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The Nature and Mode of Action of the Cellulolytic Component C_1 of *Trichoderma koningii* on Native Cellulose

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1. A purified cellulolytic component C_1 was isolated free from associated activities of the cellulase complex and shown to act as a β-1,4-glucan cellobiohydrolase on both simple and complex forms of native cellulose. 2. The enzyme releases terminal cellobiose units from cellulose, its extent of action being determined principally by the product and by the nature of the substrate. 3. Component C_x of the cellulase system is not required for the action of component C_1 (cellobiohydrolase). The enzyme synergizes extensively with cellobiase in extending the hydrolysis of native and of less-complex forms of cellulose to at least 70% with the liberation of glucose. 4. The cellobiohydrolase is relatively unstable, with an optimum at pH 5 and a K_m of 0.05 mg/ml. The enzyme is inhibited by its product, from which it is released by cellobiase. 5. Of other compounds tested against the cellobiohydrolase the metal ions Cu^{2+}, Zn^{2+}, phenylmercuric and Fe^{3+} are increasingly effective inhibitors. Glucose has no action at concentrations found inhibitory with cellobiose. 6. The relationship of the enzyme to the entire cellulase complex is discussed.

Ever since the C_1–C_x concept was proposed over two decades ago (Reese *et al.*, 1950) the nature of the C_1 component of the cellulase complex has presented a challenging problem. The existence of this component was strongly questioned some years ago (Whitaker, 1953). Other workers, however, showed that certain fungi could provide culture filtrates that were highly effective in producing extensive (Mandels & Reese, 1964) or complete (Halliwell, 1965*a*,*b*) solubilization of both native and degraded forms of cellulose, an activity previously associated only with intact and viable organisms. A newly isolated protein in addition to cellobiase and CM-cellulase was believed to be the enigmatic component C_1 (Mandels & Reese, 1964). Attempts to purify the three fractions further (see e.g. Selby & Maitland, 1967) left each associated with activity characteristic of one or more of its partners. Thus partly purified component C_1 contained also either CM-cellulase, or short-fibre-forming activity, or both (Selby, 1968; Wood, 1968). Component C_1 had no action on cellulose other than a very weak solubilizing effect except in the presence of component C_x (CM-cellulase), when its activity increased up to fivefold.

The role of the C_1 component in cellulolysis is unknown (for reviews see Reese, 1963; Selby, 1968; Hajny & Reese, 1969; Walters & Hueck-van der Plas, 1971; Whitaker, 1971). Both inhibitory and synergistic interactions between purified cellulolytic components prepared from whole culture filtrates have been described (Halliwell & Riaz, 1970). Their C_x component included the more readily separable, low-molecular-weight C_x (CM-cellulase) fraction considered 'non-essential' by some workers (Selby & Maitland, 1967; Wood & McCrae, 1972). As shown elsewhere, however, such findings apply only to that fraction when acting alone; it synergizes extensively with a further component of the cellulase system in forming short fibres (Halliwell & Riaz, 1971). Component C_1 was isolated free from the other cellulolytic components and in this state showed none of the extensive synergism shared between less pure C_1 and C_x (CM-cellulase) components. Although the effect of our purified component C_1 acting alone on undegraded cellulose was to produce only minor solubilization (Halliwell & Riaz, 1970), this was directly related to the formation of reducing sugars, the nature of which was later confirmed as cellobiose (Halliwell & Riaz, 1971). No other sugar was formed from native cellulose by the action of the purified C_1 component, free from all other enzymic activity, thus indicating it might well be a β-1,4-glucan cellobiohydrolase, a finding that was supported also by its action on simpler forms of cellulose as reported in a preliminary form the same year (Halliwell *et al.*, 1972).

Whereas there is much information showing how degraded forms of cellulose can be rapidly and extensively hydrolysed by crude or purified cellulolytic preparations containing only C_x activity (for reviews, see above), breakdown of native, undegraded, cellulose by either C_x or C_1 components has presented a far more complex problem, is little understood and is a process of considerably greater biological significance. The present work makes use of two forms of native

cellulose as model substrates for component C_1 to establish the mode of enzymic attack on cellulose in general, whether of an undegraded, partially degraded or highly degraded nature. The normally small degree of hydrolysis characteristic of the action of purified component C_1 preparations on the native substrates can be considerably enhanced without any contribution from component C_x if cellobiase is present. The resistance of different forms of cellulose to hydrolysis by component C_1, acting as a cellobiohydrolase, may then be considered in terms of an inhibition of the enzyme by products of the reaction and its release therefrom by cellobiase or excess of substrate. Further properties of the cellobiohydrolase are also described in assessing its contribution to the overall process of cellulolysis.

Experimental

Cultures and preparation of enzyme and substrates

Cultures. Sources were as described previously with cultures of *Trichoderma koningii* (Halliwell & Riaz, 1970, 1971).

Purification procedure. Component C_1 (5μg of protein/ml; measured as albumin; Halliwell, 1961) and cellobiase (10μg of protein/ml) used in the present work were purified by recycling chromatography, first on DEAE-Sephadex and then on Sephadex G-75, followed finally by dialysis (Halliwell & Riaz, 1970, 1971) until they were free from each other's activity, from CM-cellulase, and from short-fibre-forming ability. This was achieved as follows. Component C_1 after separation from culture filtrates (Halliwell & Riaz, 1970) was dialysed for periods up to 15h (overnight if necessary) in Visking tubing (Scientific Instrument Centre, London, U.K.) at 3°C against several changes of water, freeze-dried, redissolved and re-run on a similar column. The highest concentration of cellobiase activity was found in the early part of the first peak. These fractions were separated from later fractions, pooled, dialysed, freeze-dried and re-run as above. Cellobiase was finally passed through Sephadex G-75 to eliminate residual pigment (Halliwell & Riaz, 1971). After separation the purified cellulolytic components, cellobiase and C_1, showed no tendency to attack the membrane when dialysed under these conditions. Each component was used in amounts equivalent to that found in 1ml of original culture filtrate unless stated otherwise in the text. A 1ml portion of culture filtrate solubilized 40% of dewaxed cotton fibres (2mg) in 7 days at 37°C. The cellobiase component was assayed at 37°C and pH5.0 for 1–2.5h (depending upon the activity) by its ability to convert cellobiose into glucose, this being measured specifically by the glucose oxidase–peroxidase–anisidine reaction (Halliwell, 1966). In addition to a 10min assay at pH7.6, used for convenience to avoid diluting CM-cellulase activity during fractionation, the same enzyme was shown to be absent from purified preparations of component C_1 and cellobiase acting on CM-cellulose at pH4.8 (the optimum for cellulolysis and also close to the optimum of pH5.0 for the CM-cellulase of our organism) and 37°C for 1h, or longer (5h) in some cases described in the text. The presence of CM-cellulase in component C_1 was also tested for by using a natural substrate (cellulose of cotton as opposed to the synthetic substrate CM-cellulose) and measuring the enzyme's ability to promote short-fibre formation during a shaken incubation of 5 days' duration with culture filtrates or enzyme preparations low in that property (Halliwell & Riaz, 1971). None was formed.

Preparation of bacterial cellulose. Bacterial cellulose was derived from *Acetobacter xylinum* 8034 maintained on a liquid medium of bacteriological peptone (0.5% Oxoid), yeast extract (0.5%), Na_2HPO_4 anhydrous (A.R., 0.27%), citric acid monohydrate (A.R., 0.12%) and glucose (A.R., 2%), adjusted to pH7.0 with HCl or NaOH (Hestrin & Schramm, 1954) and solidified when necessary with agar (1.5% Oxoid no. 3) to provide slants. An actively growing liquid inoculum was prepared by transferring bacteria from slants into 25ml of the liquid medium described above in 250ml conical flasks and incubating for 3 days at 25°C. The culture so formed, freed from any pellicle, was used to inoculate 250ml of liquid medium in a Glaxo bottle. This was incubated at 30°C for 10–20 days. Purification of the bacterial cellulose was based on the procedure of Barclay *et al.* (1954). Pellicles from each bottle were separated from the liquid phase by filtration, homogenized in a blender (MSE Ato-Mix) with 20ml of water, filtered, resuspended in water and homogenized again. After soaking for 18h in 5% NaOH at 30°C the fibres were filtered, washed with water, 0.5% acetic acid and finally with water until the solution remained neutral overnight. By suspending the product in 20ml of water, followed by homogenizing, filtering and washing, a fine dispersion of cellulose was obtained.

Other materials

Reagents were obtained as shown: glucose, sucrose, $CuSO_4,5H_2O$ and $ZnSO_4,7H_2O$ (all A.R.) and 1,5-gluconolactone (Special Laboratory Reagent; all from Fisons Ltd., Loughborough, Leics., U.K.; $MnSO_4,4H_2O$ and $FeCl_3,6H_2O$ (both A.R.; BDH, Poole, Dorset, U.K.); cellobiose (biochemical grade), *p*-chloromercuribenzoic acid (L.R.), CM-cellulose (sodium salt, low viscosity; Laboratory Reagent) and 2-nitrophenyl β-D-glucopyranoside (all from BDH); sodium *N*-lauroylsarcosinate (pure) and sodium diethyldithiocarbamate trihydrate (A.R.; both from Koch-Light Ltd., Colnbrook, Bucks., U.K.); phenyl-

mercuric acetate (Grade 1; Sigma Ltd., Kingston-upon-Thames, Surrey, U.K.).

Enzyme assays. These were done as described by Halliwell & Riaz (1970, 1971).

Results

Action of component C_1 and cellobiase on cellulose

Despite its apparently weak cellulolytic (solubilizing) power against the complex undegraded cellulose of dewaxed native cotton fibres, our purified component C_1 possessed a new and interesting feature, the ability to form small amounts of the disaccharide cellobiose, when acting unaided, or glucose when supported by cellobiase (Halliwell & Riaz, 1971). In each case the total sugar produced was equivalent to the total soluble carbohydrate. Further, the combined enzymic reaction was synergistic in increasing the solubilizing (hydrolytic) power of component C_1 by over 50% to give an overall solubilization of 16% of the initial substrate. Since that time our best component C_1 preparations acting alone have usually attained about 10% solubilization of the standard amount and form of cellulosic substrate (2mg of dewaxed cotton fibres or 20% solubilization of 1mg in the same period of 7 days; cf. Fig. 1), a degree of solubilization which, on addition of the purified cellobiase component, invariably increased by some 20–80% or more (12% to over 16% solubilization of substrate) over the 7-day assay period at 37°C, depending on the potency of the two enzymic components involved. The effect was more marked with short fibres as a cellulosic substrate, increases of 90–110% (19–21% solubilization of substrate) being obtained irrespective of whether the substrate had been prepared from (dewaxed) cotton fibres by the action of crude culture filtrates of *Trichoderma koningii* or *Myrothecium verrucaria*. The standard assay procedures indicated no cellobiase or CM-cellulase in the C_1 component.

Periods beyond 7 days had little or no effect in promoting cellulolysis of native substrates (dewaxed cotton) by component C_1 unless cellobiase was also present, in which case almost 70% solubilization of substrate was achieved in 21 days (Fig. 1). Cellobiase failed to attack the substrate in 28 days. Hydrolytic products formed from cotton by component C_1, identified by the glucose oxidase procedure in our earlier work (Halliwell & Riaz, 1971), were now confirmed by chromatographic separation and isolation on Sephadex G-15. Throughout the incubation period cellobiose was the sole sugar released by the action of component C_1. The presence also of cellobiase in the reaction mixture permitted only glucose to accumulate. During the 3-week incubation period the most obvious action of component C_1 on dewaxed cotton fibres was to produce little apparent change in the substrate other than a gradual decrease in mass. No short fibres were found.

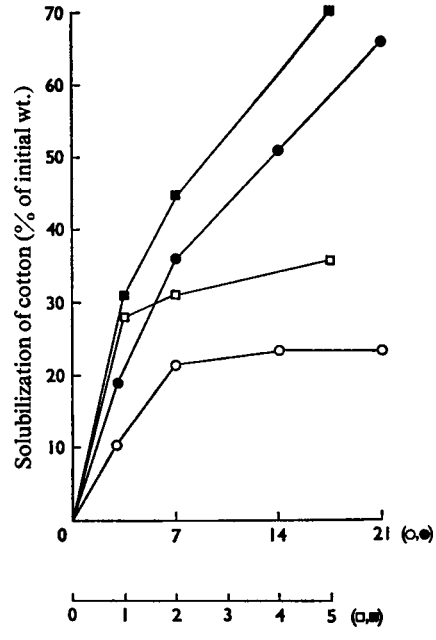

Fig. 1. *Action of component C_1 and cellobiase on native forms of cellulose*

The standard assay system was used with 1mg of dewaxed cotton fibres and 1ml of component C_1 (5μg), either alone (○) or supplemented with 1ml of cellobiase (10μg) (●) for up to 21 days, or with bacterial cellulose (1mg) and 0.2ml of component C_1, either alone (□) or supplemented with 0.2ml of cellobiase (■) for up to 5 days. The last point for bacterial cellulose represents 80% solubilization. Solubilization was measured by the phenol method at 490nm on the total soluble carbohydrates present in the supernatant after centrifuging (Halliwell & Riaz, 1971).

Replacement of the native fibrous cellulose of cotton by that of bacterial cellulose as a substrate for the combined action of component C_1 and cellobiase proved even more favourable to cellulolysis. As above with cotton, cellobiase itself was wholly ineffective also against the new and simpler cellulosic substrate throughout the incubation of 5 days' duration. Component C_1 acting alone on the same substrate rapidly attained its near-maximum hydrolysis of 28% in 1 day, increasing this solubilization by only 8% in the subsequent 4 days (Fig. 1). When reinforced by cellobiase, however, component C_1 displayed almost linear activity with time up to 80%

solubilization over the same period (Fig. 1). The pattern of hydrolysis of bacterial cellulose was almost identical with that found above with cotton cellulose, namely cellobiose from the action of component C_1 alone or glucose if cellobiase were also present. The only exception to this was observed when the extent of hydrolysis reached 36% (Fig. 1, day 5) with bacterial cellulose subjected to the action of component C_1 alone. At this point 98% of the reducing sugars released were in the form of cellobiose, and the rest as glucose.

Inhibition of component C_1 acting on native cellulose

Although cellobiase itself lacked any action on cellulose its ability to promote hydrolysis of both relatively simple and complex forms of cellulose by component C_1 suggested that the latter activity might be inhibited by its product cellobiose. Although the typical values (Table 1) with cotton fibres arose from enzymic hydrolyses extending over several days, they suggested that cellobiose could act as a competitive inhibitor. The degree of inhibition of component C_1 by cellobiose depended on the relative concentrations of substrate and inhibitor. Increasing the concentration of substrate enabled the inhibition to be gradually relieved and almost overcome; thus in the presence of the smallest amount of cellulose (0.05mg/ml), cellobiose at a concentration of 79μM was inhibitory in decreasing the extent of hydrolysis to 40% of that in the uninhibited control system. Cellobiose at the higher concentration (158μM) was completely inhibitory and this in turn was only slightly affected by increasing the quantity of substrate to 0.5mg/ml.

Table 1. *Effect of cellobiose on cellulolysis by component C_1*

The standard assay procedure was used to determine the solubilization of different amounts of cellulose (dewaxed native cotton fibres) by component C_1 at 37°C in periods up to 7 days in the absence and in the presence of cellobiose as an inhibitor. Solubilization, as a percentage of the initial amount of substrate, was measured from the formation of reducing sugars by the ferricyanide procedure (see the Experimental section).

Concn. of cellobiose (μM)	Solubilization of substrate (%) Initial concn. (mg/ml) ... 0.05	0.2	0.5
0	36	12	6
79	14	10	5
158	0	0	1

In contrast the same amount of substrate almost completely overcame the inhibitory effect of 79μM-cellobiose shown at the lower concentration of cellulose (0.05mg/ml).

Cellobiose formed as a product *in situ* may be even more effective as an inhibitor than that added as the free sugar. For example, component C_1 was incubated alone in buffer solution (sets *a* and *c*) or with cellobiose at a final concentration of 60μM (set *b*) for 1h at 37°C before addition of the same amount of disaccharide to set *c*, and cellulose (dewaxed fibres) to all three sets. After further incubation for 7 days the extent of hydrolysis of cellulose in sets *b* and *c* containing added cellobiose was 69 and 86% respectively of the control (set *a*). Evidently more intimate association of enzyme and its product cellobiose was conducive to the development of the inhibitory system. 2-Nitrophenyl β-D-glucopyranoside tested under the same conditions as were employed with cellobiose (Table 1) produced a similar degree of inhibition to that shown by the sugar at equimolar concentrations.

Other properties of component C_1 acting on native cellulose

Hydrolysis of the native cellulose of cotton by component C_1 was confined to acid media, being optimum at pH5 (Fig. 2). Enzymic activity, feeble though it was at values as low as pH3, was almost as effective as that at pH6. A somewhat similar response to acid and alkali was shown by the enzyme in testing its stability in absence of substrate (Fig. 2). The enzyme revealed marked sensitivity to neutral and alkaline media but relatively high resistance to destruction in solutions as acidic as pH2; optimum stability was shown at pH5.

Our standard assay system for component C_1 operates at 37°C, which proved to be the optimum of temperatures tested (Fig. 3). Although cellulolysis was just as effective at slightly below (30°C) as it was above (40°C) the optimum of 37°C, a further rise in temperature of only 5° to 45°C produced rapid inactivation of the enzyme by over 50%. Sensitivity to elevated temperatures was further emphasized by incubating the enzyme in the absence of substrate at 45–80°C (Fig. 4). As little as 5min exposure within this range destroyed 40–100% of the enzymic activity. Even at 45°C only slightly above that of the standard assay at 37°C, almost 70% of the initial activity was lost after incubation for 30min in the absence of cellulose.

Kinetic measurements on component C_1

The nature of the action of component C_1 as just described, particularly against the native cellulose of cotton, was less than helpful in attempting to analyse its enzymic activity in the initial stages of hydrolysis.

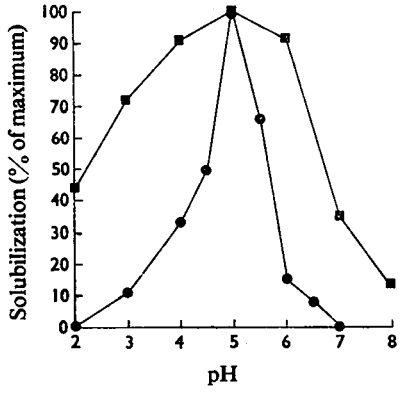

Fig. 2. *Effect of pH on the activity and stability of component C_1*

The effect of pH on the enzymic solubilization of cellulose (dewaxed native cotton fibres) was measured in the standard assay system with 0.2M-acetic acid–HCl buffer up to pH5.5 and with 0.2M-KH_2PO_4–NaOH buffer for pH6–7 (●). After incubation for 7 days at 37°C at each pH value the total soluble carbohydrates formed were separated by centrifuging and measured as described in Fig. 1. At the optimum of pH5 the degree of solubilization (set at 100%) corresponds to 24% of the 1mg of cellulose used. In determining the stability of the enzyme (■) component C_1 was incubated without substrate in buffer solutions at pH2–8 for 15min at 37°C, adjusted to pH5 with acetic acid or sodium acetate and supplied with cellulose (dewaxed cotton). Cellulolysis was measured after incubation for a further 7 days at 37°C. For pH8, 0.05M-boric acid–KCl–NaOH buffer was used. Other details are given above.

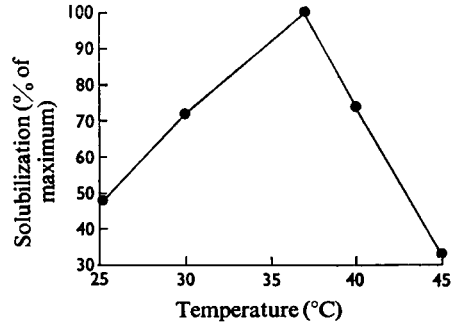

Fig. 3. *Variation in the activity of component C_1 with temperature*

The enzyme was assayed under standard conditions with cellulose (dewaxed cotton) at pH4.8 and at the temperatures shown. Other details are given in Fig. 2.

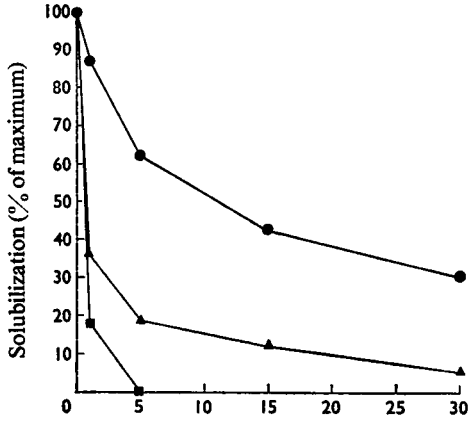

Fig. 4. *Stability of component C_1 at different temperatures in the absence of cellulose*

The enzyme in the standard acetate buffer system at pH4.8 was kept at 45°C (●), 60°C (▲) and 80°C (■) for 1–30min, and then cooled to 37°C, treated with substrate (dewaxed cotton) and incubated at that temperature for 7 days. All measurements are relative to control sets incubated under standard conditions at 37°C for 7 days. Other details are as given in Fig. 2.

Difficulties were minimized, however, by using bacterial cellulose, a relatively simpler native substrate, which had proved more amenable to hydrolysis (Fig. 1). Under the conditions shown in Fig. 5 a Michaelis constant, K_m, of 0.05mg of cellulose/ml and a maximum velocity, $V_{max.}$, of 14μg of reducing sugar/ml in the 5h assay were derived from double-reciprocal plots. At near-complete saturation of enzyme with substrate, curve 2 of Fig. 5 depicts linear hydrolysis of cellulose during a 16h incubation period in the presence of small amounts of enzyme. At higher concentrations, beyond 0.2μg of component C_1/ml, the enzyme appears both unsaturated and unstable and departs from the linear response (Fig. 5, curve 3).

Cellobiose at concentrations of 0–56μg/ml acted as a competitive inhibitor of component C_1 (Fig. 6). CM-cellulose tested under the same conditions up to 42μg/ml gave a similar family of lines. The inhibition constant (K_i) calculated from the slope or from the apparent increase in K_m of the plots for 28μg of each inhibitor gave values of 21μg of cellobiose/ml and 8μg of CM-cellulose/ml respectively, suggesting that the polymer was a somewhat more effective inhibitor of component C_1.

A number of other compounds were tested likewise as possible inhibitors of component C_1 at the same concentrations (14–56μg/ml), but only at low

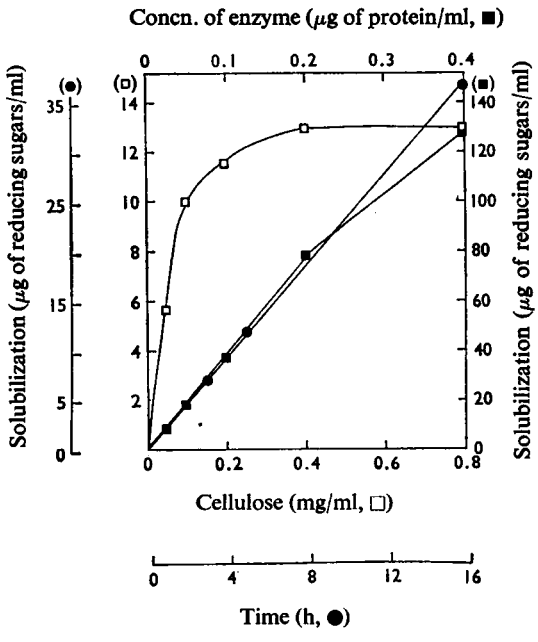

Fig. 5. *Effect of amount of substrate, period of incubation and enzyme concentration on the activity of component C_1 on cellulose*

For all lines on the figure the substrate bacterial cellulose was incubated at 37°C for various periods of time with 0.02ml of enzyme (0.1 μg of protein)/ml of assay system unless stated otherwise. In line (1) (□) where the period of incubation was 5h the substrate ranged from 0.05 to 0.8mg/ml and the activity from 0 to 13 μg of reducing sugars/ml measured at 625nm (Halliwell, 1961; Halliwell & Riaz, 1970); in line (2) (●) a constant amount of cellulose (0.3mg/ml) was incubated for periods up to 16h and provided activities from 0 to 37 μg of reducing sugars/ml; in line (3) (■) 0.3mg of cellulose/ml incubated for 16h with 0.005–0.08ml of enzyme (0.025–0.4 μg of protein)/ml gave 0–128 μg of reducing sugars/ml. All concentrations are per ml of assay system.

substrate concentration (0.1mg/ml). In marked contrast with the two inhibitory cellulose derivatives examined above glucose had no effect when employed in the same amount (twice the molarity) as that used with cellobiose above. A similar lack of response was found with 1,5-gluconolactone, sucrose, *p*-chloromercuribenzoic acid, *N*-lauroylsarcosinate, diethyldithiocarbamate and $MnSO_4$. Of the remaining compounds tested the metal salts $CuSO_4$ (390 μM, by extrapolation), $ZnSO_4$ (200 μM) and $FeCl_3$ (45 μM) were increasingly effective at the concentrations shown in giving 50% inhibition of component C_1 activity. The same degree of inhibition was obtained with the organic mercurial phenylmercuric acetate at 83 μM.

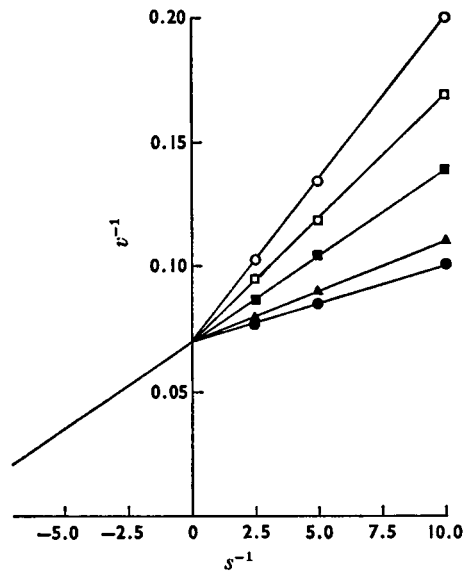

Fig. 6. *Competitive inhibition of component C_1 by cellobiose*

Component C_1 (0.02ml/ml) was incubated under the standard conditions (Fig. 5, line 1, □) with different concentrations (s) of bacterial cellulose (0.1–0.4mg/ml) as illustrated in the absence (●) and in the presence of a constant amount of the inhibitor cellobiose at 14 (▲), 28 (■), 42 (□) and 56 (○) μg/ml. All concentrations are per ml of assay system. The formation of reducing sugars as μg/ml (measured at 625nm) during the assay period of 5h is represented by v.

Discussion

Component C_1 is believed to be a cellobiohydrolase which acts weakly on native cellulose (Halliwell & Riaz, 1971), and more effectively on simpler forms of insoluble cellulose (Halliwell *et al.*, 1972). More recently Wood & McCrae (1972) have studied the action of a component C_1 preparation on a degraded acid-swollen cellulose. Since, however, they were apparently unaware of the earlier work with preparations of component C_1 freed from extraneous activities (Halliwell & Riaz, 1971; Halliwell *et al.*, 1972), the significance of their findings with a relatively impure component C_1 is difficult to assess. Thus after two treatments on DEAE-Sephadex their component C_1 (Wood & McCrae, 1972) still possessed amounts of C_x activity (CM-cellulase) readily detectable in an

hour. Although the extraction procedure followed that used by Wood (1968) the possible presence also of short-fibre-forming activity was not investigated. Further treatment of the C_1 preparation by isoelectric focusing before testing against cellulose (Wood & McCrae, 1972) did not appear to improve its purity. The presence therein of the additional cellulolytic activities could well account both for the increase in the extent of solubilization of cotton from 4 to 7% observed by Wood & McCrae (1972) on extending the incubation from 7 to 28 days, and for the further increase to 15% solubilization, also in 28 days, on trebling the concentration of component C_1. In contrast, our own unconcentrated component C_1 preparation free from C_x (and short-fibre-forming activity) achieves about 10% solubilization of the same substrate (2mg of cotton) in 7 days (Halliwell & Riaz, 1970, 1971) with little or no increase beyond this period (cf. Fig. 1).

Selby & Maitland (1967) and Wood (1968) considered short-fibre-forming activity to be a property of both component C_1 and C_x (see also Selby, 1968). C_1 and cellobiase components of both these groups of workers each contained CM-cellulase, and the C_1 component also had short-fibre-forming activity. Hence the observed synergism between such impure cellobiase and C_1 components could best be assigned to C_1–C_x interactions, as Wood (1968) himself suggests. Wood & McCrae (1972) reported that their residual CM-cellulase activity was a function of component C_1. However, our own component C_1 preparations are free from CM-cellulase (as well as short-fibre-forming activity), the former activity being measured by essentially the same procedure (ferricyanide; Halliwell, 1961) as that now adopted by Wood & McCrae (1972).

In attempting to avoid the difficulties encountered above we used component C_1 only after it had been recycled until free from other enzymic activities. Bacterial cellulose, a native substrate, was preferred to acid-swollen cellulose as a more susceptible or 'degraded' substrate than cotton, since this avoids the possibility of substituting phosphate groups in cellulose and of rendering it susceptible to other components such as CM-cellulase. Our component C_1 as a cellobiohydrolase attacks not only the most complex, undegraded forms of cellulose as in cotton but also simpler forms by releasing terminal cellobiose units (Fig. 1). This sugar alone or with trace amounts of glucose accounted wholly for the total carbohydrate solubilized, no other product being formed. Component C_1 action on different substrates is limited mainly and most effectively by its product, cellobiose, when other components of the cellulase system are absent. It is of some interest that the cellobiose found at the maximum limits of hydrolysis attained by component C_1 acting alone on simple and complex forms of cellulose respectively (Fig. 1) is of the same order (about 40–30% of the concentrations respectively) of added cellobiose found to inhibit the enzyme (Table 1, Fig. 6). The difference may be accounted for in the greater efficiency of the cellobiose formed *in situ* (see the Results section) and the heterogeneity of the enzymic reaction. Under these conditions the rate-limiting step is determined by the dissociation of the product from the enzyme–product complex, an action relieved only, so far, by the presence of cellobiase. Dialysis was unsatisfactory. In the complete cellulase system cellobiohydrolase may also be regulated by cellodextrins if their action is analogous to that shown in the present report by cellulose derivatives of greater (CM-cellulose) and lesser (cellobiose) complexity. The inhibition of cellobiohydrolase is competitive and is overcome by increasing substrate concentration. The latter effect, coupled with the increased availability of sites of attack, and the instability of the enzyme (Fig. 4) could account for the greater susceptibility of degraded substrates (cf. bacterial cellulose) to hydrolysis by this enzyme with its preference for chain ends. It also reinforces the idea of a terminal mode of attack by component C_1. Of the other compounds tested against the cellobiohydrolase the most effective was Fe^{3+} (50% inhibition at 45μM). Not unexpectedly phenylmercuric acetate was more potent (50% inhibition at 83μM) as an organic mercurial than was mercuribenzoate, its action demonstrating the presence and importance of thiol groups in this enzyme. In contrast, complete lack of inhibition was shown by glucose even at twice the molarity used with cellobiose.

In proposing a mechanism of cellulolysis Wood & McCrae (1972) make two statements requiring comment: first, that C_1 is a C_x component unable to attack highly ordered substrates; secondly, they dispute the postulate of Reese *et al.* (1950) by stating 'C_x and not C_1 initiates attack on native cellulose by providing end-groups for C_1'. The present work demonstrates that a purveyor of accessible end-groups is not essential for the action of component C_1; the enzyme itself is capable both of initiating hydrolysis and of generating end-groups from cellulosic substrates of any complexity without the aid of component C_x. A scheme for the cellulolytic process had been proposed earlier (Halliwell & Riaz, 1971) whereby two cellulases, components C_1 and C_2, had both shown their ability to initiate attack on native cellulose. The latter enzyme was highly effective in promoting the formation of short fibres (additional chain ends) in one of the first recognizable stages in the degradation of the substrate (Halliwell, 1966). The present work extends these findings in showing that relatively simple forms of cellulose, intermediate products in the form of short fibres and the parent, most complex fibrous substrate itself, are all susceptible to the action of component C_1 and further break-

down into soluble products. These may be cellobiose or glucose depending on whether component C_1 acts alone or in company with cellobiase. Under the latter conditions, and relieved from the action of cellobiose, no other component of the cellulase system is needed for the cellobiohydrolase to extend, apparently to completion, the hydrolysis of simple and complex forms of cellulose. With cellobiose, the product of hydrolysis, acting as an inhibitor and glucose forfeiting this property, the intact organism has developed an efficient regulatory system to suit its own particular metabolism and environment under conditions of dearth and plenty.

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