The mechanism of fungal cellulase action

Synergism between enzyme components of *Penicillium pinophilum* cellulase in solubilizing hydrogen bond-ordered cellulose

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Studies on reconstituted mixtures of extensively purified cellobiohydrolases I and II and the five major endoglucanases of the fungus *Penicillium pinophilum* have provided some new information on the mechanism by which crystalline cellulose in the form of the cotton fibre is rendered soluble. It was observed that there was little or no synergistic activity either between purified cellobiohydrolases I and II, or, contrary to previous findings, between the individual cellobiohydrolases and the endoglucanases. Cotton fibre was degraded to a significant degree only when three enzymes were present in the reconstituted enzyme mixture: these were cellobiohydrolases I and II and some specific endoglucanases. The optimum ratio of the cellobiohydrolases was 1:1. Only a trace of endoglucanase activity was required to make the mixture of cellobiohydrolases I and II effective. The addition of cellobiohydrolases I and II individually to endoglucanases from other cellulolytic fungi resulted in little synergistic activity; however, a mixture of endoglucanases and both cellobiohydrolases was effective. It is suggested that current concepts of the mechanism of cellulase action may be the result of incompletely resolved complexes between cellobiohydrolase and endoglucanase activities. It was found that such complexes in filtrates of *P. pinophilum* or *Trichoderma reesei* were easily resolved using affinity chromatography on a column of *p*-aminobenzyl-1-thio- β -D-cellobioside.

INTRODUCTION

There is now world-wide interest in the commercial potential of using cellulases to generate glucose feed-stocks which can be used for further chemical and biological conversion. Not surprisingly, this interest has resulted in considerable research activity in the area in recent years. Studies aimed at the elucidation of the mechanism by which the most recalcitrant hydrogen bond-ordered areas (crystalline) of the cellulose are rendered soluble have featured strongly in these investigations (Coughlan, 1985; Enari and Niku-Paavola, 1987; Wood, 1989), the rationale being that a solution to the mechanism by which these crystalline areas are solubilized is crucial to any well-controlled attempt to maximize the enzymatic conversion of cellulose for commercial and other purposes.

Cellulase preparations obtained from culture filtrates of only a few fungi have been found to have the capacity for solubilizing crystalline cellulose. Remarkable in this respect are those from *Trichoderma reesei*, *T. viride*, *T. koningii*, *Fusarium solani* and *Penicillium pinophilum/funiculosum* (Eriksson & Wood, 1985). Working with these cellulases, it has been established that enzyme systems that can degrade crystalline cellulose contain enzymes, loosely defined as exo-1,4- β -glucosidases (normally cellobiohydrolases), endo-1,4- β -glucanases and β -glucosidases. It has also been shown that each type of enzyme is usually found in these cellulase systems in multiple forms, and that the multiple forms interact synergistically to solubilize crystalline cellulose (Wood, 1985; Henrissat *et al.*, 1985; Enari & Niku-Paavola, 1987). However, despite there having been significant advances made in the understanding of the individual

enzymes, the nature of the interactions occurring between the enzymes in the synergistic process are not yet clearly understood (Wood, 1985), and the problem continues to be the subject of intense debate in the literature (Wood, 1989).

There is a consensus that synergism between cellobiohydrolase and endoglucanases is a characteristic of cellulases that can degrade crystalline cellulose. However, some workers interpret data which they have obtained with purified cellobiohydrolases and some endoglucanase fractions to indicate that endoglucanases have relatively minor roles to play, much of the activity being accounted for either by a cellobiohydrolase acting alone (Berghem *et al.*, 1975) or by the synergistic interaction of two immunologically unrelated cellobiohydrolases (Gritzali & Brown, 1979; Fagerstam & Pettersson, 1980; Enari, 1985). The reason for these differences in opinion is not immediately obvious, but it would seem that much of the uncertainty relates to the differing views that are held on the substrate specificity and mode of action of cellobiohydrolases which have been purified to apparent homogeneity in different ways. Enzyme–enzyme complexes have been shown to exist in cellulase preparations from *T. reesei* (Sprey & Lambert, 1983), and it has been suggested that failure to resolve these complexes into their individual components could account for many of the differing views published (Wood, 1989).

Whatever the explanation, most reports suggest that significant activity on crystalline cellulose is obtained only when two enzymes are present (Wood, 1985, 1988). These may be cellobiohydrolase I plus immunologically unrelated cellobiohydrolase II (Enari, 1985) or cellobiohydrolase I plus endoglucanase activity (Wood, 1985). In this report, we now show that the activity of the

cellulase of *P. pinophilum/funiculosum* cannot be described in these simple terms. After a multistage purification procedure resulting in the isolation of two highly purified cellobiohydrolases (I and II) and five major endoglucanases (I, II, III, IV and V), we have found evidence that indicates that a meaningful rate of hydrolysis of crystalline cellulose in the form of the cotton fibre is obtained only with an enzyme mixture that contains at least three enzymes; namely, cellobiohydrolases I and II and either endoglucanase III or V.

Part of this work has already been presented in a preliminary form (Wood *et al.*, 1988).

EXPERIMENTAL

Materials

P. pinophilum C.M.I. 87160iii was obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. Avicel (PH101) was purchased from Honeywell & Stein, London W.1., U.K., Immobilines from Pharmacia Ltd, Prince Regent Road, Hounslow, Middlesex TW3 1NE, U.K., and Acrylagel and Bis-Acrylagel from National Diagnostics, Highland Park, New Jersey, U.S.A. All reagents were AnalaR grade or equivalent and were bought either from BDH Chemicals, Poole, Dorset, U.K. or from Sigma Chemical Co., Poole, Dorset, U.K.

Extraction of barley β -glucan

This was done by the method of Preece & Mackenzie (1952).

Preparation of purified enzyme components

Cellobiohydrolases I and II were separated from the other components of the cellulase complex and purified to apparent homogeneity on polyacrylamide isoelectric focusing gels and SDS/polyacrylamide gels as previously described (Wood & McCrae, 1986a,b). However, a final stage involved the use of an affinity column of *p*-aminobenzyl-1-thio- β -D-cellobioside (van Tilbeurgh *et al.*, 1984).

An alternative method of preparing cellobiohydrolase II involved the use of electrofocusing in polyacrylamide gels with an immobilized pH gradient covering the range 3.5–6.0. LKB-Pharmacia Immobiline was used throughout according to LKB Application Note 321.

Acidic Immobiline mixture. A mixture of Immobilines, pK 3.6 (300 μ l), pK 4.6 (223 μ l) and pK 6.2 (158 μ l) were diluted to 7.5 ml with distilled water and adjusted to pH 3.5 with Immobiline pK 9.4.

Basic Immobiline mixture. A mixture of Immobilines, pK 3.6 (390 μ l), pK 4.6 (522 μ l), pK 6.2 (276 μ l) and pK 9.3 (722 μ l) were diluted to 7.5 ml with distilled water and adjusted to pH 6.2 with Immobiline pK 3.6.

Acidic (dense) solution. This was composed of Acidic Immobiline mixture (above) (7.5 ml), Acrylagel (2.43 ml), Bis-Acrylagel (1.13 ml), glycerol (87%, 4.2 ml) and water to give a total volume of 15 ml.

Basic (light) solution. This was composed of Basic Immobiline mixture (above, 7.5 ml), Acrylagel (2.43 ml), Bis-Acrylagel (1.13 ml) and water to give a total volume of 15 ml.

The gels (0.5 cm) were prepared exactly as described in

LKB Application note 321 using 20 μ l of *NNN'*-tetramethylethylenediamine (TEMED) (diluted 1:10) and 20 μ l of ammonium persulphate (10%, w/v).

A sample of *P. pinophilum* cellulase which had been desalted on a column of Biogel P6 DG was applied to the 15 wells in the gel [13.5 mg of protein (Folin) in total]. The enzyme was focused using constant power (5 W; 1500 V) at 10 °C for 24 h. After 24 h, two bands of protein could be seen as slightly swollen areas at points on the gel. By blotting the wells and applying fresh enzyme to the wells at 24 h intervals, droplets of protein solution proud of the gel surface were observed. These droplets were removed using a capillary tube. Finally, the gel was cut into strips and the areas which were swollen were immersed in 0.05 M-acetate buffer, pH 3.8, and left to stand at 4 °C overnight. The gel was sonicated (MSE Soniprep) for 4 min (30 s on/off intervals) at 1 °C. The suspension was filtered through Whatman GFA filters washed with 0.2 M-acetate buffer, pH 5.4. Protein in the filtrate was precipitated with (NH₄)₂SO₄ (85% saturation) at 1 °C, centrifuged (75000 *g*; 20 min) and the pellet was redissolved in 0.1 M-acetate buffer, pH 5.0. Single protein bands were detected on polyacrylamide isoelectric focusing gels (Coomassie Blue stain).

The purification of endoglucanases I, II, III, IV and V was effected by a 19-step procedure involving ion-exchange chromatography, gel filtration, isoelectric focusing and chromatofocusing: the details are published elsewhere (Bhat *et al.*, 1989).

Preparation of cell-free enzyme preparations from fungi other than *P. pinophilum*

Cultures of the fungi *Myrothecium verrucaria*, *Stachybotrys atra*, *Memnoniella echinata*, *Aspergillus awamori*, *Thermoascus aurantiacus* and *Chaetomium thermophile* were prepared in shake flask culture using 100 ml of the medium of Mandels & Weber (1969) in a 250 ml conical flask. The flasks were shaken at 150 rev./min for 14 days at 28 °C, and the enzyme was isolated from the supernatant by precipitation with ammonium sulphate until the saturation was 80%. The pellet obtained by centrifuging at 75000 *g* for 20 min was redissolved in a 0.1 M-acetate buffer, pH 5, and stored at –18 °C until used.

Enzyme assays

Activity to cotton. The activity towards cotton fibre (dewaxed; Corbett, 1963) was measured using 1, 2 or 5 mg of substrate. The 1 and 2 mg samples were incubated for 7 days at 37 °C, and then the residual cellulose was determined by potassium dichromate-sulphuric acid reagent (Wood, 1969). The 5 mg sample was incubated at 37 °C for the times stated in the Results section in a reaction mixture containing 2.5 ml of 0.2 M-acetate buffer, pH 4.8, 0.1 ml of sodium azide solution (0.05 M), and enzyme and water to give a total volume of 5 ml: the sugar solubilized was measured by reducing sugar (Nelson, 1952) and/or phenol/sulphuric acid methods (Dubois *et al.*, 1956).

Activity to Avicel. The reaction mixture, consisting of 2.5 ml of a 0.2% (w/v) suspension of Avicel (PH 101) in 0.2 M-acetate buffer, pH 4.8, 0.1 ml of NaN₃ (0.05 M) solution and enzyme and water to give a total volume of 5.1 ml, was incubated at 37 °C for 5 days. The suspension was centrifuged and 0.5 ml of the supernatant was used

for assay of reducing sugar (Nelson/Somogyi) and/or total sugar (phenol/H₂SO₄).

In another assay the incubation period was 21 days. In this case fresh enzyme was added at intervals of 7 days. This was done by removing the supernatant after centrifugation, washing with buffer, and centrifugation, and adding the enzyme to the residual Avicel.

Activity to CM-cellulose. This assay was carried out using the reducing sugar method described previously by Wood & McCrae (1977).

Other assays. Reducing sugar was determined by the method of Nelson (1952) and total sugar by the method of Dubois *et al.* (1956). In each case the reagents were calibrated using D-glucose as standard.

The effect of cellobiohydrolase and endoglucanase enzymes on barley glucan was measured as follows. Barley glucan (1 g) was suspended in distilled water (75 ml) and heated gently to effect complete solution, cooled and made up to 100 ml. An aliquot of this substrate (5 ml), 5 ml of acetate buffer, pH 5.0, 0.2 ml of sodium azide (0.05 M) and enzyme (20 µg of protein) were mixed and incubated at 30 °C in a test tube. At various time intervals, the time of outflow of the reaction mixture from a viscometer was measured (Wood & McCrae, 1972). Another sample (0.5 ml) was taken at the same time intervals, boiled for 5 min, cooled and the reducing sugar present determined by the method of Nelson (1952).

Cellobiohydrolase antiserum

Antisera to purified cellobiohydrolases I and II were prepared as previously described (Wood & McCrae, 1986a).

RESULTS AND DISCUSSION

Purity and substrate specificity of cellobiohydrolases I and II

Cellobiohydrolases I and II were purified by a series of chromatographic procedures involving gel filtration, ion exchange and chromatofocusing (Wood & McCrae, 1986a,b). The proteins were found to give single bands (pI, 4.4 for cellobiohydrolase I; pI, 5.0 for cellobiohydrolase II) after polyacrylamide-gel isoelectric focusing (Fig. 1a) and SDS/polyacrylamide-gel electrophoresis. On the basis of the Ouchterlony diffusion technique, the two cellobiohydrolases appeared to be immunologically distinct (Fig. 1b).

The ability to produce reducing sugars from CM-cellulose is accepted as being a property which is characteristic of endoglucanases. A typical specific activity for the endoglucanases of *P. pinophilum* was 16–30 i.u./mg of Folin protein. Purified cellobiohydrolases, in the main, have little capacity for hydrolysing CM-cellulose, but recently Enari & Niku-Paavola (1987) have isolated a cellobiohydrolase II component from *T. reesei* which was still relatively rich in activity to CM-cellulose.

The purified cellobiohydrolases from *P. pinophilum* isolated in the present study showed very little activity to CM-cellulose. Thus, using a CM-cellulose substrate with an apparent degree of substitution of 0.6, cellobiohydrolase I had a specific activity of only 0.06 (i.u./mg), while the value for cellobiohydrolase II was 0.05. Neither cellobiohydrolase I nor II [using 52 µg and

Fig. 1. Examination of cellobiohydrolases I and II for purity

(a) Isoelectric focusing in polyacrylamide gels. (b) Double immunodiffusion by the technique of Ouchterlony (1967). For details of the methods, see the Experimental section.

33 µg of protein (Folin-Lowry), respectively] showed any capacity for reducing the viscosity of a 1 % (w/v) solution (10 ml) of CM-cellulose in 1 h at 27 °C.

Cellobiohydrolase II of *P. pinophilum* was also isolated from the crude cellulase preparation and purified to apparent homogeneity (polyacrylamide-gel isoelectric focusing and SDS/polyacrylamide-gel electrophoresis) by repeated isoelectric focusing in Immobiline gels, as described in the Experimental section. Purified in this way, the cellobiohydrolase was still capable of hydrolysing CM-cellulose to a significant extent (specific activity of 0.7 i.u./mg using the reducing method of analysis). However, when the enzyme was applied to an

Fig. 2. The effect of purified cellobiohydrolase and endoglucanases of *P. pinophilum* on the viscosity of a solution of barley β -glucan

The incubation mixture (for details, see the Experimental section) contained 20 μ g of either cellobiohydrolase I (○) or II (●) or endoglucanases I (◆), II (△), III (▲), IV (□) or V (■). The time of outflow of water was 25 s.

affinity column of *p*-aminobenzyl-1-thio β -D-cellobioside coupled to Affigel-10 (Biorad) (van Tilbeurgh *et al.*, 1984), some CM-cellulase activity was eluted with the buffer. The cellobiohydrolase which was eluted with aqueous cellobiose (0.01 M) now had a specific activity of 0.05 to CM-cellulose, which is similar to that obtained with the more drastic purification procedure involving multiple methods (Wood & McCrae, 1986a,b).

Purified cellobiohydrolase I of *T. reesei* has been reported to degrade the β -D-glucan from barley with a random action typical of an endoglucanase (Henrissat *et al.*, 1985). As viscosity is a parameter related to chain length, a randomly acting enzyme would be expected to effect a rapid fall in viscosity while, with an end-wise acting enzyme, the fall in viscosity would be expected to be slow. As seen in Fig. 2, the viscosity of a solution of barley β -glucan was virtually unaffected by cellobiohydrolase I of *P. pinophilum*, while the effect of

cellobiohydrolase II was minimal (Fig. 2): the same amount (20 μ g of Folin-Lowry protein) of any one of endoglucanases I, II, III, IV and V enzymes, in contrast, effected a dramatic reduction in the viscosity (Fig. 2).

On the basis of these results, the mode of action of the cellobiohydrolases of *P. pinophilum* would appear to be different from that proposed to operate in *T. reesei*; and this is highly unlikely. A more logical explanation is that cellobiohydrolase has a marked tendency to form aggregates with endoglucanases and that these aggregates are extremely difficult to break up into the component parts. At this stage it is not clear whether these enzyme aggregates are adventitious or whether these are specific complexes orientated for maximum effect in solubilizing crystalline cellulose, as would seem to be the case in the cellulase from the anaerobic bacterium *Clostridium thermocellum* (Mayer *et al.*, 1987; Lamed & Bayer, 1988). However, the existence of these complexes is real, and this has been elegantly demonstrated by Sprey & Lambert (1983), who showed that a component of *T. reesei* cellulase, which gave a single band on isoelectric focusing, could be separated into six different hydrolytic activities after treatment with the dissociating agent urea-octyl glucoside. We have also obtained good evidence for the existence of enzyme-enzyme complexes during attempts to purify the cellulase components of *T. reesei*, and the purification of cellobiohydrolase II of *T. reesei* has proved particularly difficult for this reason. The cellobiohydrolase II enzyme was found to be associated with a relatively large and constant amount of CM-cellulase activity after repeated chromatography under a variety of conditions, including affinity chromatography on cellulose, as described by Nummi *et al.* (1983). However, surprisingly, a large fraction of the CM-cellulase activity was separated from the cellobiohydrolase II after affinity chromatography on a column of *p*-aminobenzyl-1-thio- β -D-cellobioside bound to Affigel-10 (Biorad), as described by van Tilbeurgh *et al.* (1984). Clearly, the tendency of these enzymes to form aggregates in solution could explain the widely held views for the substrate specificities of the cellobiohydrolases in particular.

Solubilization of crystalline cellulose by the cellobiohydrolases of *P. pinophilum* acting alone and in concert

Neither purified cellobiohydrolase I nor II of *P. pinophilum* showed a significant amount of activity on cotton fibre (Table 1), even when the incubation was allowed to proceed for 21 days and fresh enzyme was

Table 1. Solubilization of crystalline cellulose in the form of cotton fibre and Avicel by cellobiohydrolases I and II of *P. pinophilum* acting alone and in combination

Abbreviation: CBH, cellobiohydrolase. The assay involving 5 mg cotton (see Experimental section) was used. Fresh enzyme (200 μ g of CBH I and/or CBH II) was added at 7 day intervals where appropriate.

Enzyme	Substrate ... Incubation time ...	Solubilization (%)			
		Cotton		Avicel	
		7 days	21 days	5 days	21 days
CBH I		4	6	9	18
CBH II		4	4	7	13
CBH I + CBH II		13	14	25	38

added at 7 day intervals. A 1:1 mixture of the cellobiohydrolases was more effective, and solubilized the sample of cotton to the extent of 14 %. A slightly higher degree of hydrolysis was effected by the same enzyme mixture in only 5 days when Avicel was the substrate: prolonged incubation (21 days) resulted in 38 % hydrolysis. These results differ dramatically from those of Enari (1985) and Pettersson *et al.* (1981), who showed that 90 % of the activity to crystalline cellulose in the form of the cotton fibre could be accounted for by the synergistic interaction of cellobiohydrolases I and II of *T. reesei*.

Another point of difference in the studies on *T. reesei* and our study on *P. pinophilum* was in the fragmentation of the cotton into short fragments. A mixture of cellobiohydrolases I and II of *T. reesei* resulted in the extensive breakdown of the cotton into short fibres (Enari, 1985), but we can now report that a mixture of cellobiohydrolases I and II of *P. pinophilum* did not release short fibres under the same conditions. We have found that the breakdown of cotton into short fragments requires the presence of some of the endoglucanase components. Thus, the addition of only 0.01 i.u. of CM-cellulase activity from a reconstituted mixture of the five major endoglucanases of *P. pinophilum* to an assay containing 10 mg of cotton fibre, 50 µg of cellobiohydrolase II protein, 5 ml of acetate buffer, pH 5.0, resulted in the complete breakdown of the cotton to short fragments in 4 days when incubated at 37 °C with occasional inversion of the tube.

Synergism between the cellobiohydrolase and endoglucanase components in solubilizing crystalline cellulose

Highly purified endoglucanases I, II, III, IV and V of *P. pinophilum* were similar to the cellobiohydrolases in that they showed little activity on cotton fibre when acting individually (Table 2). Surprisingly, neither the individual endoglucanases nor a reconstituted mixture of the various endoglucanases showed any capacity for acting in synergism with either cellobiohydrolase I or II. Interestingly, the only circumstance in which synergism in solubilizing cotton fibre was observed was when both cellobiohydrolases I and II were acting in solution together with endoglucanase activity. However, only when cellobiohydrolases I and II and either endo-

glucanase III and V were acting in admixture was synergism really significant.

These data were unexpected. On the basis of these results it would appear that for a significant rate of attack on crystalline cellulose in the form of the cotton fibre that three enzymes are required. These are cellobiohydrolases I and II and at least one endoglucanase: cellobiohydrolase I and II must both be present. This observation is compatible with our previous hypothesis (Wood & McCrae, 1986a) that efficient hydrolysis of the different configurations of non-reducing cellulose chain end which will be generated by endoglucanase action on the chain in the first stage of attack, will require the presence of two stereospecific end-wise acting cellobiohydrolases.

Synergism between cellobiohydrolases and endoglucanases originating in different microorganisms

Although fungi such as *Myrothecium verrucaria*, *Stachybotrys atra*, *Memnoniella echinata* and *Giocladium roseum* can degrade crystalline cellulose, the extracellular cellulases that they produce is unable to do so to any significant extent (Wood, 1975). These cellulases are considered to contain endoglucanases because they can degrade soluble CM-cellulose or cellulose swollen in phosphoric acid, and it is assumed that they are deficient in cellobiohydrolase because they show little or no capacity for solubilizing crystalline cellulose (Wood & McCrae, 1979). Surprisingly, however, it has been found that when the cellobiohydrolase I of *T. koningii* or *F. solani* was added to the cellulase preparation isolated from cultures of these fungi, the synergism was still very poor (Wood & McCrae, 1979).

Wood (1981, 1985) has attempted to rationalize these data to account for these and other similar anomalous results. It now appears that the explanation is quite simple. Clearly, if the results obtained with *P. pinophilum* cellulase components in the present study and the conclusions drawn are tenable, namely that both cellobiohydrolases I and II are required for synergism with the endoglucanases, then it would now appear that in those cases where synergism between enzymes from different fungal sources was previously reported to be limited, the mixture of enzymes used was also deficient in cellobiohydrolase II. The results presented in Table 3 show that this is in all probability the case. As can be

Table 2. Synergism between cellobiohydrolases and endoglucanases in solubilizing cellulose (cotton fibre)

Abbreviations: E, endoglucanase; CBH, cellobiohydrolase. 1 mg of cotton fibre was used as substrate with the assay described in Wood (1969). Where appropriate, the amounts of enzyme added were: cellobiohydrolase I and II, 50 µg; endoglucanase, 1 i.u. CM-cellulase. When all five endoglucanases were added to the assay, 0.2 i.u. of each endoglucanase (i.e. I, II, III, IV, and V) was used.

CBH added	Solubilization (°) with and without endoglucanase (E)						
	None	+ EI	+ EII	+ EIII	+ EIV	+ EV	+ EI + II + III + IV + V
None	—	< 1	< 1	< 1	< 1	< 1	< 1
I	4	6	5	5	3	5	10
II	6	4	1	6	3	11	4
I + II	13	17	13	35	16	38	56
Increase in activity (°) when E combined with CBH I + II	—	4	0	22	3	22	42

Table 3. Synergism between cellobiohydrolases of *P. pinophilum* and the cell-free cellulases of some other fungi in solubilizing crystalline cellulose (cotton fibre)

Abbreviation: CBH = cellobiohydrolase. For details of the preparation of the extracellular cellulases, see the Experimental section. Cotton fibre (1 mg) was the substrate: the assay for activity was carried out as detailed in Wood (1969). Where appropriate, the amounts of enzyme added were CBH I, 50 μ g; CBH II, 50 μ g. The volume of cellulase added contained 1 i.u. of endoglucanase (CM-cellulase).

Cellulase used	Solubilization (%) by cellulases alone and in admixture with CBH I and/or II			
	Alone	+CBH I	+CBH II	+CBH I + II
None	—	4	6	13
<i>Thermoascus aurantiacus</i>	< 1	9	1	30
<i>Chaetomium thermophile</i>	< 1	12	2	42
<i>Aspergillus awamori</i>	0	13	20	40
<i>Myrothecium verrucaria</i>	0	10	3	35
<i>Stachybotrys atra</i>	0	8	0	33
<i>Chaetomium globosum</i>	0	13	0	33

seen from Table 3, the addition of cellobiohydrolase I to the culture filtrates of *Chaetomium globosum*, *M. echinata*, *S. atra*, *M. verrucaria* and *Chaetomium thermophile* resulted in very little synergistic activity between the enzymes in solubilizing cotton fibre, and the addition of cellobiohydrolase II to the culture filtrates was the same in this respect. However, when both cellobiohydrolases I and II were added to the culture filtrates containing endoglucanase activity, a significantly higher degree of synergistic activity was observed.

The observation that three enzymes are required for hydrolysis of crystalline cellulose in the form of the cotton fibre raises another question. Why is it, during previous studies in this laboratory (Wood, 1975; Wood & McCrae, 1979) on the cellulases of the fungi *T. koningii* and *F. solani*, that 'cross synergism' was observed between the cellobiohydrolases and (a) a fraction containing several endoglucanases (Wood, 1975) and (b) certain separated endoglucanases (Wood & McCrae, 1979)? The answer once again would appear to be quite simple, i.e. the endoglucanases were still complexed with some cellobiohydrolase II component.

Effect of different ratios of cellobiohydrolases I and II and endoglucanase activity on synergistic activity

A reconstituted mixture comprising equal amounts of the individual endoglucanases (I, II, III, IV, V) of *P. pinophilum* was tested with differing ratios of cellobiohydrolase I and II and a constant amount of endoglucanase. As Fig. 3 shows, synergistic activity was highest when the ratio of the cellobiohydrolases was 1:1. It can also be seen that providing both cellobiohydrolases I and II are present, quite a high degree of synergistic activity is observed with widely differing ratios of the two enzymes: cellobiohydrolase I or II acting independently with the reconstituted mixture of endoglucanases I, II, III, IV and V showed little capacity for solubilizing cotton.

Using cellobiohydrolase I and II in the ratio of 1:1, the effect of varying the proportion of endoglucanase in the mixture on the degree of synergistic activity obtained was tested (Fig. 4). It can be seen that the degree of hydrolysis observed continued to increase with increasing con-

centration of endoglucanase, but in fact there was little difference between the mixture containing 1 i.u. of endoglucanase and that containing 2 i.u.

Only very small amounts of endoglucanase need be added to cellobiohydrolases I and II for effective solubilization. Indeed, the assay containing only 0.25 i.u. effected a high degree of solubilization (47%), and even 0.05 i.u. produced 32% solubilization under the conditions tested. As traces of endoglucanase appear to be effective in solubilizing crystalline cellulose in the form of the cotton fibre when both cellobiohydrolases I and II are also present in the mixture, it is clear that studies on the mode of action of the individual enzymes of the cellulase complex and their interactions resulting in the solubilization of crystalline cellulose can only be carried out on extensively purified enzyme components.

Fig. 3. The effect of varying the ratio of cellobiohydrolase I/cellobiohydrolase II in the presence of a constant amount of endoglucanase on the solubilization of crystalline cellulose

Cotton fibre (1 mg) was incubated (Wood, 1969) at 37 °C for 7 days with 0.25 i.u. of each of the five purified endoglucanases (I, II, III, V and VI) isolated from *P. pinophilum* cellulase (Bhat *et al.*, 1989) and a total of 100 μ g of cellobiohydrolase made up of cellobiohydrolases I and II in different ratios.

Fig. 4. The effect of varying the ratio of endoglucanase to cellobiohydrolase of *P. pinophilum* on the solubilization of crystalline cellulose

Cotton fibre (1 mg) was incubated (Wood, 1969) at 37 °C for 7 days with 50 µg of cellobiohydrolase I and 50 µg of endoglucanase (CM-cellulase). The endoglucanase used was a reconstituted mixture of equal amounts of purified endoglucanases I, II, III, IV and V (Bhat *et al.*, 1989).

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