Biochem. J. (1981) **193**, 67–74
Printed in Great Britain

Substrate specificity and mode of action of the cellulases from the thermophilic fungus *Thermoascus aurantiacus*

Maxwell G. SHEPHERD*†, Chow Ching TONG‡ and Anthony L. COLE‡
**Department of Biochemistry, University of Otago, Dunedin, New Zealand, and*
‡Department of Botany, University of Canterbury, Christchurch, New Zealand

(*Received 10 June 1980/Accepted 8 September 1980*)

The substrate specificities of three cellulases and a β-glucosidase purified from *Thermoascus aurantiacus* were examined. All three cellulases partially degraded native cellulose. Cellulase I, but not cellulase II and cellulase III, readily hydrolysed the mixed β-1,3;β-1,6-polysaccharides such as carboxymethyl-pachyman, yeast glucan and laminarin. Both cellulase I and the β-glucosidase degraded xylan, and it is proposed that the xylanase activity is an inherent feature of these two enzymes. Lichenin (β-1,4;β-1,3) was degraded by all three cellulases. Cellulase II cannot degrade carboxymethyl-cellulose, and with filter paper as substrate the end product was cellobiose, which indicates that cellulase II is an exo-β-1,4-glucan cellobiosylhydrolase. Degradation of cellulose (filter paper) can be catalysed independently by each of the three cellulases; there was no synergistic effect between any of the cellulases, and cellobiose was the principal product of degradation. The mode of action of one cellulase (cellulase III) was examined by using reduced cellulodextrins. The central linkages of the cellulodextrins were the preferred points of cleavage, which, with the rapid decrease in viscosity of carboxymethyl-cellulose, confirmed that cellulase III was an endocellulase. The rate of hydrolysis increased with chain length of the reduced cellulodextrins, and these kinetic data indicated that the specificity region of cellulase III was five or six glucose units in length.

It is well established that the degradation of cellulose occurs by the action of both endoglucanases and exoglucanases (Streamer *et al.*, 1975; Berghem *et al.*, 1975, 1976). The exact mode of action of these cellulases is, however, incompletely understood. Evidence to date indicates that the mechanism of degradation of native cellulose involves the endocellulases penetrating the cellulose fibres and attacking at random the β-(1,4)-linkages along the cellulose chains. After this depolymerization, the exocellulase action produces cellobiose (Nisizawa, 1973; Hofsten, 1975; Pettersson, 1975). Tong *et al.* (1980) have described the isolation and purification of three cellulases and a β-glucosidase from the thermophilic fungus *Thermoascus aurantiacus*, and the present paper describes the substrate specificity of these enzymes and the mode of action of one cellulase (cellulase III).

Abbreviation used: CM, carboxymethyl.

† To whom reprint requests should be addressed.

Experimental

Enzyme

The cellulases and β-glucosidase from *T. aurantiacus* were purified as described previously (Tong *et al.*, 1980). The final preparations were homogeneous, as determined by polyacrylamide-gel electrophoresis and Bio-Gel P-60 filtration. The enzymes are referred to below as β-glucosidase, cellulase I, cellulase II and cellulase III respectively, according to the positions they occupied in the gel in descending sequence (Tong *et al.*, 1980). The protein concentrations of the purified enzyme preparations used in these studies were 70 μg/ml (β-glucosidase), 120 μg/ml (cellulase I), 218 μg/ml (cellulase II) and 680 μg/ml (cellulase III).

Substrates

Whatman no. 1 filter paper and Whatman cellulose powder (CF II) were obtained from Whatman, Maidstone, Kent, U.K. CM-cellulose 7HF was from Hercules, Wilmington, DE, U.S.A.

0306-3275/81/010067-08$01.50/1

The alkali-swollen cellulose was prepared by the method of Hash & King (1958) and the acid-swollen cellulose by the method of Rautela & Cowling (1966). Solka floc SW-40 was a gift from Brown Co., Berlin, NH, U.S.A. Acala cotton fibres and cotton yarn were kindly supplied by the Cotton Research Association, Shirley Institute, Didsbury, Manchester, U.K. Avicel micro-crystalline cellulose PH-101 was provided by the FMC Export Corp., Food and Pharmaceutical Products, Philadelphia, PA, U.S.A. Yeast glucan, barley glucan and CM-pachyman were kindly supplied by Professor B. A. Stone, Department of Biochemistry, La Trobe University, Vic., Australia. Cello-oligosaccharides and their reduced analogues were prepared as described below. All other carbohydrates were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Analytical methods

Reducing sugars were determined by the Nelson–Somogyi procedure (Nelson, 1944; Somogyi, 1952). One enzyme unit is defined as that amount of enzyme that produces an increase in A_{560} of 0.1 under the conditions defined (Tong *et al.*, 1980). In the studies of the mode of action against cellulodextrins and reduced cellulodextrins, the reducing sugars were determined with the *p*-hydroxybenzoic acid hydrazide reagent (Hurst *et al.*, 1977*a*). The anthrone/H_2SO_4 method of Herbert *et al.* (1971) was used for determination of total carbohydrate. The cellobiose activity was determined by the glucose oxidase procedure of Lloyd & Whelan (1969). β-Glucosidase activity was assayed as described previously (Tong *et al.*, 1980).

Substrate specificity

Each substrate (0.24–20 mg/ml) was incubated with the enzyme (0.28–4.1 μg/ml) at 60°C in 0.1 M-citrate/phosphate buffer, pH 5.0, in a final volume of 1.0 ml. The incubation time ranged from 0.5 to 24 h. The hydrolytic activity towards all substrates except *p*-nitrophenyl β-D-glucoside and cellobiose was measured by the appearance of reducing end groups with the Nelson–Somogyi method. β-Glucosidase and cellobiase activities were determined as described by Hurst *et al.* (1977*a*). Hydrolysis of laminarin and the cellulodextrins was monitored after gel filtration (Sephadex G-15) of the reaction mixture; this procedure was necessary because of the high background given by the substrates.

Preparation of cello-oligosaccharides

Oligosaccharides from cellulose were prepared by acetolysis–deacetylation and fractionated as described by Hurst *et al.* (1978). Reduced oligosaccharides were prepared by treating the cello-oligosaccharides with $NaBH_4$ (Hurst *et al.*, 1978).

Identification of reaction products

The products of cellulose hydrolysis were analysed by descending chromatography on Whatman no. 1 chromatography paper in a butan-1-ol/acetic acid/water (12:3:5, by vol.) solvent system. After the samples were applied, the chromatograms were developed for 22 h at room temperature. The paper sheets were then dried and the sugars were detected with alkaline silver oxide reagent (Menzies & Seakins, 1969). The brown background was removed by immersing the paper in 10% (w/v) $Na_2S_2O_3$ solution. The standards of glucose, cellobiose and the higher oligosaccharides were prepared in aq. 10% (w/v) propan-2-ol and run as reference markers.

Hydrolysis of cellulodextrins

Hydrolysis of the cellulodextrins and the reduced cellulodextrin series by cellulase III was conducted as described below, and the reaction mixture was then fractionated on a Sephadex G-15 column (1 cm × 85 cm) by elution with water at 10 ml/h. Fractions (approx. 0.6 ml; 10 drops) were collected for analysis. (*a*) Cellobiosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellobiosylsorbitol (1 mg/ml, in citrate/phosphate buffer, pH 4.5) and 0.1 ml of enzyme (82.5 μg/ml). Time of hydrolysis at 60°C was 24 h; the reaction mixture was then heated for 5 min on a boiling-water bath to inactivate the enzyme, cooled and then applied to the column. (*b*) Cellotriosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellotriosylsorbitol (1 mg/ml, as in *a*) and 0.1 ml of enzyme (82.5 μg/ml). Time of hydrolysis at 60°C was 4 h; the reaction mixture was then treated as in (*a*). (*c*) Cellotetraosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellotetraosylsorbitol (1 mg/ml, as in *a*) and 0.1 ml of enzyme (8.25 μg/ml). Time of hydrolysis at 60°C was 1 h; the reaction mixture was then treated as in (*a*). (*d*) Cellopentaosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellopentaosylsorbitol (1 mg/ml, as in *a*) and 0.1 ml of enzyme (1.03 μg/ml). Time of hydrolysis at 60°C was 0.5 h; the reaction mixture was then treated as in (*a*). (*e*) Cellotriose hydrolysis: the incubation mixture contained 0.5 ml of cellotriose (1 mg/ml, in citrate/phosphate buffer, pH 4.5) and 0.1 ml of enzyme (82.5 μg/ml). Time of hydrolysis at 60°C was 48 h; the reaction mixture was then heated for 5 min on a boiling-water bath to inactivate the enzyme, cooled and then applied to the column. (*f*) Cellotetraose hydrolysis: the incubation mixture contained 0.5 ml of cellotetraose (1 mg/ml, as in *e*) and 0.1 ml of enzyme (4.12 μg/ml). Time of hydrolysis at 60°C was 1 h; the reaction

mixture was then treated as in (*e*). (*g*) Cellopentaose hydrolysis: the incubation mixture contained 0.5 ml of cellopentaose (1 mg/ml, as in *e*) and 0.1 ml of enzyme (2.06 μg/ml). Time of hydrolysis at 60°C was 0.5 h; the reaction mixture was then treated as in (*e*).

Results and discussion

In determining the substrate specificity of cellulases, a wide range of β-glucans has been used, including the soluble derivatives CM-cellulose and CM-pachyman. The use of heterogeneously linked glucans in addition to the homogeneous types has been useful in distinguishing specific substrate requirements of the enzyme. The availability of linear β-mannans, β-xylans and β-glycol chitosans with various types of linkages makes it possible to examine the effect of altering the configuration or size of the monomer unit of the action of β-glucan hydrolases, and some purified cellulases have been tested in this way (Cole & King, 1964; Clarke & Stone, 1965; Pettersson, 1969; Streamer *et al.*, 1975; Hurst *et al.*, 1978).

The purified cellulases from *T. aurantiacus* were not capable of hydrolysing β-1,4-mannan, β-1,4-glycol chitosan or chitin. Since these substrates all contain the β-1,4-glycosidic linkage, it is apparent that the enzymes cannot accommodate changes in glucosyl residues to mannosyl, aminoglucosyl or *N*-acetyl-D-aminoglucosyl residues.

As shown in Table 1, the three cellulases exhibited considerable variation in their ability to degrade native cellulose. Filter paper was the substrate most amenable to degradation. Cellulase II, which was the most active of the three cellulases against native celluloses, had no hydrolytic activity towards CM-cellulose even when the enzyme concentration was increased to 6.25 μg/ml. This inability to degrade CM-cellulose, plus the observation that cellobiose was the primary product formed when filter paper was degraded by this enzyme, indicate that cellulase II is an exo-β-1,4-glucan cellobiosylhydrolase. On the other hand, more than 90% of the CM-cellulose-hydrolysing activity was confined to the cellulase III component; the rapid decrease in the viscosity of CM-cellulose (Fig. 1) indicates that this enzyme has an endo- rather than exo-cellulolytic mode of action.

One striking difference in the specificity of these cellulase preparations was that cellulase I, unlike the other two cellulases, readily hydrolysed the native mixed β-1,3;β-1,6-polysaccharides such as yeast glucan, laminarin and CM-pachyman (degree of substitution 0.32). With a yeast glucan substrate concentration of 0.2% (w/v), the production of reducing end groups could be detected from the action of only 6 ng of enzyme/ml. Lichenin (2 mg/ml),

Table 1. *Substrate specificity of β-glucosidase and cellulases from T. aurantiacus*

Each substrate was incubated with an enzyme at 60°C for the 24 h incubation, and at 67°C for the 0.5 h incubation, under the conditions defined. Enzyme activity was measured by the appearance of reducing sugars, except for the substrates *p*-nitrophenyl β-D-glucoside and cellobiose, which were tested as described in the Experimental section. Hydrolysis of laminarin and cellulodextrins was monitored by using Sephadex G-15 gel filtration because of the high reducing power in the blanks.

Substrate	Concn. of substrate (mg/ml)	Linkage type(s)	Incubation time (h)	Final concn. of enzyme (μg/ml)	Reducing sugar as glucose equivalent (μg)			
					β-Glucosidase	Cellulase I	Cellulase II	Cellulase III
Cotton yarn	20	β-1,4	24	4	0	3	7	0
Solka floc	20	β-1,4	24	4	0	27	32	30
Cellulose powder	20	β-1,4	24	4	0	13	42	32
Avicel	20	β-1,4	24	4	0	18	80	40
Filter paper	20	β-1,4	24	4	0	24	108	85
Alkali-swollen cellulose	2	β-1,4	24	4	0	3	6	24
CM-cellulose	6.75	β-1,4	0.5	0.8	0	21	0	315
p-Nitrophenyl β-D-glucoside	0.24		0.5	0.02	3740*	0	0	0
Cellobiose	2	β-1,4	0.5	0.28	135	0	0	0
Xylan	2	β-1,4	0.5	4	146	76	0	0
Lichenin	2	β-1,4;β-1,3	0.5	1.65	132	41	3	38
Laminarin	2	β-1,3;β-1,6	0.5	4	120	36	0	0
CM-pachyman	2	β-1,3;β-1,6	0.5	4	76	65	0	0
Yeast glucan	2	β-1,3;β-1,6	24	4	105	205	0	0

* As μg of *p*-nitrophenol.

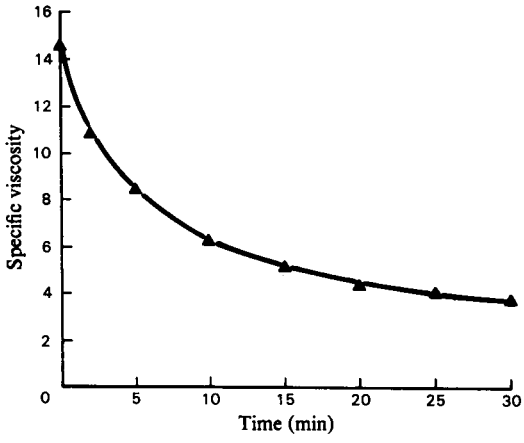


Fig. 1. *Decrease in viscosity of CM-cellulose solution by cellulase III*

To measure this, 5ml of 0.75% (w/v) CM-cellulose solution in citrate/phosphate buffer (0.1M-citric acid/0.2M-NaH_2PO_4), pH4.5, was mixed with 1.0ml of enzyme solution (0.041μg) in a viscometer and incubated at 65°C. The efflux time of the mixture was taken at the times indicated. The specific viscosity at zero time was determined by substituting buffer for the enzyme solution.

the only mixed β-1,4;β-1,3-polysaccharide tested, proved to be susceptible to degradation by all three cellulase enzymes. This mixed-glucan-hydrolysing activity was also exhibited by the three cellulase bands isolated by disc-gel electrophoresis from *Aspergillus niger* (Hurst *et al.*, 1978). An attempt was made to ascertain whether or not the ability to attack β-1,3;β-1,6-linkages was an intrinsic feature of the cellulase-I enzyme. In a previous study (Tong *et al.*, 1980) it was found that the pH–activity profiles for cellulase I acting on β-1,4- and β-1,3;β-1,6-substrates were identical, with a sharp pH optimum at pH4.5. In addition, the pH–stability and temperature–stability profiles for cellulase I on CM-cellulose and yeast glucan were similar. The observation that cellulase II and cellulase III hydrolyse the mixed β-1,4;β-1,3-linked lichenin and not the β-1,3;β-1,6-linked glucans indicates that the 4-β-glucosyl residues are specifically required in the glycosyl portion of the linkage hydrolysed by these enzymes.

A number of non-glucosidic polymers were tested as substrates for the three cellulases. They included glycol chitosan (β-1,4-glucosamine residues), mannan (β-1,4-mannose residues), xylan (β-1,4-xylose residues), chitin (β-1,4-*N*-acetylglucosamine residues) and polygalacturonic acid (β-1,4-galacturonic acid residues). Xylan was the only one of these polysaccharides to be extensively degraded, and this occurred with both β-glucosidase and cellulase I. The specific activity of the xylanase was comparable with the specific activity of the CM-pachymanase. The purified cellulase isolated from *Trichoderma reesei* (Toda *et al.*, 1971) also degraded xylan, and it was proposed that this xylanase activity occurred on the same protein as the cellulase activity. Furthermore, endocellulase showed endo-xylanase activity, whereas the exo-xylanase activity was associated with the exo-cellulase enzyme (Shikata & Nisizawa, 1975). Kanda *et al.* (1976) have also demonstrated that xylanase activity is intrinsic to a homogeneous cellulase from *Irpex lacteus*. The β-glucosidase purified from *Pyricularia oryzae*, which splits off glucose units from the non-reducing ends of β-gluco-oligosaccharides, also degrades the soluble β-1,4-glucan CM-cellulose, as well as β-1,3-glucan, β-1,6-glucan and mixed β-1,3;β-1,6-glucan (Hirayama *et al.*, 1978). Hence it is probable that the xylanase activity reported in the present paper is an inherent feature of both the cellulase I and the β-glucosidase enzymes. The cellulase preparations do not exhibit activity towards either *p*-nitrophenyl β-D-glucoside or cellobiose.

The β-glucosidase had no hydrolytic activity towards any form of cellulose tested, but degraded cellulodextrin. Although it is known that β-glucosidase activity will extend from the dimer to chains of six or perhaps even ten glucose units (Grassman *et al.*, 1933), it is doubtful whether its activity would extend to chains of the degree of polymerization found in CM-cellulose (degree of polymerization > 100). The β-glucosidase was capable of breaking down xylan and other mixed β-1,3;β-1,6-polysaccharides. This property was also exhibited by the β-glucosidase from *Pyricularia oryzae* (Hirayama *et al.*, 1978).

Synergism between separated components has been reported for a number of fungal cellulases (Selby & Maitland, 1967; Wood, 1968; Olutiola & Ayers, 1973). Therefore the activities of the purified enzyme components towards filter paper were examined singly and in various combinations such that the original cellulase complex was reconstituted in a stepwise manner. The standard assay procedures were employed with 2.4μg of each cellulase and 0.7μg of β-glucosidase, and the release of reducing sugars from the cellulose was used as an indicator of enzymic activity after incubation at 60°C for 24h. Under these conditions cellulase I, cellulase II and cellulase III produced 30, 75 and 73μg of glucose equivalent/ml respectively. When combined the three cellulases produced 176μg of glucose equivalent/ml. These data indicated that there was no synergism with the three cellulases acting on filter paper. Whenever the enzyme under test contained β-glucosidase, there was an increase in the amount of reducing sugar formed of 80–120%

over that of the expected value if no synergism occurred between components. An investigation of the products formed from cellulose indicated that the major product of hydrolysis of filter paper by each of the cellulases acting alone for the 24h incubation was cellobiose. When acting in the presence of β-glucosidase, glucose was the final product. Thus, for each cellobiose molecule formed by the action of cellulase component on filter paper, two molecules of glucose were created by hydrolysis of cellobiose by the β-glucosidase. This resulted in a 2-fold increase in the amount of the reducing sugars observed in the reaction mixtures containing β-glucosidase. When this correction is applied to the results, it is clear that there is no significant synergism between the three purified cellulases and the β-glucosidase components of *T. aurantiacus*.

The nature of the enzymic action of cellulase III on CM-cellulose was examined. A progress curve for the reaction was followed by monitoring the increase in reducing sugars. This was linear for 10min, and the catalytic-centre activity of cellulase III on CM-cellulose at 65°C was 33mol of glucose equivalent/s per mol of cellulase III. At 10min intervals, samples were removed and analysed by paper chromatography. No glucose was detected at any time during the reaction, and the products were cellobiose and higher oligosaccharides. These data indicated that cellulase III has an endo-cellulolytic mode of action, and this conclusion was supported by the rapid decrease in viscosity of CM-cellulose (Fig. 1). In this experiment, 0.041μg of cellulase III caused the specific viscosity of the CM-cellulose to decrease from 15 to 8 in 5min at 65°C under the standard enzyme-assay conditions.

Information on the mode of action of cellulase III was determined by using a series of β-1,4-linked oligosaccharides. The chemically reduced substrates permit reliable identification of the site of attack, and, where more than one bond is attacked, the relative frequencies of attack at each bond can be determined (Scheme 1). The location of the glucosyl bonds that are most susceptible to attack by cellulase III was ascertained by determining the change in the composition of the β-1,4-oligosaccharides and their reduced analogues after the hydrolysates were analysed on a column of Sephadex G-15 (Figs. 2 and 3). With the reduced oligosaccharides, the terminal reducing glucose residue has been converted into a sorbitol residue by $NaBH_4$. It was assumed that the sorbitol residue did not bias the action of the enzyme. Cellobiosylsorbitol was hydrolysed with cellobiose as the main reducing sugar, but some glucose was also produced (Fig. 2*a*). This indicated a preferential cleavage at bond 2 compared with bond 1 (numbering from the non-sorbitol end of the molecule). Hydrolysis of cellotriosylsorbitol yielded cellotriose

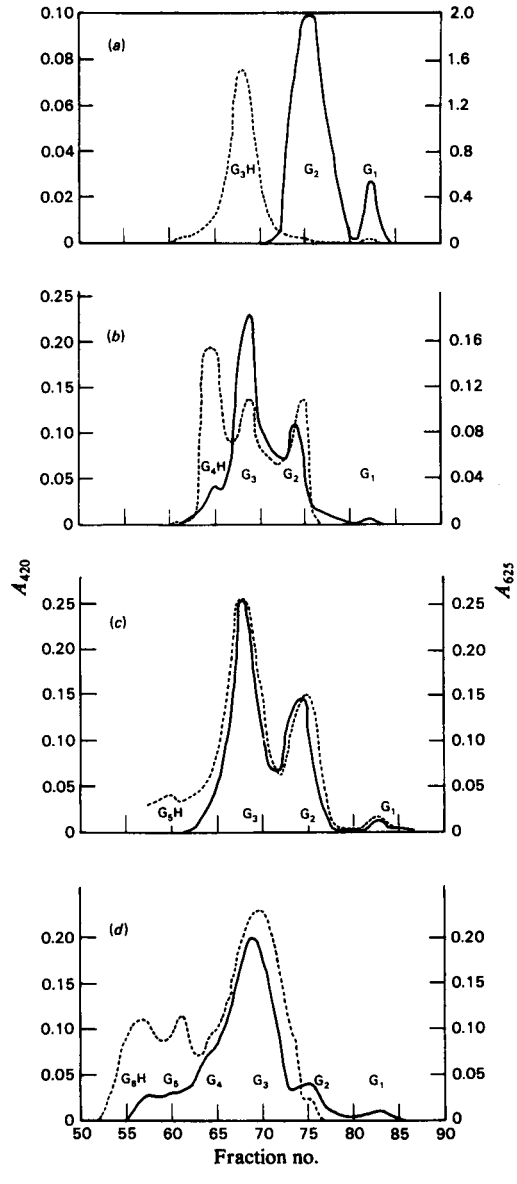


Fig. 2. *Column chromatography of reduced cellulodextrins after hydrolysis by cellulase III*

The reduced cellulodextrins were hydrolysed by cellulase III, and the hydrolysates were then fractionated on a Sephadex G-15 column as described in the Experimental section. Each fraction (0.6ml) was collected and assayed for reducing sugars (——, A_{420}) with *p*-hydroxybenzoic acid hydrazide reagent and for total carbohydrate (– – –, A_{625}) with the anthrone/H_2SO_4 reagent. Key: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose; G_3H, cellobiosylsorbitol; G_4H, cellotriosylsorbitol; G_5H, cellotetraosylsorbitol; G_6H, cellopentaosylsorbitol. (*a*) Cellobiosylsorbitol hydrolysis; (*b*) cellotriosylsorbitol hydrolysis; (*c*) cellotetraosylsorbitol hydrolysis; (*d*) cellopentaosylsorbitol hydrolysis.

and cellobiose as major products, with only a trace of glucose (Fig. 2*b*), showing that bonds 2 and 3 were preferentially cleaved. On hydrolysis of cellotetraosylsorbitol, cellotriose was the predominant reducing sugar formed, although some cellobiose and trace amounts of glucose were also evident (Fig. 2*c*), revealing that, although bonds 1, 2 and 3 are susceptible to cleavage, the penultimate glucosyl bond from the sorbitol end is the preferred cleavage point. Hydrolysis of reduced cellohexaose gave cellotriose as the major product. Other reducing sugars were also produced, but not in large amounts (Fig. 2*d*). The relative amounts of the products indicate that the central linkages are the preferred points of cleavage (Scheme 1). This clearly demonstrates the departure from the classical interpretation of random hydrolysis (i.e. the equal susceptibility of all bonds to cleavage). The observed preference for non-terminal linkages confirms the endo-cellulolytic action of the enzyme, which was previously indicated by the rapid decrease in the viscosity of a CM-cellulose solution with the simultaneous slow release of reducing sugars.

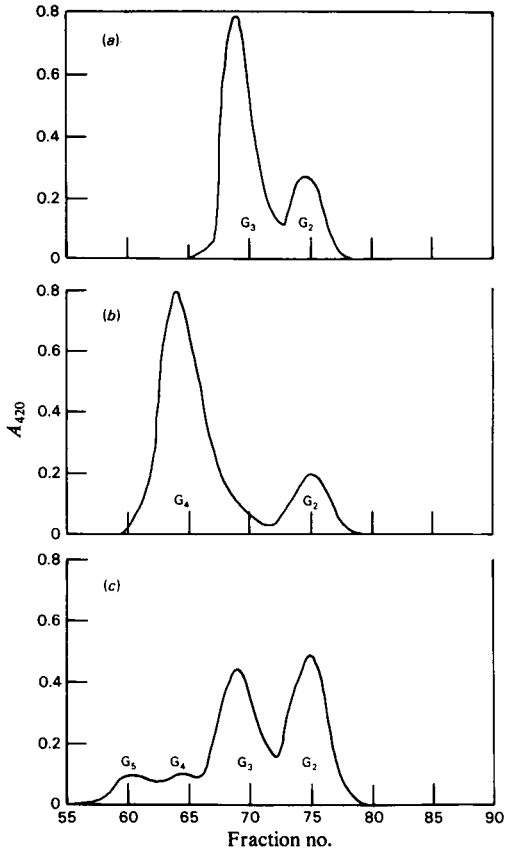
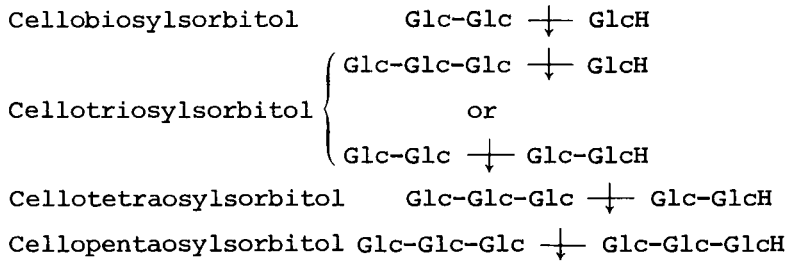


Fig. 3. *Column chromatography of cellulodextrins after hydrolysis by cellulase III*

Column conditions were as described for Fig. 2, except that fractions were analysed for reducing sugars only. Key: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose. (*a*) Cellotriose hydrolysis; (*b*) cellotetraose hydrolysis; (*c*) cellopentaose hydrolysis.

The rate of hydrolysis increased with chain length of the reduced cellulodextrins (Table 2). The relative rate increased 90-fold on going from cellobiosylsorbitol to cellotetraosylsorbitol, but there was only a 10-fold increase in going from cellotetraosylsorbitol to cellopentaosylsorbitol. The same trend in the relative rates for the reduced cellulodextrins has been observed by Hurst *et al.* (1978), working with a cellulase from *A. niger*, except that the *A. niger* enzyme would not react with cellobiosylsorbitol. The increase in hydrolytic rate was not dramatic in going from cellopentaosylsorbitol to reduced cellulodextrin with a degree of polymerization >7. From these observations it may be concluded that the enzyme has at least five or possibly six subsites in the active centre that recognize or bind glucose residues. A decrease in K_m with increasing chain length of the oligosaccharides has been observed by Li *et al.* (1965), and they reported that the optimum substrate chain length was at least six glucosyl units. Hurst *et al.* (1977*b*, 1978) and Pettersson (1969), working with cellulases from *A. niger* and *Penicillium notatum* respectively, have concluded that the specificity region of the enzyme is five glucosyl units in length. Similarly, Whitaker (1954) and Hanstein &

Scheme 1. *Site of cleavage of reduced cellulodextrins by cellulase III*



Whitaker (1963) showed that the specificity region of the *Myrothecium* cellulase was at least five glucose units in length.

In the hydrolysis of unmodified cellulodextrins, the pattern of cleavage was similar to that obtained with the reduced cellulodextrins and is shown in Scheme 2. In the hydrolysis of cellotriose, cellobiose was the only product detected (Fig. 3*a*). Clearly glucose must be present, but could not be detected by the *p*-hydroxybenzoic acid hydrazide reagent. The sensitivity of that reagent towards equimolar amounts of cellobiose and glucose was tested in a separate experiment, and it was found that it was only 25% as sensitive for glucose as for the cellobiose. The reaction products were then analysed on paper chromatography, and it was shown that both cellobiose and glucose were formed from hydrolysis of cellotriose.

Cellotetraose was cleaved to cellobiose (Fig. 3), although a trace amount of glucose was detected on paper chromatography. This result clearly indicated that hydrolysis of this substrate took place at the middle bond, in contrast with bond 3, which was the preferred site of cleavage in cellotriosylsorbitol. To account for this difference, we conclude that, with a short-chain-length substrate such as cellotetraose, it does appear that the sorbitol residue has an effect on the action of the enzyme. This effect was minimal for cellopentaose, from which the major products, cellotriose and cellobiose, were obtained in approximately equimolar amounts.

The experiment with the cellulodextrins allows us to explain the low rate of hydrolysis of highly ordered forms of cellulose by the cellulase-III component. The enzyme was found to degrade cellotriose very slowly. Thereafter the rate of degradation increased rapidly with the degree of polymerization of the oligosaccharide chain up to a limit of six glucose residues. In crystalline cellulose the polysaccharide chains are very closely packed, and it is improbable that longer segments of polysaccharide chains are accessible to the enzyme. Furthermore, after degradation of highly ordered cellulose (filter paper) for 48h, the molar ratio of glucose to cellobiose (estimated visually on paper chromatography), was 1:1, which is the ratio expected on degradation of cellotriose. The acid-swollen cellulose gave glucose and cellobiose approximately in the molar ratio 1:2, which is the expected ratio on degradation of longer oligosaccharides. These results support the hypothesis that, at least in the early stage of cellulose degradation, only short segments of polysaccharide chains in unmodified insoluble cellulose are accessible to the enzyme.

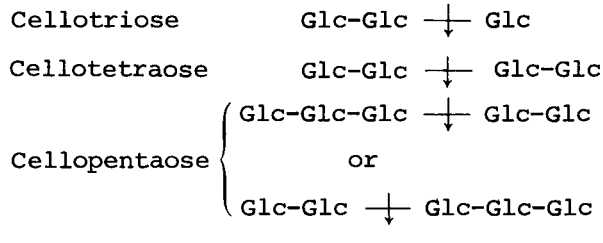
Quantitative information on the relative rates of hydrolysis of the unmodified cellulodextrins could not be obtained because of the high reducing power inherent in these substrates, which interfered with the

Table 2. *Relative rates of hydrolysis of the reduced cellulodextrins*

Reaction mixtures contained 2ml of oligosaccharides (1mg/ml) in citrate/phosphate buffer (0.1M-citric acid/0.2M-NaH_2PO_4), pH4.5, and 0.4ml of enzyme solution. After incubation at 60°C, 0.3ml samples were removed at intervals and then assayed with 2.5ml of *p*-hydroxybenzoic acid hydrazide reagent. Enzyme solutions used: for cellobiosylsorbitol and cellotriosylsorbitol, 41.2μg/ml; for cellotetraosylsorbitol, 4.1μg/ml; for cellopentaosylsorbitol and reduced cellulodextrins, degree of polymerization (D.P.)>7, 2.0μg/ml. Total period of incubation was 30min, except for cellobiosylsorbitol and cellotriosylsorbitol, where it was 2h. Initial velocities were calculated as the increase in A_{420}/min from the linear portion of the curve. Relative hydrolysis rate was calculated by normalizing for different enzyme concentrations. Results are the means of duplicate determinations.

Compound	Relative hydrolysis rate
Cellobiosylsorbitol	1
Cellotriosylsorbitol	11
Cellotetraosylsorbitol	94
Cellopentaosylsorbitol	903
Reduced cellulodextrins (D.P. >7)	1100

Scheme 2. *Deduced sites of hydrolysis of cellulodextrins by the cellulase-III component*



p-hydroxybenzoic acid hydrazide reducing-sugar assay. It was clear, however, that the rate of degradation increased with chain length.

In determining the mechanism of celluloysis, these data demonstrate that the production of accessible end groups is not essential for the action of the exo-cellulase II component; the enzyme itself is capable both of initiating hydrolysis and of generating end groups from highly ordered cellulosic substrates without the aid of an endo-cellulase such as cellulase III. Hydrolysis of cellulose by *T. aurantiacus* therefore involves a multi-enzymic process whereby degradation of cellulosic materials can be catalysed independently by each of the cellulase components. The present data indicate that the cellulase-III component of *T. aurantiacus* functions as an endo-glucanase and should be designated as a 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4).

References

Berghem, L. E. R., Pettersson, L. G. & Axïo-Fredriksson, U.-B. (1975) *Eur. J. Biochem.* **53**, 55–62

Berghem, L. E. R., Pettersson, L. G. & Axïo-Fredriksson, U.-B. (1976) *Eur. J. Biochem.* **61**, 621–630

Clarke, A. E. & Stone, B. A. (1965) *Biochem. J.* **96**, 793–801

Cole, F. E. & King, K. W. (1964) *Biochim. Biophys. Acta* **81**, 122–129

Grassman, W., Zechmeister, L., Toth, G. & Stadler, R. (1933) *Justus Liebigs Ann. Chem.* **503**, 167–174

Hanstein, E. G. & Whitaker, D. R. (1963) *Can. J. Biochem. Physiol.* **41**, 707–718

Hash, J. H. & King, K. W. (1958) *J. Biol. Chem.* **232**, 381–393

Herbert, D., Phipps, P. J. & Strange, R. E. (1971) *Methods Microbiol.* **5B**, 265–282

Hirayama, T., Horie, S., Nagayama, H. & Matsuda, K. (1978) *J. Biochem.* (*Tokyo*) **84**, 27–37

Hofsten, B. V. (1975) in *Symposium on Enzymatic Hydrolysis of Cellulose* (Bailey, M., Enari, T. M. & Linko, M., eds.), pp. 281–288, SITRA, Helsinki

Hurst, P. L., Nielsen, J., Sullivan, P. A. & Shepherd, M. G. (1977*a*) *Biochem. J.* **165**, 33–41

Hurst, P. L., Sullivan, P. A. & Shepherd, M. G. (1977*b*) *Biochem. J.* **167**, 549–556

Hurst, P. L., Sullivan, P. A. & Shepherd, M. G. (1978) *Biochem. J.* **169**, 389–395

Kanda, T., Wakabayashi, K. & Nisizawa, K. (1976) *J. Biochem.* (*Tokyo*) **79**, 989–995

Li, L. H., Flora, R. M. & King, K. W. (1965) *Arch. Biochem. Biophys.* **111**, 439–447

Lloyd, J. B. & Whelan, W. J. (1969) *Anal. Biochem.* **30**, 467–470

Menzies, I. S. & Seakins, J. W. T. (1969) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), pp. 310–315, W. Heinemann, London

Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380

Nisizawa, K. (1973) *Hakko Kogaku Zasshi* **51**, 267–276

Olutiola, P. O. & Ayers, P. G. (1973) *Trans. Br. Mycol. Soc.* **60**, 273–280

Pettersson, L. G. (1969) *Arch. Biochem. Biophys.* **130**, 286–294

Pettersson, L. G. (1975) in *Symposium on Enzymatic Hydrolysis of Cellulose* (Bailey, M., Enari, T. M. & Linko, M., eds.), pp. 255–267, SITRA, Helsinki

Rautela, G. S. & Cowling, E. B. (1966) *Appl. Microbiol.* **14**, 892–898

Selby, K. & Maitland, C. C. (1967) *Biochem. J.* **104**, 716–724

Shikata, S. & Nisizawa, K. (1975) *J. Biochem.* (*Tokyo*) **78**, 499–512

Somogyi, M. (1952) *J. Biol. Chem.* **195**, 19–23

Streamer, M., Eriksson, K.-E. & Pettersson, B. (1975) *Eur. J. Biochem.* **59**, 607–613

Toda, S., Suzuki, H. & Nisizawa, K. (1971) *Hakko Kogaku Zasshi* **49**, 499–521

Tong, C. C., Cole, A. L. & Shepherd, M. G. (1980) *Biochem. J.* **191**, 83–94

Whitaker, D. R. (1954) *Arch. Biochem. Biophys.* **53**, 439–449

Wood, T. M. (1968) *Biochem. J.* **109**, 217–227