Kinetic Properties and Substrate Specificities of Two Cellulases from Auxin-treated Pea Epicotyls*

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Two cellulases purified from growing regions of auxin-treated peas (buffer-soluble and buffer-insoluble) hydrolyze cellulose powder, partially substituted carboxymethylcellulose (CM-cellulose), higher cellodextrins, and certain mixed linkage glucans (e.g. barley β-glucan), at rates comparable to those reported for the most active fungal cellulases, and with kinetics and product formation characteristic of endohydrolase action. They are unable to cleave 1,3-linkages in β-glucans, or 1,4-linkages in dextrans containing excessive substitution at C6; α configuration, alternating β-1,3- and 1,4-linkages, or residues other than anhydroglucose. They are not active towards cellobiose or the 1,4-linkage of reducing end of cellodextrin chains. It is concluded that buffer-soluble and buffer-insoluble cellulases are true β-1,4-glucan 4-glucanohydrolases (EC 3.2.1.4).

On a molar basis, Vmax values for buffer-insoluble are higher than buffer-soluble cellulase acting towards any of the substrates tested, but Km values towards CM-cellulose and cellodextrase are essentially identical. Both cellulases were inhibited by Cu2+, Hg2+, and sulfhydryl-binding reagents. Buffer-insoluble, but not buffer-soluble, cellulase was inactivated by reagents that bind serine and threonine, which reflects differences in their amino acid composition. No major qualitative differences have been detected in the mode of action of the two enzymes. Despite marked differences in their physical and immunological properties, close similarities between buffer-soluble and buffer-insoluble enzymic properties suggest that their active sites are the same.

Cellulase, assayed by measuring hydrolysis of CM-cellulose or cellulose, occurs indigenously in growing regions of pea epicotyls (1) and is one of very few plant enzyme activities known to increase dramatically following treatment of tissue with the auxin type of growth regulator (2, 3). After purification to homogeneity, it has been established (4, 5) that this activity is due to two proteins with molecular weights of 20,000 (buffer-soluble) and 70,000 (buffer-insoluble). The proteins differ in amino acid composition, mobilities in various electrophoretic and chromatographic systems, sedimentation behavior, and immunological properties. Their chemical and physical properties were sufficiently distinct to lead to the tentative conclusion that one was not derived from the other. Antibodies raised to the two cellulases have been used to examine their subcellular locations (6) and to assay for development of mRNA which codes for the enzymes (7). At least one (buffer-soluble) appears to be generated de novo by endoplasmic reticulum following hormone treatment, and one (buffer-insoluble) becomes bound to microfibrils in walls of growing cells. None of these studies yields any explanation as to a functional significance for two celulolytic enzymes elaborated in the same tissue.

Speculation concerning the possible role(s) of cellulase activity in growing plant cells (8–10) can not be fully informed or evaluated without further knowledge of biochemical properties of the enzymes responsible. This paper records standard kinetic constants and examines the mode of action towards cellodextrins and other potential substrates of the two purified pea cellulases. It is concluded that these enzymes are both true β-glucan endohydrolases which act specifically on β-1,4-linkages.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Assay—Buffer-soluble and buffer-insoluble cellulases were purified (11) from extracts of auxin-treated apices of pea epicotyls as previously described (4, 5) to yield preparations with specific activities of 1.3 × 104 and 1.8 × 104 viscosity units per mg of protein, respectively. The pure enzymes denatured rapidly in dilute buffer (4), but when dissolved in 20 mM sodium phosphate buffer, pH 6.2, containing 0.04% bovine serum albumin, they were comparatively stable and could be stored frozen for long periods without loss of activity.

Cellulase activity was routinely assayed viscometrically (4). One viscosity unit of activity is defined here as the amount of cellulase which, in 2 h at 35°C, causes a 1% decrease in η∞, of 1.1 ml of 0.65% (w/v) CM-cellulose in 20 mM sodium phosphate buffer, pH 6.2, containing 0.2 mM NaF. Reducing power was assayed (12) in aliquots of reaction mixtures in which substrates and cellulase (150 to 600 viscosity units/ml) were incubated at 35°C in 20 mM sodium phosphate buffer, pH 6.2, containing 0.03% sodium azide. The pH was close to the optimum for activity by both cellulases towards CM-cellulose (4, 5) and cellodextrase (11).

To assay cellulase activity towards cellodextrins, substrates and products were separated by paper chromatography and identified by comparing mobilities with values for authentic dextrans (prepared by D. A. I. Goring, McGill University). Solvents were the organic phase of isooctyl alcohol:pyridine:water (1:1:1, v:v:v) or propyl alcohol:ethyl acetate:water (6:1:3, v:v:v). Products were located with alkaline AgNO3 (14) or potassium periodocuprate (15) or, when 14C-labeled, by autoradiography (Kodak, N°-Screen X-ray film). Radioactive products were quantitated by cutting spots from the paper and counting in toluene/Omnifluor (New England Nuclear Corp.).
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Substrates and Products—Soluble β-1,4-linked oligosaccharides from cellbiose to cellohexaose (G2 to G6) were prepared as described by Miller et al. (16) by fractionation on a charcoal/celite column of partial hydrolysates of cellulose powder (Whatman, standard grade) or [U-14C]cellulose (ICN Pharmaceuticals). The products co-chromatographed with authentic cellodextrins and yielded Rf values consistent with a homologous series (17). Measurements of optical rotation (25°, 589 nm) showed that the isolated higher dextrins were optically pure and linearly related in a Freudenburg plot (18). Cello-rotation (25°, 589 nm) showed that the isolated higher dextrins were consistent with a homologous series (17). Measurements of optical matographed with authentic cellodextrins and yielded Rf values by cellulase from Streptomyces and glucanase from E. T. Reese (United States Department of the Army, Natick, "CM-cellulase" activity (Fig. 2). The degree of inhibition in the cellulases is similar towards CM-cellulose and cellodextrins is provided by the fact that the dextrins competitively inhibit values for the two substrates and enzymes (cf. Table I). Nevertheless, apparent Km values for buffer-soluble and buffer-insoluble cellulase under conditions essentially identical for the two enzymes.

RESULTS

Hydrolysis of CM-cellulose (CM-cellulase activity) — Both buffer-soluble and buffer-insoluble cellulases lower the viscosity of solutions of CM-cellulose rapidly but generate reducing groups at a comparatively slow rate (Fig. 1), an action pattern characteristic of endohydrolases. Even after viscosity losses are essentially complete, reducing power continues to be generated at a linear rate which is the same for both enzymes. Eventually (by 24 to 48 h, Refs. 5 and 11), reducing power ceases to be produced, not because the enzymes denature, but because the residual substrate becomes inaccessible i.e. too highly substituted for hydrolysis to occur (26). Measurements of initial rates of hydrolysis of CM-cellulose at different substrate concentrations (11) yields linear Lineweaver-Burk plots with identical apparent Km values for buffer-soluble and buffer-insoluble cellulase (Table I). The Vmax value calculated per μmol of enzyme is higher for the larger (buffer-insoluble) enzyme (Table I). Evidently the two cellulases possess similar affinities and modes of action towards CM-cellulose, but different turnover numbers.

Substrate Specificity—Cellohexaose is the longest chain cel- lodextrin which can be obtained in solution at concentrations sufficient to measure kinetic properties of potential hydrolases. Buffer-soluble and buffer-insoluble cellulases hydrolyze cellohexaose more rapidly than CM-cellulose (Table I), probably because the cellodextrin only cleave linkages in unexcised regions of CM-cellulose (26). Nevertheless, apparent Km values for the two substrates and enzymes are essentially the same (3.5 to 3.8 mg/ml). Further evidence that affinity of the cellulases is similar towards CM-cellulose and cellodextrins is provided by the fact that the dextrins competitively inhibit "CM-cellulase" activity (Fig. 2). The degree of inhibition increases exponentially with D.P., indicating that the cellulases preferentially bind to longer chain celldextrins (see also Fig. 3).

Table II records values for the initial rate of production of reducing groups in various polysaccharides incubated with buffer-soluble and buffer-insoluble cellulase under conditions optimal for hydrolysis of CM-cellulose and cellohexaose. The enzymes hydrolyze suspensions of crystalline or partially swollen cellulose powder at rates which are lower than Vmax values towards CM-cellulose or cellohexaose (cf. Table I). However, hydrolysis continued in these tests for at least 24 h, by which time as many as 10% of the glucosidic linkages in cellulose were cleaved (11). No free glucose, cellobiose, or soluble dextrin could be detected in any of the reaction mixtures by chromatographic procedures, even after 24 h of incubation with cellodextrins, indicating that internal linkages of long chains were preferentially hydrolyzed.

Buffer-soluble and buffer-insoluble cellulases also hydrolyze certain glucans containing both β-1,3- and β-1,4-linkages, but only when these contain at least some consecutive β-1,4-linkages (Table II). Endohydrolysis of barley β-glucan can be followed viscometrically, and plots of viscosity loss versus reducing groups produced (11) yield action patterns for this substrate which are very similar to those observed with CM-cellulose (Fig. 1). The main product detected in hydrolysates of barley β-glucan at the end of 24 h has a chromatographic...
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Fig. 2. Inhibition of cellulase activity towards carboxymethylcellulose (CMC) by cellodextrins. Purified cellulases (0.1 ml, 150 units) were added to CM cellulose (0.8 ml, 0.8%, w/v) in the presence of cellodextrin (0.2 ml, 10 mM) and incubated for 15 min at 35°. CM-cellulase activity at different cellohexaose concentrations showed that inhibition was competitive with $K_i$ values for buffer-soluble (BS) and buffer-insoluble (BI) cellulases of 1.74 and 0.74 mM, respectively. Glucose does not inhibit CM-cellulase activity (5).

Fig. 3. Initial rates of hydrolysis of cellodextrins by buffer-soluble (BS) and buffer-insoluble (BI) cellulases. Reaction mixtures (0.8 ml) contained D-cellodextrins (1.0 mM) and cellulase (400 units) at 35°, pH 6.2. At 2 h, aliquots were chromatographed and radioautographed and the amount of substrate which disappeared was measured. Products generated in these systems are shown in Fig. 4.

Hydrolysis of Cellodextrins - The initial reaction rates of buffer-soluble and buffer-insoluble cellulases acting on non-limiting concentrations of $^{14}C$-cellodextrins were assayed by measuring the amount of labeled substrate which disappeared during the early linear phase of digestion. The results (Fig. 3) show that neither enzyme hydrolyzes cellobiose ($G_2$), but both degrade higher cellodextrins ($G_3$ to $G_6$) at rates which increase exponentially with D.P.

Progress curves for production of degradation products from cellodextrins ($G_3$ to $G_6$) are shown in Fig. 4. The two cellulases display similar action patterns while cleaving each of these substrates and the dextrins derived from them. The following generalizations apply to activities of both enzymes.

- Cellotriose is slowly degraded to equimolar concentrations of $G_1$ and $G_2$, indicating cleavage of one or both of the two glucosidic linkages.
- Cellotetraose is hydrolyzed more rapidly in early stages mainly to $G_2$ with $G_1$ and $G_3$ generated in smaller but equal amounts. It cannot be determined from these data whether the internal linkage of cellotetraose is most susceptible to hydrolysis with both terminal linkages cleaved at about half the rate, or whether one terminal linkage is resistant to hydrolysis while the other two linkages are cleaved at similar rates.
- Cellopentaose is degraded rapidly to equal amounts of $G_1$ and $G_3$, with $G_2$ and $G_4$ accumulating relatively slowly. Evidently one or both of the internal linkages are cleaved more readily than either terminal linkage. The predominant final product is $G_2$, presumably because $G_2$ and $G_4$ are subject to further degradation. Cellohexaose degrades very rapidly (Fig. 3) to ages or $\alpha$-1,4-linkages, nor are linear $\beta$-1,4-linked polysaccharides hydrolyzed when they contain residues other than anhydroglucosyl.

As expected from results of these tests for specificity, neither cellulase was inhibited by addition of 1% n-o-nirimycin (5-amino-5-deoxy-D-glucopyranose), an antibiotic reported (27) to strongly inhibit $\beta$-glucosidase, but not endoglucanase activity. Both enzymes were completely inactive in the presence of several noncompetitive inhibitors, including heavy metal ions (5 mM Cu$^{2+}$ or Hg$^{2+}$) and sulfhydryl reagents (2 mM p-chloromercuribenzoate, mercaptethanol, or n-ethylmaleimide). The only notable difference observed (11) in responses of the two enzymes to a variety of potential inhibitors was the inactivation of buffer-insoluble but not buffer-soluble cellulase by reagents which bind serine and threonine residues ($e.g.$ succinichydride, disopropyl fluorophosphate). This may be related to the fact that buffer-insoluble cellulase contains many more of these residues per molecule than buffer-soluble cellulase (4).

Hydrolysis of Various Polysaccharides by Pea Cellulases

| Substrate      | $V_{max}$ (Buffer-soluble) mmol Glc eq/min/µmol cellulase | $V_{max}$ (Buffer-insoluble) mmol Glc eq/min/µmol cellulase |
|----------------|-----------------------------------------------------------|------------------------------------------------------------|
| Whatman cellulose | 13                                                        | 80                                                         |
| Acid swollen cellulose | 22                                                        | 87                                                         |
| Alkali-swollen cellulose | 28                                                        | 95                                                         |
| Barley $\beta$-glucan | 93                                                        | 177                                                        |
| Lichenin     | 22                                                        | 141                                                        |

Hydrolysis of Various Polysaccharides by Pea Cellulases

Purified cellulases (0.2 ml, 600 units) were added to saturating concentrations of substrates (0.9 ml, 0.3 to 1.2%, w/v) and incubated at 35°, pH 6.2. Aliquots were removed at intervals for reducing power determination. Under these conditions, no activity was detected towards $\beta$-glucosides, laminarin, CM-pachyman, reduced SIII polysaccharide, starch, wheat straw xylan, ivory nut mannann, or chitin.

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Fig. 4 (left). Products formed from cellodextrins by buffer-soluble (BS) and buffer-insoluble (BI) cellulases. G₁ to G₅ represent dextrins with D.P. from 1 to 5 (Glc to cellobextrin). The decrease in concentrations of initial substrates was also measured in order to assay for $V_{\text{max}}$ values (Fig. 3). No other products were detected on chromatograms.

Fig. 5 (right). Labeled products formed from reduced (tritiated) cellodextrins by buffer-soluble (BS) and buffer-insoluble (BI) cellulases. Reaction mixtures (0.3 ml) contained reduced H-cellodextrins (1 mg, G₅ to G₅₅) and cellulase (200 unite) at 35°C, pH 6.2.
form $G_3$ and $G_4$ as the main products, with transitory amounts of $G_9$, $G_{14}$, and $G_{27}$ detectable in early stages of the decay (11).

There was no indication in these tests that transglucosylation occurred in the mixtures e.g. cellobextrins of higher D.P. than the substrates were never detected on chromatographs. Tests for transglucosylation, using $^{14}$C-cellobextrins as potential donors and $^{13}$C-cellobextrins or cellulose as acceptors under a variety of reaction conditions, yielded no evidence that pea cellulos could form β-1,4-linkages.

**Hydrolysis of Reduced Cellobextrins** – In order to assess the relative susceptibilities of linkages in cellobextrins to hydrolysis, the terminal reducing glucosyl residues ($G_2$ to $G_9$) were reduced (tritiated) and used as asymmetrically labeled substrates ($G_{29}$ to $G_{59}$). Fig. 5 shows progress curves for the generation of labeled products. As with other substrates (Tables I and II), buffer-insoluble cellulase was more active towards reduced $^{3}$H-cellobextrins than buffer-soluble cellulase on a molar basis but the mode of action of the two enzymes was indistinguishable. None of these substrates gave rise to labeled sorbitol, indicating that the linkage adjacent to the reduced terminal glucose is not cleaved. The other terminal linkage is readily hydrolyzed, however, as shown by the rapid initial rate of production of labeled products containing one less glucose unit than the substrates used. During early stages of hydrolysis of $G_{59}$, equimolar amounts of $G_{39}$ and $G_{49}$ are produced i.e. the central and susceptible terminal linkage are cleaved at comparable rates. These same products are generated at equal rates initially from $G_{59}$, with $G_{19}$ produced relatively slowly, suggesting a moderate preference for hydrolysis of the more central linkages in this substrate. There is clear evidence also that $G_{59}$ and $G_{49}$ formed in these preparations are subject to further degradation. Eventually, $G_{19}$ is the only labeled product remaining from all substrates.

**DISCUSSION**

Evidence has been presented (Tables I and II) that pea cellulase activities display specificity for poly- or oligosaccharide chains containing β-1,4-linkages between anhydroglucose residues. They do not possess β-glucosidase activity and they cannot hydrolyze cellubiose (Fig. 3). They are also inactive towards the linkage adjacent to a terminal glucose residue in cellobextrins when that unit is reduced (Fig. 5). A similar restricted action pattern has been observed (28) with purified *Myrothecium* cellulase. Kinetics of hydrolysis of higher soluble cellobextrins (Fig. 4) are consistent with the conclusion that the terminal linkage at the reducing end of these chains is also resistant to cleavage, whereas other linkages are hydrolyzed in an essentially random manner. Unsubstituted cellobiose is hydrolyzed at a substantial rate (Table I) in such a way that none of the reducing groups introduced in early stages of the reaction are released as glucose, cellubiose, or soluble cellobextrins i.e. the enzymes have no endohydrolase activity. The action patterns of cellulases towards CM-cellulose (Fig. 1), and the exponential relationship observed (Fig. 3) between rate of cellobextrin hydrolysis and D.P., confirm that these enzymes are true endohydrolases.

The pea cellulos can also depolymerize β-glucans that contain a limited number of β-1,3-linkages, e.g. barley β-glucan (Table I). This substrate has regions of two or three contiguous 1,4-linkages separated by 1,3-linkages (25). The pea cellulos, like many fungal cellulos (29, 30), generate several products from such substrates, the most prominent of which is the trisaccharide 4-O-β-p-laminaribiosyl-p-glucose, i.e. β-1,4-linkages are cleaved. This observation, coupled with the inability of the pea cellulos to hydrolyze alternating 1,3- and 1,4-linked β-glucan (Table II), indicates that these enzymes should be classed with cellulos of the *Streptomyces* type (25), rather than with the "mixed-linkage hydrolases" from *Bacillus* (31).

It is concluded that buffer-soluble and buffer-insoluble cellulos merit designation as true β-1,4-glucan 4-glucanohydrolases (EC 3.2.1.4) and require a minimum of a celloligosyl unit within a polymer in order to effect cleavage. Of the substrates tested, the enzymes degrade cellohexaose most effectively, as do many fungal cellulos (32, 33). This has been interpreted (34) as indicating that such cellulos possess a binding site which accommodates at least 6 glucose units. Cellubiose must also be capable of binding to pea cellulos to a limited extent since it inhibits cellulose activity towards CM-cellulose (Fig. 2), but the disaccharide is not cleaved and evidently cannot span the active site. Reduced cellobextrins are hydrolyzed less rapidly than the parent dextrins (cf. Figs. 4 and b), suggesting that opening of the terminal glucopyranose ring of these substrates interferes with binding. In view of the high rate of hydrolysis of some mixed linkage glucans (Table II), the binding site apparently does not require that all contiguous glucose units be β-1,4-linked. Excessive substitution of 6-hydroxyls in glucan chains (e.g. as in commercial DEAE-cellulose) does not prevent binding but completely blocks hydrolysis by pea cellulos (4). Accordingly, highly branched β-1,4-glucans in cell walls (e.g. xylou glucan) would not be expected to act as good substrates, although fragments may be produced from them by cellulase action (35, 36), presumably at points where no substitution occurs. It is also possible that the cellulos could degrade potential intermediates of cellulose synthesis, e.g. cellobextrin covalently linked to lipid (37, 38) or protein (38, 39).

The pea cellulos activities per unit of protein or per mole of enzyme are at least as active towards cellulose, cellobextrins, and CM-cellulose as the most active fungal cellulos (cf. 38, 40). Pea and fungal cellulos show many similarities in enzymic properties but differ in that thermal instability, pH optimum, and $K_m$ values are all generally higher for the pea enzymes. This may be related to conditions found in living plant cell walls where endogenous cellulos would have to act if it is to have any functional significance.

A requirement for auxin-induced cellulase activity in growth and development in plants is still a tentative proposition. Nevertheless, as effective endohydrolases, both buffer-soluble and buffer-insoluble cellulos possess the basic property required to fulfill the potential functions which have been suggested for them namely to promote "loosening" (2, 10) and to enhance cellulose synthesis by generating primer chain ends (9, 41) within the continuous microfibrillar framework of young expanding cells.

The most striking feature of buffer-soluble and buffer-insoluble cellulos as observed here is that so many of their enzymic properties are identical, implying that they possess similar active sites. Buffer-insoluble cellulase has a higher turnover number than buffer-soluble cellulase (Tables I and II), but the difference is hardly sufficient to explain why auxin treatment should generate two such enzymes in approximately equal amounts (4, 5). Indeed these observations justify reopening the question of whether one cellulase is a precursor of the other. The original reasons for considering this unlikely (4) were based on differences in amino acid composition and the absence of cross-reactivity between the enzymes and antibodies raised to them. However, the apparent multiplicity...
observed in many cellulases is often due to modification of a single enzyme species by culture conditions, complex formation, or proteolysis (42). Close examination of the development of pea cellulase activities in early stages after hormone treatment shows (5) that the level of buffer-soluble cellulase increases first, i.e., within a few hours, and that buffer-insoluble reaches or exceeds it only later, i.e., after 1 day. It may be that the larger cellulase (buffer-insoluble) is a form modified from the smaller during the process of secretion from the cell, an event which must occur if the enzyme is to reach its substrate in the wall. This interpretation is in accord with evidence that mRNA for buffer-soluble cellulase is synthesized de novo by rough endoplasmic reticulum polysomes following hormone treatment, while a distinct messenger for buffer-insoluble cellulase could not be located in these preparations (71, as well as ultrastructural observations (6) that buffer-soluble cellulase is concentrated in endoplasmic reticulum vesicles whereas most buffer-insoluble cellulase is bound firmly to inner surfaces of growing cell walls.

Acknowledgments—We wish to thank our colleagues who kindly provided us with substrates and diagnostic enzyme preparations that are not commercially available. We are particularly grateful for helpful advice and discussion accorded to us by Professors B. A. Stone, A. S. Perlin, and D. P. S. Verma.

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J. Biol. Chem. 1977, 252:1402-1407.

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