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ENZYMIC ACTIVITIES OF ENDO-1,4-β-D-GLUCANASES PURIFIED FROM *TRICHODERMA VIRIDE*

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Summary

Endoglucanases II, III and IV (EC 3.2.1.4) from *Trichoderma viride* are highly active in degrading CM-cellulose or phosphoric acid swollen cellulose, and only slightly active on Avicel. The specific activities of the endoglucanases increase with the length of the cellooligosaccharide substrates.

By rate and product analyses using high pressure liquid chromatography the mode of action of Endoglucanase III was differentiated from that of Endoglucanases II and IV. Endoglucanase III has a low affinity for cellobiose, reacts rapidly with cellotriose, and gradually increases in reactivity with cellooligosaccharides as degree of polymerization increases from four to six. In addition to cleaving internal glycosidic bonds of polymeric substrates, it preferentially cleaves cellobiosyl units from the non-reducing end of oligosaccharides. The cellobiosyl units are often, under initial reaction conditions, transferred to the substrate-acceptor. Endoglucanases II and IV show a preference for internal glycosidic bonds of cellooligosaccharides. The soluble products from the initial action of Endoglucanases II and IV on swollen cellulose are glucose, cellobiose, and cellotriose, which are slowly converted to glucose and some cellobiose.

Introduction

In describing the enzymic degradation of crystalline cellulose, most investigators working with purified exo- and endo-1,4-β-D-glucanases propose that the endo-1,4-β-D-glucanases (EC 3.2.1.4) act randomly on cellulose cleaving internal linkages and providing chain-ends for exo-cellobiohydrolase action [1—5]. Experiments testing this hypothesis are complicated by the insolubility of the substrate and its structural polymorphism which provide different microenvironments for enzyme action and consequently lead to non-linear kinetic patterns. Thus it has been necessary to use CM-cellulose or other soluble derivatives to study the

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mode of attack of 1,4-β-D-glucanases on polymeric substrates. Experiments using CM-cellulose have illustrated differences in modes of action among the 1,4-β-D-glucanases [2,6–8].

Oligosaccharides formed by 1,4-β-D-glucanase action on polymeric and oligosaccharide substrates reflect the action patterns of these enzymes. High pressure liquid chromatography provides a rapid, sensitive and quantitative analysis of such soluble oligosaccharides [9–12]. Many investigators have analyzed the products of cellulolysis only after prolonged incubation of substrate and enzyme, thus limiting mechanistic significance of such studies. Since the products of one enzymic cleavage may be the substrates for further reactions, the apparent kinetics will vary with the chain length of both substrates and products. In addition to these hydrolytic reactions, transglycosylation has been demonstrated for 1,4-β-D-glucanases [8,13].

In this report three endo-1,4-β-D-glucanases purified from *Trichoderma viride* [14] are further differentiated on the basis of their action patterns.

Materials and Methods

Enzymes

Endoglucanases II, III and IV [14] have specific activities of 1010, 60 and 250 units/mg protein, respectively. Units of activity are expressed in terms of the change in specific fluidity $\{\Delta\phi_{sp}$ per min$\}$ of a CM-cellulose solution [14].

Substrates

High viscosity CM-cellulose 7 HP (DS = 0.83) was obtained from Hercules Powder Company, Hopewell, Va. Phosphoric acid swollen (Walseth) cellulose was prepared from Avicel PH 101 [14]. Avicel PH 101 was obtained from FMC Corporation, Newark, Del. Cellobiose was obtained from Eastman Organic Chemicals, Rochester, N.Y.

Cellooligosaccharides (cellotriose to cellohexaose) were prepared according to the method of Miller [15]. Reduced cellooligosaccharides (cellobiitol through cellohexaitol) were prepared from the corresponding purified cellooligosaccharides by the method of Cole and King [16]. The purity of both normal and reduced cellooligosaccharides was found to be >99% by high pressure liquid chromatography [11].

Enzyme assays

The relative activities of the endoglucanases with polymeric substrates were measured by following release of reducing sugars. Enzyme was incubated with 1% (w/v) Avicel, phosphoric acid swollen cellulose, or CM-cellulose in pH 4.5, 0.05 M sodium acetate buffer at 40°C for 20 min. Soluble reducing sugar was measured as glucose equivalents [14].

The release of reducing sugar was compared graphically with the concomitant change in viscosity [6] according to the method described by Nisizawa et al. [2] in order to compare the modes of attack of endoglucanases on CM-cellulose. Enzyme was incubated at 40°C with CM-cellulose and reducing sugar release and viscosity were measured after various intervals [14]. Glucose equivalents released were plotted versus the corresponding change in fluidity. The

slope of the resulting line indicated the relative susceptibility of the internal bonds of CM-cellulose to attack by the enzyme and the relative frequency of multiple enzymic cleavage in a polymeric chain.

In the kinetic studies using cellobiose and cellotriose as substrates, enzymic activity was measured by the production of glucose [17,18].

High pressure liquid chromatography

High pressure liquid chromatography has been adapted to the separation and quantification of oligosaccharides [11]. The instrument used was a Waters Associates Model ALC 202/401 Liquid Chromatograph supplemented with a Model 6000 Solvent Delivery System and Model 660 Solvent Programmer (Waters Associates, Inc., Milford, Mass.). It was equipped with a differential refractometer. A Spectra-Physics Autolab System I Computing Integrator (Spectra-Physics, Santa Clara, Calif.) was used in kinetic studies to compute the relative areas of the chromatographic peaks. Cellooligosaccharides were separated using a Waters μBondapak® carbohydrate column with approx. 3 : 2 (w/v) acetonitrile/water solvent systems [11]. Peak area was found to be proportional to the weight percent concentration of each oligosaccharide.

Specific activities for the endoglucanases were determined with 1% (w/v) cellooligosaccharide substrates at 30°C. At short intervals after initiating reaction, samples were taken from the mixture and analyzed directly by high pressure liquid chromatography. The disappearance of substrate (μmol) was plotted as a function of reaction time. A linear disappearance (zero-order reaction) of substrate was observed in all cases where greater than 75% of the substrate remained permitting calculation of the specific enzyme activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein). The soluble products from endoglucanase action with 1% phosphoric acid swollen cellulose or Avicel were identified by high pressure liquid chromatography.

Normal and reduced cellooligosaccharides [11] were separated using a 45 min-programmed solvent flow increase from 2.0 to 4.5 ml/min with appropriate acetonitrile/water (approx. 7 : 3 to 3 : 1) solvent systems.

The cleavage of specific glycosidic bonds was investigated for each endoglucanase and the exo-cellobiohydrolase by comparing initial hydrolysis products from cellopentaitol or cellotetraitol. For this analysis, 150 μl of each substrate solution was incubated with 1—10 μl of enzyme at 40°C for short intervals in which there was less than 10% degradation. The reaction was stopped by immersion in boiling water for 3 min. The sample was evaporated to dryness under nitrogen, redissolved in 50 μl distilled water, and a 25 μl aliquot was analyzed by high pressure liquid chromatography.

Kinetic studies

For kinetic studies of the endoglucanases, either glucose production or substrate disappearance was measured. The kinetic constants, K_m and V, for cellobiose and cellotriose hydrolysis by endoglucanases were obtained by determining glucose production after enzymic action at 40°C for 30 min using the glucose oxidase assay [17]. Determination of K_m and V values with cellotetraose as substrate was attempted using high pressure liquid chromatography. The decrease in cellotetraose concentration in 150 μl of pH 4.5, 0.1 mM sodium

acetate buffer was measured after incubation with enzyme at 40°C for a period of time during which less than 20% of the substrate was degraded. This method was not sensitive enough to permit quantification of products obtained at low substrate concentrations (<1 mM).

Results

Mode of action

In order to distinguish the modes of action of the purified endoglucanases with the CM-cellulose substrate, reducing sugar production was measured concomitantly with the decrease in viscosity after incubation of substrate and enzyme (Fig. 1). Enzyme concentrations were chosen to provide equivalent units of activity (viscosimetric) on CM-cellulose. The exo-cellobiohydrolase C concentration was arbitrarily chosen to be ten times that used for Endoglucanase II. In addition, since Endoglucanases II and IV produced a much greater change in fluidity, lower enzyme concentrations had to be used for the viscosimetric assay in order to yield reliable drain times. The resulting changes in fluidity values were multiplied by the appropriate dilution factors (20 and 9, respectively) to permit comparison of these values at enzyme concentrations equivalent to those used for the reducing sugar determinations. The slopes (Fig. 1) reflect the types of enzymic action with CM-cellulose; a predominantly endo-action results in a larger positive slope. The exo-cellobiohydrolase C yielded a very small slope (0.05) indicative of its exo-action. Endoglucanases II and IV exhibited nearly identical slopes (0.32 and 0.31, respectively) reflecting similar modes of action on this substrate. A slope intermediate to that of Endoglucanases II and IV and that of exo-cellobiohydrolase C was obtained

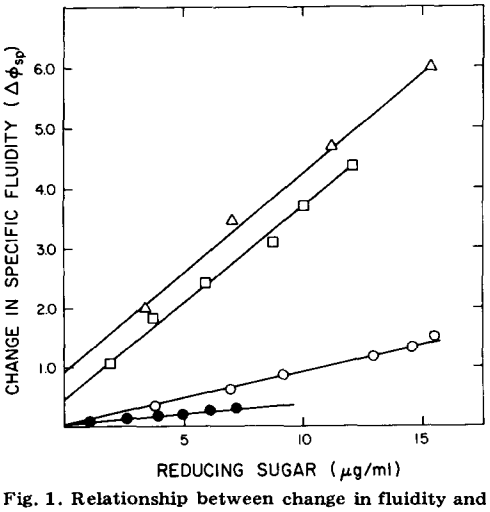

Fig. 1. Relationship between change in fluidity and production of reducing sugar during the hydrolysis of CM-cellulose by endo-1,4-β-D-glucanases. Enzyme samples were incubated with CM-cellulose at 40°C for different periods of time (5, 10, 15, 20, 25 and 30 min) and analyzed for both reducing sugar and fluidity. The values shown for Endoglucanases II (△) III (○) and IV (□) and exo-cellobiohydrolase C (●) were obtained using enzyme concentrations of 0.119 μg/ml, 0.134 μg/ml, 0.19 μg/ml and 1.22 μg/ml, respectively.

with Endoglucanase III (0.10). The latter enzyme has sufficient viscosimetric activity with CM-cellulose to be termed an endoglucanase, but produces more reducing sugar from CM-cellulose than the other endoglucanases.

Polymeric substrates

Activities of the endoglucanases were determined using 1% (w/v) solutions or suspensions of cellulosic substrates (Table I). CM-cellulose was completely soluble, and phosphoric acid swollen cellulose and Avicel were insoluble in pH 4.5, 0.05 M sodium acetate buffer. Among these substrates, Endoglucanases II, III and IV were most reactive with CM-cellulose. Furthermore, these enzymes produced less soluble reducing sugar from Avicel than from the hydrated, accessible substrate, phosphoric acid swollen cellulose.

Endoglucanase II which has enzymic properties very similar to Endoglucanase IV differs significantly in its three-fold higher activity with CM-cellulose and lower activity on Avicel. Endoglucanase II, although having a distinctively "endo" action pattern, releases reducing sugar residues from CM-cellulose and phosphoric acid swollen cellulose as effectively as Endoglucanase III.

To better describe the action of these endoglucanases on polymeric 1,4-β-D-glucans, the products formed by enzyme action on phosphoric acid swollen cellulose (Fig. 2) and Avicel were compared. After limited incubation at 40°C with phosphoric acid swollen cellulose, Endoglucanase II or IV produced glucose/cellobiose/cellotriose in molar ratios of 3 : 4 : 1 or 4 : 5 : 3, respectively (Fig. 2A, C). However, Endoglucanase II or IV, after longer reaction times (48 or 96 h, respectively), altered the product array to give molar glucose/cellobiose ratios of 8 : 1 or 3 : 1, respectively (not shown). Endoglucanase III, on the other hand, formed equimolar amounts of glucose and cellobiose after phosphoric acid swollen cellulose hydrolysis for 60 min (Fig. 2B). As Endoglucanase III reacted slowly with cellobiose, the distribution of products shifted slowly toward glucose resulting in a 2 : 1 molar ratio of glucose to cellobiose after 144 h (not shown). These results demonstrate that the extent of reaction determines the relative product distribution.

The product array formed after incubation of the endoglucanases with Avicel differed from that observed with phosphoric acid swollen cellulose. Longer incubations were necessary with the former substrate in order to obtain

TABLE I

Specific activities of endo-1,4-β-glucanases on polymeric and cellooligosaccharide substrates. Enzyme assays were carried out using 1% (w/v) substrate solutions or suspensions as described in Materials and Methods. Values are given in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein.

Substrate	II	III	IV
Carboxymethylcellulose	29.1	28.2	9.16
Phosphoric acid-swollen cellulose	9.60	9.92	7.40
Avicel	0.060	0.128	0.096
Cellohexaose	22.52	19.0	8.5
Cellopentaoase	18.61	15.8	4.8
Cellotetraose	3.86	14.2	1.89
Cellotriose	0.06	7.34	0.04
Cellobiose	<0.05	<0.05	<0.05

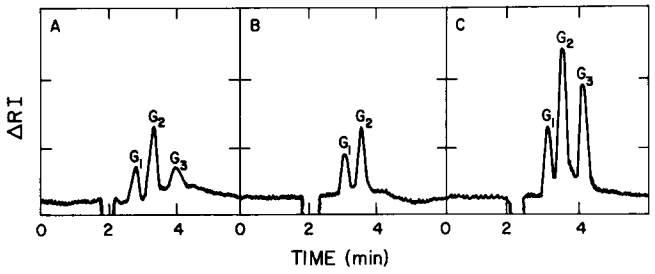

Fig. 2. Separation by high pressure liquid chromatography of soluble products from endo-1,4-β-D-glucanase reaction with phosphoric acid swollen cellulose. The soluble products from the action of Endoglucanases II, III or IV (panels A, B and C, respectively) on 1% (w/v) substrate were identified after incubation at 40°C for 60, 60 and 120 min, respectively. Concentrations of Endoglucanases II, III and IV were 93, 40 and 53 μg/ml, respectively. The mixtures were eluted at 1.0 ml/min using an acetonitrile/water (6 : 4) solvent system. Samples were deionized with Amberlite MB-3 before injection. Carbohydrate peaks detected by refractive index are G_1, glucose; G_2, cellobiose; and G_3, cellotriose.

detectable amounts of soluble carbohydrates. The action of Endoglucanases III or IV on Avicel produced molar ratios of glucose/cellobiose of 1 : 3 or 3 : 1, respectively. In contrast to Endoglucanase IV, Endoglucanase II produced no detectable cellobiose at any point during its reaction with Avicel.

Cellooligosaccharide substrates

Normal and reduced cellooligosaccharides were employed as substrates for the endoglucanases and the course of enzymic reactions was followed by high pressure liquid chromatography (Table I). With Endoglucanases II and IV, the rates tended to increase with substrate chain-length. However, the rates of reaction catalyzed by Endoglucanase III were similar for the oligosaccharides larger than cellotetraose. In addition, Endoglucanase III has a relatively high activity with cellotriose. These specific activities should be interpreted with caution since a single substrate concentration was employed and the reactions were carried out in distilled water instead of at the optimum pH 4.5. Under these conditions none of the endoglucanases demonstrated any activity on 1% cellobiose after a 48 h incubation. Endoglucanase II had a higher specific activity than Endoglucanase IV on each soluble substrate (as with CM-cellulose), and Endoglucanases II and III were nearly equal in their ability to cleave cellohexaose.

The kinetics of glucose production by Endoglucanases II, III or IV from cellobiose or cellotriose were studied using glucose oxidase; enzymic hydrolysis of cellotetraose was measured by high pressure liquid chromatography. The resulting Lineweaver-Burk plots for Endoglucanases II, III and IV permitted calculation of K_m and V values. Kinetic constants could not be obtained for Endoglucanase II action on cellotetraose or for any other endoglucanase on cellopentaose since the apparent K_m values were less than the sensitivity limit of the high pressure liquid chromatography (approx. 1.0 mM). A summary of the kinetic parameters derived by least squares regression analysis of the double reciprocal plots is shown in Table II. The values are consistent with the rates previously observed when 1% (w/v) cellooligosaccharides were used as substrates. The similarity of Endoglucanases II and IV is reflected in the kinetic

TABLE II

Kinetic constants of endo-1,4-β-D-glucanases with cellobiose, cellotriose and cellotetraose substrates. Enzyme reactions with cellobiose or cellotriose were followed by the glucose oxidase method and those with cellotetraose by high pressure liquid chromatography as described in Materials and Methods. Concns. used: for cellobiose (0.25—10 mM); cellotriose (0.25—10 mM); and cellotetraose (1—5 mM). Values for V were expressed as $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein and those for K_m as millimolar. Kinetic constants are shown with corresponding standard deviations.

Substrate	II	III	IV
Cellobiose			
K_m	1.03 ± 0.07	162.0 ± 39	1.26 ± 0.09
V	0.498 ± 0.031	3.65 ± 0.88	0.301 ± 0.021
Cellotriose			
K_m	0.339 ± 0.006	2.65 ± 0.18	0.279 ± 0.17
V	1.22 ± 0.012	24.4 ± 1.79	0.957 ± 0.029
Cellotetraose			
K_m	—	1.33 ± 0.11	2.13 ± 0.12
V	—	66.7 ± 3.1	14.1 ± 0.6

constants obtained when each acted on cellobiose or cellotriose; for each enzyme the K_m decreased and V increased with the larger substrate. Since the K_m value (2.3 mM) obtained for Endoglucanase IV with cellotetraose is near the lower detection limits of the high pressure liquid chromatography method (1.0 mM), its accuracy should be confirmed with a more sensitive experimental method.

Endoglucanase III has a high K_m value, 162 mM, for cellobiose. This value could only be estimated, because cellobiose concentrations greater than 25 mM could not be used in the high pressure liquid chromatography study. Although this enzyme has a low affinity for cellobiose, at high cellobiose concentrations, it has seven to twelve times more activity than Endoglucanase II or IV, respectively. These characteristic properties of Endoglucanase III with cellobiose suggest its identity with the "low affinity" glucanase recently postulated by Sternberg [19].

Endoglucanase III has significantly higher maximum specific activity with each of the substrates tested (Table II). As substrate chain-length increased from two to four, the K_m value decreased and V values increased. Substrate inhibition was observed with each of the endoglucanases at substrate concentrations greater than ten times the K_m values.

The mode of action of each endoglucanase on cellooligosaccharides was investigated using high pressure liquid chromatography. Since Endoglucanases II and IV yield a similar array of products at short reaction times, only results obtained with Endoglucanase IV are presented. This enzyme cleaves cellotetraose at each of the glycosidic bonds yielding a mixture of glucose, cellobiose and cellotriose (Fig. 3). A small amount of a pentasaccharide due to transglycosylation was detected with Endoglucanase IV. When cellopentaose was used as a substrate, Endoglucanase IV cleaved internal bonds to form primarily cellobiose and cellotriose with small amounts of glucose and cellotetraose (Fig. 3B).

The products of Endoglucanase IV reaction with cellohexaose after short and extended incubations are shown in Figs. 4A and 4B, respectively. Under

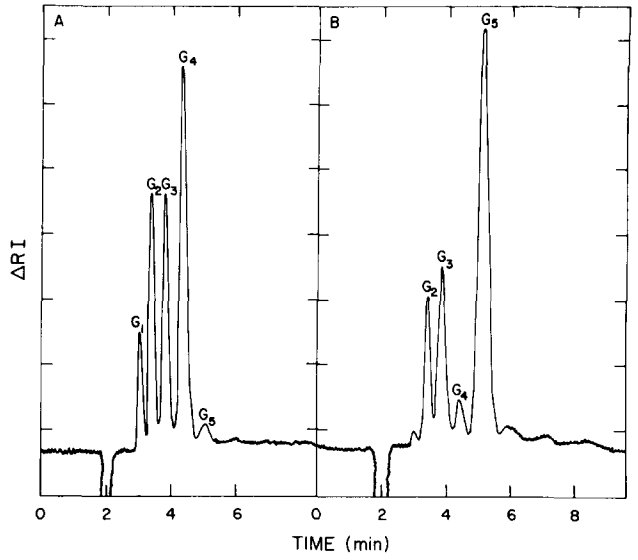

Fig. 3. Separation by high pressure liquid chromatography of products formed by Endoglucanase IV reaction with either cellotetraose (G_4) or cellopentaose (G_5). Cellooligosaccharide products from reaction of Endoglucanase IV with 1% (w/v) G_4 (A) or G_5 (B) were separated on a 30-cm μBondapak carbohydrate column (see Fig. 2). The reaction mixture contained 6.5 μg/ml of enzyme and was incubated at 30°C with G_4 or G_5 for 1444 or 300 min, respectively. At this point 44% of G_4 and 53% of G_5 remained.

initial conditions, Endoglucanase IV formed either cellotriose or cellobiose and cellotetraose (Fig. 4A). Four days of further reaction with the initial products resulted in the formation of glucose, cellobiose and cellotriose (Fig. 4B). The presence of cellobiose and cellotriose in addition to glucose is consistent both

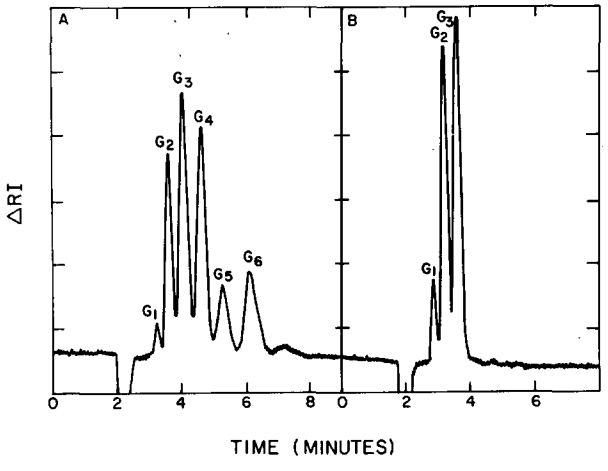

Fig. 4. Separation by high pressure liquid chromatography of products formed by Endoglucanase IV reaction with cellohexaose (G_6) after different periods of incubation. Cellooligosaccharide products from Endoglucanase IV action at 30°C on 1% (w/v) G_6 for 298 (A) or 5774 (B) min were separated as described in Fig. 2. The reaction mixture contained 6.5 μg/ml of enzyme and after 298 min, 16% of G_6 remained.

with the slight reactivity of Endoglucanase IV toward cellobiose and cellotriose and with the product distribution observed when phosphoric acid swollen cellulose serves as the substrate.

The product array formed by Endoglucanase III action on cellotriose, cellotetraose or cellopentaose (Fig. 5A, 5B, 5C) was different from that of Endoglucanase IV (Fig. 4). Endoglucanase III effects significant cleavage or transfer of cellobiosyl units.

To identify the glycosidic bonds cleaved by the endoglucanases, cellopentaitol and cellotetraitol were used as substrates. The rate of reaction with cellopentaitol or cellotetraitol was approximately the same as that with the corresponding normal cellooligosaccharides. The product distribution generated by the action of Endoglucanase II or IV with each substrate was again similar; however, the direct formation of the observed products, cellobiose, cellobiitol, cellotriose and cellotriitol without glucose or glucitol cannot be due to hydrolysis alone. One may speculate that cellotetraitol, for example, is cleaved forming cellobiitol and an enzyme-bound cellobiosyl unit which then is transferred to the substrate-acceptor, cellotetraitol. The resulting hexaitol may be rapidly cleaved forming equimolar amounts of a trisaccharide and cellotriitol. Although attempts to demonstrate the formation of cellohexaitol by Endoglucanase II or IV were unsuccessful, it may be necessary to consider both transfer and hydrolysis activity in order to explain product distributions generated by glucan-hydrolases.

The distinctive character of Endoglucanase III is demonstrated by its mode of reaction with reduced oligosaccharides. From cellopentaitol (Fig. 6B) the production of cellotriitol as the sole product indicated "endo" cleavage. The absence of corresponding amounts of cellobiose suggested the possibility of transferase action as did its endo-action on cellotetraitol (Fig. 6A) which led to

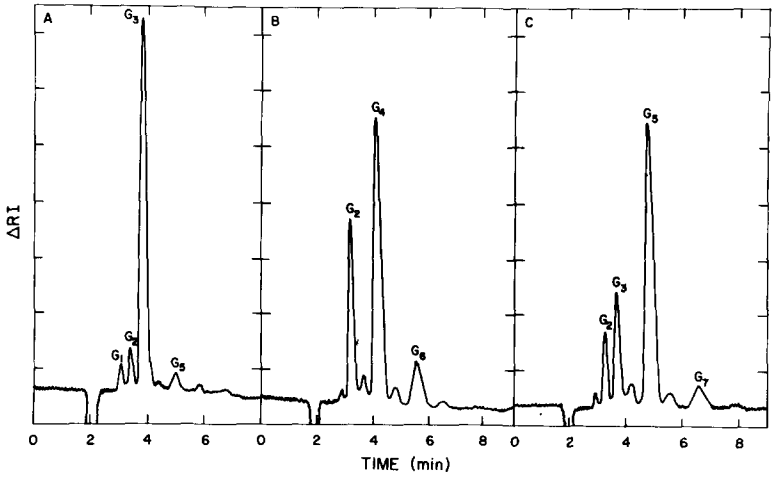

Fig. 5. Separation by high pressure liquid chromatography of products formed by Endoglucanase III reaction with cellotriose (G_3), cellotetraose (G_4) or cellopentaose (G_5). Cellooligosaccharide products from reaction of Endoglucanase III with 1% (w/v) G_3 (A), G_4 (B) or G_5 (C) were separated as described in Fig. 2. The reaction mixtures contained 6.1 μg/ml of enzyme and were incubated at 30°C with G_3, G_4 or G_5 for 246, 246 and 256 min, respectively. At this point 74% of G_3, 45% of G_4 and 50% of G_5 remained.

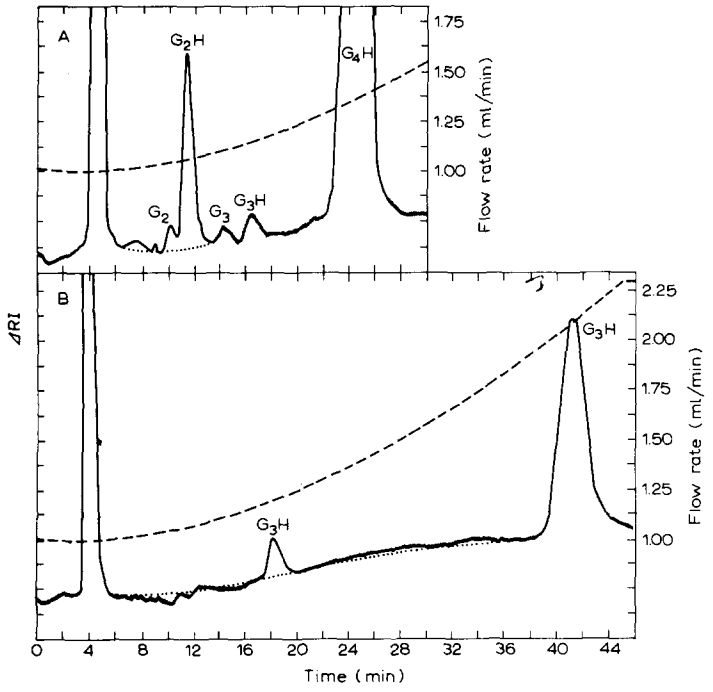

Fig. 6. Separation by high pressure liquid chromatography of products formed by Endoglucanase III reaction with either cellotetraitol (G_4H) or cellopentaitol (G_5H). Normal and reduced cellooligosaccharide products from reaction of Endoglucanase IV with 1% (w/v) G_4H (A) or G_5H (B) were separated using a 45 min flow program (- - - - -) from 1.0 to 4.6 ml/min with acetonitrile/water (3 : 1) solvent system. Minor contaminants in these substrates are indicated by the small dashed lines (· · · · · ·) which trace the relevant segment of the chromatographic pattern obtained with unreacted substrates. The reaction mixtures contained 2.0 μg/ml of enzyme and were incubated at 40°C for 30 min. Approx. 91 and 85% of G_4H and G_5H, respectively, remained after this incubation. Carbohydrate peaks are denoted as G_2, cellobiose; G_2H, cellobiitol, etc.

the production primarily of cellobiitol without an equivalent amount of cellobiose. These results are consistent with the transfer of an enzyme-bound cellobiosyl unit to cellotetraitol forming a hexaitol. When the reaction mixture of Fig. 6A was analyzed by the standard isocratic high pressure liquid chromatographic method, the existence of the postulated hexaitol was confirmed. Similar attempts to demonstrate formation of a heptaitol from cellopentaitol were unsuccessful, probably due to a lack of sensitivity for cellooligosaccharides beyond cellohexaose.

All three endoglucanases showed a preference for the second glycosidic bond (numbered from the non-reducing end) in cellotetraitol and either the second (Endoglucanase III) or the third (Endoglucanase II or IV) bond in cellopentaitol. These preferences may be compared with that of the exo-cellobiohydrolase C [2], which cleaves principally the second bond of either cellotetraitol or cellopentaitol. Although the exo-cellobiohydrolase C is clearly different from the Endoglucanases II and IV with respect to mode of action on oligosaccharides, it is similar to Endoglucanase III in this respect. However, Endoglucanase III is quite easily distingusihed from exo-cellobiohydrolase C by the high activity of the former on cellooligosaccharides and CM-cellulose.

Discussion

Specificities, rates and modes of action of the endoglucanases with polysaccharide and cellooligosaccharide substrates have served to further differentiate Endoglucanases II, III and IV from one another and from other enzymes of the cellulase system. The crystallinity of native cellulose, the substituents on soluble, derivatized cellulosic substrates and the many alternate paths newly formed products may follow all complicated interpretation of the observed activities. For example, a potential product may be transferred to an acceptor-substrate, remain enzyme-bound, be associated with an insoluble substrate, dissociate from the enzyme and remain insoluble, or be released from the enzyme as a soluble product. Only in the latter case, are the products conveniently detected. Hydrolytic products from one enzymic cleavage often serve as substrates for further reactions. Thus, products of the initial reaction may be quite different from those detected after longer periods of time.

Graphical comparison of viscosity decrease and reducing sugar production during enzyme action on CM-cellulose provides a qualitative description of the modes of enzyme attack (Fig. 1). Nisizawa et al. [2] and Okada [7,8] differentiated the multiple endo-1,4-β-D-glucanases of *Irpex lacteus* or *T. viride*, respectively, by the slopes obtained from such plots. Kanda et al. [21] interpreted the magnitude of the slopes as a measure of the "degree of randomness" of endoglucanases by assuming that the steeper the line the more random the enzymic attack. Large positive slopes reflect the ability of an enzyme to cleave internal bonds in each of a number of cellulose molecules resulting in a large decrease in viscosity (and number average molecular weight). This multi-chain attack would be more likely to occur with a single (or few) bond cleavage(s) per enzyme-substrate encounter [22]. Activity of enzymes (like the exo-cellobiohydrolase C) results in more nearly horizontal lines and represents single chain attack with few to several bonds cleaved per encounter.

The similarity of the slopes obtained for Endoglucanases II and IV indicates a similar multi-chain attack on CM-cellulose. However, at a given enzyme concentration, Endoglucanase II is much more effective both in decreasing viscosity and releasing reducing sugar. This is consistent with its low affinity for cellulose which presumably permits it to diffuse from the site of initial cleavage and to react subsequently with other cellulose chains.

Endoglucanase III yielded a lower slope value, similar to that of exo-cellobiohydrolase C. Thus Endoglucanase III may act on internal linkages of CM-cellulose molecules with subsequent multiple cleavage of the same chain. In this "single chain" attack the enzyme binds near the initial scissile bond and cleaves repeatedly while remaining in a productive complex. A multi-chain mechanism like that proposed for the α-amylases [22] is consistent with the results obtained with Endoglucanases II and IV.

High pressure liquid chromatography was used to determine the rates of reaction and products formed initially by the endoglucanases. Products of Endoglucanase III action on cellooligosaccharides indicate predominant cleavage of cellobiosyl units from the non-reducing end. Frequently, under initial conditions, the resulting cellobiosyl units are transferred to the substrate-acceptor. The relatively high activity with cellotriose and low affinity for cello-

biose of Endoglucanase III is consistent with its characteristic product distribution from polymeric substrates (Fig. 2B). Endoglucanases II and IV are similar with respect to enzymic activities with cellooligosaccharides; this is consistent with a common mode of action on CM-cellulose. The distinctive products, glucose, cellobiose and cellotriose, were formed initially from normal and reduced cellooligosaccharides and phosphoric acid swollen cellulose but, after extensive hydrolysis, the cellotriose was cleaved to cellobiose and glucose. These enzymes demonstrated similar linkage specificity in cleaving cellotetraitol and cellopentiatol.

Endoglucanases II and IV, although similar in many respects, were very different in their activities on crystalline cellulose. The inability of Endoglucanase II to bind to Avicel or react with it may be related to its relatively low carbohydrate content and molecular weight. Conversion of Endoglucanase IV by proteolysis to Endoglucanase II could account for differences in their physical and chemical properties. Nakayama et al. [23] reported that partial proteolysis of an endo-1,4-β-D-glucanase from *T. viride* yielded products with modified structure and substrate specificity. They concluded that limited proteolysis of cellulase components in culture filtrates may be responsible, in part, for the observed multiplicity and the resulting variation in carbohydrate and molecular weight among multiple enzyme forms.

Comparison of Endoglucanases II, III and IV from *T. viride* with those purified by Okada [7,8] on the basis of their respective modes of action on CM-cellulose suggests correspondence between cellulase II-A and Endoglucanase II or IV and between cellulase III and Endoglucanase III. Cellulase II-B is not similar to any of the endoglucanases reported in this paper. Okada and Nisizawa [13] and Okada [8] also have reported kinetic analyses of purified endoglucanases using cellooligosaccharides as substrates. Since kinetic constants were obtained using reducing sugar analyses, a method which does not differentiate between single and multiple reactions, this type of analysis is not sufficient to establish specific values for K_m or V. However, the relationships among the enzymes reflected by the kinetic constants obtained in this manner are consistent with those stated above. Thus, for example, Endoglucanase III like cellulase III displays generally similar K_m and V values with cellooligosaccharide substrates, particularly with cellotriose. High concentrations of cellobiose (0.1 M) as substrate were used to study the transglycosylation activity of cellulases II-A, II-B and III [8,13]. Qualitatively by paper chromatography the formation of cellotriose by cellulase II-A, and of cellotriose and cellotetraose with cellulase II-B and of cellotetraose by cellulase III was observed. The high transferase activity of cellulase III was confirmed in our laboratory with Endoglucanase III. However, since no structural characterization of cellulases II-A, II-B, and III has been reported, it is impossible to confirm conclusively the identities of these enzymes.

Wood [1] has recently purified four endoglucanases from *Trichoderma koningii* which are active in the solubilization of cotton and another low molecular weight endoglucanase which does not participate in this process. In agreement with Wood's results we find [14] two endoglucanases (Endoglucanases II and IV) present in relatively high proportions (67 and 15% of the total endoglucanase activity, respectively) and two other components (Endoglucanases I and

III) which together account for less than 18% of the total endoglucanase activity measured viscosimetrically. However, Endoglucanase III has several properties different from either Endoglucanase II or IV and may have a distinct role in cellulose degradation. Although Endoglucanase III has a mode of hydrolytic action different from that of either Endoglucanase II or IV, it is quite similar to Endoglucanase IV in its affinity for Avicel.

The capacity of the individual endoglucanases to form short fibers from filter paper indicated that affinity for the substrate may be the key to the efficacy of these enzymes. Following the procedure of Berghem et al. [24], we have observed that among the purified enzymes only Endoglucanases III and IV were able to cause disintegration of the filter paper. However, when exo-cellobiohydrolase C (which had a very high affinity for cellulose) was added to Endoglucanase II, the combined action of these enzymes was able to degrade the filter paper substantially with concomitant short fiber formation.

Because a number of forms are found for both exo-cellobiohydrolases [25] and the endoglucanases, detailed structural evaluation of these glycoproteins at different stages of enzyme production is desired. With this information we may elucidate not only the origins of the forms, but the probable genetic basis of their production and the extent to which they are individually necessary or important in cellulose degradation.

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