by gel filtration on Bio-Gel P-100 as described by Woodward *et al.* (1986). This procedure separated most of the

Abbreviations used: CBH, cellobiohydrolase; EG, endoglucanase.

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endoglucanase activity from the β-glucosidase activity. Although the endoglucanase from *Asp. niger* possesses the ability to hydrolyse soluble CM-cellulose, it exhibits no activity towards insoluble microcrystalline cellulose owing to its lack of affinity for this substrate (Klyosov *et al.*, 1986). Also, *Asp. niger* endoglucanase does not act synergistically with *Tr. reesei* CBH to hydrolyse Avicel (N. E. Lee & J. Woodward, unpublished work). The fractions containing β-glucosidase (cellobiase) activity, assayed as described previously (Woodward & Arnold, 1981), were pooled and contained 5.4 units/ml.

Hydrolysis of Avicel by EG I, EG II, CBH I and CBH II alone and in combination

Avicel (10 mg/ml) was incubated with different concentrations of EG I, EG II, CBH I and CBH II, alone and in combination, with excess β-glucosidase in 50 mM-sodium acetate buffer, pH 5.0, at 50 °C with constant stirring. The total volume of the reaction mixture was 5.0 ml, and at specified times (see the Figures) a suitably diluted sample was assayed for glucose. The cellulase components were added simultaneously to the reaction mixture. The degree of synergism is defined as the ratio of the glucose produced by the combined action of cellulase components on Avicel to the total glucose produced by their independent actions. A degree of synergism of 1 or less is indicative of zero synergism or inhibition respectively during the reaction.

Analytical methods

Glucose was measured by using the hexokinase assay reagent (Sigma Chemical Co.). Protein was assayed by using the Coomassie Blue reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.) according to the method of Bradford (1976). All absorbance measurements were made with a Carey 219 spectrophotometer possessing a variable spectral slit width in the range 0.05–3.6 nm.

RESULTS AND DISCUSSION

Synergism is dependent upon cellulase concentration

Synergism among EG II, CBH I and CBH II was observed when these components were used to hydrolyse Avicel to glucose (Fig. 1). The degree of synergism, however, was dependent upon the concentration of these cellulase components (Fig. 2). At the highest saturating concentration of cellulase used, 360 μg/ml (160 μg of EG II/ml, 100 μg of CBH I/ml and 100 μg of CBH II/ml), little synergism was observed. However, as the concentration of each component decreased, the degree of synergism increased, reaching a maximum at a cellulase concentration of 20 μg/ml (5 μg of EG II/ml, 10 μg of CBH I/ml and 5 μg of CBH II/ml). At lower concentrations than these the degree of synergism declined, suggesting that an optimum concentration of cellulase components exists for maximum synergistic effect on Avicel. At concentrations of enzyme components above those that result in maximum synergism, the lower degree of synergism could be explained by overcrowding of the substrate binding sites. This would then hinder the migration of components from one site to another, which is necessary for their co-operative action. Conversely, as the concentration of components becomes limiting, co-operation between them is decreased.

In previous work (Woodward *et al.*, 1988) we studied the binding kinetics of EG I, EG II, CBH I and CBH II

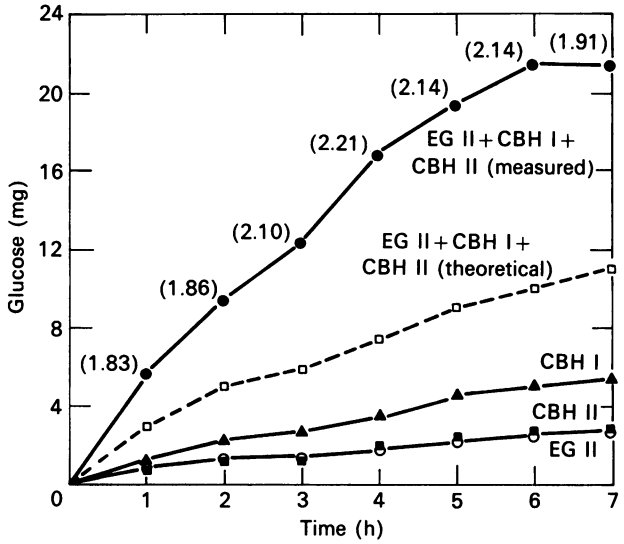

Fig. 1. Hydrolysis of Avicel by *Tr. reesei* EG II, CBH I and II alone and in combination

Avicel (10 mg/ml) was incubated with EG II (5 μg/ml), CBH I (10 μg/ml) and CBH II (5 μg/ml) alone or in combination. β-Glucosidase (1.08 units) was added to each reaction mixture to produce a total volume of 5.0 ml. The reaction was carried out in 50 mM-acetate buffer, pH 5.0, and at the times indicated glucose production was monitored by using the hexokinase assay reagent. The numbers in parentheses represent the degree of synergism. The broken line indicates the theoretical glucose production, which was determined by the summation of the glucose produced by the independent action of the cellulase components.

to Avicel. From the saturating binding data that were obtained in these earlier studies it was possible to determine, for each component, the amount of enzyme that must be added to a given quantity of Avicel in order to achieve a certain level of saturation. At the highest concentration of enzyme used in the present study the substrate will be saturated with each component, assuming that it possesses specific binding sites for the components that also bind in the presence of each other. If this assumption is true, then the maximum synergistic effect is obtained at 35, 50 and 26% saturation of substrate for EG II, CBH I and CBH II respectively. It is not possible at present to prove this, however, because it is not known how much of one component binds to Avicel in the presence of another.

Synergism between cellulase components from different as well as the same sources has been reported (Coughlan *et al.*, 1987), with the degree of synergism varying from 2.0 to 4.8 depending on the source of the components. For example, in a mixture of *Trichoderma koningii* CBH (40 μg/ml), EG (2.8 units) from *Talaromyces emersonii* and β-glucosidase (0.03 unit) from *Fusarium solani* that was incubated with Avicel (10 mg/ml) in buffer, pH 5, at 50 °C for 24 h, the degree of synergism was 4.8. It is uncertain whether the amount of the EG or CBH components used in this case was saturating the substrate. If the phenomenon, namely that the degree of synergism is dependent upon the concentration of the cellulase components, is true for cellulases from all sources (at least acting on Avicel), then it is

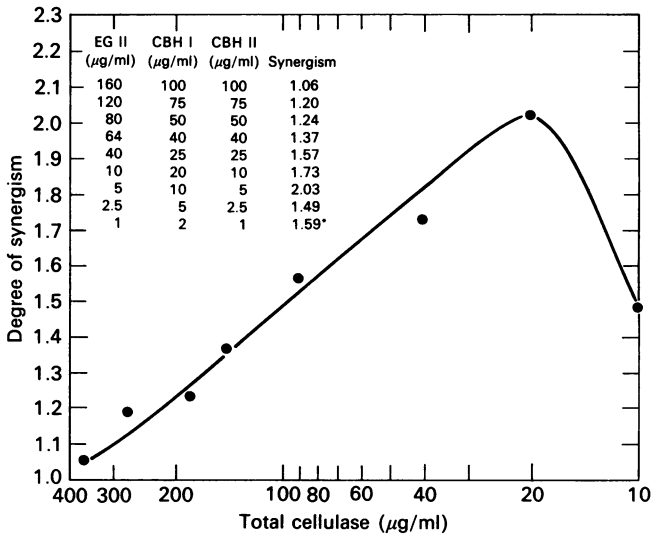

Fig. 2. Hydrolysis of Avicel by different concentrations of *Tr. reesei* EG II, CBH I and CBH II acting in combination.

Avicel (10 mg/ml) was incubated with different combinations of purified cellulase components (see Table inset) in 50 mM-acetate buffer, pH 5.0, at 50 °C, producing a total volume of 5.0 ml. Glucose formation was monitored at hourly intervals for 7 h. The degree of synergism was determined by averaging the synergism measured among the components at each hourly interval (see Fig. 1). A degree of synergism of 1 indicates that the glucose produced by the components acting alone equals that produced by their combined action. Each reaction mixture contained 1.08 units of β-glucosidase. * Not shown on graph.

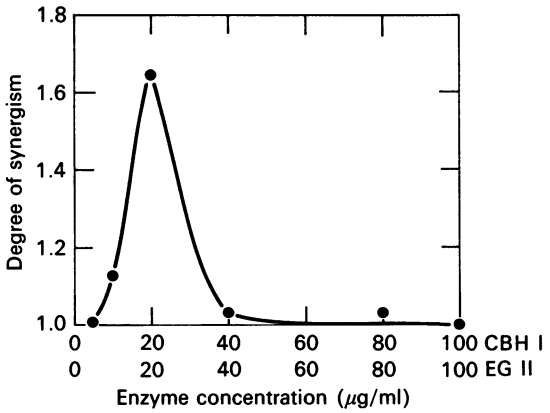

Fig. 3. Hydrolysis of Avicel by different concentrations of EG II and CBH I in combination in 1:1 ratio

For details see the legend to Fig. 2. In these experiments the total volume was 2.0 ml, and glucose formation was monitored for 4 h.

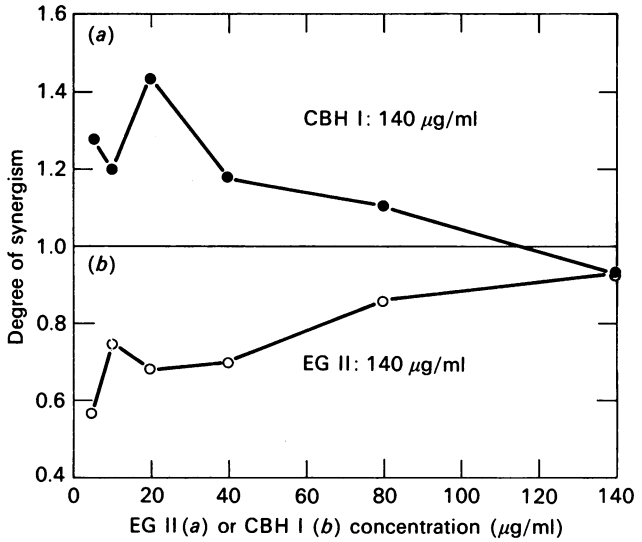

Fig. 4. Hydrolysis of Avicel by a combination of different ratios of EG II and CBH I

Avicel (10 mg/ml) was incubated (*a*) with CBH I (140 μg/ml) and various concentrations of EG II and (*b*) with EG II (140 μg/ml) and various concentrations of CBH II as indicated on the horizontal axis. For other details see the legend to Fig. 2. A degree of synergism of less than 1 indicates that the amount of glucose produced by the combination of constant EG II and various CBH I concentrations was less than that expected by the summation of the glucose produced by their independent actions.

likely that the concentration of cellulase used was insufficient to saturate the substrate. Also, the concentration of a cellulase component required to saturate a given amount of substrate may vary depending on the source. This is also true for EGs and CBHs from the same source (Beldman *et al.*, 1987).

Synergism is not dependent on the ratio of components

In a cellulase mixture approx. 70% of the protein is CBH (Reese, 1982), and thus the ratio of CBH to other proteins in the mixture, including the EG and β-glucosidase components, is approx. 2:1. Previous studies have addressed the effect of the ratio of cellulase components on the synergism obtained between them. Henrissat *et al.* (1985) found that a 1:1 ratio between CBH I and EG I or EG II and a 19:1 ratio between CBH II and EG I or EG II were required for maximum synergistic degradation of Avicel. Wood & McCrae (1986) found a 1:1 ratio between CBH I and CBH II to be most effective for Avicel hydrolysis. Neither of these studies addressed the relationship between the ratio of components at concentrations that saturated the substrate and the degree of synergism.

In the present study different concentrations of CBH I and EG II at the ratio of 1:1 have been used to hydrolyse Avicel; it was found that the degree of synergism was independent of the ratio but dependent on the concentrations of CBH I and EG II (Fig. 3). Optimum concentrations of both EG II and CBH I were apparently required for a maximum degree of synergism, in agreement with the data in Fig. 2, which also indicate a lack of relationship between the relative proportions of EG II, CBH I, CBH II and the degree of synergism.

In other experiments Avicel was hydrolysed by a combination of different ratios of EG II to CBH I in which the concentration of either enzyme was held constant (140 μg/ml; 100% saturation of Avicel) and the other varied. Between ratios of CBH I to EG II ranging from 28:1 to 1:1, with the CBH I concentration constant at 140 μg/ml, there was no obvious correlation

to the degree of synergism, although there seemed to be a tendency toward a lower degree as the ratio decreased (Fig. 4*a*).

If the concentration of EG II was held constant at 140 μg/ml while the concentration of CBH I was varied, the amount of glucose formed by different ratios of EG II and CBH I was less than that theoretically expected, as determined by the summation of the glucose formed by their independent actions. This is indicated by a degree of synergism of less than 1, indicating inhibition of the reaction occurring when EG II and CBH I are acting in combination on Avicel under these conditions (Fig. 4*b*). In this case the degree of synergism tended to increase as the ratio of EG II to CBH I decreased, i.e. the converse of the situation produced when the CBH I concentration was constant.

Ryu *et al.* (1984) have noted that when CBH is added to Avicel upon which EG is absorbed the latter becomes desorbed, suggesting that there is competition between these components for cellulose binding sites. The question then arises, why should EG II and CBH I impede the activity of each if they bind at specific catalytic sites? Chanzy *et al.* (1984) showed that gold-labelled CBH I binds along the length of cellulose microfibrils rather than just at the chain ends where this enzyme would be expected to bind and act catalytically. If EG II also binds randomly along the cellulose chain, which would be expected in view of its mode of action, then at saturation of Avicel with this component the binding of CBH I would be impeded, especially at the numerous non-reducing ends. Thus in this case a degree of synergism of less than 1 can be easily understood.

The converse situation should also be true for the case when Avicel is saturated with CBH I, according to the data of Chanzy *et al.* (1984). However, when EG II is added, synergism between these components, albeit to a small degree, is observed (Fig. 4*a*). These data could be explained by the fact that, although CBH I binds randomly along the Avicel chain length, EG II catalyses the hydrolysis of certain 'exposed' intrachain glycosidic bonds, creating new chain ends upon which CBH I can then act. Whether CBH I is able to hydrolyse internal glycosidic bonds is not known. However, White & Brown (1981) found that high-resolution electron microscopy of cellulose from *Acetobacter xylinum* (incubated with CBH for 30 min) revealed no change in structure, although the cellulose ribbon was coated with enzyme particles. This would suggest that, although CBH I binds along the length of the chain, only that enzyme which is bound to the chain ends can act catalytically. The remaining enzyme may only act once a new chain end is formed.

Extent of glucose formation in relationship to synergism

Table 1 shows the relationship between the amount of glucose formed by the action of EG II, CBH I and CBH II on Avicel as a function of the percentage of the highest concentration of these components used (360 μg/ml). This concentration of cellulase resulted in the greatest glucose production in 7 h (approx. 51 mg). About 75% (approx. 38.3 mg) of this amount of glucose was produced by 40 μg of cellulase/ml, or 11% of the maximum enzyme concentration. In the absence of synergism 40 μg of cellulase/ml would produce 22.1 mg of glucose, or about 45% of the greatest theoretical amount of glucose that would be formed (48.6 mg) by the components acting alone. These data indicate that because of synergism the enzyme requirement for the production of a given quantity of glucose can be decreased. Presumably, the glucose concentration produced by the highest enzyme concentration can be achieved with 40 μg of enzyme/ml by increasing the incubation time of substrate and enzyme.

Table 1. Hydrolysis of Avicel as a function of the percentage of maximum enzyme concentration

For details see the legend to Fig. 2. The amount of glucose produced is that after 7 h. Maximum enzyme concentration equals 360 μg/ml (160 μg of EG II/ml, 100 μg of CBH I/ml and 100 μg of CBH II/ml).

Enzyme concentration (% of maximum)	Glucose production			
	Theoretical*		Measured	
	(mg)	(% of maximum)†	(mg)	(% of maximum)†
1.1	5.8	12	9.3	18
2.8	9.8	20	14.6	28
5.6	10.4	21	21.1	41
11.1	22.1	45	38.3	74
25	23.0	47	39.1	76
40	30.7	63	42.1	82
50	28.1	58	34.8	68
75	38.5	79	46.2	90
100	48.6	100	51.5	100

* Summation of glucose produced by components acting alone.

† Percentage of glucose produced by maximum enzyme concentration.

Table 2. Hydrolysis of Avicel by EG I and EG II alone and in combination

Avicel (10 mg/ml) was incubated with EG I (20 μg/ml) and/or EG II (10 μg/ml) and with EG I (100 μg/ml) and/or EG II (160 μg/ml). For other details see the legend to Fig. 1 and Table 1. Glucose was measured after 24 h.

Enzyme concentration	Glucose concentration (mg/5 ml)		$100 \times B/A$ (%)
	Theoretical (*A*)	Measured (*B*)	
EG I + EG II (30 μg/ml)	7.3	4.8	66
EG I + EG II (260 μg/ml)	19.5	8.1	41

Hydrolysis of Avicel by EG I and EG II

Hydrolysis of Avicel was performed with a combination of EG I and EG II at low and high (saturating) concentrations. The amounts of glucose formed after 24 h were measured and compared with the theoretical totals, which were obtained by summing the glucose formed by these enzymes acting alone (Table 2).

Inhibition of Avicel hydrolysis was observed when EG I and EG II acted in combination with each other, suggesting that they compete with each other for the same binding sites on Avicel. Less inhibition was observed at the lower concentrations.

Our results also showed that, when a mixture of substrate-saturating concentrations of EG I, EG II, CBH I and CBH II hydrolyses Avicel, less glucose is produced compared with the sum of the glucose formed by their independent actions (results not shown). This apparent inhibition could be caused by competition between EG I and EG II, as noted above. Another possibility is that at saturating concentrations of EG I and EG II binding of the CBH components is impeded. The data of Fig. 4 and Table 2 lend support to these hypotheses.

CONCLUSIONS

Synergism between cellulase enzyme components acting on Avicel is dependent upon their individual concentrations and not on their relative proportions. At high concentrations of cellulase co-operation between cellulase components (synergism) will be hindered because all the binding sites, for which the components appear to compete, will be saturated. If the substrate is not saturated with enzyme, as would be the case at low enzyme concentrations, synergism occurs because, presumably, such competition is eliminated. For the efficient and economic use of cellulase to hydrolyse cellulose, it is important to use a concentration of enzyme at which synergism among components is significant.

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