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Substrate Specificity and Mode of Action of a Cellulase from *Aspergillus niger*

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The mode of action and substrate specificity of a cellulase purified from *Aspergillus niger* were examined. The enzyme showed little capacity to hydrolyse highly ordered cellulose, but readily attacked soluble cellulose derivatives and amorphous alkali-swollen cellulose. Activity towards barley glucan and lichenin was greater than with CM-cellulose. Low activity was detected with CM-pachyman (a substituted β-1,3-glucose polymer) and xylan. Activity towards yeast glucan, mannan, ethylene glycol chitin, glycol chitosan, laminarin, polygalacturonic acid and pectin could not be demonstrated. Cellobiose and *p*-nitrophenyl β-D-glucoside were not hydrolysed, whereas the rate of hydrolysis of the higher members of the reduced cellulodextrins increased with chain length. The central bonds of cellotetraosylsorbitol and cellopentaosylsorbitol were the preferred points of cleavage. Kinetic data indicated that the specificity region of the cellulase is five glucose units in length. The evidence indicates that the cellulase is an endoglucanase.

Although it is known that the hydrolysis of cellulose occurs by the action of both exoglucanases and endoglucanases (Streamer *et al.*, 1975; Berghem *et al.*, 1975, 1976), the mode of action of cellulases is incompletely understood. The mode of action of some cellulases has been determined by using a series of β-1,4-linked oligosaccharides (Cole & King, 1964; Clarke & Stone, 1965*b*; Pettersson, 1969; Streamer *et al.*, 1975). The isolation and purification of a cellulase (EC 3.2.1.4) from a commercial preparation of the extracellular enzymes of *Aspergillus niger* has been described (Hurst *et al.*, 1977). The present paper describes the substrate specificity and the mode of action of this cellulase.

Experimental

Enzyme

Cellulase (EC 3.2.1.4) from *A. niger* was purified from cellulase type II (Sigma, St Louis, MO, U.S.A.) as described previously (Hurst *et al.*, 1977). The final preparation was homogeneous in the analytical ultracentrifuge, on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and Bio-Gel P-60 gel filtration. With gel electrophoresis one major and two minor bands were revealed. The three protein bands were isolated as previously described (Hurst *et al.* 1977); all three bands exhibited cellulase activity with identical K_m values for CM-cellulose and pH optima. The protein concentration of the purified enzyme preparation was 9 mg/ml.

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Substrates

CM-cellulose 7LIXP was obtained from Hercules, Wilmington, DE, U.S.A. Cellulose powder (chromatography grade) was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Cellobiose was obtained from BDH, Poole, Dorset, U.K. Yeast glucan, barley glucan and CM-pachyman were kindly supplied by Professor B. A. Stone, Department of Biochemistry, La Trobe University, Bundoora, Vic., Australia. Avicel was from the American Viscose Corp., Marcus Hook, PA, U.S.A. Cello-oligosaccharides and their reduced analogues were prepared as described below. All other carbohydrates were obtained from Sigma. Alkali-swollen cellulose was prepared as described by Hash & King (1958).

Analytical methods

Reducing sugars were determined with the *p*-hydroxybenzoic acid hydrazide reagent (Hurst *et al.*, 1977). The orcinol method of Svennerholm (1956) was used for determination of total carbohydrate.

Substrate specificity

Each substrate (1–10 mg/ml) was incubated with cellulase (0.1–25 μg/ml) at 40°C in 0.1 M-sodium acetate buffer, pH 4.0, in a final volume of 0.6 ml. The incubation time ranged from 0.5 to 48 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 *p*-hydroxybenzoic acid hydrazide reagent. β-Glucosidase and cellobiose activities were determined as described previously (Hurst *et al.*, 1977).

Hydrolysis of laminarin and the cellulodextrins was monitored after gel filtration of the reaction mixture; this procedure was necessary because of the high background of the substrates.

Preparation of cello-oligosaccharides

Oligosaccharides from cellulose were prepared by acetolysis/deacetylation and then fractionated by ethanol gradient elution of a stearic acid treated charcoal/Celite column by the method of Miller *et al.* (1960). The column (60cm × 6cm) was filled with 2½% (w/v) stearic acid-treated adsorbent. A sample (5g) of the oligosaccharide mixture was applied to the column, which was eluted with a linear gradient composed of 3 litres of water and 3 litres of 45% (v/v) ethanol. The individual oligosaccharides were further purified by chromatography on a Sephadex G-15 column (2cm × 180cm) eluted with water. The appropriate fractions were pooled and freeze-dried.

Reduced oligosaccharides were prepared by treating the cello-oligosaccharides with $NaBH_4$. Each solution of 50mg of cello-oligosaccharide in 2ml of water, plus 200mg of $NaBH_4$ was heated on a boiling-water bath for 10min cooled and then neutralized with acetic acid. Odd- and even-numbered members of the series were combined, then purified on the Sephadex G-15 column (2cm × 180cm), and freeze-dried.

Results

Substrate specificity

A number of different glycosidic compounds were scanned as potential substrates, to obtain information about the glycosyl linkage and glycosyl-group requirements of the enzyme. Comparative quantitative data were not obtained because of the varying incubation times and enzyme concentrations. In addition, it was not established that enzyme substrate-saturation was achieved; hence extrapolations to constant incubation time and enzyme concentration would have been invalid.

The *A. niger* enzyme showed a very limited ability to degrade highly ordered celluloses (Avicel and cellulose powder). On the other hand, cellulose that was rendered amorphous by swelling with alkali was readily degraded, considering that the substrate is still insoluble after this treatment. As might be expected, activity towards cellulosic compounds was enhanced when they were freely soluble: CM-cellulose, a synthetic polymeric substrate, was rapidly hydrolysed by the enzyme (Hurst *et al.*, 1977). Of the compounds tested, the natural polysaccharides, lichenin and barley glucan, were the most amenable to hydrolysis. With a substrate concentration of 0.1% (w/v), the production of reducing end groups could be detected from the action of 5ng of enzyme/ml, which is an enzyme concentration an order of magnitude less than with 1.0% (w/v) CM-cellulose as substrate. This mixed-glucan-hydrolysing activity was not a contaminant in the cellulase preparation, since a separate experiment showed that the ability to degrade both barley glucan and CM-cellulose was common to the three protein bands isolated on gel electrophoresis. Also, the pH–activity profile with respect to barley glucan showed a similar sharp pH optimum at about pH4.0, compared with the pH–activity profile for CM-cellulose.

CM-pachyman (1mg/ml) proved to be susceptible to degradation by relatively high concentrations of cellulase (greater than 1μg/ml). Results obtained from attempts to ascertain whether or not this activity was an intrinsic feature of the cellulase enzyme were ambiguous. The β-1,3-glucanase activity was located with the main cellulase peak on polyacrylamide-gel electrophoresis. However, only 10% of the pachymanase activity was recovered from the sliced gel. (cf. 70% recovery of cellulase activity). A distinct β-1,3-glucanase protein band was not found. The pH–activity profile with CM-pachyman exhibited a peak at pH4.0, but also a shoulder in the region pH5–6, compared with the symmetrical profile obtained with CM-cellulose. Moreover, heating a solution of enzyme (60μg/ml, in 0.1M-sodium acetate buffer, pH4.0) at 65°C for 10min resulted in the loss of 90–95% of the CM-cellulase activity, although 85–90% of the β-1,3-glucanase activity was retained. The latter activity was completely destroyed by heating to 100°C or by treatment with trichloroacetic acid (100mg/ml).

In view of the identity of the linkage types found in laminarin, as compared with CM-pachyman, it was surprising to find that the former compound was completely inert to degradation. The technique of gel filtration on Sephadex G-15 was used to monitor any depolymerization of the substrate, since laminarin had a background of reducing end groups too high to be used in the usual *p*-hydroxybenzoic acid hydrazide assay. The insoluble β-1,3;β-1,6 yeast glucan was not hydrolysed by the enzyme.

Of the non-glucosidic polymers tested, xylan was the only polysaccharide degraded by the enzyme. The specific activity of the xylanase was low; it was comparable with the specific activity of the CM-pachymanase. The purified cellulase was devoid of chitinase, pectinase and mannanase activities.

Mode of action against cellulodextrins and reduced cellulodextrins

The enzymic hydrolysis of the β-1,4-oligoglucosides and the reduced analogues was followed by determining the change in composition with time, by means of gel filtration. The hydrolysates were analysed on a column (1cm × 85cm) of Sephadex

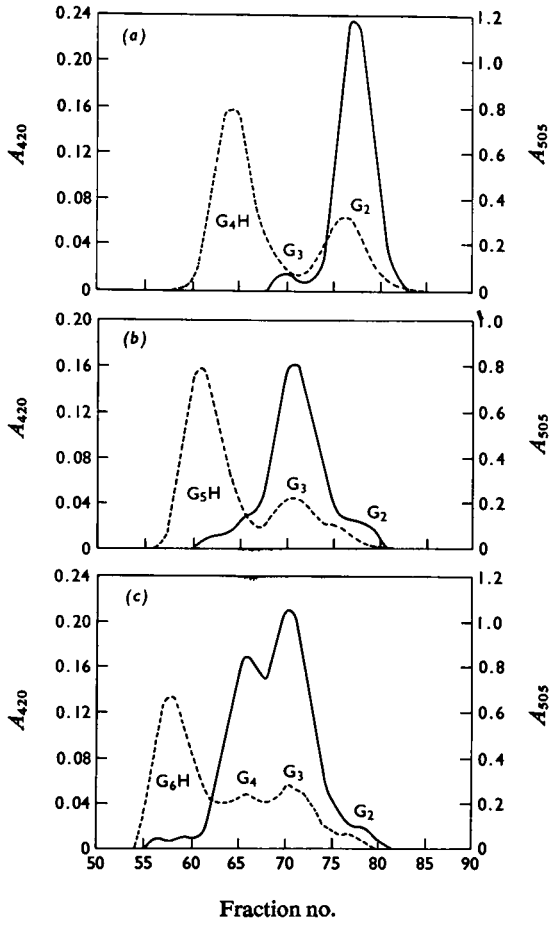

Fig. 1. *Column chromatography of reduced cellulodextrins after hydrolysis by cellulase*

Hydrolysates were analysed on a column (1 cm × 85 cm) of Sephadex G-15 eluted with water at 10 ml/h. Fractions (approx. 0.6 ml; ten drops) were collected and analysed for reducing sugars (——) with *p*-hydroxybenzoic acid hydrazide reagent (A_{420}) and total carbohydrate (– – – –) with orcinol reagent (A_{505}) as described in the Experimental section. G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_4H, cellotriosylsorbitol; G_5H, cellotetraosylsorbitol; G_6H, cellopentaosylsorbitol. (*a*) Cellotriosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellotriosylsorbitol (1 mg/ml, in 0.1 M-sodium acetate buffer, pH 4.0) and 0.1 ml of cellulase (100 μg/ml). Time of hydrolysis at 40°C was 12 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*) Cellotetraosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellotetraosylsorbitol (1 mg/ml, as in *a*) and 0.1 ml of cellulase (50 μg/ml). Time of hydrolysis at 40°C was 0.5 h; the reaction mixture was then treated as in (*a*). (*c*) Cellopentaosylsorbitol hydrolysis: the incubation mixture contained 0.5 ml of cellopentaosylsorbitol (1 mg/ml, as in *a*) and 0.1 ml of cellulase (20 μg/ml). Time of hydrolysis at 40°C was 0.5 h; the reaction mixture was then treated as in (*a*).

G-15 eluted with water. Use of the oligosaccharides, in which the terminal reducing glucose residue has been converted into a sorbitol residue by $NaBH_4$, facilitates the estimation of the relative frequency of attack to the various glucosyl bonds of the substrates (Scheme 1). Cellobiosylsorbitol was not hydrolysed at all by the enzyme. Hydrolysis of cellotriosylsorbitol produced cellobiose as the main reducing sugar and a trace of cellotriose (Fig. 1*a*), indicating preferential cleavage at bond 2 compared with bond 3 (numbering from the non-sorbitol end of the molecule).

Cellotriose was the major reducing-sugar product from the hydrolysis of cellotetraosylsorbitol. Trace amounts of cellotetraose and cellobiose were evident (Fig. 1*b*), showing that, although bonds 2, 3 and 4 are susceptible to attack, the penultimate glucosyl bond from the sorbitol end was the preferred cleavage point. Hydrolysis of cellopentaosylsorbitol gave cellotriose and cellotetraose in approximately equimolar amounts (Fig. 1*c*); cellobiose was also produced, but not in large amounts. Hence, again the central bonds in the glucose oligomer were the most favoured points of hydrolysis.

Clearly there was a pronounced tendency for the rate of hydrolysis of the reduced oligosaccharides to increase with chain length. A separate experiment was therefore undertaken in which the initial velocities of the hydrolysis of the individual reduced oligosaccharides were determined by using the standard assay procedure (Table 1). In contrast with cellobiosylsorbitol, the reducing glucosidic trimer, cellotriose, is hydrolysed by the cellulase, albeit very slowly (Fig. 2*a*). Cellotetraose was more rapidly cleaved to yield approximately equimolar amounts of cellotriose, cellobiose and glucose (Fig. 2*b*). Hydrolysis of cellopentaose occurred readily, with the production of cellotriose and cellobiose in equimolar quantities, and traces of glucose and cellotetraose (Fig. 2*c*). Quantitative data on the relative rates of hydrolysis of the unmodified cellulodextrins could not be obtained because of the high background obtained with these substrates, which interfered with the *p*-hydroxybenzoic acid hydrazide reducing-sugar assay. Clearly, however, the rate of degradation increased with chain length.

Hydrolysis of lichenin by cellulase

A sample of lichenin (1 mg/ml) was hydrolysed by the enzyme for 2 h. After termination of the reaction by heating on a boiling-water bath for 5 min, the hydrolysate was fractionated on the Sephadex G-15

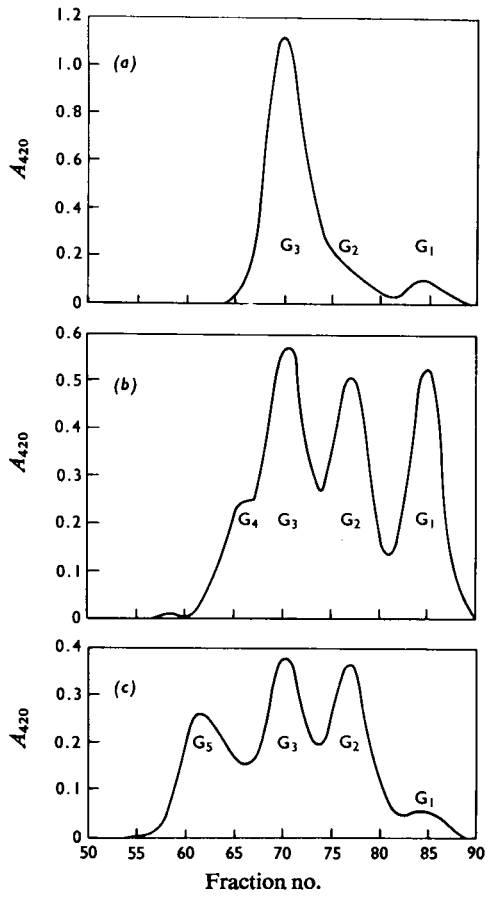

Fig. 2. *Column chromatography of cellulodextrins after hydrolysis by cellulase*

Column conditions are as described for Fig. 1, except that fractions were analysed for reducing sugars only. G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose. (*a*) Cellotriose hydrolysis: the incubation mixture contained 0.5ml of cellotriose (1mg/ml, in 0.1M-sodium acetate buffer, pH4.0) and 0.1ml of cellulase (100μg/ml). Time of hydrolysis at 40°C was 75h; the reaction mixture was then heated for 5min on a boiling-water bath to inactivate the enzyme, cooled and then applied to the column. (*b*) Cellotetraose hydrolysis: the incubation mixture contained 0.5ml of cellotetraose (1mg/ml, as in *a*) and 0.1ml of cellulase (100μg/ml). Time of hydrolysis at 40°C was 12h; the reaction mixture was then treated as in (*a*). (*c*) Cellopentaose hydrolysis: the incubation mixture contained 0.5ml of cellopentaose (1mg/ml, as in *a*) and 0.1ml of cellulase (50μg/ml). Time of hydrolysis at 40°C was 1h; the reaction mixture was then treated as in (*a*).

column (1cm×85cm). The appropriate control incubation and separation was carried out concurrently. A reducing sugar that chromatographed slightly ahead of cellotriose was the predominant product of hydrolysis (Fig. 3). The peak was rather broad; shoulders, indicating the presence of smaller and larger saccharides, were observed. Glucose was

Table 1. *Relative initial rates of hydrolysis of reduced cellulodextrins*

Reaction mixtures contained 2ml of oligosaccharide (1mg/ml) in 0.1M-sodium acetate buffer, pH4.0, and 0.4ml of enzyme solution. After incubation at 40°C 0.3ml samples were removed at intervals and then assayed with 2.5ml of *p*-hydroxybenzoic acid hydrazide reagent. Enzyme solutions used: for reduced cellulodextrin (degree of polymerization >7) and cellopentaosylsorbitol, 10μg/ml; for cellotetraosylsorbitol, 20μg/ml; and for cellotriosylsorbitol and cellobiosylsorbitol, 100μg/ml. Total period of incubation was 30min except for cellotriosylsorbitol and cellobiosylsorbitol, where it was 3h. 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	Hydrolysis rate
Reduced cellulodextrin	185
Cellopentaosylsorbitol	160
Cellotetraosylsorbitol	46
Cellotriosylsorbitol	1
Cellobiosylsorbitol	0

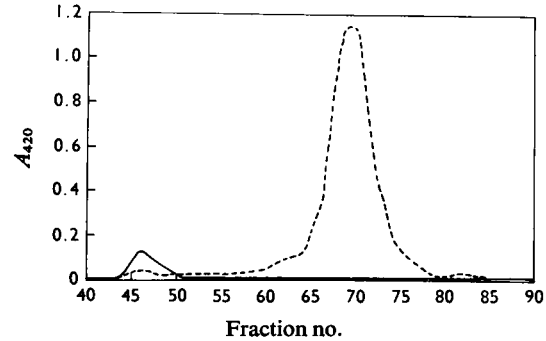

Fig. 3. *Column chromatography of lichenin after hydrolysis by cellulase*

Column conditions are as in Fig. 1. Fractions were analysed for reducing sugars with *p*-hydroxybenzoic acid hydrazide reagent as in the Experimental section. The incubation mixtures contained 0.5ml of lichenin (1mg/ml) in 0.1M-sodium acetate buffer, pH4.0, and 0.1ml of cellulase (100μg/ml). After appropriate time each mixture was heated on a boiling-water bath for 5min to inactivate the enzyme, cooled and then applied to the column. ——, Zero-time control; ----, after 2h hydrolysis time.

almost entirely absent from the chromatogram. Definitive peaks corresponding to higher oligosaccharides were not found. It was estimated, from the chromatogram, that approximately two-thirds of the lichenin had been fully degraded in the 2h incubation period.

Discussion

Commercial enzyme preparations derived from culture filtrates of *A. niger* have been found to hydrolyse a wide range of carbohydrates, including yeast glucan, xylan, laminarin, pectin, polygalacturonic acid, chitin, lichenin and CM-pachyman, as well as cellulosic compounds (Holden, 1950; Li & King, 1963; Clarke & Stone, 1965*a*).

Of the non-cellulosic compounds tested, only xylan and CM-pachyman were hydrolysed. The specific activities with respect to these substrates, however, were low compared with that of CM-cellulase. For the CM-pachyman-hydrolysing activity, inconclusive evidence for the existence of a separate enzyme was obtained. Laminarin, which contains the same linkage types as CM-pachyman, was not hydrolysed. Laminarin differs from CM-pachyman, however, in that it is a much smaller molecule and it contains mannitol (Barras *et al.*, 1969). A distinct β-1,3-glucanase band was not found on gel electrophoresis. Only some 10% of the β-1,3-glucanase activity was recovered from the sliced gels, compared with the 70% recovery of cellulase activity. The β-1,3-glucanase activity, however, was much more stable to heat-treatment than was cellulase. Furthermore, the pH–activity profile for β-1,3-glucanase showed a broad optimum about pH 4–6 compared with the sharp pH 4 optimum of the cellulase (Hurst *et al.*, 1977). These latter results indicate that the two types of activity observed are due to different components. From the difference in heat-stability, Clarke & Stone (1965*a*) came to a similar conclusion about the residual β-1,3-glucan-hydrolysing activity in a purified β-1,4-glucan hydrolase from *A. niger*.

It has been reported that some cellulase components from *Trichoderma viride* show xylanase activity: furthermore endocellulase showed endoxylanase activity (Toda *et al.*, 1971), and exocellulase was associated with exoxylanase activity (Shikata & Nisizawa, 1975). Kanda *et al.* (1976) have shown conclusively that xylanase activity is intrinsic to a homogeneous cellulase from *Irpex lacteus*. Thus it is likely that the xylanase activity reported here is an inherent feature of the cellulase. The cellulase from *A. niger* described by Clarke & Stone (1965*b*), however, did not have the capacity to hydrolyse xylan. In contrast, however, mannan was hydrolysed by that enzyme.

Barley glucan and lichenin were found to be excellent substrates for the cellulase. Although these substrates contain β-1,3- as well as β-1,4-bonds, these glucans were more susceptible to hydrolysis than was CM-cellulose. Because of the carboxymethyl substitutions, most of the CM-cellulose is unavailable for hydrolysis. Assuming a random distribution of the unsubstituted anhydro-glucose units the frequency of adjacent unsubstituted sequences can be predicted. For a CM-cellulose of degree of substitution 0.79 it has been calculated that the numbers of one, two, three, four and five adjacent unsubstituted residues per thousand glucose residues are 147, 44.1, 13.2, 4.0 and 1.2 respectively (Wirick, 1968). Hence if at least three adjacent unsubstituted glucosyl residues are required for hydrolysis (Eriksson & Hollmark, 1969), only 2% of the substrate can be readily hydrolysed. Lichenin is considered to be composed of cellotriose units joined by β-1,3-linkages (Peat *et al.*, 1957) and barley glucan has a similar content (70%) of β-1,4-linkages (Clarke & Stone, 1966). Hence their rapid depolymerization by cellulase can be explained simply in terms of the availability of glucosyl linkages.

Pettersson (1969) has shown that the major products from lichenin formed by the action of a *Penicillium* cellulase were oligosaccharides with degree of polymerization 5 or 6. Clarke & Stone (1966) noted that a trisaccharide was the main product from exhaustive hydrolysis of barley glucan, oat glucan and lichenin by an *A. niger* cellulase. Fig. 3 shows that the predominant product of hydrolysis of lichenin by this *A. niger* cellulase was an oligosaccharide with an elution volume similar to that of cellotriose.

The preferred points of cleavage of the reduced cellulodextrins, determined from the chromatography described in Figs. 1 and 2, are shown in Scheme 1.

Since there was no evidence for glucose being released in the early stages of hydrolysis of the reduced oligosaccharides, it was concluded that the cellulase cannot act as an exocellulase, successively removing glucosyl units from the non-sorbitol end of the oligomer. The observed preference for non-terminal linkages confirms the endo-action of the enzyme, which was previously indicated by the demonstration that the cellulase rapidly decreases the

Glc-Glc-Glc ‡ Glc-Glc-GlcH or Glc-Glc-Glc-Glc ‡ Glc-GlcH	Cellopentaosylsorbitol
Glc-Glc-Glc ‡ Glc-GlcH	Cellotetraosylsorbitol
Glc-Glc ‡ Glc-GlcH	Cellotriosylsorbitol
Glc-Glc-GlcH	Cellobiosylsorbitol

Scheme 1. *Site of hydrolysis of reduced cellulodextrins*

viscosity of CM-cellulose solutions with the simultaneous slow release of reducing sugars (Hurst *et al.*, 1977). Inspection of the elution profiles (Fig. 1) shows that the preference was not absolute for a particular bond. For example, a small amount of cellotriose was produced from cellotriosylsorbitol, indicating cleavage at the glucosyl–sorbitol bond. Both cellotetraose and cellobiose were obtained in small amounts with cellotetraosylsorbitol as substrate, showing that bonds 2 and 4 were cleaved (bonds are numbered from the non-sorbitol end of the molecule). Clarke & Stone (1965*b*) reported a similar preference for non-terminal linkages with a cellulase from *A. niger*, but attack at the glucosyl–sorbitol linkage by that enzyme did not occur. Component 1 from *A. niger* appeared to remove successive glucosyl moieties from the non-reducing end of cellotetraosylsorbitol (Cole & King, 1964).

The rate of hydrolysis of the reduced cellulodextrins increased with chain length (Table 1). Since the increase is not drastic in going from cellopentaosylsorbitol to reduced cellulodextrin (degree of polymerization of 7), it is postulated that the enzyme has at least five and possibly six subsites in the active centre that recognize or bind glucose residues. Considering, however, that the enzyme displayed equal preference for bonds 3 and 4 in the hydrolysis of cellopentaosylsorbitol, it might be argued that the hexamer is one residue too large to be completely accommodated in the active centre. Hence it is concluded that the specificity region of the *A. niger* cellulase is five glucose units long.

Although quantitative data on the rate of hydrolysis of the native oligosaccharides could not be obtained, it was clear that the susceptibility to attack also increased with chain length. With these reducible substrates the trimer was hydrolysed, although extremely slowly. From the results obtained with the reduced substrates and the elution profiles (Fig. 2), the points of hydrolysis of the native oligosaccharides were deduced and are shown in Scheme 2.

The pentamer appeared to be hydrolysed in a straightforward manner, yielding cellobiose and cellotriose as major products. Hydrolysis of cellotetraose, however, gave glucose, cellobiose and cellotriose in approximately equimolar amounts. To account for this result it must be concluded that cleavage at the terminal bond (1 or 3) occurred at twice the frequency of cleavage at the internal bond. Since glucose was not produced from cellotriosylsorbitol, bond 3 is the bond cleaved. Cellotetraose is a poor substrate compared with cellopentaose, and the lack of a preferred site of hydrolysis implies that the tetramer has no strong productive binding mode. This is further indication that the specificity region of the enzyme is greater than four glucose units in length.

Cellopentaose	Glc-Glc-Glc ‡ Glc-Glc	→	Cellobiose and cellotriose (1:1)
Cellotetraose	Glc-Glc ‡ Glc-Glc or Glc-Glc-Glc ‡ Glc	→	Glucose, cellobiose and cellotriose (1:1:1)
Cellotriose	Glc-Glc ‡ Glc	→	Glucose and cellobiose

Scheme 2. *Deduced sites of hydrolysis of cellulodextrins*

Designating the proposed subsites A–E, it is possible to conclude that the substrate is aligned with the non-reducing end towards subsite A and that bond cleavage occurs between subsites C and D. These present findings may be compared with work from other cellulases in which the kinetics of the splitting of β-1,4-oligoglucosides are similarly dependent on chain length. Pettersson (1969) has shown that the specificity region of a *Penicillium notatum* endocellulase is five glucose units long. Similarly, Whitaker (1954) and Hanstein & Whitaker (1963) showed that the *Myrothecium verrucaria* cellulase had at least five substrate-recognition subsites in the active centre. A decrease in K_m values with increasing chain length of oligoglucosides has been observed by Li *et al.* (1965) with a cellulase from *T. viride*, and they reported that the optimum substrate chain was at least six glucosyl units long.

Large active centres, containing several subsites, appear to be a general feature of hydrolytic enzymes acting on polymeric substrates. Abramowitz *et al.* (1967) and Berger & Schecter (1970) concluded that the active centres of carboxypeptidase A and papain consist of five and seven substrate-binding sites respectively. Lysozyme has six subsites (Blake *et al.*, 1967; Phillips, 1967).

The present data indicate that this particular *A. niger* cellulase functions as an endoglucanase with five subsites and should be designated as a 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4).

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