The action on cellulose and its derivatives of a purified 1,4- β -glucanase from *Trichoderma koningii*

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The specific properties have been examined of the 1,4- β -glucanase component of *Trichoderma koningii* that participates in an early and effective stage of random breakdown of native cellulose to short fibres. The enzyme was purified and freed from associated components of the cellulase complex (particularly β -glucosidase) that interfere with, and complicate interpretation of, the action of such enzymes. Purification increased the specific activity 25-fold over culture filtrates; the enzyme hydrolysed CM-cellulose faster than the purified β -glucosidase from the same organism hydrolysed any of its substrates (cellobiose or cellodextrins). The specificity of the glucanase was directed towards soluble derivatives of cellulose, CM-cellulose and cellodextrins, and not to insoluble cellulose or α -linked polymers. The approximate K_m was 2.5 mg of CM-cellulose $\cdot$ ml⁻¹ at 37°C at the optimum pH, 5.5, where enzymic activity was maximal with 6–7 mg of CM-cellulose $\cdot$ ml⁻¹ and inhibited by higher concentrations. The temperature optimum was 60°C. The glucanase attacked larger cellodextrins (cellohexaose to cellotetraose, in that order) much more readily than smaller dextrins (cellobiose and cellotriose) and released a mixture of products, glucose up to cellopentaose, which was quantitatively determined after chromatography on charcoal. Similar examination of hydrolysates of the reduced cellodextrins showed clearly the high specificity of the enzyme for the central bond of its natural substrates (the cellodextrins), whatever their chain length, and indicated the nature of the enzyme as an endoglucanase. Outer bonds shared a weaker, but similar, susceptibility to enzymic cleavage. Transferase activity was absent and no larger dextrins than the initial substrate were formed.

Cellulosic materials have long played an important role in everyday life as constituents of wood, paper, cloth, rayon, film, plastics, rope and fillers. More recently, the polysaccharide has been recognized as a valuable source of energy, chemicals and microbial protein, available in vast quantities of continuously renewed biomass (Bailey *et al.*, 1975; Ghose, 1977; Linko, 1977; Flickinger & Tsao, 1978; Halliwell, 1979). Realization of this potential however, requires a thorough understanding of cellulolytic microbes and their enzymes. The latter have included, for many years, three distinct activities, β -glucosidase or cellobiase, glucanase (CM-cellulase) and Component C₁. The last component is a cellobiohydrolase and releases G₂ from

cellulose (Halliwell & Riaz, 1971; Halliwell *et al.*, 1972), a reaction confirmed elsewhere in related *Trichoderma* species (Wood & McCrae, 1972; Berghem & Pettersson, 1973). The role of β -glucosidase has been studied in detail on cellobiose (Berghem & Pettersson, 1974; Gong *et al.*, 1977) and on cellodextrins also (Halliwell & Vincent, 1977).

The role of glucanase(s), however, is unknown. In some way it co-operates with glucosidase and cellobiohydrolase in stimulating saccharification of cellulose (for reviews, see Halliwell, 1979; Lee & Fan, 1980). In *Trichoderma koningii*, two main glucanase (CM-cellulase) components have been recognized, one associated with Component C₂ (Halliwell & Riaz, 1971; Halliwell & Griffin, 1978). These interact to convert native cellulose into short fibres, a property of random segmentation not shared by other components, cellobiase and cellobiohydrolase, of the cellulase complex. Cellobio-

Abbreviations used: G₂, G₃, ...G₆, cellobiose, cello-triose, ... cellohexaose; D.S., degree of substitution; G₁H, G₂H, ...G₆H, sorbitol (glucitol), cellobiitol, ... cello-hexaitol.

hydrolase from *T. viride* likewise failed to form free fibres from filter paper, whereas two CM-cellulases both had that property, one showing some ability to decrease the viscosity of cotton (Berghem *et al.*, 1976). Elsewhere, three purified CM-cellulases from *T. viride* showed that their individual activity on celloextrins differed, but increased from G_2 to G_6 (Shoemaker & Brown, 1978). The contribution of the enzymes to cellulolysis was not investigated. The role of glucanases thus remains obscure, but of considerable interest. In one of the reactions reported above, CM-cellulase showed a specific synergistic association leading to random degradation of native cellulose to short fibres. The present work concerns the isolation, purification and nature of that glucanase (CM-cellulase) and its contribution to cellulose metabolism.

Materials and methods

Enzyme

Cultures of *Trichoderma koningii* were grown on cleaned non-dewaxed cotton fibres and inorganic-salts medium for up to 34 days, filtered, clarified by centrifuging and sterilized by filtration (Halliwell & Riaz, 1970, 1971).

Methods of enzymic analysis

For the present work it was desirable to minimize interference from other components of the cellulase complex produced by this organism. This was aided by increasing the sensitivity of available assay procedures as follows.

Cellobiase activity (EC 3.2.1.21). This was measured specifically as glucose liberated from G_2 by using reagents for the glucose oxidase/peroxidase procedure (Halliwell, 1965, 1966), modified thus: 0.1 ml of 0.1 M-acetate buffer, pH 5.5, 0.4 ml of 0.1% G_2 and enzyme (0.1 ml) in a total volume of 0.6 ml were incubated for 1 h (or longer, see below) at 37°C, followed by addition of 1 ml of 0.9 M-Tris-buffer, pH 7, 0.25 ml of glucose oxidase and 1.25 ml of peroxidase/dianisidine solutions. After the mixtures had been incubated for 1 h at 37°C, absorbances were read at 436 nm.

CM-cellulase (glucanase) activity (EC 3.2.1.4). This was measured on CM-cellulose (low viscosity, D.S. 0.7; BDH Chemicals, Poole, Dorset, U.K.) by using 0.15 ml of 0.1 M-acetate buffer, pH 5.5, 0.35 ml of substrate (1%) and 0.1 ml of enzyme (total volume of 0.6 ml), incubated for up to 1 h, or as stated below, at 37°C before determining reducing sugars as glucose in the whole sample by the direct ferricyanide procedure (Halliwell & Riaz, 1970). For present purposes this involved adding 0.4 ml of 1% (w/v) Na_2CO_3 , 1 ml of water, 1 ml of cyanide plus carbonate mixture and 2 ml of ferricyanide reagent

before boiling for 25 min, cooling 5 min, and reading absorbances at 420 nm (Halliwell, 1961).

Protein determinations were made by the Folin-Lowry procedure, with citrate replacing tartrate (Halliwell, 1961).

Determination of substrates

Glucose (A.R. grade) and cellobiose (G_2) (both from BDH), soluble dextrins and reprecipitated cellulose (prepared as described below) were all measured by the chromic acid procedure (Halliwell, 1958). The phenol/ H_2SO_4 procedure was also used for determining total soluble carbohydrates (Halliwell & Riaz, 1971) and the ferricyanide/ferric alum $[NH_4Fe(SO_4)_2]$ method for reducing sugars (Halliwell, 1961).

Reduced sugars, sorbitol (G_6H) to cellohexaitol (G_6H), were purchased (sorbitol; BDH) or prepared as described below and, as expected, did not react with the ferricyanide reagent, but all (equal amounts) responded equally in the chromic acid procedure; each reduced sugar showed a different response in the phenol/ H_2SO_4 method compared with the corresponding reducing sugar. Equal amounts of reducing sugars (glucose to G_6) gave progressively less response in the ferricyanide procedure as the chain length increased. These differences were useful in identifying different products during fractionation of enzymic hydrolysates, by using calibration curves prepared for each sugar, glucose to G_6 (reducing and reduced) by the phenol/ H_2SO_4 procedure, and for the same reducing sugars by the ferricyanide method.

Preparation and purification of soluble dextrins and reprecipitated cellulose

Soluble celloextrins from G_3 to G_6 were prepared from cellulose by a procedure adapted from Miller (1963); CO_2 and concentrated HCl replaced fuming HCl, and an alternative form of charcoal improved the resolution and permitted separation and recovery of celloextrins and reduced celloextrins (not described in the original procedure) in a quantitative manner. Essential details are as follows. Cellulose powder (15 g; Whatman no. 1 or CF 11) was added with vigorous stirring to concentrated HCl (A.R. grade, sp.gr. 1.18, 200 ml) precooled to -30°C with solid CO_2 . Stirring was continued (15–20 min) at this temperature until the mixture was homogeneous and had become slightly yellow. The temperature of the reaction mixture was then raised to, and maintained at, 25°C with continuous stirring for 0.75–1 h (for mainly the larger dextrins, G_5 and G_6) or 2–3 h (for predominantly shorter dextrins, G_3 and G_4). A heavy white precipitate appeared when, at the end of the hydrolysis period, the reaction mixture was poured into ice-cold distilled water (600 ml). Partial neutralization to

pH 4–5 required 180 g of solid NaHCO_3 (A.R. grade).

Reprecipitated cellulose. The white precipitate described above was filtered (Whatman no. 1 filter paper) and the filtrate (800 ml) retained for soluble dextrins; the precipitate was washed repeatedly with distilled water to remove much-soluble dextrins and, especially, G_2 and glucose. Residual soluble sugars were then best removed by dialysing the precipitate for 7 days at 4°C with daily changes of distilled water. Portions of suspension were removed at intervals and tested for reducing sugars by the ferricyanide/ferric alum method (see above) until absent. Total carbohydrates were determined by the chromic acid method and the suspension diluted to 10 mg/ml in 0.05% chlorbutol (BDH). The yield of reprecipitated cellulose was 73–79%.

Soluble dextrins. (a) Initial separation on charcoal. The 800 ml filtrate from the insoluble dextrin precipitate was concentrated to 100 ml by rotary evaporation at 45°C . The precipitate of NaCl was removed by filtration (Whatman no. 1 filter paper) with no loss of soluble dextrins (determined as reducing sugars). Chromic acid determination of carbohydrates showed the yield of soluble dextrins was 6% of the initial cellulose.

Charcoal (120 g; Norit A; Sigma, Poole, Dorset, U.K.) and Celite Analytical Filter Aid (120 g; BDH) were gently stirred with 960 ml of 2.5% stearic acid (s.p. grade; BDH) in ethanol (96%, v/v) for 30 min (dissolution of stearic acid in ethanol required 30 min at 45°C). The adsorbent was sucked down on a Buchner funnel to remove ethanol and free stearic acid before resuspension in 960 ml of 50% ethanol, stirring for 15 min and re-filtering. The precipitate was finally resuspended in 300 ml of 10% ethanol to give a uniform slurry and was pumped (type MHRE pump; Watson Marlow Ltd., Falmouth, Cornwall, U.K.), while being stirred, into a Pyrex column ($5\text{ cm} \times 60\text{ cm}$) fitted at each end with a bung carrying inlet or outlet tubes. The lower bung with the outlet tube was covered with a glass-wool plug overlaid with two thicknesses of glass-fibre filter-paper (Whatman GF/A). While the column was being packed, continuous stirring of the adsorbent passing to the pump was essential to prevent its sedimentation in the stock vessel and in the rubber connecting tubes. Before dextrins were separated, the column was washed with 4 litres of water to remove ethanol.

A portion (50 ml; 500 mg) of the concentrated dextrin solution above was applied to the top of the column and pumped into it (compressed-air line) through the inlet bung. At this stage both bungs were wired on firmly to the ends of the column, the upper bung of which was connected by rubber tubing via the Watson Marlow pump to a 3-litre aspirator (mixing vessel), magnetically stirred, containing 2.2

litres of water. The upper (inlet) end of this otherwise closed vessel was connected to a similar aspirator (reservoir) open to the atmosphere at its upper end. The column was then washed with water (5–6 litres) to remove all glucose from the sample (verified in the eluate by the ferric alum method described above). The empty reservoir was then filled with 3 litres of 50% ethanol, the mixing vessel with 2.2 litres of water, the alcohol gradient was commenced and 12 ml fractions collected. The flow rate of the pump was adjusted throughout the separation to maintain the starting value of 50–55 ml/h. A 0.1 ml portion of every fifth fraction was tested for reducing sugars by the ferric alum method (see above). Each dextrin peak was pooled and concentrated by rotary evaporation at 45°C to a volume of about 30 ml for further purification (see below). When the final eluates showed less than $5\text{ }\mu\text{g}$ of reducing sugar/ml by the ferric alum method, the column was washed with water until free from alcohol and ready for re-use.

(b) Purification of cello-dextrins (G_3 , G_4 , G_5 and G_6). A 0.6 ml portion ($400\text{ }\mu\text{g}$ of sugars) determined by the chromic acid method of the partially purified cello-dextrins above showed, on micro-column analysis (below), that each was contaminated with the higher and lower member of the series. Removal of these contaminating sugars was effected by passing the concentrated samples through smaller charcoal columns ($3\text{ cm} \times 30\text{ cm}$), exactly as above, except that the mixing vessel contained 500 ml of water and fractions were collected as 6 ml volumes. Each main dextrin fraction so obtained was re-run two to three times on similar columns until micro-column analysis of $400\text{ }\mu\text{g}$ portions showed a single purified peak of the main dextrin, which was concentrated to small volume and stored in glass-stoppered flasks at -10°C .

(c) Micro-column analysis of sugars, dextrins and their reduced derivatives (up to $400\text{ }\mu\text{g}$). Charcoal/celite adsorbent, prepared as above, was dried at 18°C in a vacuum desiccator over silica gel to a fine greyish-black powder. When needed, 2 g was suspended in 5 ml of 20% ethanol, poured into a glass column ($1.3\text{ cm} \times 5\text{ cm}$) and packed by using compressed air. At its lower end the column carried a bung bearing a small glass outlet tube covered by a glass-fibre filter-paper disc (Whatman GF/A). The adsorbent was washed with water until free from ethanol. Sugars or dextrins in 0.6 ml samples (up to $400\text{ }\mu\text{g}$ of total carbohydrates) were applied to the top of the charcoal bed by using compressed air, the space above the adsorbent was filled with water and the bung bearing an inlet tube replaced. Carbohydrates were eluted sequentially in an alcohol gradient prepared in a mixing vessel (200 ml, containing 100 ml of water), stirred magnetically, and sealed except for connections to the column and

a reservoir of ethanol (96%). A Watson Marlow pump between the column and mixing vessel maintained a flow rate of 20 ml/h. Fractions (0.6 ml) were collected and determined by the ferric alum method for reducing sugars, and the phenol/ H_2SO_4 method for total carbohydrates. By using both methods of determination it was possible to locate and differentiate reducing and reduced compounds (see text and Fig. 2 below).

Preparation of reduced cellodextrins. G_2 and purified dextrins up to G_6 were converted into the corresponding reduced derivatives with NaBH_4 (A.R. grade, BDH) as described by Cole & King (1964). The reduced sugar was finally deionized on a mixed-bed resin, concentrated by rotary evaporation at 45°C to 1 mg/ml (determined by the chromic acid method) and stored in a glass-stoppered flask at -10°C . Before use, 400 μg portions of each reduced carbohydrate was checked by micro-column analysis to ensure that no reducing sugar was present and that the sample consisted only of a single reduced cellodextrin.

Other substrates

The following were purchased: methylcellulose (low substitution, D.S.1.6, and high substitution, D.S.1.9) and soluble starch (A.R. grade), from BDH; oxycellulose and mannan from Sigma; amylose, amylopectin, dextran and nigeran from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Buffer solutions

The effect of pH on enzymic activity and stability was measured in these buffers; pH 1–3 with 0.2 M-sodium acetate/HCl; pH 4–5.5 with 0.1 M-sodium acetate/acetic acid; pH 6–8 with 0.05 M- KH_2PO_4 /NaOH; pH 8–10 with 0.05 M-boric acid/KCl/NaOH.

Results and discussion

Purification of a 1,4- β -glucanase (CM-cellulase)

In studying any one purified component, for example a CM-cellulase, of the cellulase complex, it is essential to ensure the absence of other cellulolytic components, not only in the period of their own standard assays, but also whenever this period is exceeded in reactions involving, in this example, purified CM-cellulase. Here the amounts of β -glucosidase present may be too small to be detected in a 1–2 h assay but still sufficient to hydrolyse any oligosaccharides (G_2 up to G_6 ; Halliwell & Vincent, 1977) in the longer periods used to study the mode of action of β -glucanases on such cellodextrins or on insoluble but reactive celluloses. This problem was overcome in the present work by re-cycling chromatography until the required enzyme purity was attained.

A 5 ml portion of 10-fold-concentrated freeze-dried culture filtrate from *T. koningii* was fractionated by affinity chromatography on a column (15 mm $\times$ 900 mm) of Cellex MX or Sigmacell type 38 polymer eluted at 25 ml/h with 20 mM-phosphate buffer, pH 6.8 (Halliwell & Griffin, 1978). As described by those authors, most of the CM-cellulase activity was recovered in two related components, the main CM-cellulase fraction (64% recovery) and Component C_2 (15% recovery); the cellobiase and cellobiohydrolase components contained 3 and 17% respectively of the total CM-cellulase. The main CM-cellulase component contained, in addition, weak activities, namely cellulase (2% solubilization of 2 mg of clean non-dewaxed cotton fibres in 7 days) and cellobiase (only 14% of that in culture filtrates, but readily detected over 18 h). The contaminating activities were removed by rechromatography of the dialysed, concentrated, main CM-cellulase component once more under the same conditions. Weak cellobiase activity now displayed only with a 48 h incubation was completely removed by a third treatment on the column. This final purified preparation was used in the ensuing work. It hydrolysed CM-cellulose at an initial rate of 1 μg of reducing sugar (as glucose) $\cdot \text{min}^{-1} \cdot (\mu\text{g of protein})^{-1}$, an increase in specific activity of 25-fold over that of culture filtrates. The rate of hydrolysis of CM-cellulose by this purified glucanase is thus 4–5-fold greater than that of the same amount of purified cellobiase protein acting on G_2 or cellodextrins (Halliwell & Vincent, 1977).

Further evidence of its purity was illustrated by its action on related substrates. In the standard assay on CM-cellulose at pH 5.5, the purified enzyme (2.9 μg of protein) hydrolysed its substrate readily to 2% in 30 min (8% in 4 h). The same amount of other cellulosic substrates, particularly insoluble forms, were more resistant, and even when exposed to 29 μg of enzyme protein, only methylcellulose D.S. 1.6 (4% hydrolysis in 4 h) approached the reactivity of CM-cellulose. In 18 h incubations, no saccharification occurred with methylcellulose D.S. 1.9, oxycellulose, reprecipitated cellulose, bacterial cellulose or in 7 days with cotton fibres. α -Linked polymers starch, amylose, amylopectin, dextran, nigeran and mannan showed no saccharification in 18 h.

Lack of activity by the purified β -glucanase against reactive celluloses (reprecipitated cellulose from HCl, bacterial cellulose) is interesting, since whole culture filtrates of this organism and related species are particularly effective against these and similar reactive forms of cellulose such as those swollen in phosphoric acid. Activity against the latter substrate is, however, characteristic of other purified CM-cellulases. Four such enzymes solubilized 29–83% of the acid-swollen cellulosic sub-

strate Avicel (2 mg) in 4 h (Wood & McCrae, 1978). Three endoglucanases from *T. viride* were highly active towards CM-cellulose and swollen Avicel but weakly active towards Avicel itself (Shoemaker & Brown, 1978). A homogeneous CM-cellulase from culture filtrates (of undefined cellulolytic ability) of *Aspergillus niger* was likewise relatively inert towards Avicel and cellulose powder, but readily degraded alkali-swollen cellulose (Hurst *et al.*, 1978). In the present work, although enzymic activity against reactive cellulose was present in culture filtrates, but absent from the purified CM-cellulase, that activity was found in the cellobiohydrolase component isolated (by chromatography on cellulose) at the same time as the CM-cellulase. The cellobiohydrolase attacked both our reactive celluloses above (23% soluble in 18 h) in the characteristic manner of cellobiohydrolases isolated by other workers, when hydrolysing acid-swollen Avicel. From our reactive cellulose the cellobiohydrolase liberated 93% of the products as G_2 , 6% as glucose, both measured by chromatography on charcoal columns; the same procedure showed that, in 24 h, G_3 was attacked to an extent of 17%, the products, being equimolar amounts of glucose and G_2 . G_2 , G_2H and G_3H were not hydrolysed in 48 h but recovered unchanged from the column.

No transferase activity was associated with our purified β -glucanase. Incubation of the enzyme as in the standard assay for up to 48 h with 100 μ g of glucose and 200 μ g of G_2 or G_3 , replacing CM-cellulose, showed, after separation and analysis on charcoal columns, a quantitative recovery of the original carbohydrates.

Kinetics

Typical saturation curves of the purified β -glucanase acting on CM-cellulose at the optimum pH indicated that saturation was achieved at approx. 6–7 mg of CM-cellulose/ml [4 mg/assay (Table 1)]. Higher concentrations were inhibitory, decreasing enzymic activity to 60% of maximum at 17 mg of CM-cellulose/ml. Approximate values for V_{max} and K_m were 0.88 (0.82) μ g of reducing sugar produced $\cdot$ min⁻¹ $\cdot$ (μ g of protein)⁻¹ and 2.5 (2.5) mg of CM-cellulose $\cdot$ ml⁻¹ respectively, obtained by double-reciprocal plots and (in parentheses above) by direct linear plots (Eisenthal & Cornish-Bowden, 1974). With 3.5 mg of CM-cellulose in the standard assay, the purified enzyme continued hydrolysis at the same rate as in Table 1 for up to 30 min and fell short of this rate by only 11% in 45 min.

Effect of pH and temperature on the activity and stability of the enzyme

Hydrolysis of CM-cellulose by the purified enzyme was limited to the range pH 2–9, with the

Table 1. *Effect of CM-cellulose as a substrate and inhibitor of β -glucanase activity*

The standard assay was used with 2.9 μ g of purified enzyme protein for 15 min with up to 10 mg of CM-cellulose in the standard total volume (0.6 ml). Reducing sugars were measured as glucose by the standard method for CM-cellulase activity.

CM-cellulose (mg)	Reducing sugars produced (μ g)
0.54	10.0
1.00	15.7
1.80	20.0
4.00	26.2
6.00	25.7
8.00	20.0
10.00	15.3

optimum at about pH 5.5 (Fig. 1, $\square$), whereas maximum stability, in the absence of substrate, was found at pH 5.0, in line with a general displacement of stability towards acidic regions (Fig. 1, $\blacksquare$). Similar measurements in the absence of substrate, at the optimum pH, but at 25–70°C, indicated maximum stability of the enzyme extended only up to 30°C, thereafter falling rapidly at 60°C to only 29% of its maximum value and losing all activity at 70°C (Fig. 1, $\bullet$). Even at 37°C some 7% of the activity was lost in the absence of substrate. In contrast, at 60°C in the presence of substrate, the glucanase showed maximum activity, some 42 and 127% greater than that at the standard assay temperature of 37 and 25°C respectively (Fig. 1, $\circ$).

Mode of action against cellodextrins

The action of the purified β -glucanase was examined on G_2 and soluble reducing dextrins up to G_6 and on the corresponding non-reducing (reduced) sugars (G_2H to G_6H), the latter each possessing a terminal reduced group to facilitate recognition of the site of action of the enzyme. All these compounds as mixtures or as enzymic hydrolysates (without pretreatment) can be separated and determined quantitatively on charcoal micro-columns in amounts from 10 to 400 μ g (see the Materials and methods section and Fig. 2). Fraction numbers of the peaks were readily reproducible and the method was more effective in separating higher cellodextrins than columns (up to 90 cm length) of Bio-gel P2 or Sephadex G-15 operated at temperatures up to 60°C, though such columns are satisfactory for separating mono- and di-saccharides (Halliwell & Griffin, 1973).

The nature and quantity of the separated hydrolytic products enables estimates to be made of the frequency of attack on the different glucosyl bonds of cellodextrins. For convenience these bonds are numbered, starting (no. 1) at the non-reducing end of

Fig. 1. Effect of pH and temperature on the activity and stability of β -glucanase

□, Effect of pH on enzymic activity was measured over 15 min under standard conditions with CM-cellulose in buffers at pH 1–10 (see the Materials and methods section), followed by neutralization (where necessary, with carbonate, as described in the Materials and methods section) and determination of reducing sugars by the standard procedure. An enzymic activity of 100% corresponds to 30 μ g of reducing sugars. ■, Effect of pH on enzymic stability was measured by incubating the enzyme in the same buffers as above, in absence of substrate, for 30 min at 37°C, followed by adjusting the pH to 5.5 with a predetermined quantity of buffer and removal of a portion for assay on CM-cellulose by the standard method for 15 min. ○, Effect of temperature on enzymic activity was determined over 15 min under standard conditions with CM-cellulose but at 25, 30, 37, 45, 60, 70 and 80°C. ●, Effect of temperature on enzyme stability was measured by incubating the enzyme at the same seven temperatures, as above, in the absence of CM-cellulose for 30 min before addition of substrate, followed by the standard enzyme assay over 15 min. Enzyme activities at 70 and 80°C were zero; enzyme activities at different temperatures are expressed as a percentage of the activity at the standard assay temperature 37°C (100%).

the molecule. Success in this work, however, depends on the absence (throughout the entire reaction period of the purified enzyme) of any contribution from other components of the cellulase

complex, particularly β -glucosidase [see above under 'Purification of a 1,4- β -glucanase (CM-cellulase)']. This enzyme acts directly on dextrans and indirectly on products of dextrans that may be released by CM-cellulase. Insufficient reaction time between CM-cellulase and dextrans, however, fails to allow this enzyme to develop its full potential against less readily available bonds of such substrates (see below).

G_2 , G_2H , G_3 and G_3H were not attacked in 24 h by the purified glucanase, all four being recovered quantitatively unchanged in their appropriate fractions after chromatography. In 48 h, only G_3 was attacked (2% hydrolysis) and released equimolar amounts of glucose and G_2 . Hence this is a real but feeble property of the glucanase, since its purification (see above) had excluded any contribution from cellobiase over 48 h. G_4 , G_5 and G_6 were hydrolysed (58–89%) with increasing rapidity in 7.5 h, a period that provided sufficient products for separation and a complete quantitative determina-

Fig. 2. Separation of cellulose hydrolysates on charcoal columns

Approx. 200 μ g of total soluble cellodextrins (determined by the chromic acid procedure) prepared from an acid hydrolysate of cellulose (see the Materials and methods section) was applied to a charcoal micro-column (1.3 cm $\times$ 3 cm) and fractionated as described in the Materials and methods section. ●, Reducing compounds were determined by the ferric alum procedure for reducing sugar (glucose, G_2 etc., up to G_6). ○, Separation of reduced cellulodextrins, G_2H up to G_6H , prepared from the corresponding pure reducing sugars, and applied in similar amounts (double quantity for G_6H) to the mixed reducing dextrans. Recoveries were quantitative (see also Table 2).

Table 2. *Quantitative analysis of the products of hydrolysis of cellodextrins and reduced cellodextrins by the β -glucanase* G_4 , G_5 , G_6 and the corresponding reduced dextrins, G_4H , G_5H and G_6H in 400 μ g amounts were incubated for 7.5 and 24 h, as shown, with the purified enzyme (2.9 μ g of protein), but otherwise as in the standard assay on CM-cellulose. Products and residual substrate (combined recovery from each substrate was 99–100%) were separated on charcoal micro-columns and measured by the phenol/ H_2SO_4 and ferric alum procedures. No product larger than the substrate was detected. The second value (in parentheses) in the columns for the substrates G_5 and G_5H is the calculated value for the first bond cleavage of the molecule (see the text). The extent of hydrolysis of substrates were: for G_4 , in 7.5 and 24 h, 58 and 100%; for G_4H , in 7.5 and 24 h, 36 and 100%; for G_5 and G_5H , 87 and 68%; for G_6 and G_6H , 89 and 78%.

Substrate Incubation period (h) ... Product	Products and residual substrate (nmol) from substrates below:							
	G_4		G_4H		G_5	G_5H	G_6	G_6H
	7.5	24	7.5	24	7.5	7.5	7.5	7.5
G_1	133	194	0	11	{ 106 (106)	{ 43 (43)	94	55
G_2H			213	573		{ 137 (137)		56
G_2	427	790	222	558	{ 351 (319)	{ 158 (139)	133	64
G_3H			0	16		{ 139 (139)		147
G_3	129	198	0	14	{ 319 (319)	{ 135 (135)	305	151
G_4H			382	0		{ 37 (37)		47
G_4	255	0	0	0	{ 76 (92)	{ 29 (38)	99	39
G_5H						136		30
G_5					62	0	91	38
G_6H								87
G_6							44	0

tion (Table 2, 99% recovery). The corresponding reduced dextrins (G_4H – G_6H) were slightly less susceptible to attack, an effect that diminished progressively with increasing chain length and had almost disappeared with G_6H .

Although G_4 was the least susceptible reducing dextrin in Table 2, it was fully hydrolysed in 24 h. Here, as in 7.5 h, the enzyme attacked both terminal (no. 1 or 3) and central (no. 2) bonds, with marked preference for the latter and release of G_2 (Table 2). This was emphasized with G_4H , as substrate, where equimolar quantities of both disaccharides (G_2H and G_2) were sole products in 7.5 h and were released in total amount as great as from G_4 , which had achieved 22% additional hydrolysis. Even complete hydrolysis (24 h, Table 2) failed to encourage enzymic approach to G_4H other than mainly at bond 2, from which it liberated more (total) disaccharides than from G_4 itself. Apparently the

catalytic region of the enzyme needs to accommodate at least four unaltered glucosyl subsites to achieve maximum bond-breaking potential, as is further suggested by the very small equimolar amounts of glucose (and G_3H) and G_3 that arise from the limited hydrolysis of bonds 1 and 3 of G_4H on complete hydrolysis (24 h, Table 2, contrast G_4). Hydrolysis of the central bond (nos. 2 and 3) was again preferred with pentasaccharide substrates, where approximately equimolar amounts (see below) of G_2 and G_3 (plus their reduced counterparts from G_5H) were recorded as principal products in Table 2. Terminal bonds (1,4) of both substrates were less, but equally, amenable to enzymic breakdown and provided glucose and G_4 (plus the corresponding reduced analogues from G_5H). Of all the primary products from pentasaccharides, only the tetrasaccharides (G_4 , G_4H) were possible secondary substrates for the enzyme preparation (G_2 , G_2H , G_3 ,

G_3H were not attacked, see above). Those tetrasaccharides were cleaved mainly to (additional) G_2 (in the same manner that G_4 and G_4H acted as primary substrates above), thus accounting for the observed small excess of G_2 over G_3 from the pentasaccharides in Table 2. This is seen more clearly in the hydrolysis products from G_5H , namely G_3H , G_3 , G_2H and G_2 , where only the last sugar appears in excess. Its partner on cleavage from G_5H is G_3H and is inert to hydrolysis by the enzyme and can therefore be used to recalculate yields of products in terms of the first cleavage of the pentasaccharide(s) as shown in Table 2 (values in parentheses under observed values). The revised values from G_5H indicate more clearly the preferred and equal attack on bonds 2 and 3 (139 nmol for G_2/G_3H , 136 nmol for G_3/G_2H) and much weaker action on bonds 1 and 4 (40 nmol for glucose/ G_4H , 38 nmol for G_4).

Enzymic preference for hydrolysing internal bonds extended to the hexasaccharides (G_6 and G_6H), where, as with G_4 (but not G_5), activity could again be directed to a single bond (now no. 3), with the release of trisaccharides (G_3H and/or G_3 , Table 2) as principal products. With G_6 as the substrate, other primary hydrolytic products were approximately equimolar amounts (allowing for secondary hydrolysis of G_4 to G_2) of G_2 and G_4 , as well as similar equimolar amounts of glucose and G_5 , indicating less concentrated but almost equal attack on bond numbers 2(4) and 1(5). Thus all bonds, whether at the non-reducing or reducing end of molecules, from G_4 to the G_6 , are available and susceptible to the enzyme. This is better illustrated with G_6H as substrate, where breakage also at bond 3 was favoured, followed less frequently by weaker activity against bonds 1 and 2 (and 5 and 4), releasing glucose and G_2 (and G_5 and G_4) respectively, plus the corresponding reduced moieties (Table 2). Subsequent attack, to a small extent, then commenced on the susceptible products of the primary hydrolysis (G_5 , G_4 and their reduced counterparts) in the manner found when these were primary substrates (see above), but was kept minimal by limiting the incubation period. This allowed reasonable estimates to be made of the primary scissions of G_6H by the enzyme, since (a) pentasaccharides (G_5 and G_5H) and tetrasaccharides arise solely or almost so (respectively) as primary products from G_6H and (b) all other hydrolysis products are unattacked by the enzyme (see above). In terms of the first cleavage(s) of G_6H , each complementary pair of products split from the dextrin gives a mean value of 43 and 38 nmol for bonds 1 and 5 respectively, 56 and 48 nmol for bonds 2 and 4, and 150 nmol for bond 3, indicating, as with G_6 , the availability of all the molecule to the enzyme.

Little detailed quantitative information is available on the mode of action of β -glucanases (CM-cellulase) on cellodextrins, particularly with organisms of known cellulolytic ability, with enzyme preparations freed from β -glucosidase and with unambiguous separation and identification of products. Hydrolysis of cellodextrins, for example G_5 at bonds 2 and/or 3, or G_6 at bonds 2 and/or 4 is indistinguishable in each case, without recourse to labelled (reduced) dextrins. The use of high-pressure liquid chromatography in this field by Shoemaker & Brown (1978) has provided interesting results with three purified β -glucosidase-free endoglucanases from *T. viride*. All were increasingly active on G_3 up to the G_6 and on CM-cellulose, as also is our glucanase, but their three enzymes also hydrolysed reactive cellulose (acid-swollen cellulose). Acting on cellodextrins, enzyme IV (and enzyme II) appeared to cleave, equally well, bonds 2 and 3 (or 1) of G_4 and bonds 2 (or 4) and 3 of G_6 . In contrast, enzyme III reacted more like a transferase in removing cellobiose units from G_4 , G_5 and their reduced derivatives. CM-cellulases from fungi of weak or undefined cellulolytic ability include a purified, homogeneous preparation from *Aspergillus niger* that was weakly active towards Avicel but highly active towards a reactive amorphous alkali-swollen cellulose (Hurst *et al.*, 1978). Elsewhere three separate cellulases have been isolated from *Thermascus aurantiacus* (Shepherd *et al.*, 1981). The three combined, without synergism, to hydrolyse 1% of filter paper or 0.05% of cotton yarn in 24 h at 60°C; two of the enzymes also acted on CM-cellulose. Cellulase III was tested on reduced cellodextrins (G_4H – G_6H), but only the reducing-sugar products were separated and examined, semiquantitatively, by chromatography on Sephadex G-15. The response of all three dextrins suggested that hydrolysis occurred preferentially at bond 3, irrespective of chain length. The CM-cellulase from *A. niger* behaved likewise towards G_5H and G_6H , but, in addition, the latter substrate appeared susceptible also at bond 4. The more detailed examination undertaken with both reducing and reduced products arising from the action of our β -glucanase on cellodextrins makes it clear that this enzyme differs from those above in showing definite preference for the central bond, whether this occupies a single or two-position site in even or odd-numbered dextrins respectively.

Conclusions

One of the most intriguing problems in the metabolism of cellulose is the means whereby truly cellulolytic microbes and their enzymes can effectively saccharify native fibrous substrates as well as simpler degraded forms of the polymer. That

property was originally ascribed to a component (C_1) that was thought to initiate early changes in the substrate thus permitting more rapid secondary changes of solubilization and saccharification. Component C_1 , however, was subsequently shown to be exocellulase in cleaving cellobiose units from cellulose (Halliwell & Riaz, 1971; Halliwell *et al.*, 1972). The same reports indicated that an early change brought about by whole culture filtrates of *T. koningii* during cellulolysis was a random and effective breakdown of fibrous cellulose to short segments in a mechanism dependent on a CM-cellulase and a less stable activity, Component C_2 , commonly associated with weaker CM-cellulase activity in that organism (Halliwell, 1979). Cellobiohydrolase was not involved in this reaction. The specific role of the principal CM-cellulase component that contributes to the segmentation process has been examined in the present report. Acting alone it was unable to attack (insoluble) cellulose at all, but was highly effective in hydrolysing soluble cellulose derivatives such as CM-cellulose and the larger celloedextrins. The dextrins apparently associated with the enzyme in a symmetrical manner, so that all glucosyl units were made available to the catalytic site, but only central bonds received preferential treatment in an endo-type cleavage. Products of hydrolysis of all three dextrins by the β -glucanase were small amounts of glucose, which can serve as an immediate source of energy for the microbe, larger amounts of G_2 , which acts also as an inducer of some cellulolytic components, and G_3 , which provides a temporary store of glucose, being subject to slower hydrolysis by the intracellular cellobiase of the organism (G. Halliwell & J. Lovelady, unpublished work).

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