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# The Formation of Short Fibres from Native Cellulose by Components of *Trichoderma koningii* Cellulase

By G. HALLIWELL and M. RIAZ
*Sub-department of Microbiology, Department of Botany, University College, Swansea, S. Wales, U.K.*

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Cellulolytic enzyme components of culture filtrates of *Trichoderma koningii* were fractionated on ionic and non-ionic forms of Sephadex and on cellulose powder (Whatman) and examined for their ability to hydrolyse soluble carboxymethylcellulose, and to saccharify, solubilize and form short fibres from native undegraded cellulose of the type found in cotton. DEAE-Sephadex provided two CM-cellulase components and a $C_1$ component; the $C_1$ component acted weakly and solely on cotton, forming soluble products but not short fibres. The ability to form short fibres was confined almost wholly to one of the CM-cellulase components which completely degraded cotton, minimally to soluble products and extensively to short fibres. The latter action was unaffected by the presence of the other two components. The two CM-cellulase components solubilized cellulose synergistically whereas the short-fibre-forming component and $C_1$ component were inhibitory.

Although the activities of intact cellulolytic micro-organisms were recorded as early as 1912 (Pringsheim, 1912) it is only recently that cell-free cellulase systems have been obtained capable of achieving similar extensive and complete hydrolysis of native cellulose to sugars (Mandels & Reese, 1964; Halliwell, 1965). For 15 years much effort was directed towards seeking a solution to the mechanism of action of cellulase in terms of either a uni- or a multi-enzymic system of $C_1$ and $C_x$ components (Reese, Siu & Levinson, 1950; Reese & Levinson, 1952). The road to this goal was, and still is, beset with many obstacles. Previously microbial filtrates were examined for their activity mainly on chemically or physically treated cellulose (cellulose degraded to various degrees or even soluble in character) because of their incapacity, or at best marginal enzymic facility, to attack and saccharify native natural cellulose, the substrate of ecological and economic importance. Crude and purified culture filtrates of *Myrothecium verrucaria* belong to this class, being particularly active against degraded forms of cellulose but producing no more than a 13–18% loss in weight of the native cellulose of cotton fibres in periods of up to 3 months (although accompanied by complete loss of the original fibre structure; Halliwell, 1962, 1966), or a 13% weight loss with daily replenishment of enzyme over 30 days (Mandels & Reese, 1964). Processed cotton, as yarn (previously autoclaved at 140°C in 1% sodium hydroxide for 6h to remove impurities), is evidently more susceptible to solubilization by daily fresh replacements of culture filtrate from the same organism but suffered no more than a 30% loss of weight in 16–35 days (Selby, Maitland & Thompson, 1963). In contrast, a single application of culture filtrate from certain species of *Trichoderma* produced extensive solubilization (59% in 45 days; Mandels & Reese, 1964) or complete solubilization (in 17 days; Halliwell, 1965) of cotton fibres, depending on the amount of substrate present.

Before these *Trichoderma* cellulase preparations became available, it was suggested that the early action of cellulase might engender physical changes in cellulose that were not reflected in the formation of soluble products (Halliwell, 1962). Recent investigations on $C_1$ and $C_x$ components isolated from *Trichoderma* species support this belief. Thus several workers using similar techniques have found that the $C_1$ and $C_x$ components display only feeble activity on cellulose unless mixed; independently $C_x$ component hydrolyses only the soluble CM-cellulose, whereas $C_1$ component has little or no action on cellulosic substrates but may prepare cellulose for attack by $C_x$ component (Mandels & Reese, 1964; Wood, 1968).

One of the earliest stages in the breakdown of native cellulose of cotton fibres by culture filtrates of *T. koningii* is illustrated by the appearance of very short insoluble fibres. These increase in amount for some time as the reaction proceeds, then disappear in the form of soluble sugars (Halliwell, 1965). The present investigation was to determine the place of short fibres in microbial cellulolytic systems and their relationship to purified enzymic components, such as $C_1$ and $C_x$, separated from crude culture filtrates.

## MATERIALS AND METHODS

*Source of enzymes.* Cultures of *T. koningii* were grown on non-dewaxed Texas-cotton fibres in shaken or stationary flasks on approximately 20 times the scale described for the shaken cultures in Fig. 1 in Halliwell (1961). Culture filtrates used as a source of cellulolytic enzymes for chromatography were clarified by centrifuging and sterilized by filtration (Halliwell, 1965).

*Column chromatography.* Culture filtrates were concentrated, applied to columns (25mm × 450mm) of Sephadex G-75, DEAE-Sephadex, CM-Sephadex or cellulose powder (Whatman CC31), eluted with appropriate buffers (see Figs. 1–6) and collected in 4ml fractions; the protein contents of the fractions were calculated from their extinction values at 280nm before their enzymic activities were determined.

*Substrates and methods of enzymic analysis.* Non-dewaxed and dewaxed native Texas-cotton fibres and sodium CM-cellulose were as described by Halliwell (1961, 1962).

Cellulolytic activity of separated fractions on native cellulose was measured by shaking 2mg of dewaxed fibres, 5ml of sterile 0.2M-sodium acetate–0.2M-acetic acid buffer, pH4.8, and up to 4ml of enzyme fraction in a final volume of 9ml for 1–2 days at about 25°C. Prolonged assays involving 7 days' incubation were not shaken (Fig. 4). Any residual mass of unattacked cotton was carefully removed, leaving in some cases a suspension of short fibres that were estimated turbidimetrically at 600nm, then colorimetrically, where desirable, with a dichromate reagent. Soluble products were measured in total from the loss in weight of initial substrate (determined colorimetrically with dichromate) or as reducing sugars (up to 14$\mu$g in 3ml of solution, determined by the ferricyanide–ferric alum method) as described by Halliwell (1965).

Separated fractions were sufficiently enzymatically active against cotton in some experiments to permit the use of a modification of the standard ferricyanide–ferric alum procedure described above for estimating reducing sugars (as glucose). This used the same reagents, was quicker and covered a wider range of reducing-sugar concentrations. Briefly, the sample containing up to 40$\mu$g of sugar in not more than 2ml of solution was treated with 1ml of cyanide–carbonate and 2ml of ferricyanide, boiled for 25min and cooled for 10min. Residual ferricyanide was determined from the extinction at 420nm, the degree of reduction of the reagent being proportional to the amount of sugar taken.

Enzymic hydrolysis of the soluble cellulose derivative, CM-cellulose, to reducing sugars was measured colorimetrically after undiluted samples had been incubated at pH7.6 with 0.2M-sodium phosphate buffer for 10min at 25°C, but otherwise was as described previously (Halliwell, 1961, 1962). By using the assay techniques given above, the following cellulolytic enzyme activities are recognized: (1) carboxymethylcellulase (CM-cellulase) hydrolysing the soluble cellulose derivative CM-cellulose to reducing sugars; (2*a*) short-fibre formation from native undegraded cellulose of cotton; (2*b*) solubilizing activity, converting cotton principally into soluble products and reducing sugars without the intervention of short fibres.

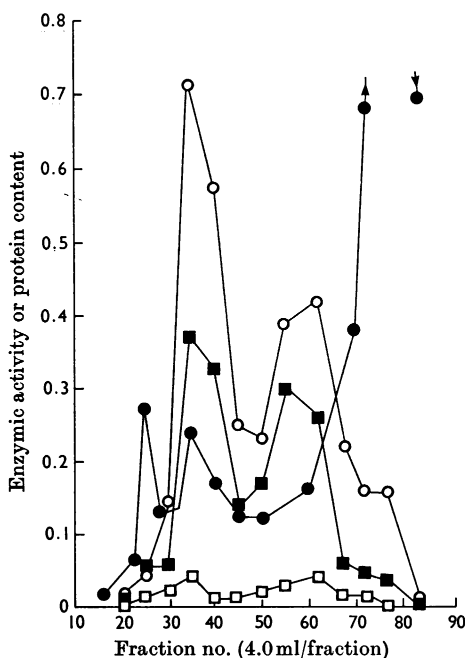


Fig. 1. Separation of components of culture filtrates of *T. koningii* on Sephadex G-75. Columns were eluted with 0.01M-sodium phosphate buffer, pH6.5, containing 0.1M-NaCl. Determinations of protein content and enzymic assays were made by the standard procedures (see the Materials and Methods section) and are shown in extinction units. ●, Protein content ($E_{280}$); ■, CM-cellulase activity ($E_{420}$). Cellulolytic activity with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$).

## RESULTS

Culture filtrates of *T. koningii* applied to columns of Sephadex G-75 and eluted with phosphate buffer, pH6.5, provided the pattern of outflow shown in Fig. 1. Three 'protein' peaks (extinction at 280nm) were obtained, in fractions 25, 35 and 77, but only the second of these was associated with major cellulolytic activity. Almost all of the cellulolytic enzymes recovered from the column were located between fractions 35 and 62, where the protein content was relatively low. Fractions at these two points had the greatest, almost equal, activity on cotton in producing numerous insoluble short fibres, but fraction 35 possessed by far the greater solubilizing power on cotton (measured as reducing sugars) as well as somewhat greater effectiveness in hydrolysing CM-cellulose. Thus short-fibre-forming activity on cotton appears to be independent of CM-cellulase or of $C_1$ activity (proportional to solubilization if synergism is involved) or of both activities. Break-

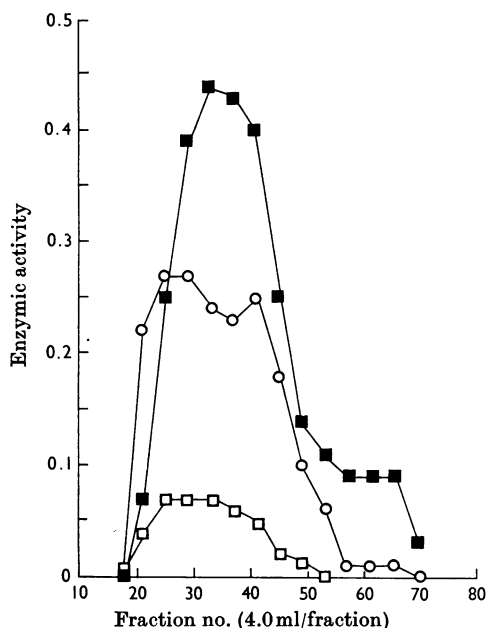


Fig. 2. Separation of components of culture filtrates of *T. koningii* on CM-Sephadex. Columns were eluted with 0.02M-sodium phosphate buffer, pH 6.9, and examined as described in Fig. 1. ■, CM-cellulase activity ($E_{420}$). Cellulolytic activity with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$). For clarity, values for protein contents are omitted.

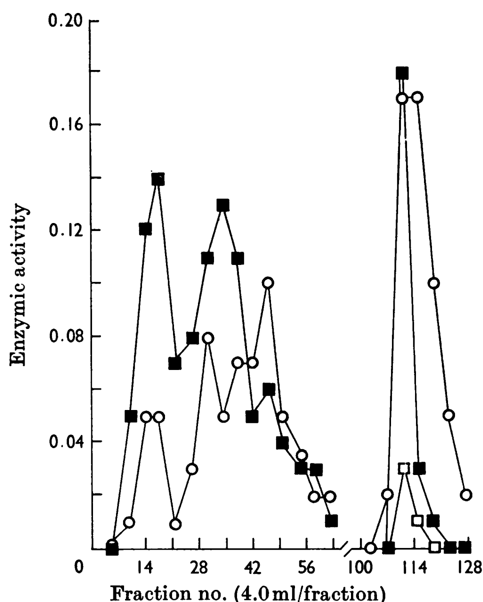


Fig. 3. Separation of components of culture filtrates of *T. koningii* on cellulose powder. Columns were eluted as described in Fig. 2 with 0.02M-sodium phosphate buffer, pH 6.9, up to fraction 100 and then with water. ■, CM-cellulase activity ($E_{420}$). Cellulase activity with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$).

down of cellulose by fractions 35 and 62 approximates to 25% conversion into insoluble products in the form of short fibres (turbidimetric assay) in addition to 40% and 23% solubilization respectively to sugars. Unlike earlier portions of the eluate, fractions subsequent to no. 62 were progressively relatively richer in activity solubilizing cotton than in activity attacking CM-cellulose (cf. fractions 35 and 50 with 69).

Further indication of the existence of the short-fibre-forming activity as at least a partial separate entity is suggested by the separation achieved in Fig. 2 with the cation-exchanger CM-Sephadex. Four protein peaks (not shown) were obtained, at fractions 26, 34, 46 and 66, the first three of which, and possibly the first part of fraction 66, were associated with one or other of the three phenomena: solubilization, CM-cellulase activity and short-fibre formation. Material eluted early from the column (fraction 20) possessed much solubilizing action compared with its short-fibre-forming activity and CM-cellulase content, but the latter increased considerably in amount over solubilization in ensuing fractions (20–34) until the final fractions (57–66) consisted of relatively pure CM-cellulase (CM-cellulase/solubilizing activity ratio 10:1). These late fractions were free from short-fibre-forming activity, which was confined mainly to the middle portion of the eluate in fractions 26–40 and associated with the main peaks of CM-cellulase and cellulose-solubilizing activity. In this region one of the most active cellulolytic fractions, no. 26, provided complete breakdown of the original cotton to short fibres accompanied by only 15% solubilization, in contrast with the 40% solubilization by the most active fraction (no. 35 in Fig. 1). Independence of short-fibre-forming ability from CM-cellulase or $C_1$ activity (or total solubilization) is implied in fractions 26 and 46. Here fractions of identical CM-cellulase content show a large difference in short-fibre-forming activity that cannot be ascribed (either in a non-synergistic or, even more so, in a synergistic system) to varying quantities of $C_1$ component at the small differences in solubilization shown by the two fractions.

Cellulolytic enzymes of *M. verrucaria* are strongly but reversibly adsorbed on cellulose powder (Halliwell, 1961). This property was used here to provide supporting evidence for the entity of the short-fibre-forming component of *T. koningii* as presented in Fig. 3, where elution with phosphate buffer removed

most of the protein together with brown pigment in a peak with maximum absorption at fraction 16.

After the outflow of protein from the column had ceased, buffer was replaced by water and further quantities of enzyme were liberated, free from pigment, with a peak of absorption at fraction 111.

Samples eluted in buffer possessed only CM-cellulase and saccharifying power without any clear relationship between the two activities. Short-fibre-forming capacity, absent from these fractions, was confined entirely to the water eluate, particularly at fraction 111. This was the only sample to segment cotton completely to short fibres, possibly accounting for the accompanying large amount of reducing sugars (9% of initial substrate) relative to adjacent fractions. Further, compared with fraction 111, the subsequent fraction, no. 115, has an equally high solubilizing action (and $C_1$ activity if synergism involves CM-cellulase) but comparatively feeble short-fibre-producing ability, again suggesting the independence of this last activity from the $C_1$ component. Fractions 115–128 in general operate principally against cotton, and not against CM-cellulose, to form reducing sugars without short fibres, and in this respect have relative enzymic activities closely parallel to those found in the $C_1$ component described below.

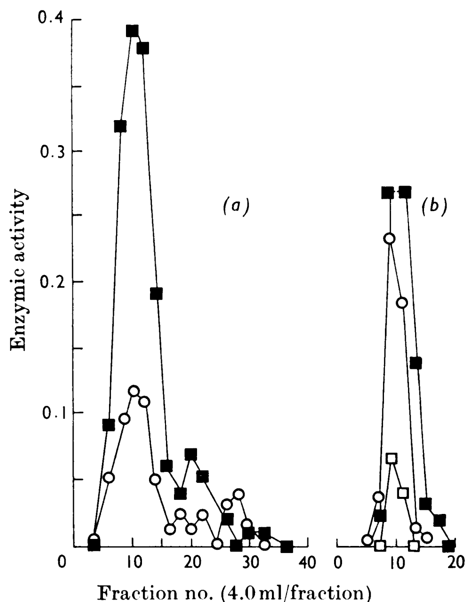


Fig. 4. (*a*) Chromatography on CM-Sephadex of pooled and concentrated fractions 7–63 of Fig. 3. (*b*) Pooled and concentrated fractions 100–128 of Fig. 3. Columns were eluted and assayed as in Fig. 2. ■, CM-cellulase activity ($E_{420}$). Cellulase activity with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$).

In experiments identical with those described in Fig. 3 the whole of each of the two types of fractions obtained with buffer and with water respectively were collected separately, concentrated, sterilized by filtration and refractionated on a second column of a different supporting material, CM-Sephadex. Each sample, namely 'buffer concentrate' and 'water concentrate', was eluted in turn with buffer as described (Fig. 4). Most of the enzymic activity of the 'buffer concentrate' was released in two peaks, the first possessing CM-cellulase activity as well as the ability to form reducing sugars from cotton (Fig. 4*a*). This was followed by a smaller but reproducible second peak of saccharifying activity against cotton, at about fraction 28, where CM-cellulase activity was insignificant. In contrast, application of the 'water concentrate' to a column of CM-Sephadex and elution with phosphate buffer showed that all three original enzymic activities of Fig. 3 (fractions 100–128) had been recovered in the eluate but with an important change in the pattern of outflow (Fig. 4*b*). Whereas fractions 100–128 of Fig. 3 had short-fibre-forming activity (□) running independently of $C_1$ activity, Fig. 4(*b*) suggests the former activity (□) could well be independent of CM-cellulase.

Thus relative enzymic activities of certain fractions strongly suggest that short-fibre-forming activity is distinct from $C_1$ or CM-cellulase activity or both. The practical separation of the activity producing short fibres from that associated with $C_1$ activity was accomplished by eluting culture filtrates from columns of the anion-exchanger DEAE-Sephadex with a salt gradient of 0–0.36M-sodium chloride in 0.02M-phosphate buffer, pH 6.9 (Fig. 5). Recycling of the $C_1$ fraction may be necessary to remove residual short-fibre-forming activity. Most of the protein was eluted in three peaks with maxima at fractions 20, 37 and 43 by using phosphate buffer alone. Addition of sodium chloride to 0.27M concentration was required to remove the small remaining amount of protein possessing slight solubilizing activity at about fraction 140. This component solubilized cotton slightly, forming reducing sugars without short fibres, and failed to hydrolyse the soluble CM-cellulose even when assayed at ten times the concentration used for fractions 10–50. This component, with slight solubilizing action, resembles the partly purified, more rapidly flowing, portion of the eluate obtained from CM-Sephadex columns, as in fraction 20 in Fig. 2. In Fig. 5, short-fibre formation is confined essentially to the first portion (fractions 30–37) of the second and major protein peak with an optimum at fraction 37; such enzymic activity was characterized by a high ratio of short-fibre-forming capacity to saccharification. The formation of short fibres by fractions 30–33 of the second peak may be

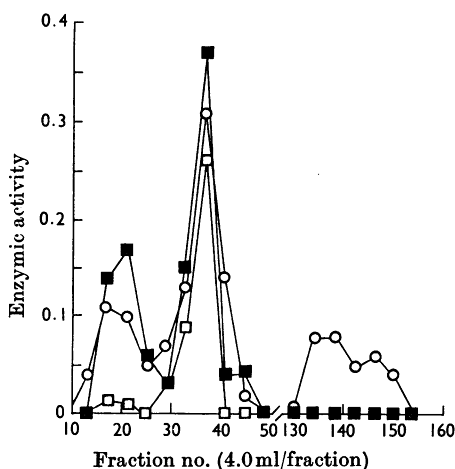


Fig. 5. Separation of components of culture filtrates of *T. koningii* on DEAE-Sephadex. Columns were eluted with 0.02M-sodium phosphate buffer, pH 6.9, until fraction 100. A salt gradient was then applied to elute fractions 130–150 in buffer containing approx. 0.27M-NaCl. All fractions were examined as described in Fig. 1. ■, CM-cellulase activity ($E_{420}$). Cellulolytic activity with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$).

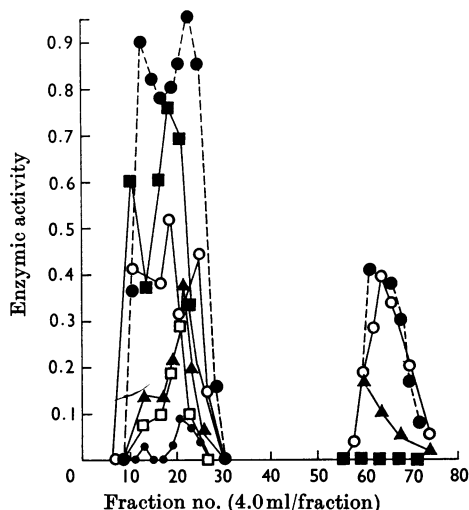


Fig. 6. Separation of components of culture filtrates of *T. koningii* on DEAE-Sephadex. Columns were eluted and fractions assayed for CM-cellulase activity as described in Fig. 5. Assays on cotton were made after incubation for 18h with and without shaking and for 7 days in stationary vessels. ■, CM-cellulase activity ($E_{420}$). Cellulolytic activities in the shaken assay with cotton as substrate: ○, production of reducing sugars ($E_{420}$), and □, production of short fibres ($E_{600}$). Cellulolytic activities in an 18h stationary assay with cotton: ▲, production of reducing sugars (no short fibres formed). Cellulolytic activities in the 7-day stationary assay with cotton as substrate: ●, production of reducing sugars ($E_{420}$), and •, production of short fibres ($E_{600}$). Extinctions are shown at twice the values found, except for ●.

contrasted with the action of fractions obtained in the first peak. Thus fractions 15–21 have saccharifying powers (○, Fig. 5) as well as CM-cellulase activities greater than or very similar to those found in fractions 30–33 but have little or no ability to yield short fibres, indicating that the components of the first peak bear no relationship to the short-fibre-forming components of the second peak. The activity of the latter in fraction 37 produced complete loss of fibre structure in converting cotton into short fibres together with relatively small but still the greatest extent of saccharification by any fraction, amounting to 18% of the initial weight of cotton.

Wheareas the general pattern of elution from DEAE-Sephadex illustrated in Fig. 5 was readily reproducible, occasional differences were encountered, mainly in older cultures. These showed some degree of separation in the second main enzymic peak, now free from $C_1$ activity, of maximum CM-cellulase activity (■) from the ability to yield short fibres (□) (Fig. 6, shaken fractions 18 and 21, 10 and 17 respectively), a finding similar to that reported above in Fig. 4(*b*). Although no short fibres appeared within the same period (18h) in the presence of enzyme in unshaken assays, reducing-sugar formation was only 30% less than that furnished by agitation. However, under the latter conditions enzymic activity was directed towards transforming all the initial cotton into large amounts of short fibres plus small amounts of soluble products; the latter, as sugars, accounted for only 5–7% of the initial substrate (fractions 18–21 in Fig. 6). In the undisturbed incubations of Fig. 6 the amount of reducing sugars increased steadily over 7 days, whereas the short fibres present represented only one-third of those found in shaken assays incubated for only 18h (□ in Fig. 6).

$C_1$ fractions eluted lastly from the column in the expected position (Figs. 5 and 6) were without cellobiase, CM-cellulase or short-fibre-forming activity on cotton; they had little effect on this substrate, producing less than 7% solubilization (and recovery of cellulase activity) in 7 days for the whole pooled $C_1$ fraction compared with 11% solubilization (and recovery of activity) from pooled fractions 9–30 (Fig. 6). Combination of these two components gave 93% recovery of the cellulase activity present in an equivalent portion of unfractionated culture filtrate producing 50% solubilization (and 100% recovery of cellulase). The longer assay period of Fig. 6 is much less conducive to short-fibre formation than the shorter periods (Figs. 1–5) as a result of the increasing

Table 1. *Degradation of cotton by components separated on DEAE-Sephadex*

Components A (fractions 10–25), B (fractions 26–50) and $C_1$ (fractions 130–155) were isolated, pooled and assayed as described in Fig. 5 by using amounts proportional to the volume of culture filtrate fractionated.

| | Solubilization (to nearest 0.5%) | Recovery of cellulase activity (%) |
|---|---|---|
| A | 2.5 | 18 |
| B | 7.5 | 50 |
| $C_1$ | 9.0 | 61 |
| A+B | 20.0 | 136 |
| A+$C_1$ | 11.5 | 75 |
| B+$C_1$ | 12.5 | 86 |
| A+B+$C_1$ | 15.0 | 100 |
| Culture filtrate (1 ml) | 15.0 | 100 |

predominance of other enzymic activities as the incubation proceeds. Hence the effect of separated components on short-fibre-forming activity was examined under conditions most suited to this action, as shown for example in Fig. 5.

The first and second CM-cellulase peaks and the $C_1$ fraction were prepared as shown in Fig. 5, pooled separately and assayed as described in the Materials and Methods section. Table 1 presents the activities of the various fractions alone and in combination. Although the degree of solubilization achieved by components B and $C_1$ was similar, short-fibre formation was confined to only one (component B) of the three components and gave a breakdown of substrate of about 50%, which remained unchanged in the presence of components A or $C_1$ and matched that in the whole culture filtrate. Whereas addition of component A to component B had a synergistic effect in doubling the expected degree of solubilization, the addition of component $C_1$ (containing neither short-fibre-forming activity nor CM-cellulase) to component B produced about 22% inhibition. In contrast, the admixture of components $C_1$ and A resulted in no change. The inhibitory effect of $C_1$ component is again evident on combining all three components, the sum of whose individual activities is reduced in the presence of $C_1$ component to that of the unfractionated culture filtrate. Components A and B themselves together are over 30% more effective in solubilizing cotton than are all three components combined or are whole culture filtrates.

## DISCUSSION

In this discussion attention is focussed primarily on cellulolysis of the native undegraded substrate, particularly of cotton fibres, by enzymes of the cellulase complex. Other types of cellulose, such as cellulose powders and filter papers prepared by physical or chemical treatments of native cellulose, are of doubtful composition and vary in their extent of degradation. Their use as substrates leads to greater speculation and less certainty in interpreting experimental results about cellulolysis in Nature. An earlier account of a cell-free cellulase system implicated short fibres as an initial stage in the attack on cotton (Halliwell, 1965). The number of fibres increased to a maximum and disappeared gradually by conversion into glucose; agitation promoted enzymic breakdown of the substrate. The process is readily distinguished from the enzymic disintegration of filter paper (1 cm squares cut from Toyo no. 2 circular or square sheets) into its component fine fibres described by Toyama (1960, 1963). Corresponding papers of British origin are Whatman no. 2 filter paper and no. 2 chromatography paper. In our hands a 1 cm square (9 mg) of each of the Whatman papers in water (9 ml) alone suffered at least 50% disintegration, producing innumerable fine fibres within 5 s of vigorous shaking by hand, a preliminary treatment of our earlier assay procedure with cotton, to wet the substrate with reagents immediately before the assay proper commenced (Halliwell, 1965). Under the same conditions cotton itself showed no change even with enzyme added. Application of the treatment to 1 cm squares of other types of filter or chromatography papers (Whatman) solely in water likewise produced countless fine fibres associated with the following degrees of disintegration: no. 1 chromatography paper in sheet form (90%) (this paper is comparable with Toyo filter paper no. 51A used by some Japanese workers); no. 1 chromatography paper in roll form (70%); no. 1 filter paper (90%).

The solution to the cellulase problem has been sought also in the role of a $C_1$ component that Reese & Levinson (1952) proposed demarcated cellulolytic micro-organisms from non-cellulolytic species that possessed only CM-cellulase activity. Fractionation of crude culture filtrates of *T. viride* by Mandels & Reese (1964) with DEAE-Sephadex disclosed a fast-moving $C_x$ component associated with $\beta$-glucosidase, a second, slower-moving, $C_x$ component (neither $C_x$ component acted appreciably on cotton) and a strongly adsorbed component designated $C_1$. This was found in the major protein peak, had weak CM-cellulase activity but acted on cotton to form reducing sugars. After further purification, the activity of $C_1$ component was increased as much as sixfold in the presence of either of the $C_x$ components. The technique of isolating $C_1$ and other cellulolytic components on DEAE-Sephadex has been extended by others with cotton fibres (Wood, 1968) and yarn (Selby & Maitland, 1967) as substrates for enzyme preparations from culture filtrates of different *Trichoderma* species. These workers used Sephadex G-75 to remove 'non-essential' $C_x$ material and permit a faster-flowing $C_x$ component to be applied to

DEAE-Sephadex. After the strongly adsorbed $C_1$ component had been eluted from the column it had no action on cellobiose or CM-cellulose and solubilized yarn to only 1–6% (Selby & Maitland, 1967); alternatively it emerged with the main protein peak, associated with traces of CM-cellulase (Wood, 1968). In both investigations, although most of the $C_x$ component was separated from $C_1$ component, it retained much cellobiase or *o*-nitrophenyl $\beta$-glucosidase. Chromatography of the mixed $C_x$ fraction on SE-Sephadex provided in one case CM-cellulase- and cellobiase-enriched components respectively, with each containing small amounts of the other (Fig. 8 in Selby & Maitland, 1967), and in the other case a double CM-cellulase component and a mixed CM-cellulase–$\beta$-glucosidase fraction (Wood, 1968).

Clearly different workers isolate apparently similar components with notable differences in properties. Culture filtrates may contain interfering proteases, purified CM-cellulase and cellobiase fractions retain various amounts of one another, and in certain cases separated $C_1$ fractions do not readily release associated CM-cellulase. Although some of the differences are probably attributable to variations in the enzymic formulation of the cellulase system by distinctive micro-organisms grown under particular conditions, others undoubtedly result from individual preferences for particular assay procedures. These include the use of different forms of cotton and of culture filtrates of various age, of cellobiose and *o*-nitrophenyl $\beta$-glucoside as substrates for cellobiase, of different reductimetric and viscometric methods for the determination of CM-cellulase, and variations in small losses in weight of substrate for cellulase estimations.

Whereas prolonged incubation of cotton with filtrates from *T. koningii* achieves complete solubilization, short periods of treatment are characterized by extensive short-fibre formation from cotton accompanied by relatively little solubilization (Halliwell, 1965). To study the solubilization reaction with components isolated in the present work, cellulolysis was measured by reducing-sugar formation, readily detectable by micro methods. Where necessary the standard dichromate procedure was used to relate saccharification to total solubilization. The relationship between early changes during cellulolysis and known $C_1$ and $C_x$ components in the overall picture of the cellulase complex is noteworthy and of considerable practical importance. Attempts to assign short-fibre-forming activity to a single known cellulolytic component have not been successful. Activity was found in both the $C_x$ and $C_1$ fractions, mainly in the $C_x$ fraction; no synergistic effects occurred between the two (Wood, 1968).

The relative enzymic activities shown in Figs. 1–4 suggest strongly that short-fibre formation was not wholly dependent on solubilization, whether that activity be considered a function of CM-cellulase or $C_1$ activity or both (synergism). Figs. 5 and 6 and Table 1 confirm this by illustrating separation of short-fibre-forming ability from other components, including $C_1$, and its restriction in young culture filtrates to the second CM-cellulase peak (Fig. 5 and Table 1). Our $C_1$ component had typically weak solubilizing action on cotton and additionally was free from all traces of CM-cellulase and short-fibre-forming properties. The interaction of these last two activities with the purified $C_1$ component during optimum conditions for short-fibre production is noteworthy (Table 1). The solubilizing power of the CM-cellulase with short-fibre-forming capacity (B) was enhanced by the presence of the CM-cellulase free from short-fibre-forming activity (or vice versa) but not by $C_1$ component, which inhibited. $C_1$ component likewise failed to synergize with component A and further, in combination with components A and B, decreased the enhanced solubilization characteristic of that binary mixture to the solubilization characteristic of unfractionated culture filtrate.

Possible explanations of the differences between our cellulolytic components and those of others may include the following. (1) The properties of our $C_1$ fraction, which was free from both CM-cellulase and short-fibre-forming activity. (2) The nature of the reaction studied, mainly short-fibre production and its allocation to known components $C_x$ or $C_1$. (3) Conditions of growth, age of culture filtrates and secretion of particular cellulolytic components. Wood's (1968) procedure resembled that of Selby & Maitland (1967) and provided similar but not identical findings for the $C_1$ and $C_x$ components. We were unable to use the same preliminary separation on Sephadex G-75 owing to the presence of short-fibre-forming activity in both CM-cellulase peaks from this column. Hence whole-culture filtrates were not deprived of the slow-moving CM-cellulase before further separations were attempted. It is noteworthy that although the low-molecular-weight CM-cellulase component obtained by Wood (1968) from Sephadex G-75 was free from short-fibre-forming ability, it retained some 'S factor' activity. (4) Finally, it may well be that some cellulolytic micro-organisms have evolved pathways that cannot yet be expressed solely in terms of our limited knowledge of a synergistic reaction involving $C_1$ and component $C_x$.

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## REFERENCES

Halliwell, G. (1961). *Biochem. J.* **79**, 185.
Halliwell, G. (1962). *Biochem. J.* **85**, 67.
Halliwell, G. (1965). *Biochem. J.* **95**, 270.

Halliwell, G. (1966). *Biochem. J.* **100**, 315.
Mandels, M. & Reese, E. T. (1964). *Devs ind. Microbiol.* **5**, 5.
Pringsheim, H. (1912). *Hoppe-Seyler's Z. physiol. Chem.* **78**, 266.
Reese, E. T. & Levinson, H. S. (1952). *Physiologia Pl.* **5**, 345.
Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950). *J. Bact.* **59**, 485.
Selby, K. & Maitland, C. C. (1967). *Biochem. J.* **104**, 716.
Selby, K., Maitland, C. C. & Thompson, K. V. A. (1963). *Biochem. J.* **88**, 288.
Toyama, N. (1960). *Mem. Fac. Agric. Miyazaki Univ.* **2**, 100.
Toyama, N. (1963). In *Advances in Enzymic Hydrolysis of Cellulose and Related Materials*, p. 235. Ed. by Reese, E. T. Oxford: Pergamon Press Ltd.
Wood, T. M. (1968). *Biochem. J.* **109**, 217.