CHARACTERIZATION OF STRUCTURE AND ENZYMIC ACTIVITY OF ENDO-1,4-β-D-GLUCANASES PURIFIED FROM TRICHODERMA VIRIDE

by

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LIST OF ABBREVIATIONS

Abbreviation	
C_1	- Exo-cellobiohydrolase; used by some authors to denote a protein required for the degradation of crystalline cellulose.
C_x	- Any of a number of enzymes which hydrolyze amorphous cellulose or derivatized cellulosic substrates.
CAPS	- Cyclohexylaminopropane sulfonic acid.
DS	- Degree of Substitution: Average number of hydroxyl groups derivatized per glucosyl residue in carboxymethylcellulose.
$E_{280}^{1\%}$	- Extinction coefficient at 280 nm of a 10 mg/ml solution.
$G_1, G_2 \ldots G_6$	- Glucose, cellobiose...cellohexaose.
$G_1H, G_2H \ldots G_5H$	- Glucitol, cellobiitol...cellopentaitol.
HEPES	- N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid.
HPLC	- High pressure liquid chromatography.
NP	- p-Nitrophenol.
NPG	- p-Nitrophenyl glucoside.
NPC	- p-Nitrophenyl cellobioside.
PSA	- Phenol sulfuric acid.
PSC	- Phosphoric acid-swollen cellulose.
SDS	- Sodium dodecyl sulfate.

INTRODUCTION

Theoretically, cellulose, our largest renewable resource, is capable of being used as a raw material for food and energy. Agricultural, industrial and municipal cellulosic waste materials could be utilized because of their availability and low cost. Such processes then would improve the environment by recycling this material. Until recently, this has not been economically feasible, but with the rising cost of food and energy, the enzymic conversion of cellulose to glucose has been receiving much attention.

Laboratories at Natick, Massachusetts and the University of California at Berkeley are attempting enzymic saccharification of waste cellulose on an industrial scale. In Japan and Sweden, studies on the enzymic saccharification of lignin-containing cellulosic waste are being actively pursued. Moreover, a group at Louisana State University is converting cellulose to single cell protein using microbial fermentation. In the food industry, cellulases are being used as cell wall disintegrators to decrease fiber content and increase digestibility of foodstuffs.

In order to use the cellulase system to its full extent in any of these health-related projects, the individual enzyme properties and overall enzyme action must be understood. The cellulase system of *Trichoderma viride* offers an ideal system for study, since this organism produces one of the most potent and stable cellulase systems. If the full utilization of the capabilities of this organism is to be realized then better understanding of the enzymes involved in the breakdown of cellulose is essential. Although some of the enzymes synthesized by *Tricho-*

derma viride have been purified and characterized, little is known about the endo-1,4-β-D-glucanases of its cellulase system. Therefore, a study of the structural characteristics and enzymic properties of purified endo-1,4-β-D-glucanases from *Trichoderma viride* has been undertaken.

1,4-β-D-GLUCANASE

LITERATURE REVIEW

Cellulose makes up more than half of the total organic carbon in the biosphere with an annual net yield from photosynthesis estimated at 10^{11} tons. This 1,4-β-D-glucan is arranged in parallel chains with both inter- and intra-chain hydrogen bonds forming a highly resistant and most insoluble material (1). It functions as the major structural component in the cell walls of plants (2). Polysaccharidases that cleave cellulose are termed 1,4-β-D-glucanases. They may be classified as endoglucanases if enzyme action is directed toward internal bonds or exoglucanases if exposed chain ends are the principal site of attack. Exoglucanases may act to remove either one monosaccharide (glucose) or one disaccharide (cellobiose) unit at a time from the non-reducing end of cellulose chains.

Cellulase

Cellulases are synthesized by a variety of higher plants, invertebrates, bacteria and fungi. The enzymes provide invertebrates, bacteria and fungi with a soluble carbon source (oligosaccharides and glucose) or modify cell wall structures in growing plants (3,4). True cellulolytic organisms produce all the enzymes needed to degrade crystalline cellulose to glucose (4,5). Representative of this cellulolytic group are *Trichoderma viride*, *Trichoderma koningii*, *Sporotrichum pulverulentum*, *Fusarium solani*, and *Irpex lacteus* (4,6).

The Cellulase System - Originally thought to be one enzyme (7), cellulases of true cellulolytic organisms are now well established to be multicomponent enzyme systems (4,8-16). The 1,4-β-D-glucanases act cooperatively to degrade native cellulose to cellooligosaccharides and glucose. These enzyme components include the 1,4-β-D-glucan 4-glucanohydrolases (E.C. 3.2.1.4) and the 1,4-β-D-glucan cellobiohydrolases (E.C. 3.2.1.91) (17). The 1,4-β-D-glucan 4-glucanohydrolase denotes an endoglucanase which attacks modified soluble cellulose in a random manner; the terminal linkages being less susceptible than the internal linkages (4,6,16,18,19). Several investigators (5,6,19,22) have shown that endoglucanases actively degrade phosphoric acid swollen cellulose (PSC) and carboxymethylcellulose (CM-cellulose) but have little hydrolytic activity on highly oriented forms of cellulose (*e.g.* cotton, Avicel). The 1,4-β-D-glucan cellobiohydrolase is an exoglucanase which cleaves cellobiosyl units from the non-reducing end of cellulose chains (5,16,23-25), and has been found to act on highly oriented forms of cellulose, to degrade PSC to cellobiose, and to have very little activity on CM-cellulose (6,25-27). A detailed discussion of cellulosic substrates used in assessing 1,4-β-D-glucanase activity has been given by Emert (28).

The products of 1,4-β-D-glucanase action are the substrates for the final enzyme of this multicomponent system, the β-glucosidase (E.C. 3.2.1.21). This enzyme exhibits cellobiase activity, splitting cellobiose into two molecules of glucose, and in addition, is active on cellooligosaccharides cleaving glucose units from the non-reducing end (4,28,29). The enzyme has little activity on the polymeric substrates

CM-cellulose, PSC, or Avicel (5,29). Reese *et al.* (30) differentiated glucosidases from exoglucanases on the basis of oligosaccharide chain-length specificity, inhibition by D-gluconic acid δ-lactone, and the anomeric configuration of the sugar released. Exoglucanases have been found to proceed with inversion of anomeric configuration, whereas glucosidases do so with retention. In addition, Barras (31) has reported that endoglucanases act with retention of configuration.

Glucose present after saccharification of crystalline cellulose by the cellulase system has been attributed to the action of β-glucosidase (5,32). Sternberg (32) has postulated that *Trichoderma viride* produces an enzyme other than β-glucosidase that acts on cellobiose. The enzyme was shown to have a low affinity for cellobiose (K_m=50mM) and hence, its activity was seen only with high concentrations of cellobiose. No further attempts at identification or purification of this enzyme activity have been reported.

There has been some evidence supporting an exo-1,4-β-D-glucanase which acts by removing successive glucosyl units from the non-reducing end of cellulose chains in a manner analogous to glucoamylase action on 1,4-α-D-glucans (33,34). However, firm evidence for such an enzyme has not been established and it is believed that the "exoglucanase" served only to convert cellooligosaccharides to glucose (4) and is probably a β-glucosidase.

Cellulase Nomenclature - A proliferation of trivial nomenclature for the 1,4-β-D-glucanases has occurred as investigators have attempted to classify these enzymes on the basis of their activity on specific

substrates or on the basis of their individual "roles" using hypothetical models (Table I). Thus Nisizawa *et al.* (6) and Kanda *et al.* (20,35) named their "purified" enzymes on the basis of the substrates which they hydrolyzed. One enzyme preparation was characterized by its ability to hydrolyze Avicel (microcrystalline cellulose) and it was termed an Avicelase, whereas another enzyme preparation was characterized by its activity on CM-cellulose and termed a CM-cellulase. Reese *et al.* (8) named the 1,4-β-D-glucanases involved in the degradation of crystalline cellulose according to a hypothetical model based on a sequential mode of action. According to this model C_1-enzyme acted on highly oriented native cellulose altering its structure to permit C_x enzyme(s) acting randomly to convert the hydrated polyanhydroglucose chains to cellobiose and glucose. Siu (36) and later Selby (37) proposed that the role of C_1 was that of a "hydrogen bondase" disrupting hydrogen bonds between parallel cellulose chains.

Leatherwood (38) assigned names to these enzymes according to an alternative model where the conversion of cellulose to cellobiose in *Ruminococcus* involved a hydrolytic factor and an affinity factor. The hydrolytic factor had many of the properties of an endoglucanase and the affinity factor was similar to the exo-cellobiohydrolase. Accor-ding to this theory, in order to degrade crystalline cellulose the affinity factor must bind the hydrolytic factor to cellulose and permit multiple attacks. This mode of action involving an enzyme-substrate complex is not new and has been suggested by Bailey and French (39) for the β-amylases.

TABLE I

Trivial and Systematic Nomenclature for the 1,4-β-D-Glucanases of the Cellulase System

1,4-β-D-Glucan 4-Glucanohydrolase (E.C. 3.2.1.4)		1,4-β-D-Glucan Cellobiohydrolase (E.C. 3.2.1.91)	
Endo-1,4-β-D-glucanase	(22,47)	Exo-cellobiohydrolase	(17)
CMCase	(6)	Avicelase	(6,27,35)
C_x	(8,9)	C_1	(8,9,17)
Hydrolytic factor	(38)	Affinity factor	(38)
		Hydrogen bondase	(36,37)

Measurement of 1,4-β-D-Glucanase Activity - Assays which measure the depolymerization of polysaccharide substrates by 1,4-β-D-glucanases may reflect the extent of reaction by the increase in reducing sugar residues or by the decrease in viscosity. For endoglucanases the cumbersome viscosimetric procedure is highly sensitive to changes in molecular weight effected by initial enzymic attack, whereas the reducing sugar assay is sensitive to products from repetitive or multiple enzymic attack (40). Spectroscopic measurements of soluble reducing sugars have been described by Nelson (41) and Somogyi (42) using an alkaline copper reagent and by Miller (43) using a dinitrosalicylic acid reagent. Although measurement of the release of reducing sugars by these techniques has been used commonly to follow the action of polysaccharidases (31), these methods alone give no indication of the chain-lengths of the products.

The most widely used method for determining the degree of polymerization of the products of β-glucanase action has been viscosimetric measurements of CM-cellulose solutions (4,31). It is the preferred assay for the rapid measurement of the depolymerization effected by endo-1,4-β-D-glucanases. However, an inherent limitation to the method is the requirement for a soluble and thus derivatized cellulosic substrate. The substituent groups on CM-cellulose which render it soluble have been shown to affect the rate of hydrolysis; the greater the degree of substitution (DS) the less susceptible CM-cellulose becomes to enzymic hydrolysis (44). Furthermore, Almin and Eriksson (45) have found that the DS of CM-cellulose must be in the range $0.8<DS<1.0$ for accurate and repro-

ducible results. Viscosity data can also be used to calculate the activity of β-glucanases in absolute terms, as recently described (45-47).

Purification of 1,4-β-D-Glucanases

Since many of the 1,4-β-D-glucanase preparations reported in the literature have had contaminating activities associated with them, results obtained with these materials have been difficult to interpret. Only recently with the advent of advanced chromatographic and electrophoretic techniques have such glucanases been purified to homogeneity (2,16). Since impure preparations can lead to incorrect or ambiguous results, the following discussion will be limited to those 1,4-β-D-glucanases for which adequate evidence of homogeneity has been presented.

Exo-cellobiohydrolase - Chromatography on several forms of ion-exchange Sephadex, which is one of the most widely used methods for purifying cellulolytic enzymes, has been applied at least once in each of the purification procedures used to obtain pure exo-cellobiohydrolases from *Trichoderma viride* (24,27,48-51) and *Trichoderma koningii* (5,26). Although the exo-1,4-β-D-glucanase of *Sporotrichum pulverulentum* (25) is reported to lack carbohydrate and to produce both glucose and cellobiose from crystalline cellulose, it may be similiar in its function to these cellobiohydrolases. When ion-exchange chromatography was used after initial separation of the cellulolytic enzymes by gel filtration (5,26,48) or after affinity chromatography on crystalline cellulose (Avicel) (12, 24, 49,51), excellent purification was achieved. Final purification of the exo-cellobiohydrolase using isoelectric focusing (5,26), preparative

gel electrophoresis (49,51) or affinity chromatography (25) yielded a homogeneous enzyme preparation.

In addition, multiple forms of the exo-cellobiohydrolase have been demonstrated from *Trichoderma koningii* (26) and *Trichoderma viride* (49). Wood and McCrae (26) separated a minor (pI=3.8) and a major (pI=3.95) isozyme using narrow-pH-range electrofocusing. Gum (51) separated one major form (C) from two minor forms (A,B) using a cellulose (Avicel) adsorption column and subsequently purified them using preparative gel electrophoresis. Gum (51) later demonstrated a fourth form (D) of the exo-cellobiohydrolase from submerged cultures of another strain of *Trichoderma viride*.

In all cases, adequate criteria for homogeneity were established using analytical polyacrylamide electrophoresis and ultracentrifugation (24,25,27,48-51) or narrow-pH-range electrofocusing (5,26). The exo-cellobiohydrolases from these sources displayed some activity on Avicel, but very little activity on CM-cellulose as measured by viscosity or on cellobiose using the glucose oxidase assay (52). Cellobiose was essentially the sole product found from enzyme hydrolysis of PSC (26, 27,48,51).

Endo-1,4-β-D-glucanases - Several investigators have purified endo-1,4-β-D-glucanases to varying degrees of homogeneity from a variety of cellulolytic organisms including *Trichoderma viride* (19,21,53), *Trichoderma koningii* (5), *Fusarium solani* (54), *Sporotrichum pulverulentum* (55), and *Irpex lacteus* (6,35). The endoglucanases were purified according to their viscosimetric and saccharifying activities

using CM-cellulose as substrate. The activities varied among these enzymes, but all were significantly higher than that of the exoglucanases. In all cases, multiple endo-1,4-β-D-glucanases were demonstrated from culture filtrates. However, adequate evidence of purity has been established only for the endoglucanases from *Trichoderma viride* (19,21), *Sporotrichum pulverulentum* (55) and *Irpex lacteus* (6,35). In the purification of the endoglucanases from these organisms, a combination of gel filtration and ion-exchange chromatography gave the most successful results (6,19,35,55). Eriksson and Pettersson (55) purified five endo-1,4-β-D-glucanases from *Sporotrichum pulverulentum* using primarily these two techniques. However, in their purification of the one carbohydrate-free endoglucanase, an affinity column of Concanavalin A-Sepharose was used along with ion-exchange and gel filtration.

Berghem *et al.* (21) employed additional techniques in the separation and purification of two endo-1,4-β-D-glucanases from *Trichoderma viride*. After initial separation of a low-molecular-weight component by gel filtration, this component was further fractionated by chromatography on a dipolar adsorbent (arginine-Sepharose 6B) and purified by isoelectric focusing. The high-molecular-weight component from the initial gel filtration step was passed through two consecutive ion-exchange columns and one Avicel adsorption column, for which it showed low affinity. It was subsequently purified by repeated isoelectric focusing.

Several criteria were used to demonstrate the purity of the low- and high-molecular-weight endo-1,4-β-D-glucanases. Free zone electrophoresis of the low-molecular-weight enzyme and sedimentation equili-

brium analysis of the high-molecular-weight enzyme indicated each to be homogeneous proteins. Unlike sedimentation equilibrium analysis, free zone electrophoresis has been found to be a rather insensitive test for homogeneity (56,57), and therefore the purity of the low-molecular-weight protein has not been firmly established. The demonstration of a single band using polyacrylamide electrophoresis which is one of the best criteria for homogeneity (2,57) was shown for the other endoglucanases. In most cases, a second physical test using sedimentation analysis (6,35) or isoelectric focusing (55) was employed to confirm purity.

Characterization of 1,4-β-D-Glucanases

The purified exo- and endo-1,4-β-D-glucanases have been partially characterized with respect to their chemical, physico-chemical and enzymic properties.

General Properties - Initial characterization of an enzyme usually includes a study of the factors affecting its activity. These factors may include pH optimum and stability, temperature optimum and stability, and inhibition. Berghem *et al.* (58) investigated the effect of pH and temperature on the activity of the exo-cellobiohydrolase purified from *Trichoderma viride*, by following the enzymic release of reducing sugar residues from Avicel. They found that the enzyme had an optimum pH of 4.8 and was sensitive to temperatures greater than 40°. Other investigators have used a range of pH (4.5-5.0) and temperature ($30-40^{o}$) when assaying for exo-cellobiohydrolase activity (25-27,50).

Okada (19) has investigated the effect of pH, temperature and inhibitors on the saccharification of CM-cellulose by two endo-1,4-β-D-

glucanases purified from *Trichoderma viride*. These endoglucanases were found to be stable between pH 5 and 7 (at 40°) with an optimum pH for catalytic activity of 4.5-5.0 and to have a temperature optimum of 50-60°. In addition, the endo-1,4-β-D-glucanases, unlike the exo-cellobiohydrolases, were not thermolabile and possessed 27-41% of their original CM-cellulose-saccharifying activity after ten minutes incubation at 100°. Other investigators (20-22,35,55) have measured endoglucanase activity between pH 4 and 5 and temperatures of 30-40°.

Okada (19) studied the effect of metal ions and organic reagents on endo-1,4-β-D-glucanase activity. He found that each endoglucanase was inactivated completely by one millimolar mercuric ions and to a lesser extent by other metal ions, but not by EDTA or "sulfhydryl reagents." This lack of inhibition by the organic reagents suggested that metals and sulfhydryl groups do not play an essential role in the enzyme-substrate reaction (19). Moreover, for no 1,4-β-D-glucanase has there been any report of a cofactor requirement like that for calcium ions to stabilize α-amylase digests (40).

Structural Characterization - Although there have been few studies concerning the structure of purified endo-1,4-β-D-glucanases (19,21,35), the exo-cellobiohydrolase C (25,50) has been more extensively investigated. Both the exo- and endoglucanases have exhibited many similar structural features. Gum (51) found that antiserum prepared against exo-cellobiohydrolase C reacted immunologically but gave non-identity spurs with the other forms of the exo-cellobiohydrolase (A,B,D) and an endo-1,4-β-D-glucanase. In addition, amino acid analyses obtained for the 1,4-β-D-

glucanases indicate they contain high levels of acidic and hydroxylated amino acids and glycine, but are low in basic amino acids (12,23-25,35, 50,51,58).

The 1,4-β-glucanases from *Trichoderma viride, Trichoderma koningii* and *Irpex lacteus* are glycoproteins and contain 9-33% carbohydrate (13, 20,22,27,35,49,50,58). Multiple forms of the exo- and endoglucanases from these sources contain different amounts and types of monosaccharide constituents (19,21,26,50). Gum (51) found that the carbohydrate content of exo-cellobiohydrolase forms increased in the order $A<B\sim D<C$ and that, whereas all forms contained mannose and glucose, only form C contained galactose. He proposed that these differences in carbohydrate content may be responsible for the non-identity immunoprecipitation spurs. Other investigators have found that their purified exo-cellobiohydrolase contains mannose, glucose and galactose (27,58). Unlike the more complete structural analyses of the exo-cellobiohydrolases, only differences in total carbohydrate content among multiple endo-1,4-β-D-glucanases has been reported (19,21).

The molecular weights of the 1,4-β-D-glucanases from *Trichoderma viride* and *Irpex lacteus* have been determined using either sedimentation equilibrium analysis (12,21,24,49,50) or gel filtration (19,21,27,35). Molecular weights ranging from 42,000 to 52,000 have been reported for the exo-cellobiohydrolase from *Trichoderma viride* using the more accurate method of sedimentation equilibrium where the partial specific volume was either assumed (13,22,25) or calculated from the composition of the enzyme (58). In addition, Gum[1] has found by thin-layer gel filtration

[1]Gum, E. K.,Jr. (1976) Personal Communication.

of exo-cellobiohydrolases in six molar guanidine hydrochloride that the forms have similar apparent molecular weights of 53,000 ± 5,000. The individual endo-1,4-β-D-glucanases have exhibited different molecular weights. Berghem *et al.* (21) reported the isolation from *Trichoderma viride* of an enzyme of low-molecular-weight (12,500) as measured by gel filtration and an enzyme of high-molecular-weight (50,000) as determined by sedimentation equilibrium. Okada (19) reported that two endoglucanases from *Trichoderma viride* had molecular weights of 30,000 and 43,000 as determined by gel filtration. The endoglucanases from *Irpex lacteus* also have dissimilar molecular weights of 35,600 and 56,000 (35).

The five endo- and one exo-1,4-β-D-glucanases purified from *Sporotrichum pulverulentum* have slightly different structural features (25, 55). Like the enzymes previously discussed, these glucanases are high in acidic and hydroxylated amino acids and glycine and low in basic amino acids. The molecular weights as determined by sedimentation equilibrium were found to be higher for the exoglucanase (48,600) than the endoglucanases (28,300-37,500). Again the multiple endoglucanases exhibited differing molecular weights. One structural distinction between the *Sporotrichum* glucanases and the glucanases from other sources rests with the carbohydrate. The five endoglucanases contained varying amounts of carbohydrate (0-10.5%) and the exoglucanase contained no carbohydrate. The low level or absence of carbohydrate in these enzymes distinguishes them from the other 1,4-β-D-glucanases reported in the literature.

The multiplicity of the 1,4-β-D-glucanases, particularly among the endo-1,4-β-D-glucanases, has led some investigators (55,59) to explore the possibility of proteolytic modification in culture filtrates. Par-

tial proteolysis of an endo-1,4-β-D-glucanase component from *Trichoderma viride* and subsequent studies of substrate specificities of the isolated fractions has been performed by Nakayama *et al.* (59). Such modifications resulted in some changes in the saccharifying and viscosimetric activities of the endocellulase components on CM-cellulose and in one case caused a loss in immunological response toward antiserum prepared against the original component. Analysis of some of the low-molecular-weight products of proteolysis demonstrated the release of glycopeptides during proteolysis. These investigators 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 enzymes.

Specificity - The activities of 1,4-β-D-glucanases studied thus far generally have shown narrow linkage and monomer type specificity (31). Recent reports have indicated broader specificity with purified endo-1,4-β-D-glucanases from *Myxobacter* (60), *Trichoderma viride* (27) and *Irpex lacteus* (61). These glycoenzymes have demonstrated either chitosanase (60) or xylanase (27,61) activity in addition to endo-1,4-β-D-glucanase activity. The endo-1,4-β-D-glucanases have significant activity on CM-cellulose (6,19,21,35,55), whereas the exo-cellobiohydrolases have essentially no activity on this same substrate (24,27,48). This finding has led Tomita *et al.* (27) to suggest that there is a specificity requirement at the sixth carbon atom of the pyranose ring for the exo-cellobiohydrolase since CM-cellulose is substituted most frequently at this position. Moreover, Tomita has reported that endo-1,4-β-D-glucanases from *Tricho-*

derma viride and *Irpex lacteus* hydrolyzed xylan, whereas the exo-cellobiohydrolase from these organisms showed no activity toward this polysaccharide (27).

The exo-cellobiohydrolase has been identified by some investigators (6,27) by its low but significant activity on Avicel. Wood and McCrae (26) have noted only a 15% degradation after incubating the exo-cellobiohydrolase with Avicel for 28 days. This low extent of degradation by the exo-cellobiohydrolase and the lack of any significant endoglucanase activity with this substrate is due to the inaccessibility of the cellulose chains to enzyme action (26), rather than specificity requirements. The exo-cellobiohydrolase and the endo-1,4-β-D-glucanase have high activity and react to a large extent on PSC where the highly crystalline structure has been loosened by treatment with acid (21,23, 25,49). Studies by Wood and McCrae (26) on the linkage type have demonstrated a narrow specificity of the exo-cellobiohydrolase for 1,4-β-D-linkages. Similar investigations with the endo-1,4-β-D-glucanases from cellulolytic organisms have not been reported.

Studies using p-nitrophenyl-β-D-cellobioside have indicated that there exists different spedificities among the endo-1,4-β-D-glucanases (6,62). Okada and Nisizawa (62) have found that one endoglucanase from *Trichoderma viride* (II-A) cleaved the holosidic bond giving glucose as a product, whereas the other (II-B) cleaved the glycosidic bond yielding cellobiose. Nisizawa *et al.* (6) found that each of the two endoglucanases from *Irpex lacteus* also showed different specificities toward the two possible cleavage sites of this substrate.

Action Patterns - The term action pattern refers to the chemical and structural relationships between substrates, intermediates and products in an enzyme-catalyzed reaction. It involves the relative susceptibilities of different bonds to enzymic attack, the identification of intermediates and products formed during the course of the enzyme catalyzed reaction, and the kinetics of the reaction (40). Because cellulose is insoluble, it has been necessary to use CM-cellulose or other soluble derivatives to study the mode of attack of 1,4-β-D-glucanases on polymeric substrates. Interpretation of results obtained using this substrate is limited by the effects of the substituents and is less definitive than in comparable experiments with the amylases for which substrate modification is not necessary to attain solubility. Experiments using CM-cellulose, nevertheless, have illustrated differences in modes of action among the 1,4-β-D-glucanases.

Gilligan and Reese (9) differentiated the activities of several "cellulase" fractions of *Trichoderma viride* on CM-cellulose according to the slopes obtained from a plot of the change in fluidity versus the corresponding increase in reducing sugars. In such graphical analysis the endo-1,4-β-D-glucanases which preferentially cleave internal bonds of CM-cellulose gave straight lines with large positive slopes, whereas more nearly horizontal lines were indicative of exoglucanase action. Nisizawa *et al.* (63) interpreted the magnitude of the slopes as a measure of the "degree of randomness" of endo-1,4-β-D-glucanases; the steeper the line the more random the enzymic attack. Nisizawa *et al.* (6) and Okada (19) differentiated multiple endo-1,4-β-D-glucanases from *Irpex lacteus* and *Trichoderma viride*, respectively, by the slopes ob-

tained from such curves. Barras *et al.* (31) discussed the different modes of attack toward CM-cellulose, in relation to the events occurring at the active sites of these enzymes. Thus a "more random" attack could be indicative of a single bond cleavage per encounter of enzyme and substrate, whereas a "less random" attack could reflect multiple cleavages. In analogy to the work of Thoma (40) on the amylases, multi-chain attack would be more likely to occur with a single bond cleavage per encounter and single-chain attack with enzymes that cleave more than one bond per encounter with substrate. Experiments designed to define the events occurring at the active site of 1,4-β-D-glucanases and test these theories of enzymic attack on polymeric substrates have not been reported.

Chromatographic analysis of the oligosaccharides formed from 1,4-β-D-glucanase action on polymeric and oligosaccharide substrates has provided useful evidence concerning the action patterns of these enzymes. After partial enzymic hydrolysis of 1,4-β-D-glucan polymers, exo-action gives a single soluble product, whereas endo-action gives a series of products (2). These hydrolysis products have been separated and identified using paper chromatography (22,26,49), gel filtration (2,58), and most recently, high pressure liquid chromatography (HPLC), which has provided a rapid, sensitive and quantitative analysis of soluble oligosaccharides (64-66).

Many investigators have analyzed hydrolysis products only after prolonged incubation of substrate and enzyme, thus limiting significance of such kinetic studies. Since the products of one enzymic cleavage may be the substrates for further reactions, the apparent kinetics

of the reaction will vary greatly depending on the chain-length of both the substrate and products. In addition to these hydrolysis reactions, transglycosylation reactions have been demonstrated for both the exo-cellobiohydrolase (51) and the endo-1,4-β-D-glucanases (62).

Chromatographic analysis of the hydrolysis products after prolonged incubation with the exo-cellobiohydrolase has shown predominantly cellobiose with a trace of glucose (22,26,27,48). On the other hand, incubation with the endo-1,4-β-D-glucanases has resulted principally in the products, glucose, cellobiose and cellotriose (16,22,62) together with, in some cases, traces of higher oligosaccharides (6,35). When purified cellooligosaccharides were used as substrates, the exo-cellobiohydrolase yields predominantly cellobiose (26,27,49). Gum[2] has followed the time course of exo-cellobiohydrolase cleavage of purified cellooligosaccharides using the highly sensitive HPLC method. He found after prolonged incubation with such substrates that this enzyme produced about 95 percent, by weight, cellobiose and about five percent glucose, whereas after more limited hydrolysis he observed a different array of products. In one such case, after cleavage of 39 weight percent of the cellotetraose substrate, Gum[2] observed principally cellobiose, and low levels of glucose and cellotriose. The transglycosylation products, cellopentaose and cellohexaose, were also present in small quantities in the early stages of hydrolysis where most of the substrate-acceptor was still present.

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[2]Gum, E. K., Jr. (1975) Personal Communication.

Paper and thin-layer chromatographic analysis of the soluble products of endo-1,4-β-D-glucanase action on cellooligosaccharides has shown an array of products which differ in relative amount depending on the incubation time and the specific endoglucanase. After prolonged incubation Wood and McCrae (16) found cellobiose and cellotriose as major products, whereas Okada and Nisizawa (62) demonstrated mainly glucose and cellobiose. Okada and Nisizawa (62) observed transglycosylation activity in one of two endoglucanases (II-B) with the formation of cellotetraose from cellobiose during early stages of incubation. The cellotetraose was later cleaved to form glucose and cellotriose.

Kinetic studies of 1,4-β-D-glucanase action on cellooligosaccharides would provide important information in understanding the action patterns of these enzymes; yet very few such reports are available. Gum[3] has determined specific activities of exo-cellobiohydrolase C with one percent, by weight, cellooligosaccharides using HPLC. In general, he found no activity with cellobiose and approximately equal activities on the cellooligosaccharides of greater chain-length. Okada and Nisizawa (62) have attempted to define the kinetic constants, for two endo-1,4-β-D-glucanases by measuring the reducing sugar produced from cello-oligosaccharides. However, because of the several types of reaction possible with these enzymes, this method does not yield an accurate and meaningful evaluation of the extent or kinetics of the reactions taking place. The relative "V_{max}" values were seen to increase with the chain-length of the substrate for both of his endoglucanases, but only with

[3]Gum, E. K., Jr. (1975) Personal Communication.

endo-1,4-β-D-glucanase II-B was there demonstrated a "V_{max}" on cellotriose. Neither enzyme cleaved cellobiose. Although differences between the two endoglucanases were also apparent from comparison of the respective "K_m" values for each cellooligosaccharide, the significance of these results is not clear.

Mechanism of Action

The mechanism(s) of action of 1,4-β-D-glucanases is not known. However, indirect evidence identifying the active site of an *Aspergillus wentii* β-glucosidase by Legler (67,68) points toward a reaction mechanism similar to that describing the action of lysozyme. Lysozyme is unique among the β-glucan hydrolases in that both its three-dimensional structure and that of the enzyme-substrate complex are accurately known so that the enzymic mechanism of action may be explained in great detail (69). The carboxylic acid groups of glutamic acid-35 and aspartic acid-52 that are present at the active site of lysozyme have been implicated in the bond breaking and bond making process. There is evidence that the glutamic acid-35 carboxylic acid group is unionized whereas the aspartic acid-52 carboxylic acid group is ionized in the optimum pH range for enzymic catalysis (70,71). Bause and Legler (68) have demonstrated the presence of aspartic acid at the active site of β-glucosidase by isolating a hexadecapeptide containing an aspartyl residue bound to an active site directed inhibitor. The aspartic acid carboxylate group was proposed to function in a manner analogous to aspartic acid-52 of lysozyme.

Similarities between the lysozyme and β-glucosidase systems have been extended to describe a possible mechanism for the 1,4-β-D-glucanases

(Figure 1)[4]. According to this mechanism, the enzyme and substrate bind in such a way that the active site of the 1,4-β-D-glucanase is in juxtaposition to the glucosidic bond to be cleaved. An unionized acidic group, perhaps a carboxylic acid group, on the enzyme acts as a general acid catalyst and donates a proton to the glucosidic oxygen of the substrate. This leads to the breaking of the glucosidic bond (C-1 oxygen bond) and formation of an oxo-carbonium ion intermediate. Formation of this intermediate is promoted by distortion of the hexose ring into a half chair conformation in which the C-1, C-2, 0-5 and C-5 atoms lie in a plane and is stabilized by electrostatic shielding from a carboxylate group on the enzyme. It is subsequently attacked by water leading to hydrolysis or by hydroxylic acceptors such as oligosaccharides in which case transglycosylation occurs.

The mechanism by which crystalline cellulose is degraded by cellulase systems has yet to be elucidated. The current theory supported by most investigators working with purified exo- and endo-1,4-β-D-glucanases is that the endo-1,4-β-D-glucanase acts randomly on native cellulose chains cleaving internal linkages and thereby providing chain-ends for exo-cellobiohydrolase action (5,6,16,18,22). Experiments to test this theory are complicated not only by the insolubility of the substrate but by its non-uniform three-dimensional structural polymorphism. The amorphous and crystalline regions of native cellulose provide different micro-environments for enzyme action and consequently produce nonlinear kinetic patterns.

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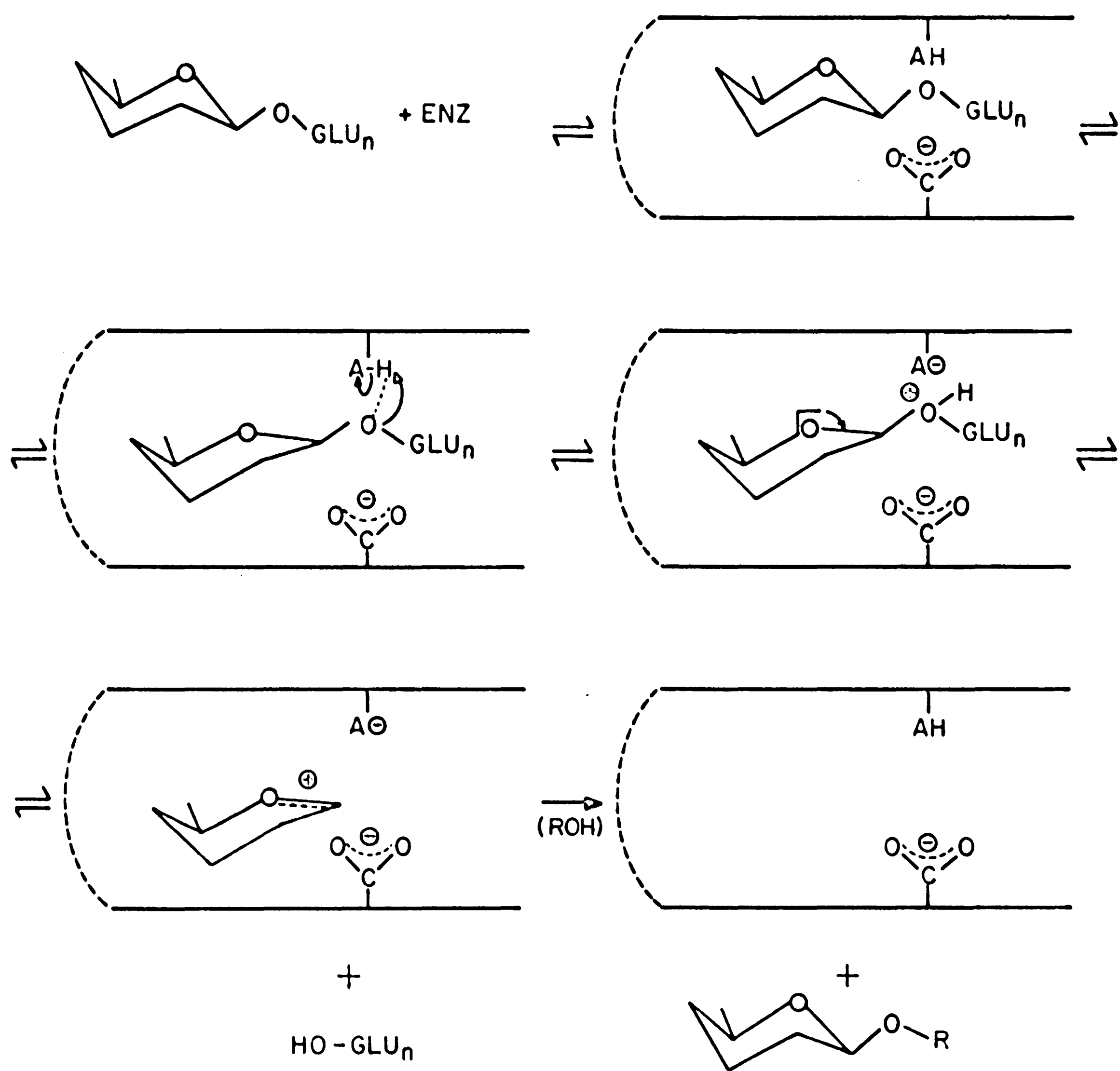
[4]Brown, R. D., Jr. (1976) Personal Communication.

FIGURE 1

Hypothetical Mechanism for Glucan Hydrolysis Catalyzed by β-D-Glucanases



O
GLU$_n$
+ ENZ
AH
A-H
AΘ
H
(ROH)
+
HO - GLU$_n$
O
R

Evidence for the mechanism of action of the endo- and exoglucanases has been based on release of reducing sugar residues from cellulosic substrates. It has been found that, when the purified enzymes are incubated together with native or crystalline cellulose, the extent of degradation of these substrates is much greater than when incubated with each enzyme separately (5,6,16,20,22,27). This synergistic action of the 1,4-β-D-glucanases was not observed when non-crystalline substrates, CM-cellulose or PSC, were used (6,22,27). These findings support the present hypothesis describing the mode of action involving the 1,4-β-D-glucanases in the degradation of native cellulose; however, experimental confirmation has yet to be reported. The mechanism of action of these enzymes will be elucidated only when it is possible to conduct precisely designed experiments using purified and characterized exo- and endo-1,4-β-D-glucanases, making possible an accurate physico-chemical analysis of substrates, intermediates and products during the course of the enzymic reaction.

EXPERIMENTAL PROCEDURES

Materials

Enzymes - Chymotrypsinogen-A from beef pancreas (six times crystallized, salt free, lot #W1426); Schwartz/Mann, Orangeburg, New York.

Exo-cellobiohydrolase C from *Trichoderma viride*; prepared by E. K. Gum, Jr. (July, 1973).

β-Glucosidase from *Trichoderma viride*; prepared by G. H. Emert (May, 1973).

Lysozyme from egg white (three times crystallized, salt free, lot #W1656); Schwartz/Mann, Orangeburg, New York.

Pancellase SS from *Trichoderma viride* (lot #203006); Yakult Biochemicals Co., Ltd., Shingikancho, Nishinomiya, Japan.

Trypsin-TRL from bovine pancreas (two times crystallized, salt free, lot #U4187); Mann Research Laboratories, New York, New York.

Substrates - Amylose (lot #43183); Calbiochem, Los Angeles, California.

Avicel-PH-101 (microcrystalline cellulose N.F.); American Viscose Division, FMC Corporation, Newark, Delaware.

Cellobiose (lot #2936); Eastman Organic Chemicals, Rochester, New York.

Cellooligosaccharides (cellotriose through cellohexaose); prepared according to the method of Miller (72).

CM-cellulose 7HP; Hercules Powder Company, Wilmington, Delaware.

Filter paper (Whatman No. 3MM); W. & R. Balston, Ltd., England.

p-Nitrophenyl-β-D-glucopyranoside (lot #24C-2610); Sigma Chemical Company, St. Louis, Missouri.

p-Nitrophenyl-β-D-cellobioside; prepared according to the method of Dea (73).

Walseth Cellulose (phosphoric acid-swollen cellulose); prepared from Avicel-PH-101 according to the method of Wood (54).

4-*O*-Methylglucuronoxylan from beechwood; gift from M. Sinner, Institut für Holzchemie and Chemische Technologie des Holzes, Hamburg, West Germany.

Chromatographic Materials - Amberlite MB-3; (Analytical Reagent Grade - ion-exchange resin); Mallinckrodt Chemical Works, St. Louis, Missouri.

μ-Bondapak-carbohydrate column; Waters Associates, Milford, Massachusetts.

DEAE-Sephadex A-50; Pharmacia Fine Chemicals, Piscataway, New Jersey.

Dowex I-X8 (Analytical Grade - anion-exchange resin, regenerated in acetate form); Bio-Rad Laboratories, Richmond, California.

OV-225 (Cyanopropylmethyl phenyl-methyl silicone), Chromosorb G-HP (80-100 mesh); Varian Aerograph, Walnut Creek, California.

Buffers - CAPS (cyclohexylaminopropane sulfonic acid, A grade, lot #010099; Calbiochem, San Diego, California.

Disodium succinate hexahydrate (97% pure); Aldrich Chemical Company, Milwaukee, Wisconsin.

Glycine (Reagent Grade); Fisher Scientific Company, Fairlawn, New Jersey.

HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid, lot #8485); Nutritional Biochemical Corporation, Cleveland, Ohio.

Imidazole (99% pure); Aldrich Chemical Company, Milwaukee, Wisconsin.

Tris(hydroxymethyl)aminomethane (Trizma Base, Reagent Grade); Sigma Chemical Company, St. Louis, Missouri.

Chemicals - Acetic anhydride; redistilled, b.p. 137°.

Acetonitrile (Nanograde); Mallinckrodt Chemical Works, St. Louis, Missouri.

Basic Fuchsin; National Aniline Division, Allied Chemical and Dye Corporation, New York, New York.

Constant boiling hydrochloric acid (Sequanal Grade); Pierce Chemical Company, Rockford, Illinois.

D-Galactosamine hydrochloride, Glucosamine hydrochloride, D-xylose; Sigma Chemical Company, St. Louis, Missouri.

Glucostat Special Reagent Set; Worthington Biochemical Corporation, Freehold, New Jersey.

D-Mannose (A-Grade); Calbiochem, Los Angeles, California.

Periodic acid; G. Frederick Smith Chemical Company, Columbus, Ohio.

Sodium borohydride (lot #S71B-226); Sigma Chemical Company, St. Louis, Missouri.

N,N,N',N'-tetramethylethylene diamine, N,N-methylene-bis-acrylamide, acrylamide (Analytical Grade), ammonium persulfate, riboflavin, Bromphenol

Blue, Coomassie Blue RDS-L Concentrate, Canalco, Inc., Rockville, Maryland.

Other chemicals were reagent grade.

Methods

Ultrafiltration - Cellulase solutions were concentrated or dialyzed using Amicon ultrafiltration cells (Amicon Company, Lexington, Massachusetts). The non-cellulosic membranes obtained from Amicon Company and used in these cells had either molecular exclusion limits of 30,000 (Membrane Type PM-30) or 10,000 (Membrane Type PM-10 or UM-10). The PM and UM membrane series have slightly different properties; UM membranes possess ionic sites whereas PM membranes are made of an inert, non-ionic polymer. Flow rates were more rapid with the PM series, and during the purification procedure, it was observed that certain proteins would penetrate PM membranes but would be retained on UM membranes. Ultrafiltration Dia-Flow Cells, Models 12 and 202, were used routinely except for large scale concentration procedures where the Amicon High Flow Ultrafiltration Cell, Model 2000, with RDS-4 reservoir was employed.

Protein Determination - Protein concentrations in cellulase solutions were determined using an ultraviolet absorption method based on the work of Warburg and Christian as described by Bailey (74). The absorbances at 280 nm and 260 nm of protein solutions were measured on a Hitachi 124 Double Beam Spectrophotometer and the concentration was calculated using the expression introduced by Kalckar (75):

$$1.45(A_{280}) - 0.74(A_{260}) = \text{mg protein/ml solution.}$$

The utility of this technique for cellulase solutions has been repeatedly

demonstrated by Emert (28) and Gum (24). Concentrations of solutions of purified endo-1,4-β-D-glucanases and exo-cellobiohydrolase C were determined using experimentally determined extinction coefficients (cf. p.41).

Avicel Column - An Avicel column as described by Gum (24) was used in the initial separation of cellulase components from Pancellase SS. A dialyzed enzyme solution of four grams protein in 500 ml pH 5.0, 0.05 M sodium acetate buffer was applied to a column (15.5 x 6.5 cm: diameter x height) containing 70 grams of Avicel. The non-adsorbed proteins which were eluted by washing with three liters of this buffer were termed the "buffer fraction" enzymes. The adsorbed enzymes were subsequently eluted with two liters of distilled water and the resulting turbid "water fraction" eluate was clarified by the incubation procedure of Gum (24). Buffer and water fractions from three Avicel columns were combined and concentrated by ultrafiltration using a PM-30 membrane.

DEAE-Sephadex Batch Separation - The cellulase components of the buffer and water fractions from the Avicel column were further separated by the batch process of Gum (24). The resulting fractions I, II and III were eluted with 0.05 M sodium succinate buffer at pH 5.35, 5.0 and 3.6, respectively. All buffers contained 3.0 mM sodium azide and the pH 3.6 buffer had been adjusted to an ionic strength of 0.5 M with sodium chloride. These fractions were concentrated by ultrafiltration using a PM-10 membrane. Fraction I contained most of the endo-1,4-β-D-glucanase activity as measured by the viscosimetric assay.

DEAE-Sephadex Column Chromatography - For the purification of cellulase components by ion-exchange chromatography, the choice of column size, flow rate and ionic strength of the eluting buffer varied with the sample. Using the procedure outlined by Pharmacia Fine Chemicals (76), DEAE-Sephadex A-50 was equilibrated in pH 6.0, 5.0 mM imidazole-3.0 mM sodium azide buffers adjusted to the desired ionic strength with sodium chloride. The ionic strength of buffers and eluates was determined as sodium chloride equivalents from a standard curve of log sodium chloride concentration versus log resistance. Resistance was measured using a Serfass Conductivity Bridge (A. H. Thomas Company, Philadelphia, Pennsylvania). The gel slurry was degassed under aspirator vacuum and poured to a height of either 40 or 60 cm in Pharmacia columns of 2.5 x 4.5 cm and 1.5 x 90 cm, respectively. After allowing the gel to settle for 30 minutes, it was equilibrated with degassed buffer flowing at the rate of 18-20 ml/hour (short column) or 8-10 ml/hour (long column) until the pH of the effluent buffer was 5.8-6.0.

For the purification of endo-1,4-β-D-glucanases from the buffer fraction, 280 mg of concentrated fraction I protein dissolved in 0.01 M ionic strength buffer was applied to the short column. The second protein peak eluted from this column at an ionic strength of 0.07 M and contained most of the endoglucanase activity. This partially purified endoglucanase was concentrated on a PM-10 membrane and further purified to homogeneity by elution from an ion-exchange column (long column) with a step gradient from 0.01 M to 0.07 M.

Endo-1,4-β-D-glucanases from the water fraction were purified in a similar manner. In this case, 150 mg of concentrated fraction I protein

from the DEAE-batch separation dissolved in 0.07 M ionic strength buffer was added to the short column. Two endoglucanases were eluted from this column when a step gradient from 0.07 M to 0.15 M to 0.3 M was applied. Although a small amount of a purified enzyme could be obtained by isocratic elution, much larger amounts of this partially purified material were combined to increase the yield of activity. A second enzyme was obtained in pure form by elution in 0.3 M ionic strength buffer. Contaminants were separated from the enzyme eluted by 0.07 M ionic strength buffer by means of further chromatography on the long column using a single step ionic strength gradient from 0.01 M to 0.07 M.

Column chromatography was carried out at 4-6° and the protein content of the eluate was monitored at 280 nm using an ISCO Model UA-4 Ultraviolet Analyzer (Instrumentation Specialties Company, Lincoln, Nebraska). Fractions were collected every ten minutes in an automatic ISCO fraction collector. The protein concentration of fractions to be assayed for enzymic activity was calculated by the Kalckar equation from the absorbance readings at 280 nm and 260 nm. Fractions of interest within each eluted protein peak were combined and concentrated on PM-10 or UM-10 membranes and stored in the freezer.

Disc Gel Electrophoresis - The discontinuous buffer systems No. 1 (pH 8.9) described by Maurer (77) and modified by Emert (28), No. 6 (pH 7.5), No. 8 (pH 4.3) and No. 9 (pH 2.9) described by Maurer were used for electrophoretic studies of cellulases in cylindrical polyacrylamide gels. The power supply used in these studies was a Model 200 Canalco Electrophoresis Constant Rate Source. Protein was stained with Coomassie

blue prepared as described by Gum (24), and carbohydrate was stained with periodic acid-Schiff reagent (PAS) using the method as described by Lang (49) and modified by destaining in bisulfite:acetic acid (0.1%: 7%) for one hour before storing in 7% acetic acid. Gel systems No. 6 and No. 9 were used to demonstrate the homogeneity of the purified endo-1,4-β-D-glucanases in a pH region where the enzyme is active. However, gel system No. 1 gave the best resolution of protein bands and was the system used routinely.

Slab Gel Electrophoresis - A Dual Vertical Slab Gel Electrophoresis Unit-Model 220 and Power Supply-Model 400 (Bio-Rad Laboratories, Richmond, California) was used to analyze purified cellulase-dodecyl sulfate complexes. The sodium dodecyl sulfate (SDS) system described by Neville (78) and modified by K. C. Harich[5] to give a 15% acrylamide system utilized a sulfate-borate discontinuous buffer system that permitted high resolution of the protein complexes. This system examined the relative molecular weights and the possibility of subunits among the purified enzymes. Protein bands were stained with Coomassie blue and analyzed using a Schoeffel Model SD-3000 Recording Spectrodensitometer with Model SDC-300 Density Computor (Schoeffel Instrument Corporation, Westwood, New Jersey).

Endo-1,4-β-D-glucanase Assay - A viscosimetric assay was used to measure endoglucanase activity. The decrease in viscosity upon incubation of the enzyme with a soluble cellulose derivative, CM-cellulose (DS=0.83), was measured by the procedure of Hash and King (79) as modi-

[5]Harich, K. C. (1976) Personal Communication.

fied by Liu (12). In the work reported here, the method was further modified with regard to both the preparation of substrate and the range of drain times used to calculate activities. The procedure for the preparation of a 0.22% solution of CM-cellulose in pH 4.5, 30 mM sodium acetate-5.0 mM sodium azide buffer was studied to determine the effect of temperature or filtration technique on the viscosity of the polymer solution. Filtering the CM-cellulose solution through glass wool as had been done previously, left fine glass fibers in the filtrate. Heating the buffer solution in order to speed up dissolution of CM-cellulose had no significant effect but did yield a solution of higher viscosity than did a non-heated solution. The CM-cellulose solution was prepared by the slow addition of CM-cellulose to buffer solution with constant stirring. This process routinely took 1-1.5 hours and was performed at room temperature. The solution was filtered through a 1000 ml coarse sintered glass filter using water aspirator vacuum and stored in the refrigerator until use.

Using distilled water as the reference liquid the relative viscosity (η_r) of the CM-cellulose solution can be calculated from the ratio of the viscosities in the following equation:

$$\eta_r = \frac{\eta_1}{\eta_2} = \frac{\rho_1 T_1}{\rho_2 T_2}$$

where η_1 = relative viscosity of the CM-cellulose solution (40°)

η_2 = relative viscosity of water (40°)

ρ_1 = density of CM-cellulose solution (40°)

ρ_2 = density of water (40°)

T_1 = drain time (sec) of CM-cellulose solution (40°)

T_2 = drain time (sec) of water (40°)

Liu (12) found a negligible difference between the densities of water and a 0.2% solution of CM-cellulose and so the equation for the relative viscosity (η_r) may be simplified to $\eta_r = T_1/T_2$. The change in specific fluidity ($\Delta\phi_{sp}$) can then be calculated using the relationship for the specific viscosity (η_{sp}) and specific fluidity (ϕ_{sp}):

$$\eta_{sp} = \eta_r - 1$$

$$\phi_{sp} = 1/\eta_{sp}$$

$$\Delta\phi_{sp} = \phi'_{sp} - \phi^o_{sp}$$

where $\phi'_{sp} = \phi_{sp}$ of the CM-cellulose solution after incubation with enzyme for 30 minutes

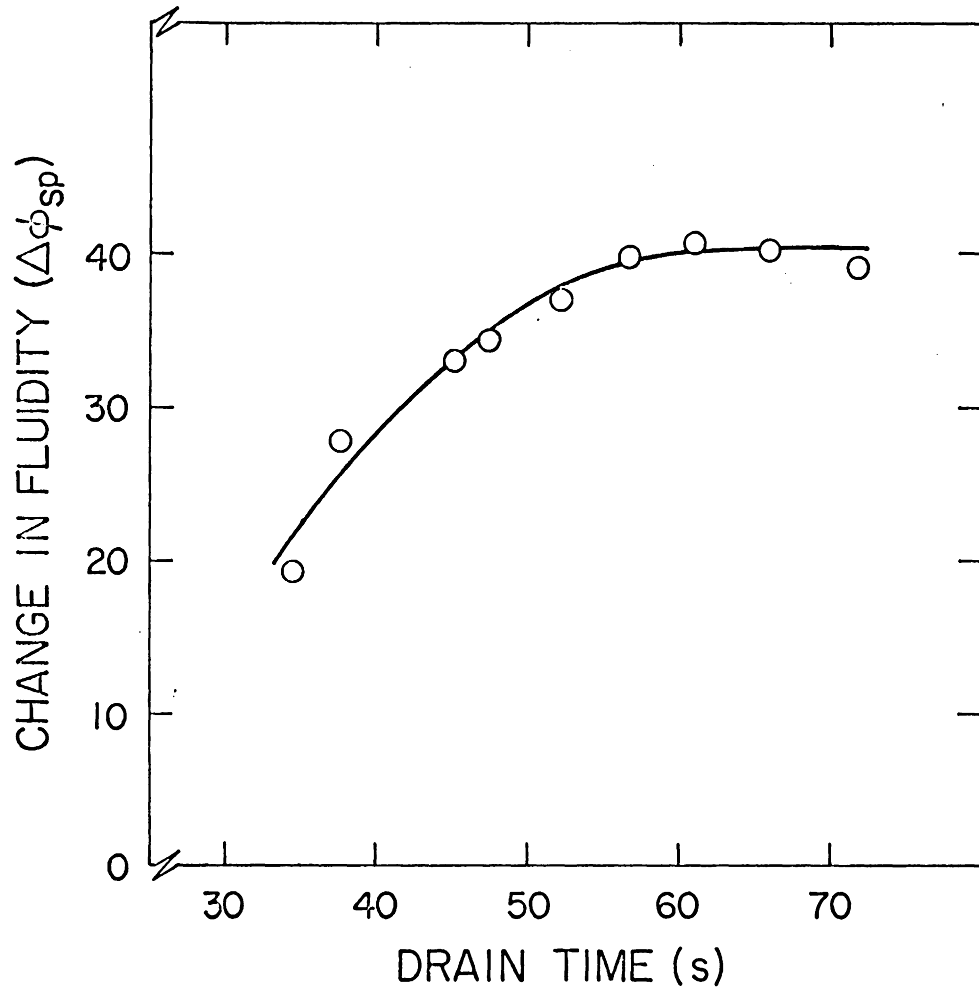
$\phi^o_{sp} = \phi_{sp}$ of CM-cellulose substrate solution

The specific activity (Z) of the enzyme solution can then be calculated as $Z = \Delta\phi_{sp}$/min-mg protein. The endo-1,4-β-D-glucanase activity was expressed in these units. Although Liu (12) had found that $\Delta\phi_{sp}$ was directly proportional to the amount of enzyme present, a further study was performed to test the range of reliable drain times. The drain times of water and CM-cellulose were 22-24 seconds and 100-115 seconds, respectively. The relationship of drain time to $\Delta\phi_{sp}$ was calculated for a series of dilutions of an endo-1,4-β-D-glucanase solution. After correcting the $\Delta\phi_{sp}$ with the appropriate dilution factor in order to compare activities at equal effective enzyme concentrations, it was found that reliable, reproducible activities could only be obtained where drain times were in the range of 45-70 seconds (Figure 2). Below this range the substrate became less sensitive to additional internal cleavages giving an apparently lower $\Delta\phi_{sp}$. Long drain times indicated too few cleavages of

FIGURE 2

Evaluation of the Useful Range of Drain Times for the Viscosimetric Assay of Endoglucanase Activity

The viscosimetric assay using a series of dilutions of an endo-1,4-β-D-glucanase solution yielded different drain times from which could be calculated the change in fluidity. The values corrected for enzyme concentration by the corresponding dilution factor were plotted versus the observed drain times.


CHANGE IN FLUIDITY ($\Delta\phi_{sp}$)
0
10
20
30
40
30
40
50
60
70
DRAIN TIME (s)

internal bonds of the substrate to give consistent results. Thus the endoglucanase activities reported were obtained using an enzyme concentration such that the drain time after 30 minutes incubation at 40° was between 45-70 seconds.

Reducing Sugar Determination - The spectrophotometric measurement of the release of reducing sugar residues has been employed extensively in following the action of polysaccharide hydrolases. This method has been described by Nelson (41) and Somogyi (42). The Somogyi reagent was filtered before use and the absorbance at 510 nm was measured within a half-hour after addition of the chromagen. When CM-cellulose, amylose or xylan were used as substrates, centrifugation after addition of chromagen was necessary to remove precipitate before absorbances could be measured.

The relative activities of the endo-1,4-β-D-glucanases with different polymeric substrates was measured using this method. Enzyme was incubated with 1% (w/v) amylose, xylan, Avicel, phosphoric acid swollen cellulose (PSC), or CM-cellulose in pH 4.5, 0.05 M sodium acetate buffer at 40° for 30 minutes. Soluble reducing sugar was measured as glucose equivalents. Laminarin and pustulan were also investigated as substrates but the absorbance yield of 1% substrate blanks was too high for use in this study.

The release of reducing sugar was compared graphically with the concomitant change in viscosity according to the method described by Nisizawa *et al.* (6) in order to compare the modes of attack of different 1,4-β-D-glucanases on CM-cellulose. In each experiment, a purified enzyme

was incubated at 40° with CM-cellulose and both reducing sugar release and viscosity were measured at 5, 10, 15, 20, 25 and 30 minutes. The corresponding values obtained for glucose equivalents and change in fluidity at each time point were plotted. The slope of the resulting line indicated the relative susceptibility of the internal bonds of CM-cellulose to attack by the enzymes.

Glucose Determination - Glucose concentration was determined using the glucose oxidase procedure outlined in the Worthington Enzyme Manual (52) and described by Emert (28). This determination was used to assay β-glucosidase activity after incubation of enzyme with ten millimolar cellobiose in pH 5.0, 0.05 M sodium acetate buffer for 20 minutes. It was also used in kinetic studies when cellobiose and cellotriose were used as substrates.

Extinction Coefficient - A method was devised which permitted accurate determination of protein extinction coefficients using only milligram quantities of purified endo-1,4-β-D-glucanases. For this determination an Abderhalden Drying Pistol Unit, a Cahn electrobalance (Ventron Instrument Corporation, Paramount, California) and a Hitachi 124 Double Beam Spectrophotometer were used. Into acid-washed one milliliter tared glass beakers trimmed to 3/4 size and weighing 0.89-0.91 grams was pipetted 0.2-0.5 ml of a filtered solution of enzyme in distilled water. Samples of the three purified endoglucanases and a blank of 0.5 ml of distilled water were added to separate beakers. These were placed in an aluminum foil boat partitioned in such a way to hold the beakers in place. This was placed in the double jacketed long arm compartment of the drying

pistol unit. The side arm containing dry phosphorous pentoxide (P_2O_5) was connected to the drying apparatus and the sample chamber was heated by boiling acetic acid (b.p. 117°) vapors. Since application of vacuum was found to cause spattering of the protein solutions, the enzyme solutions were allowed to evaporate at atmospheric pressure over P_2O_5 until no visible liquid remained in the beakers. A vacuum then was applied to the system for an additional 24-48 hours to remove any remaining moisture from the samples. Before the beakers were removed from the apparatus, the sample chamber was cooled to room temperature and the vacuum released slowly over a period of 30 minutes. The beakers were transferred with forceps to a small desiccator and weighed immediately on a Cahn Gram Electrobalance (sensitivity at 0.1 µg: 0.01% error). The weight of the beaker containing no protein varied by only 0.3 µg before and after the procedure. The dry weights determined from duplicate aliquots of solutions of each endo-1,4-β-D-glucanase were in excellent agreement (Table II).

In a separate experiment, the absorbance at 280 nm of different dilutions of enzymes dissolved in water were determined using the Hitachi 124 Double Beam Spectrophotometer. The absorbance value corresponding to each specific dilution of the enzyme was corrected by multiplying by the appropriate dilution factor. The resulting values for each protein were averaged and extinction coefficients ($E_{280}^{1\%}$) and corresponding standard deviations for endo-1,4-β-D-glucanases II, III and IV were determined to be 11.97 ± 0.35, 10.32 ± 0.37 and 13.12 ± 0.18, respectively.

The method was further checked by comparison of reported extinction coefficients obtained by this procedure. Exo-cellobiohydrolase C, chymo-

TABLE II

Comparison of Duplicate Experiments in Otaining Dry Weight of Purified Endo-1,4-β-D-glucanase Solution

Endo-1,4-β-D-glucanase	Amount of Enzyme Solution Added to Beaker (ml)	Dry Weight of Enzyme		
		Run #1 (mg)	Run #2 (mg)	Average (mg)
II	0.5	1.268	1.305	1.287
III	0.5	1.637	1.601	1.619
IV	0.3	1.328	1.331	1.330

trypsinogen, trypsin and lysozyme were examined and the results are shown in Table III. All values other than that obtained for chymotrypsinogen were in agreement with published values, leading credence to the validity of this method for the determination of extinction coefficients when only small amounts of enzyme are available.

Amino Acid Analysis - A sample containing about 0.3 mg of purified enzyme in solution was evaporated to dryness under nitrogen and hydrolyzed in constant-boiling HCl (6N) at 110° for 24, 48 or 72 hours. After removal of the HCl the hydrolyzed sample was redissolved in 0.2 N sodium citrate buffer containing standard amino acids, and analyzed for amino acids using a Model 121 Automatic Amino Acid Analyzer and Beckman System AA Computing Integrator (Beckman Instruments, Inc., Palo Alto, California) according to the method of Spackman, Stein and Moore (81) as outlined in the Model 121 Instruction Manual (82). Values for serine and threonine were extrapolated to zero hydrolysis time. The analyses for cystine and methionine were obtained from 24 hour hydrolysates of samples subjected to performic acid oxidation (83). Tryptophan was calculated from the molar ratio of tyrosine to tryptophan obtained using the spectrophotometric method of Bencze and Schmid (84).

Carbohydrate Determination - The amino sugar and neutral carbohydrate composition of the endo-1,4-ß-D-glucanases were determined according to the methods of Walborg *et al.* (85) and Metz *et al.* (86), respectively, as modified by Gum (24). After completion of the extinction coefficient determination, the resulting dried enzyme samples were used in carbohydrate analyses. The beakers containing samples of the enzymes were placed in a

TABLE III

Comparison of Literature and Experimental Values for Extinction Coefficients ($E_{280}^{1\%}$)

Enzyme	Reference	Extinction Coefficient, $E_{280}^{1\%}$	
		Literature Value	Experimental Value
Exo-cellobiohydrolase C	24	14.2	14.07
Chymotrypsinogen	80	20-20.2	17.51
Trypsin	80	12.9-17.2	15.21
Lysozyme	80	25.3-26.9	24.50

five milliliter reaction vial with the appropriate concentration of HCl and hydrolyzed as described in the above methods for either neutral carbohydrate or amino sugar determination. The vials were shaken every half-hour to ensure adequate hydrolysis of the entire sample. The weight of the reaction vial and contents before and after hydrolysis indicated no loss due to leakage. Quantification of amino sugar and neutral carbohydrate components was based on the internal standards, galactosamine in amino sugar analyses or xylose in neutral carbohydrate determinations.

The identity of alditol acetates of the neutral sugars were confirmed by use of a Varian MAT-112 Gas Chromatograph-Mass Spectrometer equipped with a Varian 620/L Computer. The instrument was used in the multiple ion selection mode. The eight characteristic mass ions associated with the alditol acetates were examined in each of the peaks obtained from gas chromatographic analysis. Total neutral carbohydrate was determined both by gas chromatography of the alditol acetates, and by the phenol sulfuric acid (PSA) method of Dubois *et al.* (87) as modified by Lang (49) using mannose as standard.

Ultracentrifugation Analysis - Sedimentation velocity and sedimentation equilibrium studies were performed on the purified endo-1,4-β-D-glucanases according to the procedure outlined by Lang (49). Molecular weights were determined from sedimentation equilibrium data using a partial specific volume from amino acid and carbohydrate composition calculated by the method of Cohn and Edsall (88).

pH Optimum and pH Stability - The optimum pH for enzymic activity using CM-cellulose or PSC as substrates was determined using the visco-

simetric and reducing sugar assays, respectively. Enzyme was incubated with either CM-cellulose or PSC solutions, which had been adjusted to different pH values, for 30 minutes at 40° and assayed. Control blanks (without addition of enzyme) at each pH value were used as a means for comparison and evaluation of results.

The stability of the endo-1,4-β-D-glucanases was examined over a pH range of 3 to 11. Buffer solutions of 0.05 M citrate (pK_{a_2}=4.7, pK_{a_3}=5.4), HEPES (pK_{a_1}=7.55) and CAPS (pK_{a_1}=10.4) were used over a pH range of 3.0-6.0, 6.0-9.0 and 9.0-11.0, respectively. Possible buffer effects were tested by comparing the activities obtained from incubation with two different buffers at either pH 6 or 9. The effect of pH on the stability of the endoglucanases was measured by the viscosimetric assay at pH 4.5 after pre-incubation at 40° for one hour at the different pH values with the appropriate buffers.

High Pressure Liquid Chromatography - High pressure liquid chromatography (HPLC), a technique which allows rapid and efficient analysis of microgram quantities of material, has been adapted in this laboratory to the separation and quantification of oligosaccharides (66). 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, Massachusetts). It was equipped with both a differential refractometer and a differential ultraviolet detector. The differential refractometer was the mode of detection except for studies involving p-nitrophenyl derivatives, in which case both detection systems were used. A Spectra-Physics Autolab System

I Computing Integrator (Spectra-Physics, Santa Clara, California) was used in kinetic studies to compute the relative area of the chromatographic peaks.

Separations were accomplished using a Waters' μBondapak carbohydrate column with acetonitrile:water solvent systems. The cellooligosaccharide series was resolved using approximately 3:2 (w/w) acetonitrile:water solvent system (Figure 3). As chain-length of oligosaccharide increased from glucose to cellohexaose, peak width increased; however, peak area was found to be proportional to the weight percent concentration of the oligosaccharide.[6] Cellooligosaccharides of at least 99% purity were prepared by the method of Miller (72) for use as substrates.

The soluble hydrolysis and transfer products formed by enzymic action with these substrates were identified and quantified by this HPLC system. Determination of specific activities for the endo-1,4-β-D-glucanases with one percent (w/v) cellooligosaccharide substrates at 30° was accomplished in the following manner. At short intervals after initiating enzymic reactions, samples were taken from the reaction mixture for HPLC analysis. The resulting oligosaccharide peaks were identified by their corresponding retention times and from the peak areas were calculated the weight fraction of each oligosaccharide. The weight of the substrate was converted to μmoles and its disappearance plotted as a function of reaction time. A straight line indicating zero-order reaction time with respect to substrate resulted in cases where greater

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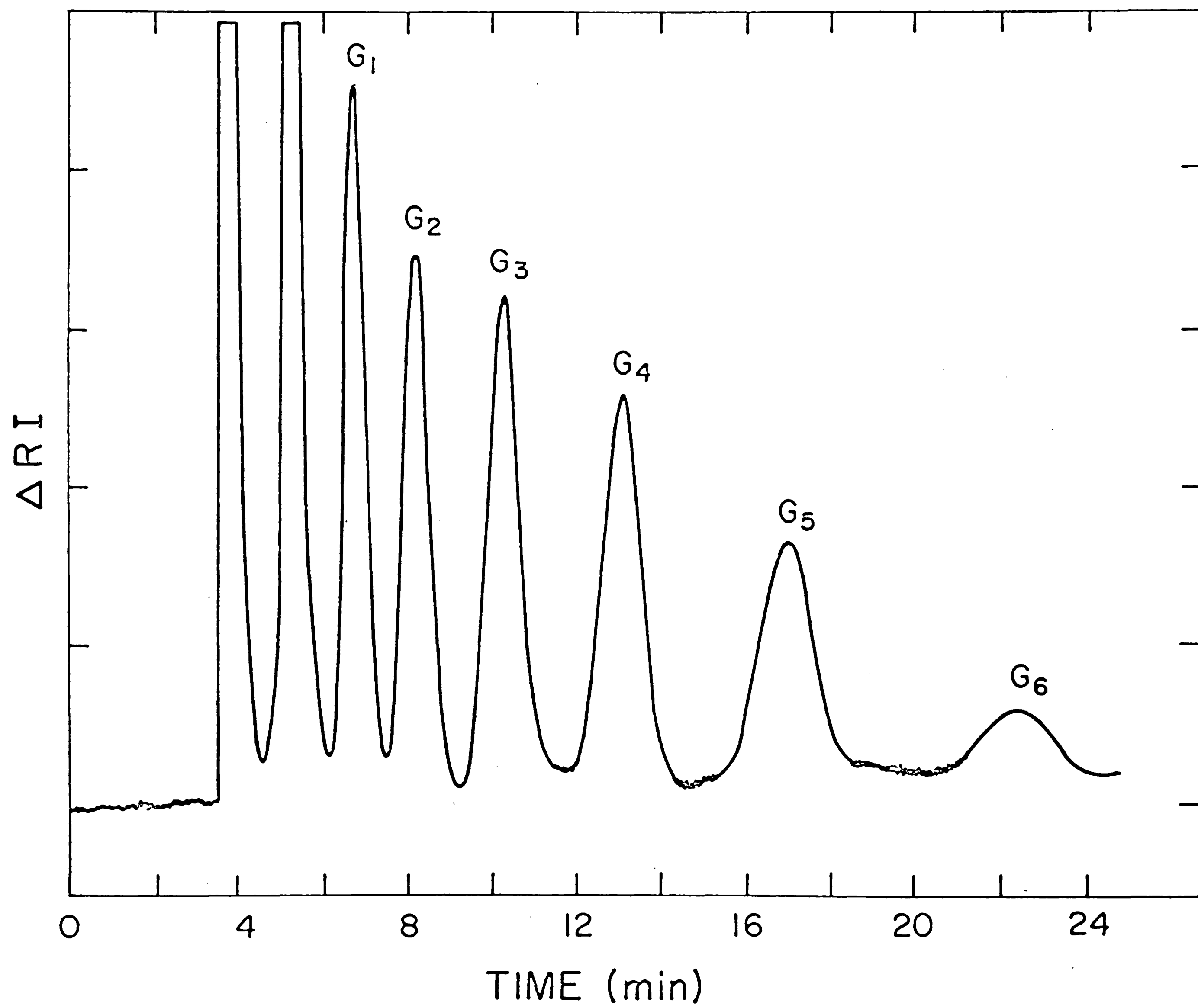
[6] Greenberg, B. (1976) Personal Communication.

FIGURE 3

Separation of Cellooligosaccharides by High Pressure Liquid Chromatography

Cellooligosaccharides formed by acid hydrolysis of cellulose powder were separated on a 30 cm μBondapak carbohydrate column. The mixture was eluted at 1 ml/min using an acetonitrile:water (6:4) solvent system. Carbohydrate peaks detected by refractive index are G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose; and G_6, cellohexaose. The two initial peaks represent water from the solvent and an artifact from an Amberlite MB-3 column used to deionize the hydrolysate.



G1
G2
G3
G4
G5
G6
ΔRI
0
4
8
12
16
20
24
TIME (min)

than 75% of the substrate still remained. The least squares slope (-dS/dt) of this line divided by the enzyme concentration gave the specific activity (μmole/min/mg protein). In addition, the soluble products from endo-1,4-β-D-glucanase action with one percent PSC and Avicel were identified by HPLC using the standard retention times of the cellooligosaccharides.

Cellooligosaccharides, as well as reduced cellooligosaccharides prepared by Gum[7] according to the procedure described by Cole *et al.* (90) and Storwick *et al.* (91) were separated using a 45 minute flow program from 40-92% of 2.5-5.0 ml/min with acetonitrile:water (7:3 - 3:1) solvent systems. An example of the separations achieved is shown in Figure 4 with a mixture of reduced (glucitol through cellopentaitol) and non-reduced oligosaccharides (glucose through cellotetraose).

Bond specificity was investigated for the endo-1,4-β-D-glucanases and the exo-cellobiohydrolase by comparing initial hydrolysis products from cellopentaitol and cellotetraitol. For this analysis, 150 μl of substrate was incubated with 1-10 μl of enzyme at 40° for short times resulting in less than 10% degradation. The reaction was stopped by immersion in boiling water for three minutes. The sample was evaporated to dryness under nitrogen, redissolved in 50 μl distilled water, and a 25 μl aliquot was injected into the HPLC. The initial products then were identified according to the retention times of corresponding standards.

In addition, excellent separation of glucose, cellobiose, p-nitrophenol, p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-cellobioside was achieved using an acetonitrile:water azeotrope (16% water; b.p. 75-

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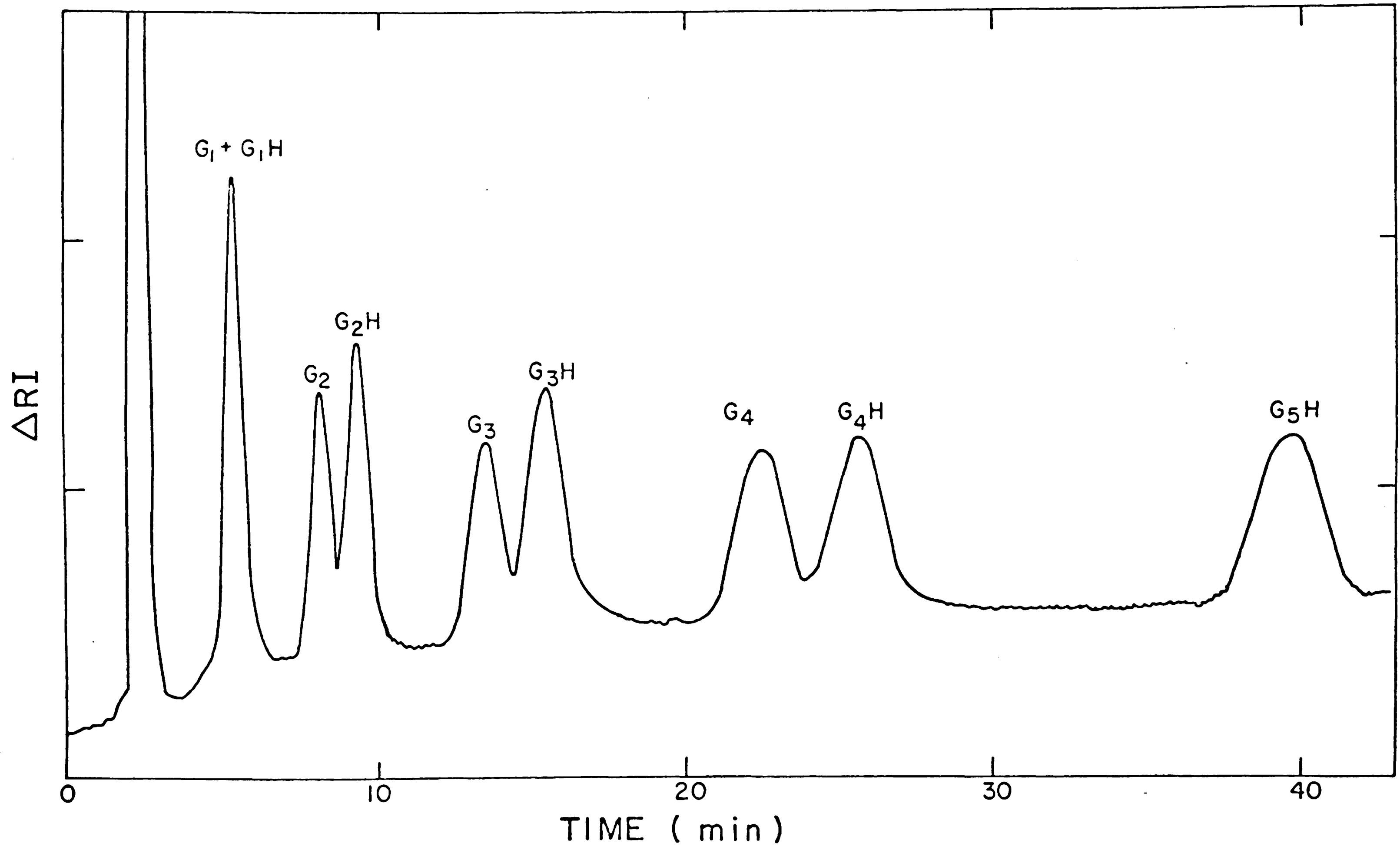
[7]Gum, E. K., Jr. (1976) Manuscript in Preparation.

FIGURE 4

Separation by High Pressure Liquid Chromatography of Reduced and Non-reduced Cellooligosaccharides

Separation of reduced and non-reduced cellooligosaccharides on a 30 cm μBondapak carbohydrate column was achieved using a 45 minute flow program from 40-90% of 5.0 ml/min with an acetonitrile:water (3:1) solvent system. Carbohydrate peaks detected by refractive index are G_1, glucose; G_1H, glucitol; G_2, cellobiose, G_2H, cellobiitol; G_3, cellotriose; G_3H, cellotriitol; G_4, cellotetraose; G_4H, cellotetraitol; and G_5H, cellopentaitol. The initial peak represents water from the solvent. Glucose and glucitol were not resolved with this flow program.


G1 + G1H
G2
G2H
G3
G3H
G4
G4H
G5H
ΔRI
0
10
20
30
40
TIME (min)

76°) and both the differential ultraviolet and refractive index detection systems applied sequentially to the effluent stream (Figure 5). This separation allowed the study of bond specificity toward p-nitrophenyl-β-D-cellobioside among the endo-1,4-β-D-glucanases. In this study enzyme was incubated with 1.5 mM p-nitrophenyl-β-D-cellobioside at 30° in pH 4.5, 0.05 M sodium acetate-3.0 mM sodium azide buffer for 23 hours. The reaction was stopped by immersion in boiling water for five minutes. The sample was deionized with Amberlite MB-3, evaporated to dryness under nitrogen and redissolved in 50 μl of distilled water. A 10 μl aliquot was injected into the HPLC, and the resulting p-nitrophenyl derivatives and oligosaccharides were detected in the effluent stream by ultraviolet absorbance and refractive index.

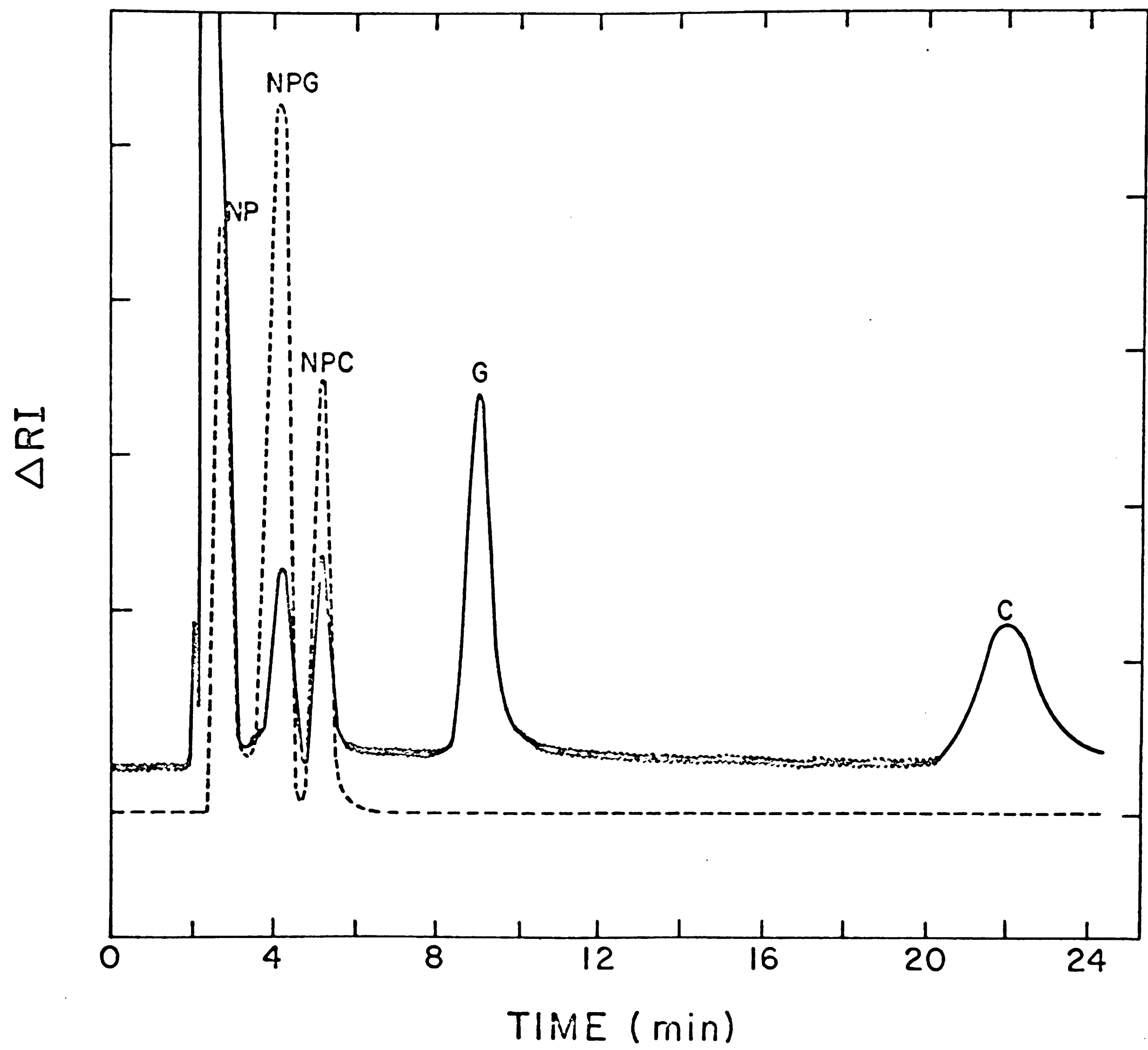
Determination of Kinetic Constants with Cellooligosaccharide Substrates - Kinetic studies of the reaction of endo-1,4-β-D-glucanases with cellooligosaccharide substrates utilized one of two methods. The kinetic constants, K_m and V_{max}, for cellobiose and cellotriose hydrolysis by endoglucanases were obtained by determining glucose production after enzymic action at 40° for 30 minutes using the glucose oxidase assay (52).

Attempts to determine K_m and V_{max} values for cellotetraose were made using a HPLC method. In this method, the reaction was followed by measuring the decrease in substrate concentration according to the following procedure. The cellotetraose in 150 μl of pH 4.5, 0.1 mM sodium acetate buffer solution was incubated with enzyme at 40° for a suitable period of time during which less than 20% of the substrate was degraded.

FIGURE 5

Separation by High Pressure Liquid Chromatography of p-Nitrophenyl Glycosides of Glucose and Cellobiose

Chromatography was performed on a 30 cm μBondapak carbohydrate column with elution at 2.0 ml/min using an acetonitrile:water azeotrope (15% water; b.p. 75-76°) as the solvent. *p*-Nitrophenyl derivatives were detected by ultraviolet absorbance at 254 nm (---), whereas carbohydrate peaks were detected by refractive index (——). The order of elution was *p*-nitrophenol, NP; *p*-nitrophenyl-β-D-glucoside, PNG; *p*-nitrophenyl-β-D-cellobioside, PNC; glucose, G; and cellobiose, C. The large initial peak is due to the solvent (water).


NPG
NP
NPC
G
C
ΔRI
A254
0
4
8
12
16
20
24
TIME (min)

The reaction was stopped by immersing an aliquot in boiling water for three minutes, after which the mixture was evaporated to dryness under nitrogen. The sample was redissolved in 25 μl water and an aliquot injected into the HPLC. The resulting peaks were quantified by the Spectra-Physics Autolab integrator. Reaction velocities (-dS/dt) were determined by comparison with substrate concentration before enzyme addition. This method did not permit quantification of products obtained at low substrate concentrations (<1 mM).

Rate Studies Using *p*-Nitrophenyl Derivatives - Enzymic hydrolysis of *p*-nitrophenyl glucosides was measured spectrophotometrically. The *p*-nitrophenol formed in the one milliliter reaction mixture during incubation at 40° in pH 4.5, 0.05 M sodium acetate buffer for one hour was measured at 405 nm after addition of 0.4 ml of 7.5% (w/v) potassium phosphate. Specific activities of the endo-1,4-β-D-glucanases with 4.0 mM *p*-nitrophenyl-β-D-glucoside and 1.5 mM *p*-nitrophenyl-β-D-cellobioside were determined as μmole *p*-nitrophenol/min/mg protein. In experiments to determine kinetic constants, 0.2-2.0 mM *p*-nitrophenyl-β-D-glucoside was incubated with enzyme for 30 minutes, after which the reaction was terminated by immersion of an aliquot in boiling water for five minutes.

Short Fiber Forming Activity - To compare the short fiber forming activity of purified endoglucanases with that of the two endoglucanases purified by Berghem *et al.* (21) the procedure described by the above authors was followed as closely as possible. In a cuvette tube containing 20 mg of Whatman No. 3MM filter paper cut into ten pieces of one square centimeter each was added three milliliters of pH 4.5, 0.05 M

sodium acetate-3.0 mM sodium azide buffer. Following addition of the purified endoglucanases to three of the four tubes, the reaction mixtures were incubated at 40°. The absorbance of the resulting fiber suspensions was measured at 400 nm periodically for four days using a Spectronic 20 Spectrophotometer (Bausch & Lomb, Rochester, New York). Before each absorbance reading the sample was mixed and any large pieces of filter paper were allowed to settle to the bottom of the cuvette. The soluble carbohydrates accompanying endo-1,4-β-D-glucanase action were identified by HPLC and analyzed quantitatively for reducing sugars.

RESULTS AND DISCUSSION

Although this investigation has emphasized the characterization of endo-1,4-β-D-glucanases in the cellulase system of *Trichoderma viride*, it was originally intended to purify an endo-1,4-β-D-glucanase from *Trichoderma viride*, study its properties, and compare it to the other types of cellulase enzymes previously purified in our laboratory (24,28). When this project began there had been no reports of endo-1,4-β-D-glucanases purified to homogeneity from true cellulolytic organisms. However, during the course of this work four other laboratories reported the purification of endoglucanases from *Trichoderma viride* (19,21), *Sporotrichum pulverulentum* (55), and *Irpex lacteus* (35,61). These studies provided only limited data on the structure and properties of the endoglucanases (19-22,35,55,61,62). Since the present work, together with previous reports, agree that there is more than one type of endoglucanase, attempts were made to purify each of the enzymes exhibiting endoglucanase activity as measured by the viscosimetric assay. Three of the four electrophoretically distinct endo-1,4-β-D-glucanases identified by this investigator were purified to homogeneity and subsequently characterized with respect to structural and enzymic properties. The endoglucanases were named as isozymes according to their electrophoretic mobility in polyacrylamide gels. For example, Endoglucanase I was so named since it migrated most rapidly and was thus apparently the most acidic protein.

Purification of Endo-1,4-β-D-glucanases

Previous work in our laboratory by Gum (24) indicated that the combined pH 5.35 and 5.0 fractions (fraction I and II) from a DEAE-Sephadex

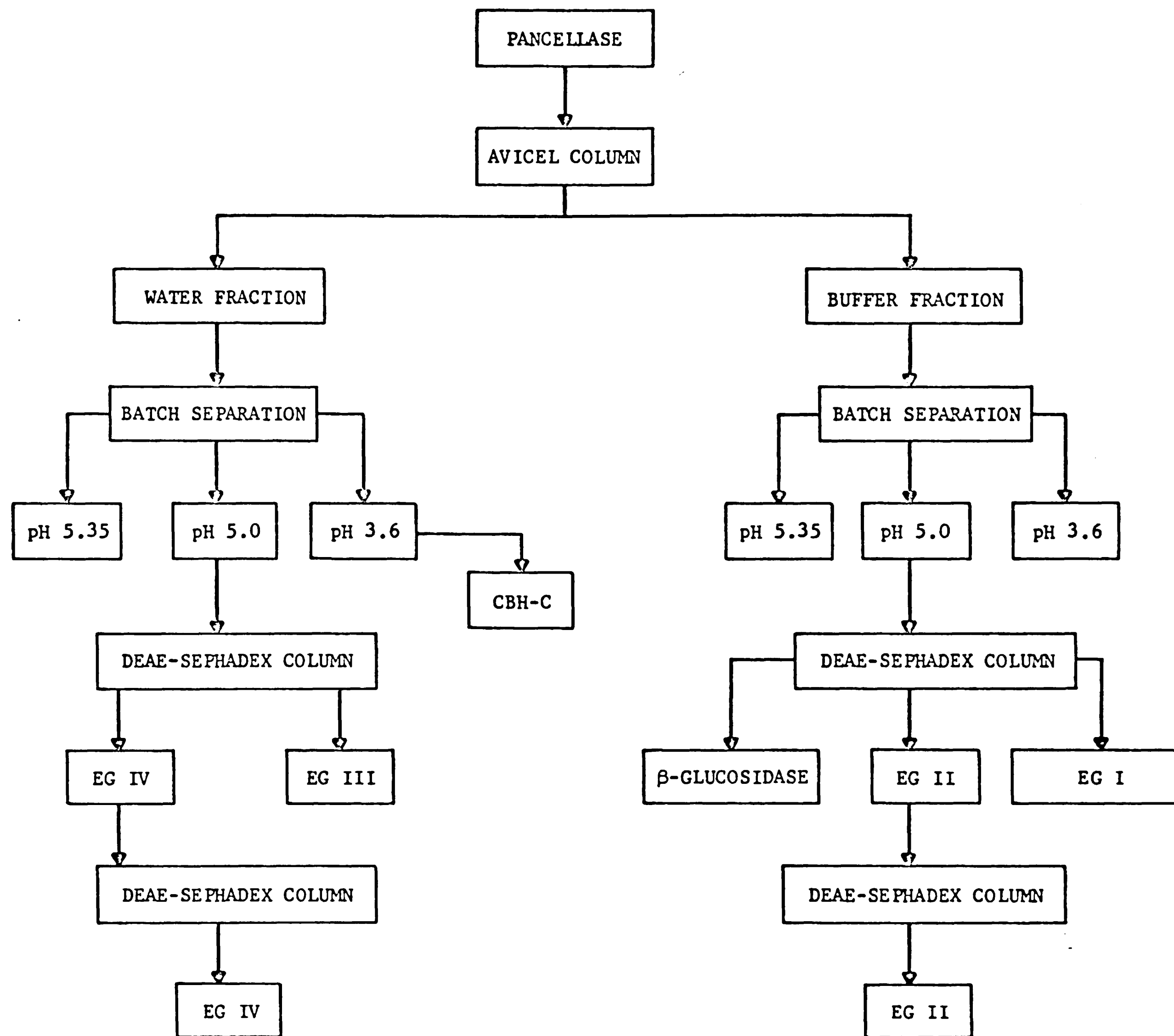
batch separation of the water fraction had relatively high endoglucanase activity. This fraction became the starting point for investigating the endo-1,4-β-D-glucanases. Initial attempts at purification of endoglucanases from this fraction using preparative gel electrophoresis and affinity chromatography failed. The endoglucanase activity of these enzymes was destroyed under the alkaline conditions employed during preparative gel electrophoresis and the use of this system at lower pH values was not successful. Likewise, elution from a Concanavalin-A-agarose column of the combined fractions I and II did not separate the cellulase components. Microheterogeneity associated with the glycoprotein components in this fraction contributed to the difficulty in use of conventional purification methods. However, after many attempts two endoglucanases were purified to homogeneity using ion-exchange chromatography.

After successful methods had been developed for the purification of two endoglucanases, effort was directed toward quantification of both protein and endoglucanase activity yield at each purification step. Pancellase, a commercial enzyme preparation derived from *Trichoderma viride*, was the source of the cellulases to be purified in this study. Purification of endo-1,4-β-D-glucanase activity was followed using the viscosimetric assay and by disc gel electrophoresis. A flow diagram of the overall purification procedure is shown in Figure 6.

Enzyme Source - Pancellase SS which contains enzymes derived from *Trichoderma viride* has been prepared commercially from solid (Koji) cultures of the fungus. In this process the 24 hour submerged seed culture had been spread over aerated wheat bran and incubated under a water spray

FIGURE 6

Flow Diagram of the Procedure Used in Purifying Endo-1,4-β-D-glucanases from Pancellase, a Commercial Enzyme Preparation Derived from *Trichoderma viride*


PANCELLASE
AVICEL COLUMN
WATER FRACTION
BUFFER FRACTION
BATCH SEPARATION
BATCH SEPARATION
pH 5.35
pH 5.0
pH 3.6
CBH-C
pH 5.35
pH 5.0
pH 3.6
DEAE-SEPHADEX COLUMN
DEAE-SEPHADEX COLUMN
EG IV
EG III
β-GLUCOSIDASE
EG II
EG I
DEAE-SEPHADEX COLUMN
DEAE-SEPHADEX COLUMN
EG IV
EG II

for two to three days. This culture had been washed, filtered and the filtrate proteins precipitated with tannic acid, after which polyethylene glycol, added to dissociate the tannic acid formed an oil layer. Cellulase activity remaining in the water layer which was collected, spray dried and bulked with lactose. The resulting yellowish powder constituted the commercial material from which the endoglucanases were purified.

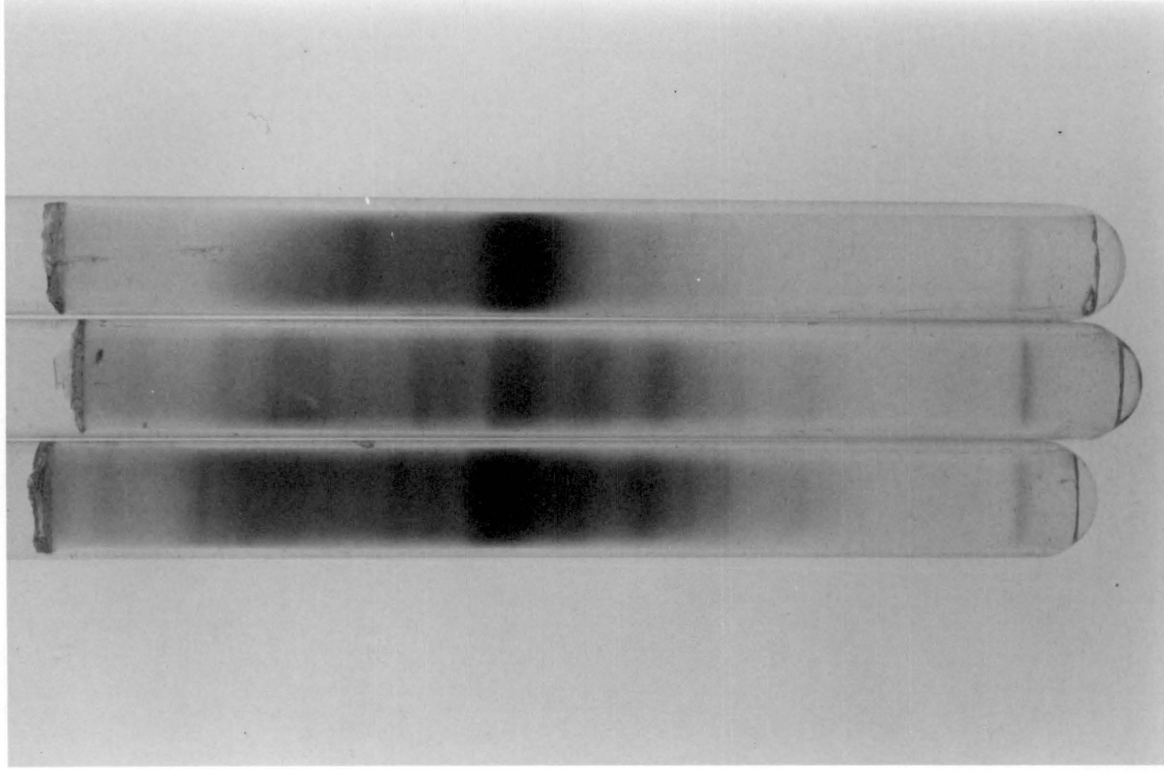
Avicel Column - As a first step the components in Pancellase were separated according to their affinity for crystalline cellulose. The crude cellulase dissolved in pH 5.0, 0.05 M sodium acetate-3.0 mM sodium azide buffer was chromatographed on Avicel columns in which the weight ratio of protein to cellulose was 1:10 as described by Gum (24). The non-adsorbed enzymes including a β-glucosidase (24) were eluted with buffer, whereas the adsorbed enzymes including an exo-cellobiohydrolase C were eluted with water. Most of the protein was associated with the buffer fraction (78%) which is in agreement with Gum's findings (24). Disc gel electrophoretic analysis of the buffer and water fractions (Figure 7) illustrated that the majority of the protein components present in Pancellase were not adsorbed onto cellulose under the conditions of this column. The adsorbed enzymes present in the water fraction included an exo-cellobiohydrolase C which constituted about 50% of the protein in this fraction and which upon electrophoresis yielded a dense band near the middle of the gel (Figure 7).

Assaying for endoglucanase activity in these fractions gave rather unexpected results. Instead of finding most of the activity in the water fraction, 77% of this activity was found to be associated with the

FIGURE 7

Disc Gel Electrophoresis of Protein Fractions Obtained from an Avicel Column

The protein samples applied to these gels were from left to right 198 μg of Pancellase SS, 160 μg of buffer fraction, and 128 μg of water fraction. The gels were stained for protein with Coomassie blue. The separator gel contained 7.5% acrylamide at pH 8.9 and stacking and sample gels contained 2.5% acrylamide and were buffered at pH 6.9.



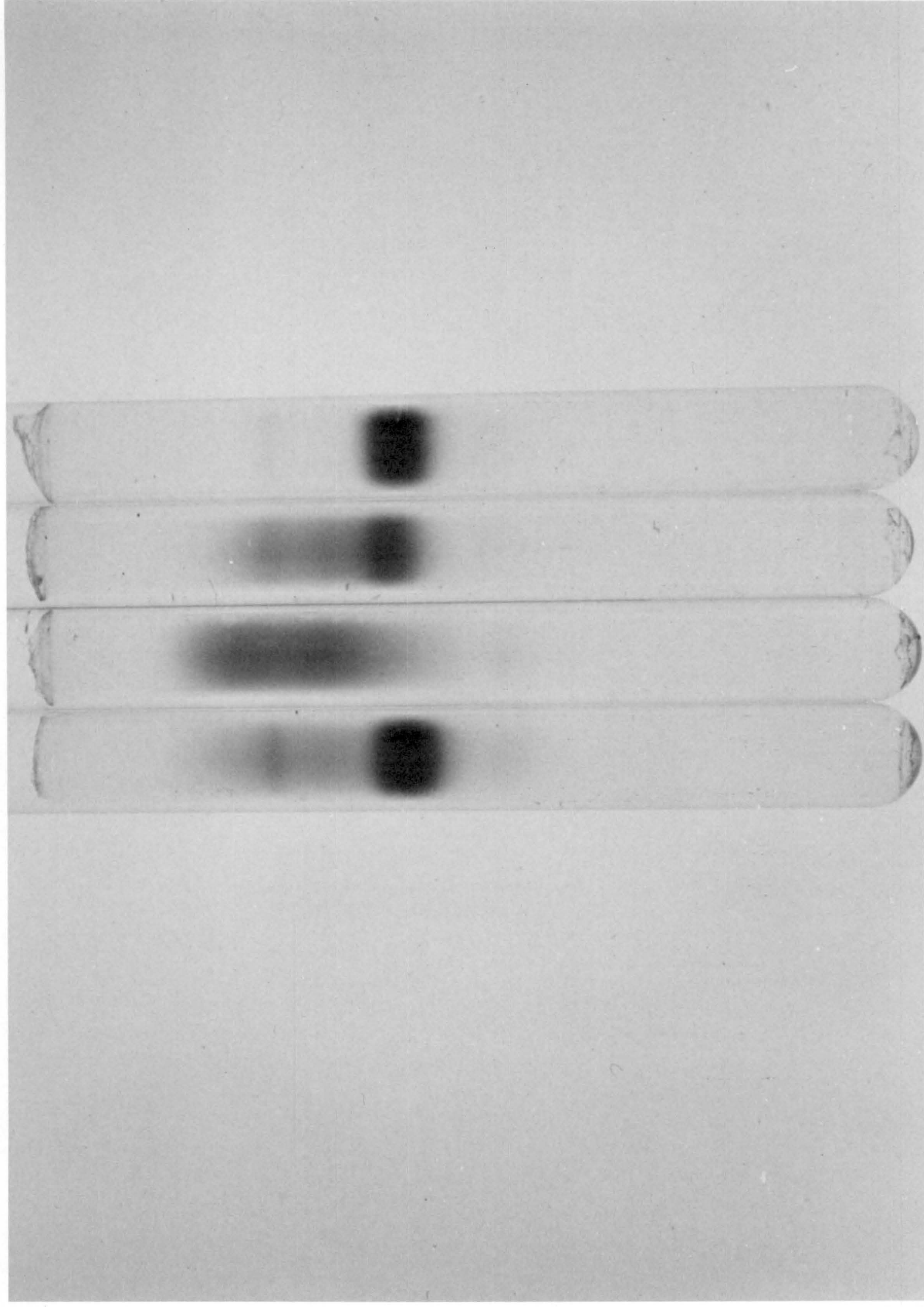
buffer fraction. Although endoglucanases which bind to cellulose (water fraction) should be important in the overall mechanism of cellulose degradation, the fact that the majority of endoglucanase activity resides with the non-adsorbed fraction could not be overlooked. Thus it was decided to attempt purification of these buffer fraction endoglucanases, in addition to the water fraction enzymes.

DEAE-Sephadex Batch Separation of the Water Fraction - Water fractions from three Avicel columns were combined and concentrated on a PM 30 membrane. The retentate contained greater than 90% of the endoglucanase activity and was the starting material for a batch separation as described by Gum (24). Approximately 1.45 grams of protein was dissolved in two liters of pH 5.35, 0.05 M sodium succinate buffer and equilibrated at 0° for 20 minutes in 300 ml of DEAE-Sephadex A-50. The proteins that were not bound to this anion-exchange resin at pH 5.35 were removed by filtration and termed fraction I. Subsequent lowering of pH first to 5.0 and then to 3.6 at an ionic strength of 0.5 M eluted the more tightly bound acidic proteins in fractions II and III, respectively. Disc gel patterns illustrating the protein components in each of these fractions are shown in Figure 8. The position of the bands in these gels further indicates the presence of proteins which are more anionic and/or of lower-molecular-weight as the elution pH was decreased from 5.35 to 5.0 to 3.6. Most (ca. 75%) of the endoglucanase activity was associated with the first fraction; whereas, in agreement with Gum (24), the exo-cellobiohydrolase C which comprised 50% of the protein in the water fraction, eluted in the third fraction. This batch separation procedure was

FIGURE 8

Disc Gel Electrophoresis of Protein Fractions Obtained from DEAE-Sephadex Batch Separation of the Water Fraction

The protein samples applied to these gels were from left to right, 122 μg of water fraction, 102 μg of fraction I (pH 5.35), 98 μg of fraction II (pH 5.0), and 76 μg of fraction III (pH 3.6). The gels were stained for protein with Coomassie blue. Electrophoresis conditions were the same as in Figure 7.



successful in separating endoglucanase activity from the exo-cellobiohydrolase C.

<u>DEAE-Sephadex Column Chromatography of the Water Fraction Endoglucanases</u> - The retentate from fraction I of a batch separation which had been dialyzed in 0.07 M ionic strength, 0.05 M imidazole buffer was applied to a DEAE-Sephadex column (2.5 x 45 cm) as described in Experimental Procedures. The elution pattern from this column is shown in Figure 9. Two protein peaks were obtained from this column. The first protein peak (fractions 20-60) which eluted at the initial ionic strength of 0.07 M was high in endoglucanase activity. This enzyme, termed Endoglucanase IV, gradually eluted from this column and attempts to sharpen this protein peak by elution with a higher ionic strength buffer only resulted in poor separation from the other components. Most of the protein (peak 2, fractions 110-130) was eluted with 0.15 M ionic strength buffer and contained very little endoglucanase activity. At the point where a negative slope was recorded for this peak (fraction 130) the eluting buffer was changed to 0.3 M. A second endoglucanase (Endoglucanase III) of lower specific activity than Endoglucanase IV eluted with the end of peak 2 (fractions 145-155). Varying pH, ionic strength and/or flow rate did not yield better purification of either peak 2 proteins or Endoglucanase III.

Disc gel electrophoretic patterns of the peak 2 proteins and of the two endoglucanase fractions from this column are shown in Figure 10. Peak 2 exhibited two bands upon electrophoresis which were never completely resolved. Endoglucanase IV was completely separated from other components

FIGURE 9

Elution Patterns from DEAE-Sephadex Column Chromatography of the Proteins in the Water Fraction-Derived Fraction I Retentate

The retentate from fraction I of a batch separation was applied to a DEAE-Sephadex column (2.5 x 45 cm). The proteins were eluted using a flow rate of 18-20 ml/hr and a step gradient (ionic strength) from 0.07 M to 0.15 M at fraction 80 to 0.30 M at fraction 130 in pH 6.0, 5.0 mM imidazole-3.0 mM sodium azide buffer. Protein concentration is indicated by absorbance at 280 nm (-□-□-□-). Specific endoglucanase activity (-o-o-o-) and ionic strength (I) of the effluent (---) are also given. Fractions were approximately 2.5 ml each and collected every ten minutes.

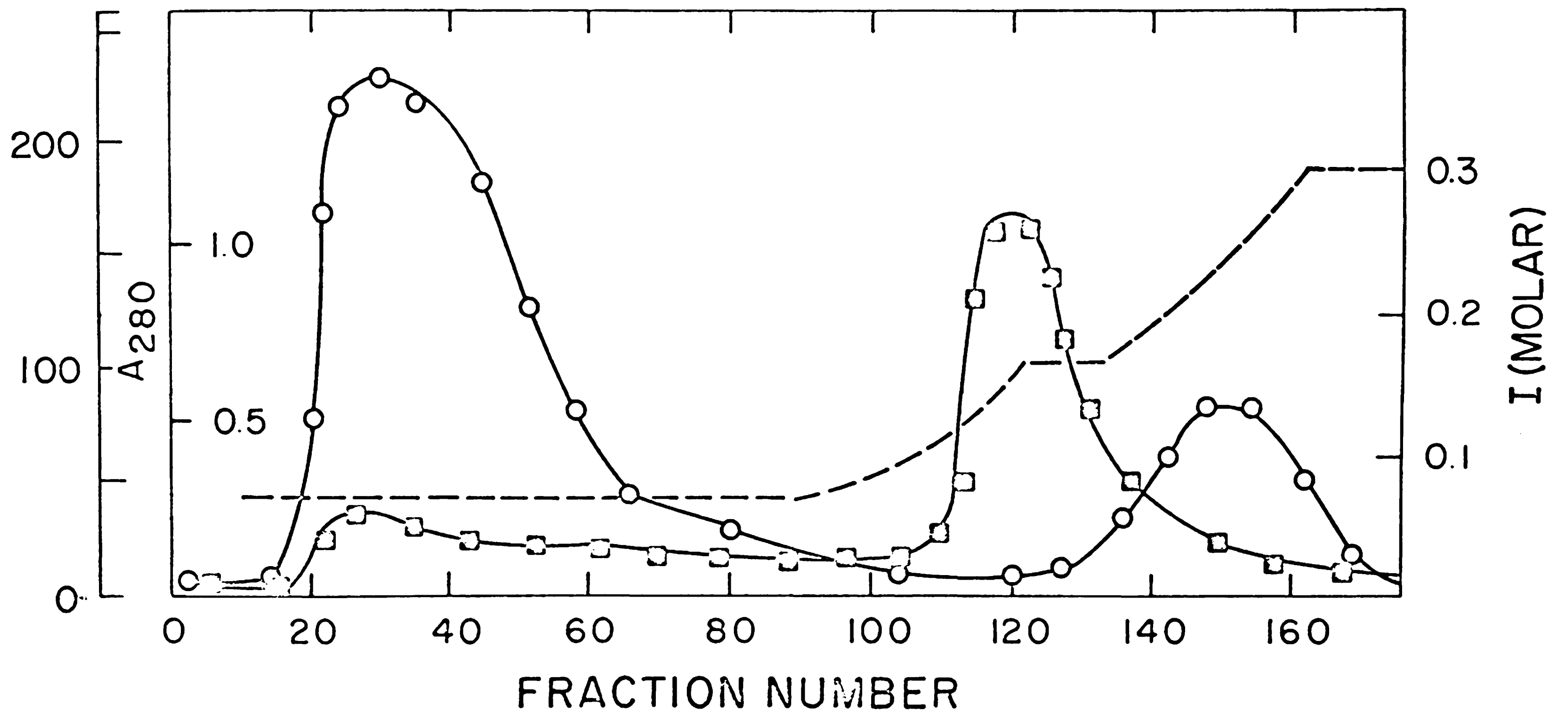
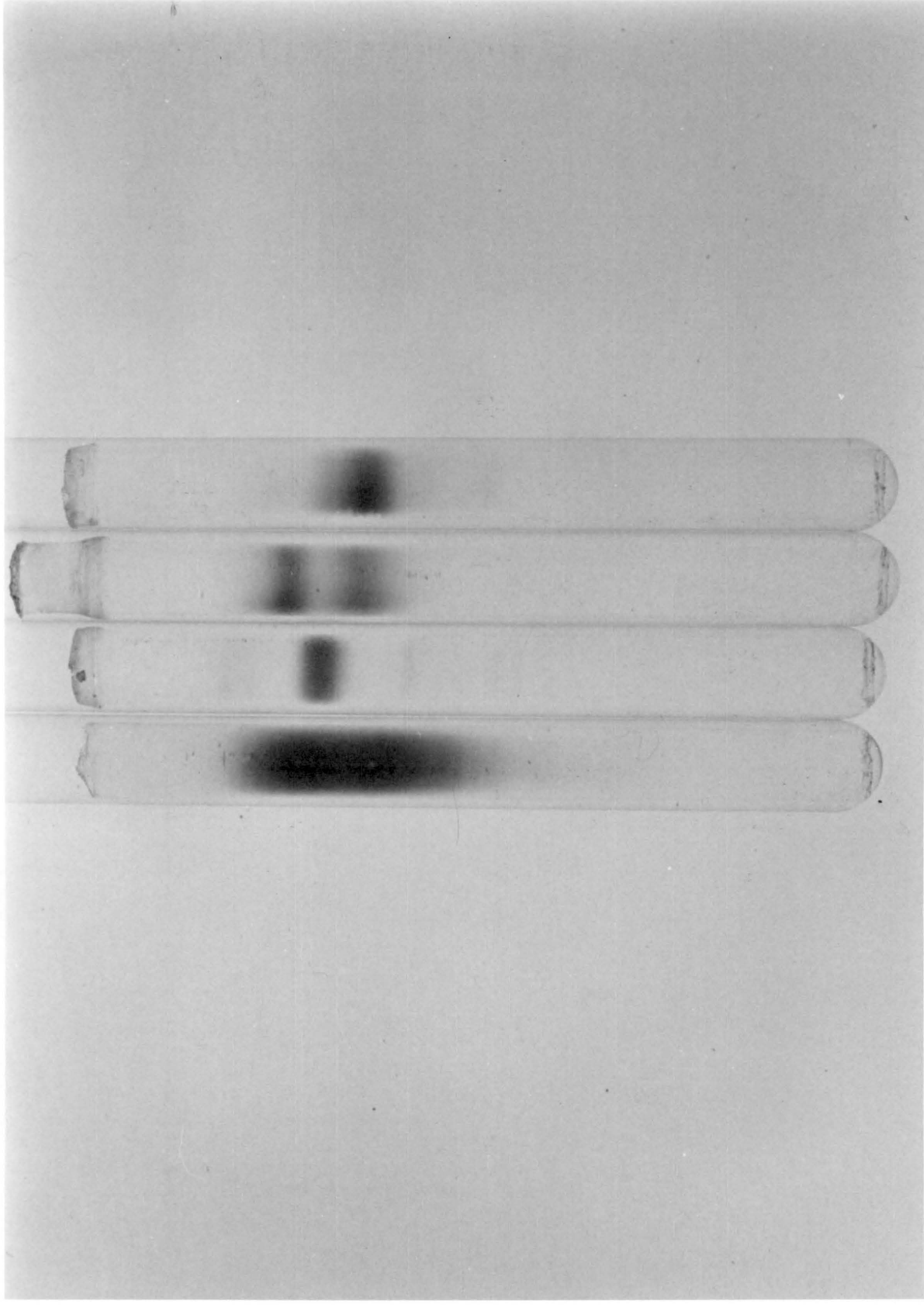

SPECIFIC ACTIVITY ($\Delta\phi_{sp}$/min/mg PROTEIN)
A_{280}
I (MOLAR)
FRACTION NUMBER
0
100
200
0.5
1.0
0.1
0.2
0.3
0
20
40
60
80
100
120
140
160

FIGURE 10

Disc Gel Electrophoresis of Combined Fractions Obtained from DEAE-Sephadex Column Chromatography of the Water Fraction

Protein samples which had been applied to these gels were from left to right, 106 μg of fraction I starting material from a batch separation, 35 μg of fraction 30 (Endoglucanase IV), 48 μg of fraction 120 (peak 2), and 43 μg of fraction 150 (Endoglucanase III). The gels were stained with Coomassie blue. Electrophoretic conditions were the same as those for the experiment illustrated in Figure 7.



in the starting material, whereas incomplete separation of Endoglucanase III from the large protein peak was always observed. Only after most of the protein had eluted from the column was this endoglucanase resolved.

Endoglucanase III and IV represent two electrophoretically distinct proteins. A quantity of Endoglucanase III sufficient for further characterization was purified to homogeneity using this column procedure; however, in obtaining a reasonable quantity of Endoglucanase IV a small amount of contaminant was found in the combined fractions (fractions 25-35). This contaminant was successfully removed using a second DEAE-Sephadex column (1.5 x 90 cm). Forty milligrams of this material were added to the column in 0.01 M ionic strength buffer. When the ionic strength was increased to 0.07 M, an initially sharp but later tailing peak was observed. Disc gel electrophoretic analysis of an equal amount of protein from each fraction within this peak was used to indicate the purity of the enzyme (Figure 11). Most of the protein (61%) was collected in the first fraction (gel #1) which yielded a single band upon disc gel electrophoresis. Since contaminant proteins were found only at the end of the peak, this method permitted isolation of quantities of purified Endoglucanase IV sufficient for further characterization studies.

<u>Summary of Purification of Water Fraction Endoglucanases</u> - Quantification of protein and enzymic activity in each of the purification steps of water fraction endoglucanases from 12.1 grams of Pancellase protein is presented in Table IV. The water fraction contained only 12 and 18% of the total Pancellase protein and endoglucanase activity, respectively. Enzymes purified from this fraction, although constituting only a small

FIGURE 11

Disc Gel Electrophoretic Patterns of Successive Fractions of Endoglucanase IV Obtained from Chromatography of Peak 2 Proteins on a Second DEAE Sephadex Column

Peak 2 proteins from DEAE-Sephadex column chromatography were separated on a DEAE-Sephadex column at lower ionic strength (cf. p.73).

Gels #1 through #10 represent successive fractions from the protein peak which was eluted at an ionic strength of 0.07 M from the second DEAE-Sephadex column. The gels, to each of which had been applied 34 μg of protein, were stained with Coomassie blue. Most of the protein (61%) applied to the column is present in the first fraction from the peak (gel #1). Gels #9-12 illustrate the delayed elution of contaminating proteins. Electrophoretic conditions were the same as those employed in the experiment illustrated in Figure 7.

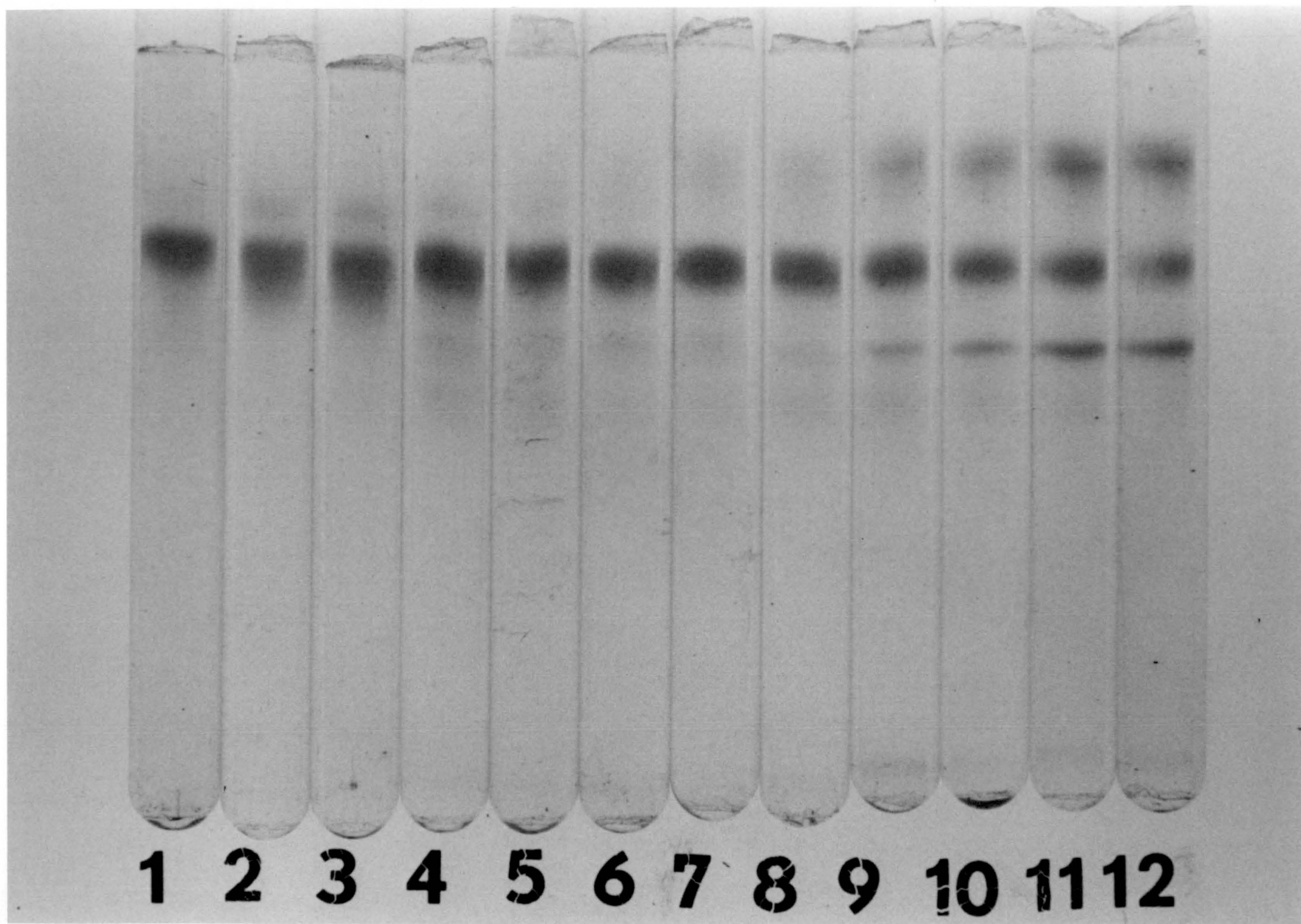

1 2 3 4 5 6 7 8 9 10 11 12

TABLE IV

Summary of Purification of Water Fraction Endoglucanases

		PROTEIN (g)		TOTAL ACTIVITY ($\Delta\phi_{sp}$/min)		SPECIFIC ACTIVITY ($\Delta\phi_{sp}$/min/mg protein)
AVICEL COLUMN:	PANCELLASE	12.1		128,260		10.6
	BUFFER FRACTION	9.43	(77.9%)[a]	98,072	(76.5%)	10.4
	WATER FRACTION	1.45	(12.0%)	23,345	(18.2%)	16.1
BATCH SEPARATION:	WATER FRACTION	1.42		24,850		17.5
	FRACTION 1, pH 5.35	0.368	(25.9%)	18,400	(74.0%)	50
	FRACTION 2, pH 5.0	0.145	(10.2%)	2,900	(11.7%)	20
	FRACTION 3, pH 3.6	0.718	(50.6%)	2,154	(8.7%)	3
DEAE COLUMN:	FRACTION 1	0.26		13,910		53.5
	ENDOGLUCANASE IV	0.02	(7.7%)	4,800	(34.5%)	240
	PEAK 2	0.2	(77.0%)	5,000	(35.9%)	25
	ENDOGLUCANASE III	0.02	(7.7%)	1,200	(8.6%)	60
DEAE COLUMN:	ENDOGLUCANASE IV	0.041		9,840		240
	ENDOGLUCANASE IV	0.025	(61.0%)	6,250	(63.5%)	250

[a]( ) = % recovery of protein or activity in that step.

proportion of the total protein and endoglucanase activity, are important because of their affinity for crystalline cellulose. Batch separation of the water fraction proteins resulted in a three-fold purification of endoglucanase activity and provided excellent separation of the endoglucanases from exo-cellobiohydrolase C, the major protein component of the water fraction. When fraction I proteins from batch separation were applied to a DEAE-Sephadex column, a five-fold purification was obtained for Endoglucanase IV (fractions 25-35, Figure 9). Purified Endoglucanase III, which eluted last on this column, had one-fourth the specific activity of Endoglucanase IV. The endoglucanase activity associated with peak 2 protein is probably due to Endoglucanase III. In addition to the proteins listed in Table IV, some partially purified Endoglucanase IV was eluted between fractions 25-35, (Figure 9). A second DEAE-Sephadex column eliminated minor contaminating proteins from the combined Endoglucanase IV (fractions 25-35, Figure 9), although sacrificing yield of this enzyme at the same time. Approximately thirty milligrams each of Endoglucanase III and IV were purified by this procedure and used for further study.

<u>DEAE-Sephadex Batch Separation of the Buffer Fraction Endoglucanases</u> - Concentration and dialysis of the buffer fraction proteins from an Avicel column on a PM 30 membrane effected significant purification of endoglucanase activity. The filtrate contained almost all of the yellow color associated with the fraction and a small amount of endoglucanase activity (<10%), whereas most of the protein and activity remained in the retentate. Thus after ultrafiltration and dialysis with pH 5.35, 0.05 M sodium succinate buffer, the retentate proteins exhibited a two-and-a-

half-fold purification of endoglucanase activity. This apparent purification could be the result of eliminating the yellow substances from the solution since its absorbance may have interferred with determination of protein in this fraction. Similar electrophoretic protein patterns before and after this filtration procedure indicated that the apparent purification was not due to the elimination of any specific protein component.

Since an ion-exchange batch separation was successful in further purification of the water fraction, it was utilized in an analogous manner with the buffer fraction retentate. A two-and-a-half-fold purification of endoglucanase activity was obtained in the fraction I (pH 5.35) eluate. Disc gel electrophoretic patterns of the proteins resulting from the batch separation are shown in Figure 12. Unlike the water fraction in which most of the protein was associated with fraction III, batch separation of the buffer fraction yielded nearly equal amounts of protein in fractions I and III. In the latter case significantly more protein (50% of buffer fraction protein compared to 12% of water fraction protein) was recovered with fraction I. Forms A and B of the exo-cellobiohydrolase had been identified previously in fraction III of the buffer fraction[8] and probably are responsible for the lower protein bands in this gel. Disc gel electrophoretic patterns of the β-glucosidase present in the buffer fraction (28) indicated that it is the most slowly migrating protein represented by the top bands in fraction I and is associated with the endoglucanase-rich fraction. The gels further illustrate the useful-

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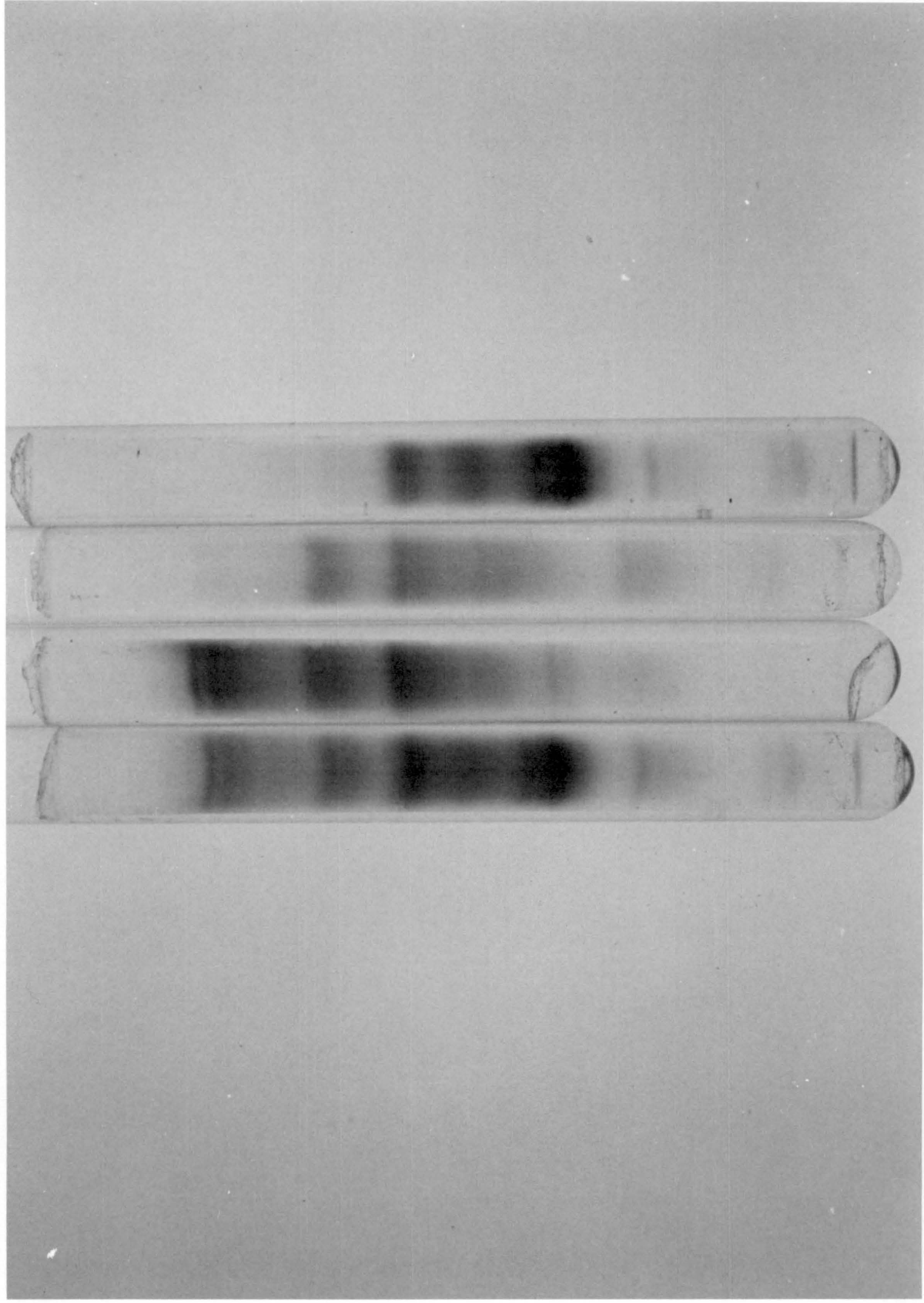
[8] Gum, E. K. Jr. (1976) Personal Communication.

FIGURE 12

Disc Gel Electrophoresis of Protein Fractions Obtained from DEAE-Sephadex Batch Separation of the Buffer Fraction

Protein samples which had been applied to these gels were from left to right 175 μg of buffer fraction, 124 μg of fraction I (pH 5.35), 67 μg of fraction II (pH 5.0), and 135 μg of fraction III (pH 3.6). The gels were stained for protein with Coomassie blue. Electrophoretic conditions were the same as in Figure 7.



ness of this ion-exchange batch process in separating protein components based on their net ionic charge.

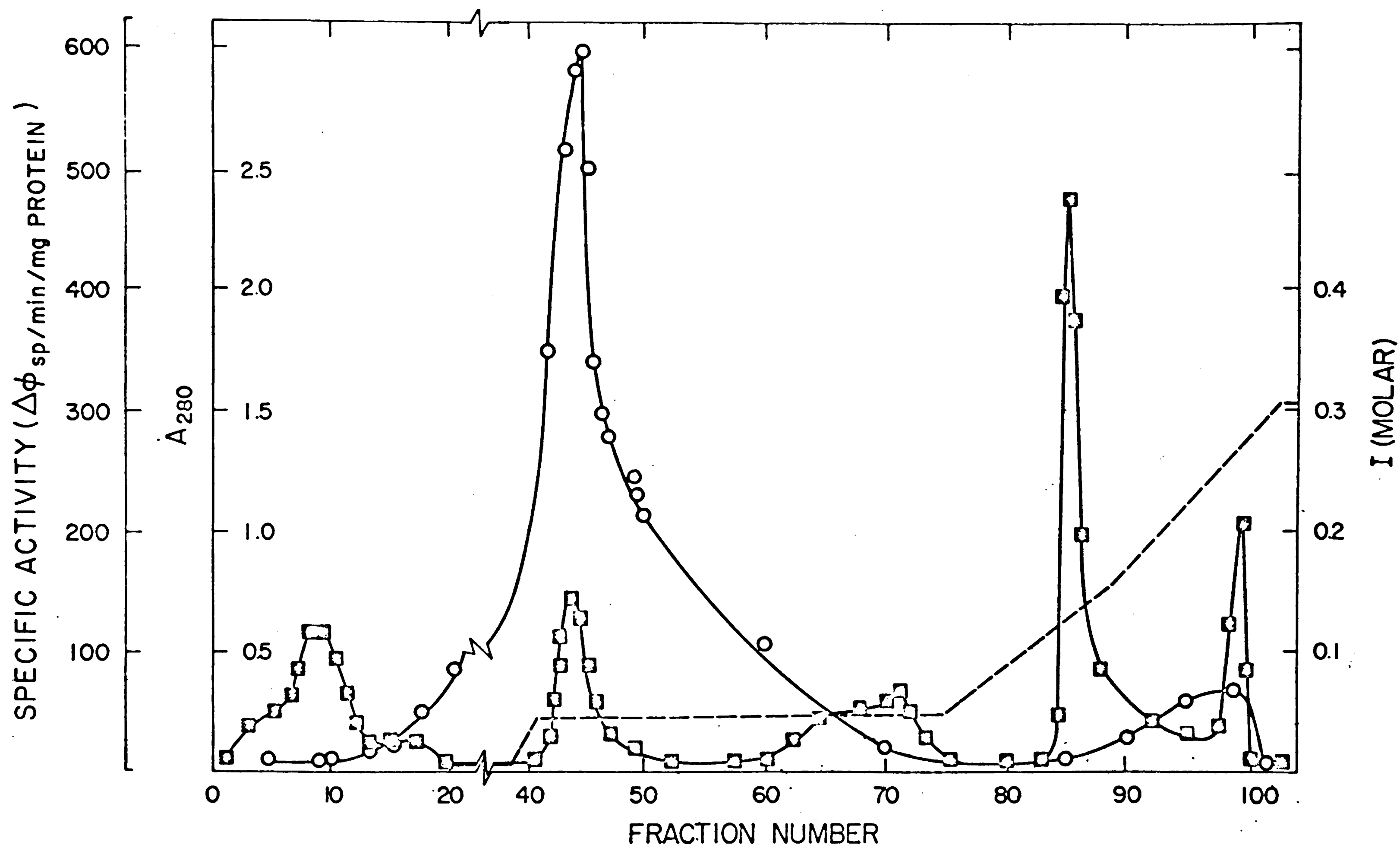
DEAE-Sephadex Column Chromatography of the Buffer Fraction Endoglucanases - Fraction I from a DEAE-Sephadex batch separation was concentrated and dialyzed with pH 6.0, 0.05 M imidazole-3.0 mM sodium azide buffer on a PM 10 membrane. Again some purification of endoglucanase activity was noted during the process and some 20% of the protein was found in the ultrafiltrate; whereas, the retentate exhibited a one-and-a-half-fold increase in specific endoglucanase activity. Initial attempts to purify endoglucanases from this retentate fraction, using conditions identical to those employed for purification of the water fraction gave poor separation of β-glucosidase and endoglucanase activities.

Lowering the initial ionic strength of the buffer to 0.01 M and then stepping up to 0.07 M gave excellent separation of β-glucosidase and endoglucanase activities (Figure 13). The β-glucosidase was eluted under initial buffer conditions of 0.01 M ionic strength (fractions 0-12) which is consistent with its position on analytical disc gels. Protein fractions (41-45) that were highly active in reducing the viscosity of CM-cellulose solutions, eluted after the ionic strength had been increased to 0.07 M. This endoglucanase fraction (Endoglucanase II) yielded two protein bands of approximately equal concentration when analyzed by disc gel electrophoresis. A third protein peak (fractions 60-75) eluted from this column at the same ionic strength but contained neither β-glucosidase or endoglucanase activity. Further increases in ionic strength to 0.15 M and 0.03 M caused the elution of a sharp protein peak (fractions 83-90)

FIGURE 13

Elution Patterns from DEAE-Sephadex Column Chromatography of the Proteins in the Buffer Fraction-Derived Fraction I Retentate

The retentate from fraction I of a batch separation was applied to a DEAE-Sephadex column (2.5 x 45 cm). The proteins were eluted using a flow rate of 18-20 ml/hr and a step gradient (ionic strength) in pH 6.0, 0.05 M imidazole-3.0 mM sodium azide buffer. The initial ionic strength of this buffer was 0.01 M which was followed by subsequent increases in ionic strength from 0.07 M (fraction 35) to 0.15 M (fraction 72) and finally to 0.3 M (fraction 92). Protein concentration is indicated by absorbance at 280 nm (-□-□-□-). Specific endoglucanase activity (-o-o-o-) and ionic strength (I) of the effluent are also given. Fractions were approximately 2.5 ml each and were collected every ten minutes.



SPECIFIC ACTIVITY (Δφ sp/min/mg PROTEIN)
600
500
400
300
200
100
A280
2.5
2.0
1.5
1.0
0.5
I (MOLAR)
0.4
0.3
0.2
0.1
0
10
20
40
50
60
70
80
90
100
FRACTION NUMBER

and a second endoglucanase (fractions 97-100), respectively. Disc gel electrophoresis of this second endoglucanase fraction (Endoglucanase I) demonstrated two protein bands in approximately equal amounts, each of which was apparently slightly more anionic than the two bands from Endoglucanase II.

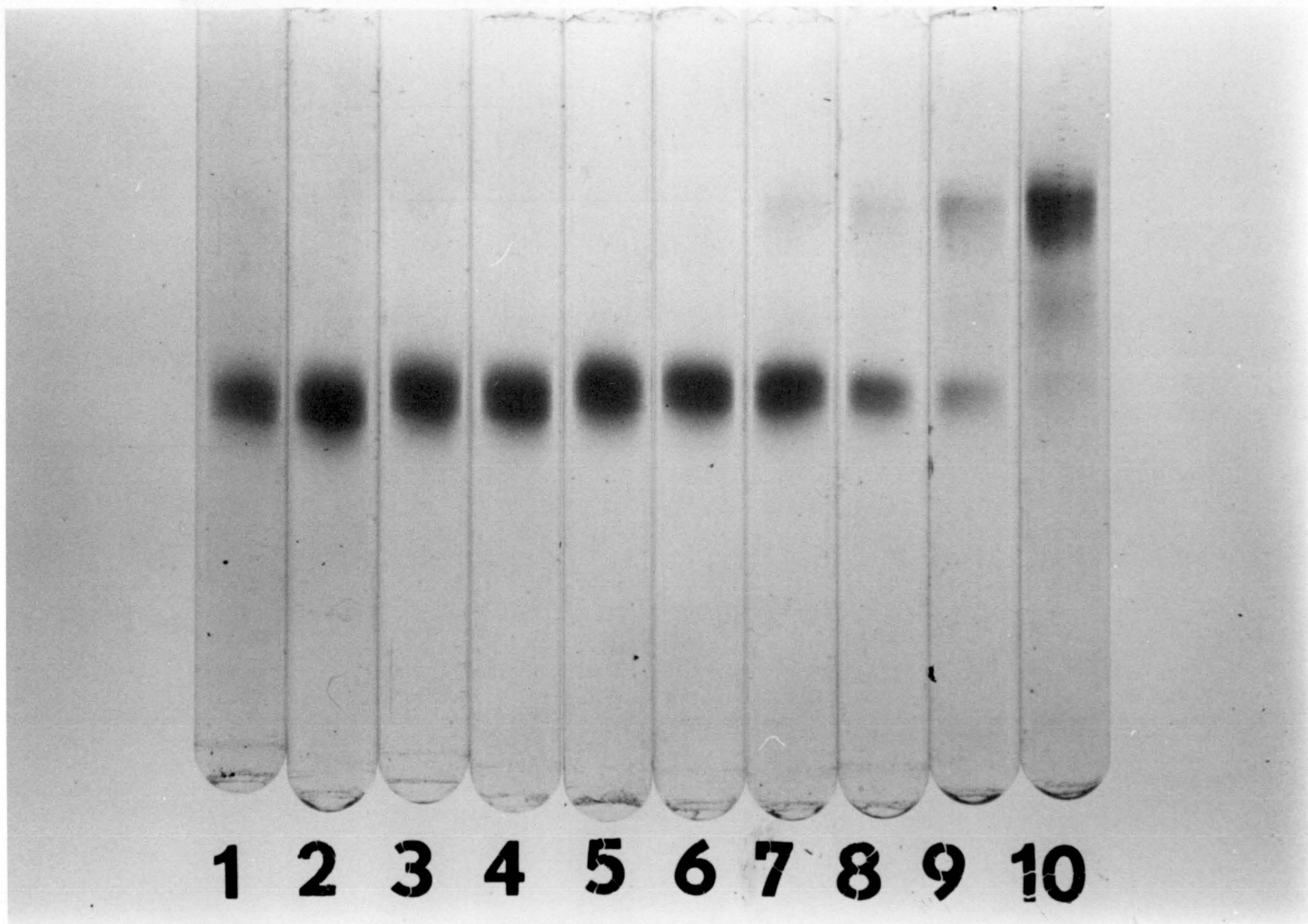
Fractions 41-45, which contained Endoglucanase II, were combined, concentrated and dialyzed with pH 6.0, 0.05 M imidazole-3.0 mM sodium azide buffer (ionic strength = 0.01 M) and applied to a DEAE-Sephadex column. After elution of minor amounts of protein, the ionic strength was stepped up to 0.07 M and a nearly symmetrical protein peak eluted from this column. Evaluation of enzyme homogeneity was accomplished by disc gel electrophoresis of equal amounts of protein from each fraction collected within this peak. The resulting protein patterns (Figure 14) illustrate the excellent separation of the two proteins. Endoglucanase activity was associated with fractions exhibiting the lower protein band. Fractions represented by gels #3-6 were combined to give purified Endoglucanase II.

Attempts to purify several milligrams of Endoglucanase I by further ion-exchange chromatography have been unsuccessful. A small quantity of this endoglucanase was obtained from one such ion-exchange column and subsequent disc gel analysis indicated that the lower protein band was indeed the one associated with endoglucanase activity. The specific activity of the purified material was found to be 250 $\Delta\phi_{sp}$/min/mg protein. However, quantities of this enzyme sufficient for its structural characterization were not obtained.

FIGURE 14

Disc Gel Electrophoretic Patterns of Successive Fractions of Endoglucanase II Obtained from Chromatography of Fractions 41-45 from a DEAE-Sephadex Column on a Second DEAE-Sephadex Column

Proteins in fractions 41-45 from DEAE-Sephadex column chromatography were separated on a second DEAE-Sephadex column (cf. p.84). Gels #1 through #10 represent successive fractions from the protein peak which eluted at an ionic strength of 0.07 M from the second DEAE-Sephadex column. The gels, to each of which had been applied 38 μg of protein, were stained with Coomassie blue. Electrophoresis of the starting material applied to this column gave two protein bands corresponding in position to bands in gel #1 and gel #10. Electrophoretic conditions are the same as in Figure 7.


1 2 3 4 5 6 7 8 9 10

Summary of the Purification of a Buffer Fraction Endoglucanase - Two endo-1,4-β-D-glucanases were identified in the buffer fraction and both were initially associated with a second protein which ran more slowly on analytical disc gels. A substantial quantity (30 mg) of only one of these endoglucanases (Endoglucanase II) was purified to homogeneity. Quantification of protein and enzymic activity in its purification from Pancellase protein are presented in Table V. Most of the protein and endoglucanase activity were associated with the buffer fraction from an Avicel column, although no increase in specific activity resulted from this step. The batch elution of buffer fraction protein adsorbed on DEAE-Sephadex at pH 5.35 (fraction I) resulted in a two-fold increase in specific endoglucanase activity. Ultrafiltration of the buffer fraction either after elution from an Avicel column or following the anion-exchange batch separation at pH 5.35 (fraction I) provided a significant increase in endoglucanase activity in the retentate. This was, in part, due to removal of low-molecular-weight proteins.

Principal separation of endoglucanases from other protein components in fraction I of the buffer fraction was accomplished by anion-exchange column chromatography. This step resulted in a five- to six-fold increase in specific endoglucanase activity. A second DEAE-Sephadex column provided an additional three-fold purification yielding a homogeneous protein upon disc gel electrophoretic analysis.

It may be noted that similar elution patterns on ion exchange columns were obtained for water and buffer fractions. In both fractions, the endoglucanases with the highest specific activity eluted at an ionic strength of 0.07 M, a large protein peak eluted at 0.15 M, and a second

TABLE V

Summary of Purification of Buffer Fraction Endoglucanase

		PROTEIN (g)		TOTAL ACTIVITY ($\Delta\phi_{sp}$/min)		SPECIFIC ACTIVITY ($\Delta\phi_{sp}$/min/mg protein)
AVICEL COLUMN:	PANCELLASE	12.1		128,260		10.6
	BUFFER FRACTION	9.43	(77.9%)[a]	98,072	(76.5%)	10.4
	WATER FRACTION	1.45	(12.0%)	23,345	(18.2%)	16.1
BATCH SEPARATION:	BUFFER FRACTION	1.59		40,227		25.3
	FRACTION 1, pH 5.35	0.863	(54.3%)	40,820	(101%)	47.3
	FRACTION 2, pH 5.0	0.126	(7.9%)	3,591	(8.9%)	28.5
	FRACTION 3, pH 3.6	0.476	(30.1%)	1,317	(3.3%)	2.75
DEAE COLUMN:	FRACTION 1	0.277		18,559		67
	ENDOGLUCANASE II	0.025	(9.0%)	9,000	(48.5%)	360
	ENDOGLUCANASE I	0.035	(12.6%)	2,100	(11.3%)	60
DEAE COLUMN:	ENDOGLUCANASE II	0.036		11,880		330
	ENDOGLUCANASE II	0.009		9,090	(76.5%)	1010

[a]( ) = % recovery of protein or activity in that step.

endoglucanase, with approximately one-third the specific activity of the first, eluted at 0.3 M. The specific activities of the buffer fraction endoglucanases (Endoglucanases I and II) were four times that of the corresponding water fraction endoglucanases (Endoglucanases III and IV). The main differences between buffer and water fractions are that there are pairs of protein bands apparently associated with impure Endoglucanases II and I.

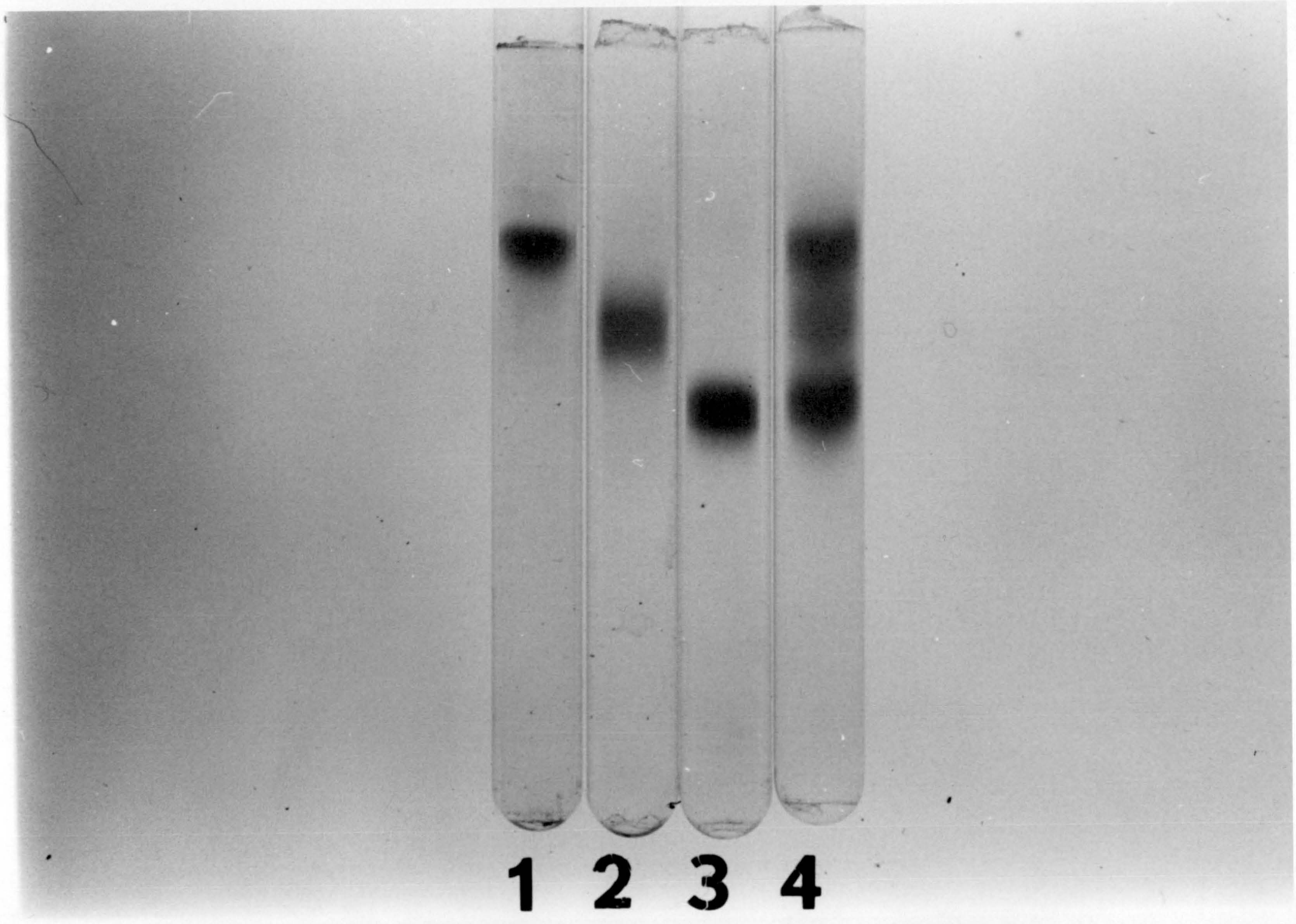
Electrophoretic Evidence for the Purity of Endo-1,4-β-D-glucanases from *Trichoderma viride* - Four electrophoretically distinct endo-1,4-β-D-glucanases, two from the water fraction (Endoglucanases III and IV) and two from the buffer fraction (Endoglucanases I and II), have been identified from *Trichoderma viride*. Three of these endoglucanases (Endoglucanases II, III and IV) have been purified to homogeneity as evidenced by a single protein band when analyzed by disc gel electrophoresis (Figure 15). Endoglucanase II originating from the buffer fraction migrated further than the two water fraction enzymes suggesting a more acidic and/or lower-molecular-weight protein. Further attempts to use low pH gel systems (pH 4.3 or pH 2.9) described by Maurer (77) to demonstrate enzyme homogeneity were not successful, since the proteins were not resolved and remained at the top of the separator gel even after 15 hours of electrophoresis. Although protein bands migrated into the separator gel using a pH 7.5 gel system (77), protein bands of these enzymes were wider and not as well resolved as in the high pH gel system usually employed.

In a duplicate experiment to that illustrated by Figure 15, staining for carbohydrate instead of protein revealed that the water fraction endo-

FIGURE 15

Disc Gel Electrophoretic Protein Patterns of Three Endo-1,4-β-D-glucanases Purified from *Trichoderma viride*

The protein samples applied to these gels were from left to right 29 μg of Endoglucanase IV, 29 μg of Endoglucanase III, 33 μg of Endoglucanase II, and 33, 29, and 29 μg of Endoglucanases II, III and IV, respectively. The gels were stained for protein with Coomassie blue. Electrophoretic conditions were the same as in Figure 7.


1
2
3
4

glucanases, which are cellulose binding proteins, contain a significant amount of carbohydrate. Only a minute amount of carbohydrate is associated with Endoglucanase II, a buffer fraction enzyme (Figure 16). The higher affinities of Endoglucanase III and IV for crystalline cellulose was associated with higher levels of carbohydrate in these glycoprotein enzymes.

In order to examine the endo-1,4-β-D-glucanases for the presence of subunits, verify homogeneity and establish relative molecular weights, they were denatured in one percent SDS and subsequently analyzed by slab gel electrophoresis (Figure 17). The single protein bands which were observed and confirmed by spectrodensitometry for each of the cellulase enzymes (taken together with the molecular weights determined by sedimentation equilibrium), indicate a lack of enzyme subunits or heterogeneity. The position of the protein bands on SDS gels is often useful for the determination of the molecular weights of proteins provided appropriate standard proteins also have been run. The relative positions of the cellulase bands indicated that Endoglucanase II is of lower-molecular-weight. The exo-cellobiohydrolase C and the β-glucosidase, which have molecular weights of approximately 51,200 (28) and 48,000 (50), respectively, appeared to be similar in size to the water fraction endoglucanases (Endoglucanases III and IV). However, estimates of the molecular weights of glycoproteins based on mobility in SDS gels have been questioned (91,92) since the carbohydrate portion of the protein causes decreased binding of SDS when compared to standard proteins (92) and results in an erroneously higher apparent molecular weight. Thus this analysis suggests

FIGURE 16

Disc Gel Electrophoretic Patterns of Carbohydrate Associated with Three Endo-1,4-β-D-glucanases Purified from *Trichoderma viride*

The protein samples applied to these gels were the same as in Figure 15. The gels were stained for carbohydrate with periodic acid-Schiff base. Electrophoretic conditions were the same as in Figure 7.

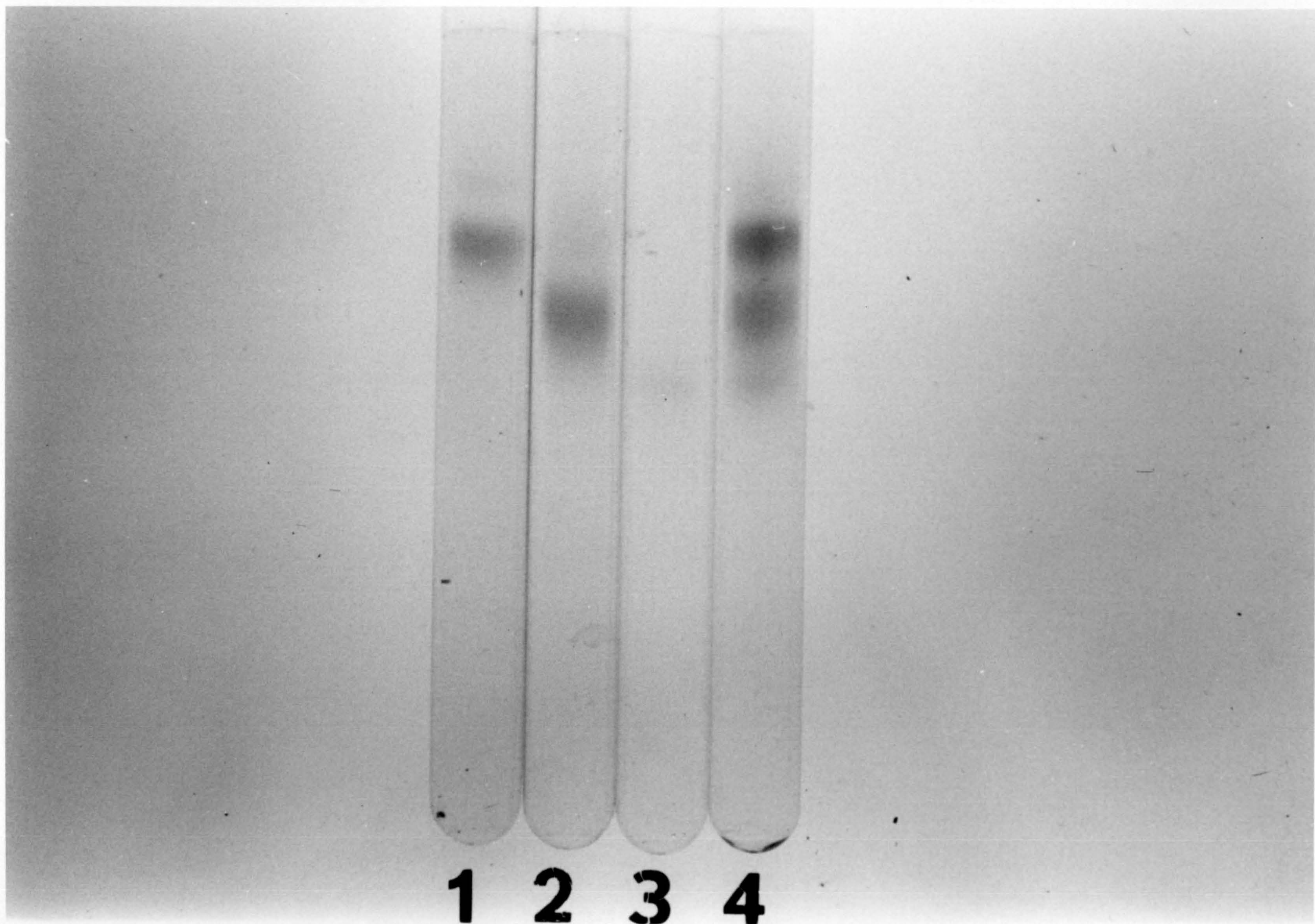
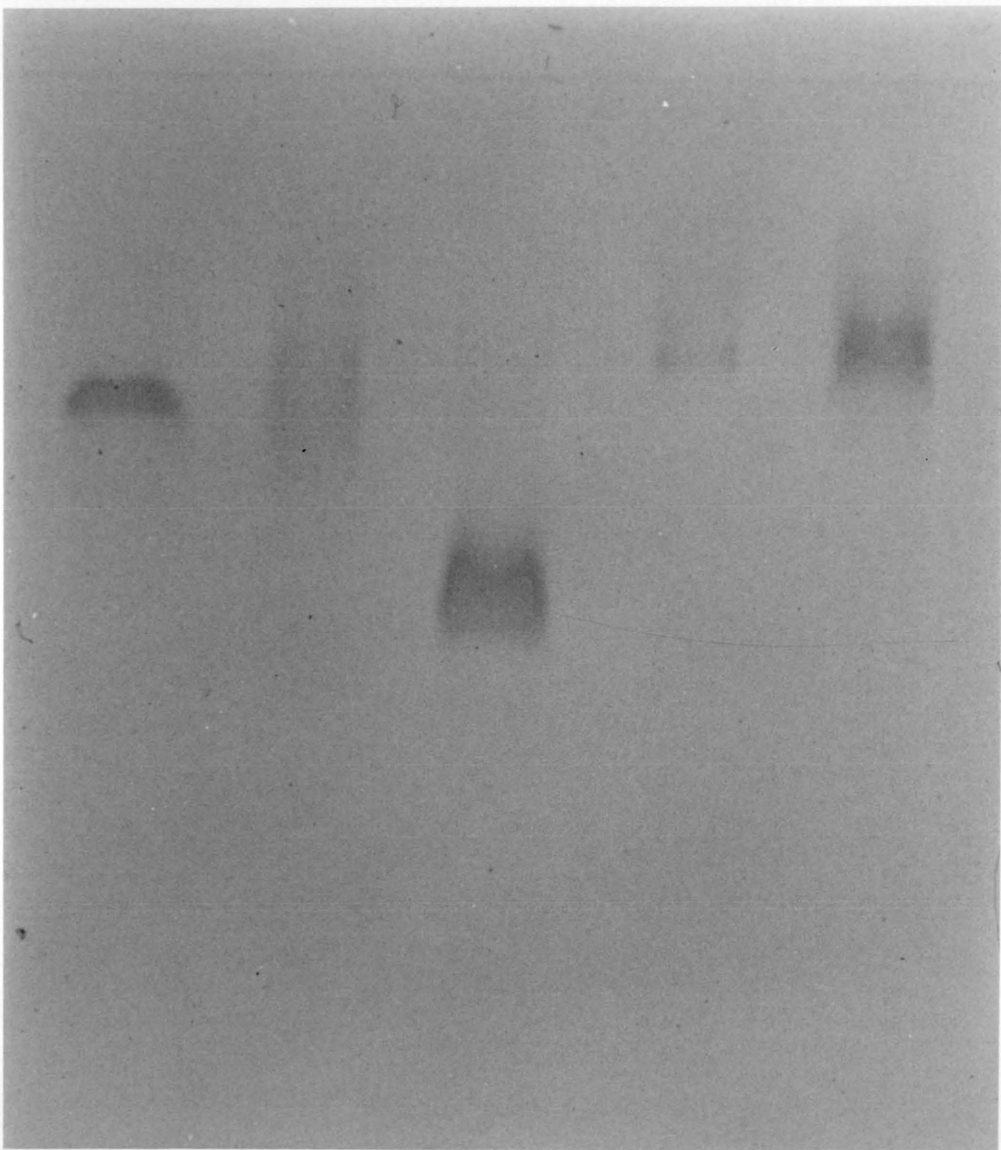

1 2 3 4

FIGURE 17

Slab Gel Electrophoresis of SDS-Protein Complexes with Purified Cellulase Components

Protein samples subjected to electrophoresis in a one percent SDS, 15% polyacrylamide slab gel were from left to right 23.9 μg of Endoglucanase IV, 17.4 μg of Endoglucanase III, 14.2 μg of Endoglucanase II, 4.5 μg of β-glucosidase (molecular weight = 51,200) and 16 μg of exo-cellobiohydrolase C (molecular weight = 48,300). The gels were prepared as described in Experimental Procedures (cf. p.34), stained for protein using Coomassie blue, and the position of bands determined using a Schoeffel Model SD-3000 Recording Spectrodensitometer. The running time was three hours at a constant current of 30 milliamps.



only that the molecular weight of Endoglucanase II is somewhat lower than that of the other cellulase enzymes.

Multiple forms of glycoprotein endoglucanases have been reported for endoglucanases purified from *Sporotrichum pulverulentum* (22,55) and *Trichoderma viride* (19,21). Two endoglucanases recently have been purified from *Trichoderma viride* by Okada (19) and by Berghem *et al.* (21). Okada (19) purified endo-cellulases II-A and II-B using mainly gel filtration and ion-exchange chromatography and found 12 and 14% carbohydrate associated with these endoglucanases. This substantial carbohydrate content is consistent with results on the two water fraction enzymes. Berghem *et al.* (21) used a variety of chromatographic techniques in the purification from *Trichoderma viride* of two endoglucanases which they termed " endoglucanases I (low-molecular-weight) and II (high-molecular-weight)." They observed that their "endoglucanase II" readily passed through an Avicel column on which an exo-cellobiohydrolase was retained. Thus it may be analogous to the buffer fraction enzyme reported here. However, carbohydrate analysis using the orcinol-sulfuric acid method indicated that their "endoglucanase II" contained 12% carbohydrate, which was much higher than found with the buffer fraction enzyme, Endoglucanase II (*v.i.*)

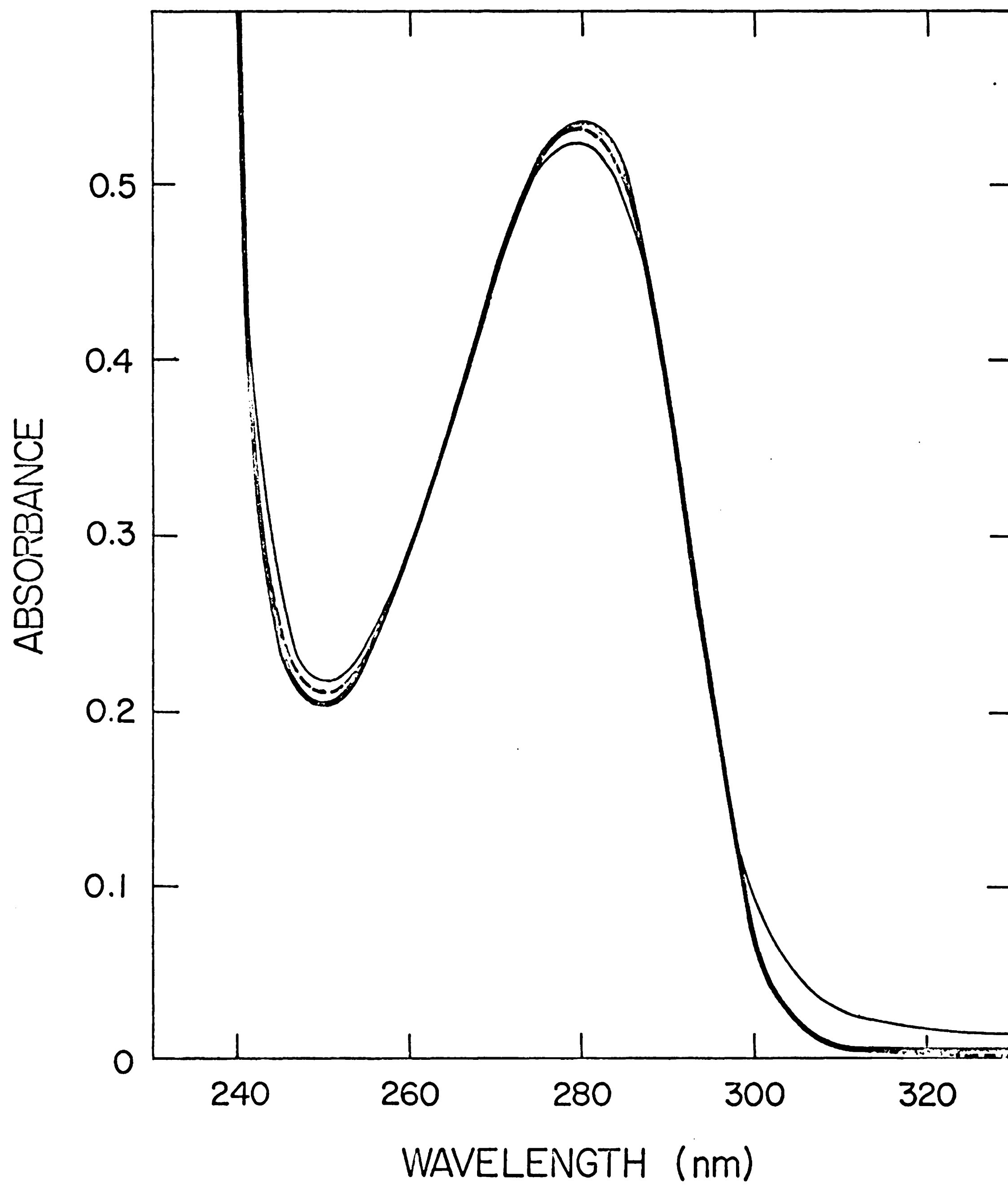
Structural Characterization of Endo-1,4-β-D-glucanases

Absorption Spectra Analyses of Endo-1,4-β-D-glucanases - The ultraviolet light absorption characteristics of the purified endo-1,4-β-D-glucanases were examined initially to identify any significant differences among these glycoprotein enzymes. The ultraviolet absorption spectra of Endoglucanases II, III and IV were almost identical (Figure 18) with ab-

FIGURE 18

Ultraviolet Absorbance Spectra of Purified Endoglucanases II, III and IV in Water

The concentration of endoglucanases II (▬▬), III (——) and IV(---) were 0.56 mg/ml, 0.57 mg/ml and 0.57 mg/ml, respectively, in distilled water. Each spectrum was determined using a Beckman Spectrophotometer, Model ACTA-MVI.


0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
240
260
280
300
320
WAVELENGTH (nm)

sorbance maxima at wavelengths ranging from 279.5 to 280.3 nm and minima from 249.7 to 250.4 nm. The quantitative relationship between the absorbance at 280 nm of a one percent enzyme solution and its actual dry weight may be expressed in terms of an extinction coefficient ($E_{280}^{1\%}$). The extinction coefficients for Endoglucanases II, III and IV were determined by the method outlined in Experimental Procedures (cf. p. 40) and found with associated standard errors to be 11.97 $\pm$ 0.35, 10.32 $\pm$ 0.37 and 13.12 $\pm$ 0.18, respectively. This may be compared to an $E_{280}^{1\%}$ equal to 14.2 that was previously determined for the exo-cellobiohydrolase C (24).

Ultracentrifugation Studies - Both sedimentation velocity and sedimentation equilibrium experiments were conducted using purified endo-1,4-β-D-glucanases II, III and IV on the Beckman-Spinco Model E Ultracentrifuge. Sedimentation velocity studies were performed at a single enzyme concentration in order to estimate sedimentation coefficients and establish further the homogeneity of the endoglucanases. The amount of purified enzyme needed for each analysis (1.5 mg) limited this study to only one concentration.

Endoglucanases II, III and IV solutions in 0.1 M potassium chloride (KCl), contained 2.55, 3.09 and 3.56 mg enzyme/ml, respectively. The rotor velocity was 59,780 rpm, and Schlieren photographs depicting the concentration gradient were taken every eight minutes. Analysis of the Schlieren patterns of these endoglucanases revealed no macro-heterogeneity since a single sedimenting boundary was observed at all times. Sedimentation coefficients were calculated from the peak positions at different times and found to be 3.76S, 4.08S and 3.95S for Endoglucanases II, III

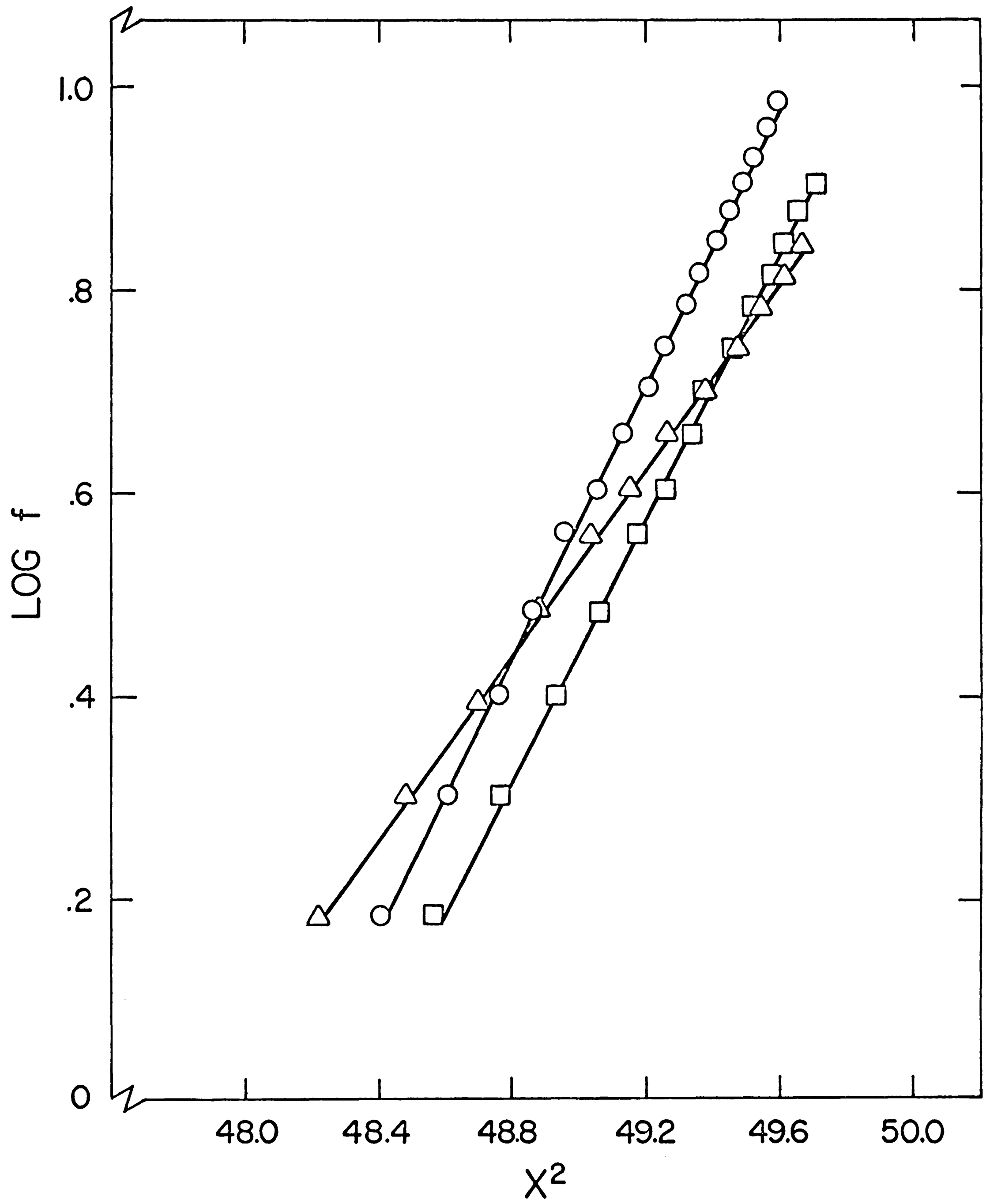
and IV, respectively. These values must be viewed with caution as only one enzyme concentration was used for their determination. However, they do give some indication of the relative size of these macromolecules and suggest that Endoglucanase II is probably a lower-molecular-weight molecule.

Sedimentation equilibrium studies were conducted (20,410 rpm) to determine the molecular weight and state of aggregation of each purified endoglucanase. The samples used in this analysis were 0.1 ml of each enzyme solution containing 1.01 to 2.33 mg enzyme/ml in 0.1 M KCl. Fringe displacements obtained by Rayleigh interference optics were measured using a Nikon Shadograph, Model 6 C. A straight line was obtained for each endoglucanase when the logarithm of the fringe displacement was plotted versus the square of the distance from the center of the rotor (Figure 19). This relationship confirmed the absence of protein aggregates and indicated a monodisperse enzyme solution. A weight-average molecular weight was calculated for each endoglucanase using the long-column menicus depletion technique of Yphantis as modified by Chervenka (93). The partial specific volumes of Endoglucanase II, III and IV were calculated from amino acid and carbohydrate compositions by the method of Cohn and Edsall (88) and found to be 0.711, 0.696 and 0.698, respectively. Computer analysis yielded weight-average molecular weights of 37,200 $\pm$ 2,400, 52,000 $\pm$ 2,600 and 49,500 $\pm$ 2,200 for Endoglucanases II, III and IV, respectively. This method of molecular weight determination is thought to be the most accurate physical method available (94). Thin layer gel filtration and SDS-slab gel electrophoresis systems served to confirm the

FIGURE 19

Sedimentation Equilibrium Analysis
of Purified Endoglucanases II, III and IV

This plot represents the logarithm of fringe displacement (log f) versus the square of the distance from the center of the rotor (X^2). The sample concentrations of Endoglucanases II (Δ), III (o) and IV (◘) were 1.16, 1.53 and 2.02 mg/ml, respectively, in 0.1 M KCl solution. The rotor speed was 20,410 rpm.


1.0
.8
.6
.4
.2
0
LOG f
48.0
48.4
48.8
49.2
49.6
50.0
X^2

relative molecular weights of the endoglucanases but were unsuccessful in providing reliable absolute molecular weights.

Amino Acid Composition of Endo-1,4-β-D-glucanases - The amino acid compositions of Endoglucanases II, III and IV, expressed as mole percent, are presented in Table VI. These enzymes were shown to be high in acidic and hydroxylated amino acids and glycine but, like other 1,4-β-D-glucanases (12,23-25,35,50,51,58), the three *Trichoderma* enzymes are low in basic amino acids. Endoglucanase III differed most in its amino acid composition. Among the three endoglucanases it contained the lowest percentage of arginine, glutamic acid (or amide), isoleucine and phenylalanine, and the highest percentage of threonine and proline. The compositions of Endoglucanases II and IV differed by less than 0.5 mole percent for all but four amino acids. Of these four amino acids, Endoglucanase II contained a higher percentage of leucine and lower percentages of threonine, proline and half cystine. Of the amino acid constituents, proline content varied most widely among these endoglucanases.

Carbohydrate Composition of Endo-1,4-β-D-glucanases - The carbohydrate composition of the endoglucanases was investigated as described in Experimental Procedures (cf. p.43) and the results are shown in Table VII. Since amino sugar analysis revealed the presence of glucosamine, but not galactosamine, in each of the endoglucanases, galactosamine was used as an internal standard from which recovery of glucosamine was calculated. Glucosamine was assumed to be present in its N-acetylated form. Endoglucanase III contained twice as much N-acetyl-glucosamine as either Endo-

TABLE VI

Amino Acid Composition of Endoglucanases II, III and IV

Amino Acid	II[a]	III[a]	IV[a]
Lysine	2.0	1.7	1.8
Histidine	1.5	1.1	1.4
Arginine	2.6	1.4	2.7
Aspartic acid	13.8	12.8	12.3
Threonine	8.8	11.1	10.2
Serine	9.4	11.9	9.5
Glutamic acid	8.6	6.6	8.6
Proline	4.5	7.6	5.7
Glycine	11.1	11.0	11.8
Alanine	7.8	7.4	8.1
Half cystine	2.0	3.3	2.9
Valine	5.7	5.2	5.3
Methionine	1.1	1.3	0.8
Isoleucine	5.1	3.5	4.7
Leucine	6.8	5.7	5.4
Tyrosine	3.5	3.9	3.3
Phenylalanine	3.4	2.1	3.1
Tryptophan	2.4	2.5	2.5

[a]Mole percent, determined as described in Experimental Procedures.

TABLE VII

Carbohydrate Composition (Weight Percent) of Endo-1,4-β-D-glucanases[a]

	II	III	IV
Total Amino Sugar			
N-Acetyl Glucosamine	0.28	0.44	0.20
Total Carbohydrate (PSA)	3.2	14.0	15.9
Neutral Carbohydrate (GC)			
Total	4.5	15.0	15.2
Mannose	2.49	11.4	10.9
Galactose	1.05	1.68	2.20
Glucose	0.95	1.92	2.13

[a]Determined as described in Experimental Procedures.

glucanase II or IV, and thus, as with amino acid content, it was also the most distinctive endoglucanase in regard to its content of N-acetyl glucosamine.

Total carbohydrate content measured by the phenol-sulfuric acid method using mannose as standard was in agreement with the previous results from carbohydrate staining procedures. Thus Endoglucanase II possessed much less carbohydrate (3.2%) than either Endoglucanase III or IV (14.0 or 15.9%, respectively). These results were in good agreement with the weight percent of total carbohydrate determined by gas chromatography of the alditiol acetate derivatives (Table VII). After identification of the neutral sugar derivatives by gas chromatography and confirmation using gas chromatography-mass spectrometry, these compounds were measured quantitatively by gas chromatography using xylose as an internal standard. Like the exo-cellobiohydrolase C (24), all three of the endoglucanases contained mannose, galactose and glucose. Mannose was the predominant neutral sugar in each enzyme. The water fraction endoglucanases (Endoglucanases III and IV) have nearly identical neutral sugar compositions, whereas the buffer fraction endoglucanase (Endoglucanase II) contained a much smaller amount of mannose. Unlike the results from amino acid and amino sugar determinations, Endoglucanase III did not differ remarkably from Endoglucanase IV in its neutral carbohydrate composition. On the contrary, Endoglucanase II, a buffer fraction enzyme, exhibited a much lower proportion of total neutral sugars (particularly mannose) than either Endoglucanases III and IV.

Summary of Structural Characterization Studies of Endo-1,4-β-D-glucanases - By use of the molecular weights calculated for the enzymes from sedimentation equilibrium data, the compositions of Endoglucanases II, III and IV were expressed as mole of constituent per mole of endoglucanase (Table VIII). In this determination amino acids, glucosamine and neutral sugars were assumed to constitute all of the dry weight of the enzymes although these components apparently accounted for only 68.2%, 104.3% and 88.5% of the dry weight of Endoglucanases II, III and IV, respectively. Repeated amino acid analysis failed to correct this discrepancy; however, consistent results were obtained and the basis for the less-than-complete aggregate compositions of Endoglucanases II and IV is not known, but may be due, in part, to adsorbed ionic materials.

The composition of the water fraction enzymes (Endoglucanases III and IV) can be compared directly because of their similar molecular weights. The significantly different amounts of arginine, serine, proline and glucosamine in Endoglucanases III and IV justifies naming these enzymes as isozymes.

Endoglucanase II is also named as an isozyme; however, on the basis of its composition alone this nomenclature could be questioned. This buffer fraction enzyme is smaller and thus, could have arisen from proteolytic modification of precursor molecules. Endoglucanase II has significantly less carbohydrate, especially mannose, but is relatively high in phenylalanine, leucine, isoleucine, glutamic acid, aspartic acid and arginine. In addition, its lack of an affinity for crystalline cellulose yet high endoglucanase activity with CM-cellulose reflects unique activity and specificity relationships when compared to the water fraction endoglucanases.

TABLE VIII

Composition of Endoglucanases II, III and IV

	Mole/Mole Endoglucanase[a]		
	II	III	IV
Lysine	6	7	7
Histidine	5	5	6
Arginine	9	6	11
Aspartic Acid	46	54	49
Threonine	29	47	40
Serine	31	50	38
Glutamic Acid	28	28	34
Proline	15	32	23
Glycine	37	45	47
Alanine	26	31	32
Half Cystine	7	14	12
Valine	18	22	21
Methionine	4	5	3
Isoleucine	17	15	19
Leucine	23	24	21
Tyrosine	12	17	13
Phenylalanine	11	9	12
Tryptophan	8	11	10
Glucosamine	0.5	1.1	0.5
Mannose	5.9	36.6	33.2
Galactose	2.4	5.4	6.7
Glucose	2.2	6.2	6.5
Molecular Weight	37,200	52,000	49,500

[a]Determined as described in Experimental Procedures.

These endoglucanases, in general, were found to be very similar in size and composition to the exo-cellobiohydrolase C (24,50) and were in agreement with literature reports of molecular weight and total carbohydrate of purified endo-1,4-β-D-glucanases from *Trichoderma viride* (19,21). The "endoglucanase II" purified by Berghem *et al.* (21) and initially thought to be like the buffer fraction Endoglucanase II was found to have a higher molecular weight (50,000) by sedimentation equilibrium and thus, is probably not the same enzyme. A second endoglucanase, "endoglucanase I", was found by gel filtration to have an extremely low-molecular-weight (12,500). Thus the two endoglucanases purified by Berghem *et al.* (21) do not appear identical to any of the endoglucanases from our laboratory. One possible explanation for these "different" enzymes could be strain variation within the *Trichoderma* species.

In addition, Okada (19) reported molecular weights of 30,000 and 43,000 for endoglucanases II-A and II-B determined by gel filtration on a Sephadex G-100 column. This procedure has been found to give erroneous results with glycoproteins (95). Since no other attempts to characterize endoglucanases II-A and II-B have been reported, it is impossible to compare further these endoglucanases based on structural properties.

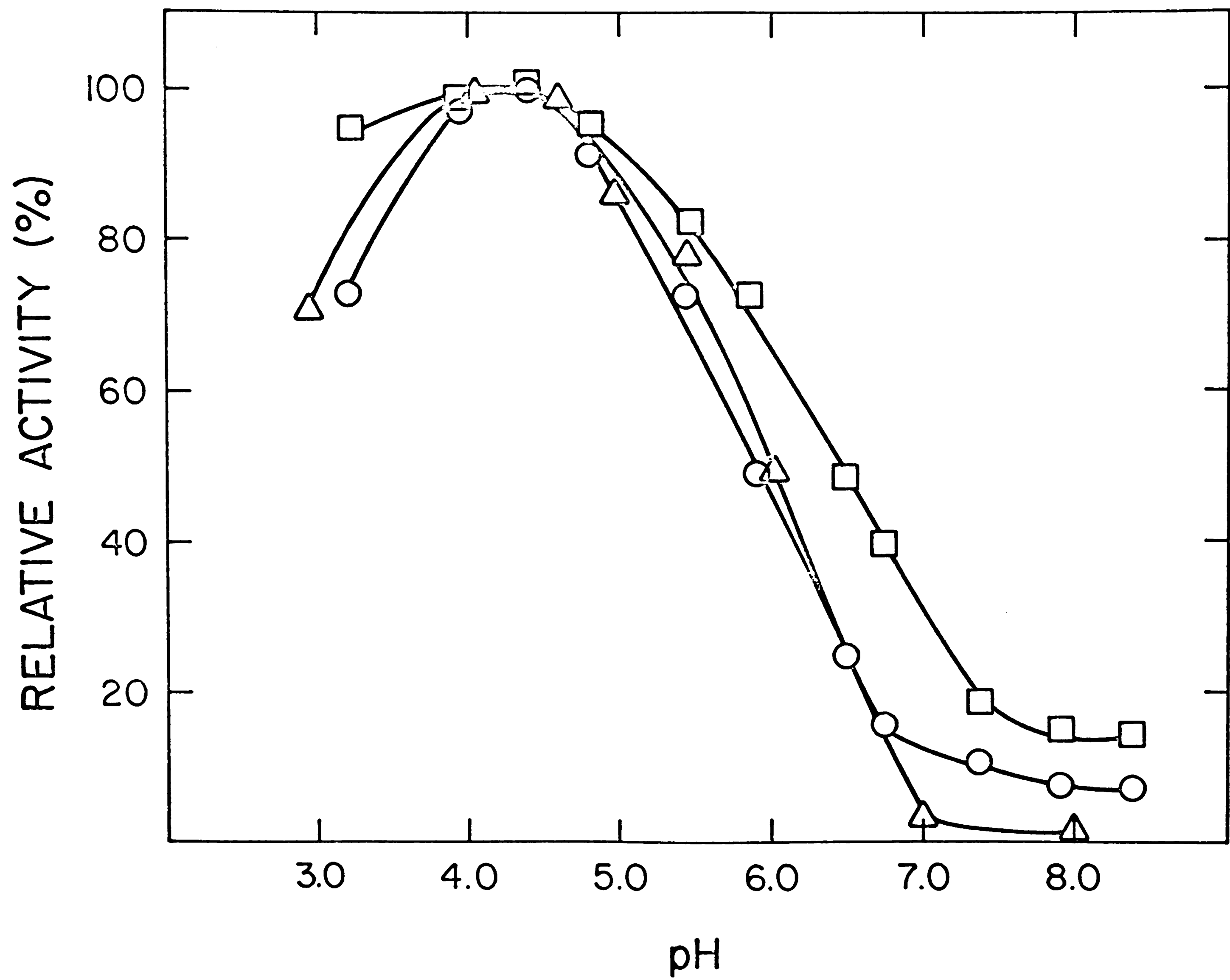
Enzyme Activity and Specificity

pH Optimum and pH Stability - The optimum pH for enzymic activity was investigated using both phosphoric acid swollen cellulose (PSC) and carboxymethylcellulose (CM-cellulose) as substrates. Endoglucanases II, III and IV exhibited similar pH dependence, giving optimum activities at pH 4.5 with PSC (Figure 20), whereas a slightly different pH dependence

FIGURE 20

pH Optima for Endoglucanases II, III and IV with Phosphoric Acid Swollen Cellulose as Substrate

Enzyme samples were incubated with phosphoric acid swollen cellulose suspensions at different pH values at 40° for 30 minutes (cf. p.46) and assayed for activity using the reducing sugar method with glucose as standard. The curves represent Endoglucanases II (Δ), III (o) and IV (□).


RELATIVE ACTIVITY (%)
100
80
60
40
20
3.0
4.0
5.0
6.0
7.0
8.0
pH

among the endoglucanases was observed with CM-cellulose (Figure 21). In this case, the pH optimum was found to be lower for Endoglucanase II (pH 3.2-4.2) than for Endoglucanases III (pH 4.5) and IV (pH 4.0-4.5). These observed optima are in agreement with those reported for endoglucanases II-A and II-B (pH 4.5-5.0) (19). To permit direct comparison of results using each enzyme with a variety of substrates, all assay reaction mixtures were buffered at pH 4.5.

In preliminary experiments with crude cellulase solutions, both during this investigation and earlier by Gum[9], it was noted that endoglucanases were inactivated above pH 9.0. Furthermore, Sternberg[10] had observed a rapid decrease in endoglucanase activity when *Trichoderma* cultures increased to pH values above 8.0. Therefore, it was important to ascertain the effect of pH on the purified endoglucanases. The activity remaining after incubation of each enzyme at 40° for one hour at each of several pH values was determined by use of the viscosimetric assay. Similar pH stability patterns were found with each of the endoglucanases as shown in Figure 22. Above pH 7.0 each of the purified endoglucanases demonstrated marked lability. Thus the purified endoglucanases may be more sensitive to the effects of pH than are the same enzymes together with the other components of the cellulase system. No specific buffer effect was noted at pH values where different buffers were used (i.e. pH 6.0 and 9.0). Okada (19) has also demonstrated alkali lability at 45° with endoglucanases II-A and II-B over a limited pH range.

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[9]Gum, E. K., Jr. (1976) Personal Communication.

[10]Sternberg, D. (1974) Personal Communication.

FIGURE 21

pH Optima for Endoglucanases II, III and IV with Carboxymethylcellulose as Substrate

Enzyme samples were incubated with carboxymethylcellulose (CM-cellulose) solutions at different pH values at 40° for 30 minutes (cf. p.46) and assayed for activity using the viscosimetric assay. Control drain times for CM-cellulose solutions were between 80 and 120 seconds. The curves represent Endoglucanases II (Δ), III (o) and IV (□).

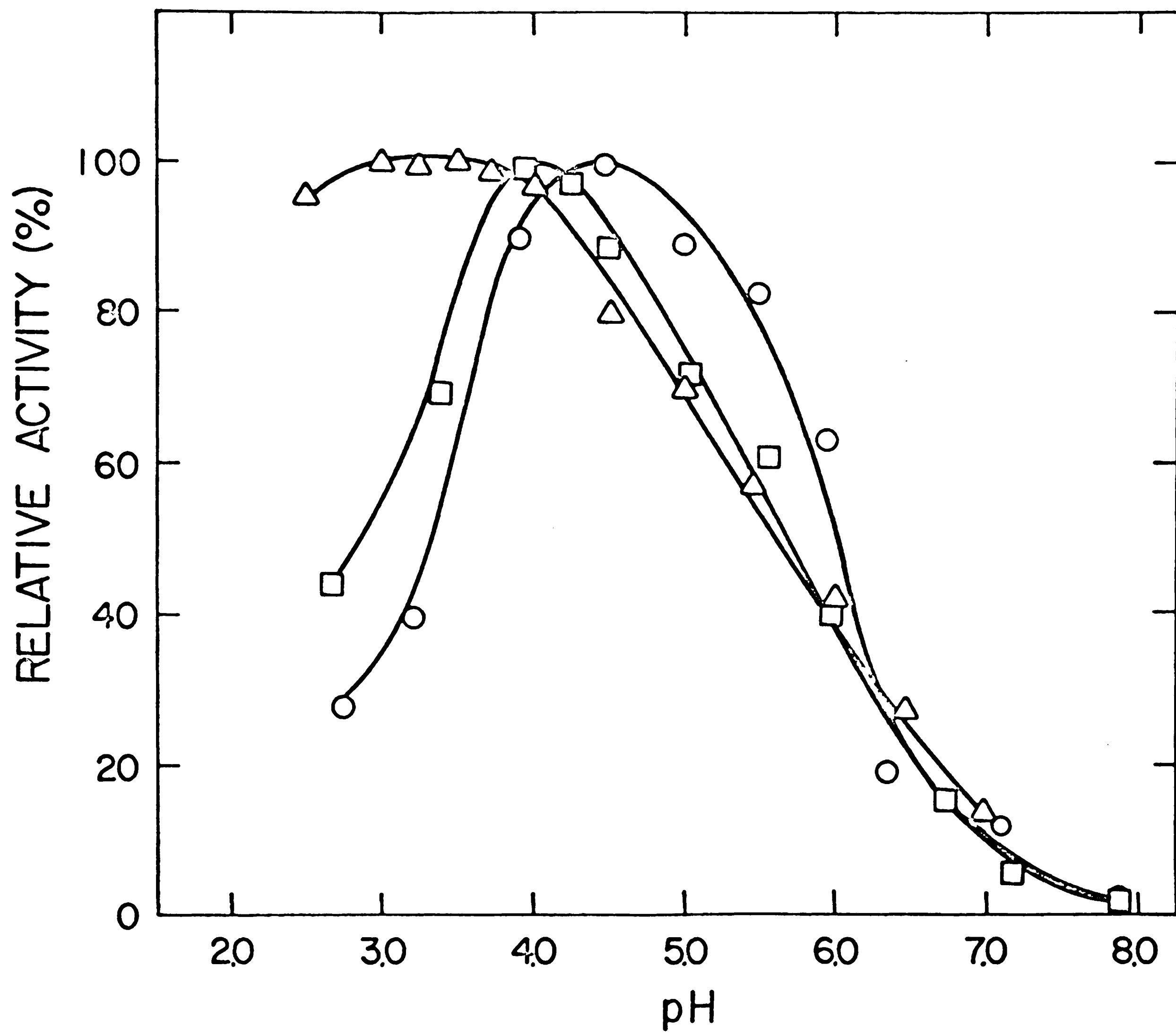
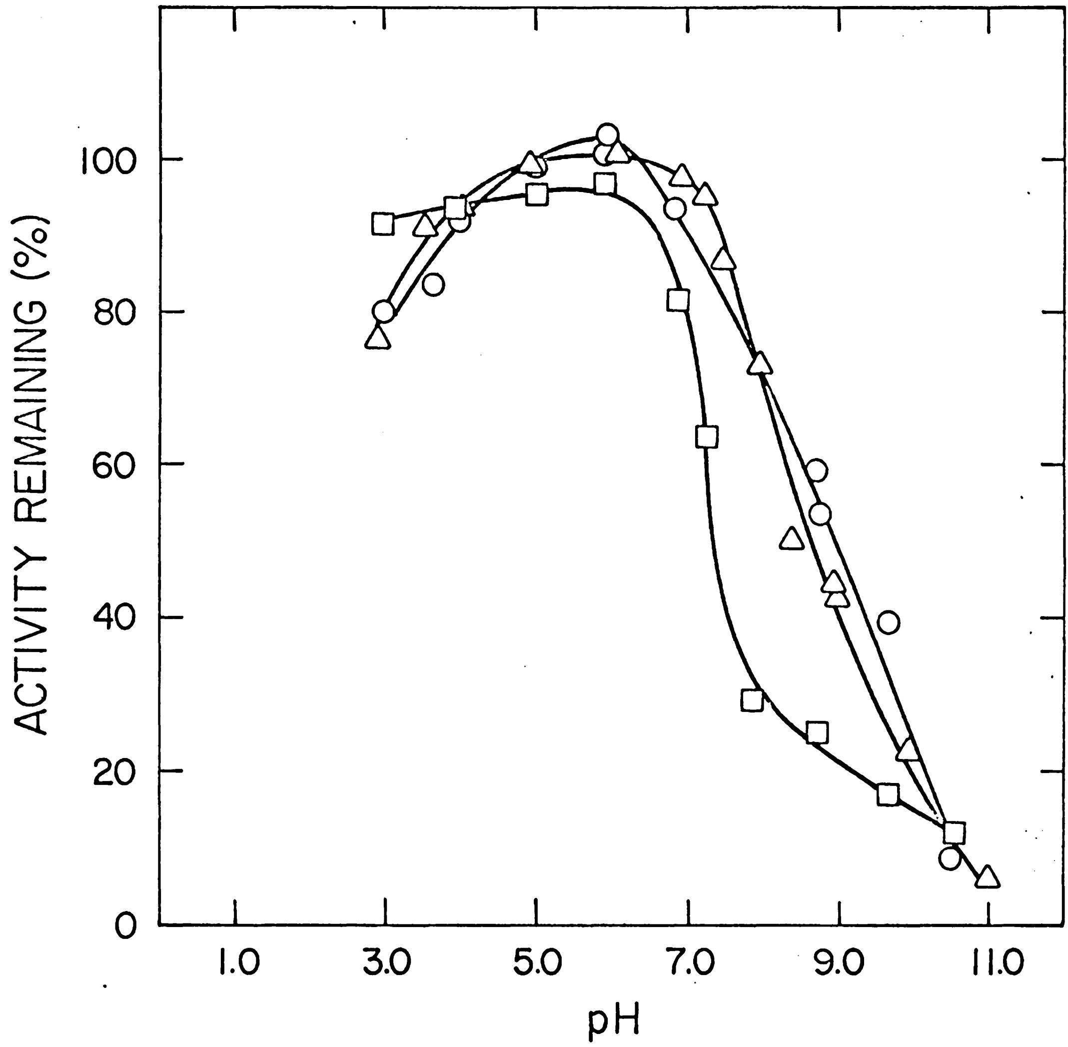

RELATIVE ACTIVITY (%)
100
80
60
40
20
0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
pH

FIGURE 22

pH Stability of Endoglucanases II, III and IV Using the Viscosimetric Assay

Enzyme samples were incubated in different pH buffer solutions at 40° for one hour, and then assayed for activity using the viscosimetric assay. Buffer solutions of 0.05 M citrate, HEPES, and CAPS were used over a pH range of 3.0-6.0, 6.0-9.0 and 9.0-11.0, respectively (cf. p.46). At pH 6.0 and 9.0 samples were analyzed in two different buffers. The curves represent endoglucanases II (Δ), III (o) and IV (□).


ACTIVITY REMAINING (%)
100
80
60
40
20
0
1.0
3.0
5.0
7.0
9.0
11.0
pH

Mode of Action - Three endoglucanases were purified from *Trichoderma viride*, each of which exhibited different viscosimetric activities with CM-cellulose. Their specific activites, in terms of $\Delta\phi_{sp}$/min/mg protein, were found to be 1010, 250 and 60 (Endoglucanases II, III and IV, respectively). The highest specific activity (viscosimetric) was that of Endoglucanase II, the buffer fraction endoglucanase. Perhaps since this enzyme does not bind to cellulose, it may readily diffuse away from the CM-cellulose substrate after an initial cleavage, and subsequently cleave different cellulose chains in a multi-chain type of attack mechanism. This type of mechanism would explain the extraordinarily high viscosimetric activity of this endoglucanase with CM-cellulose.

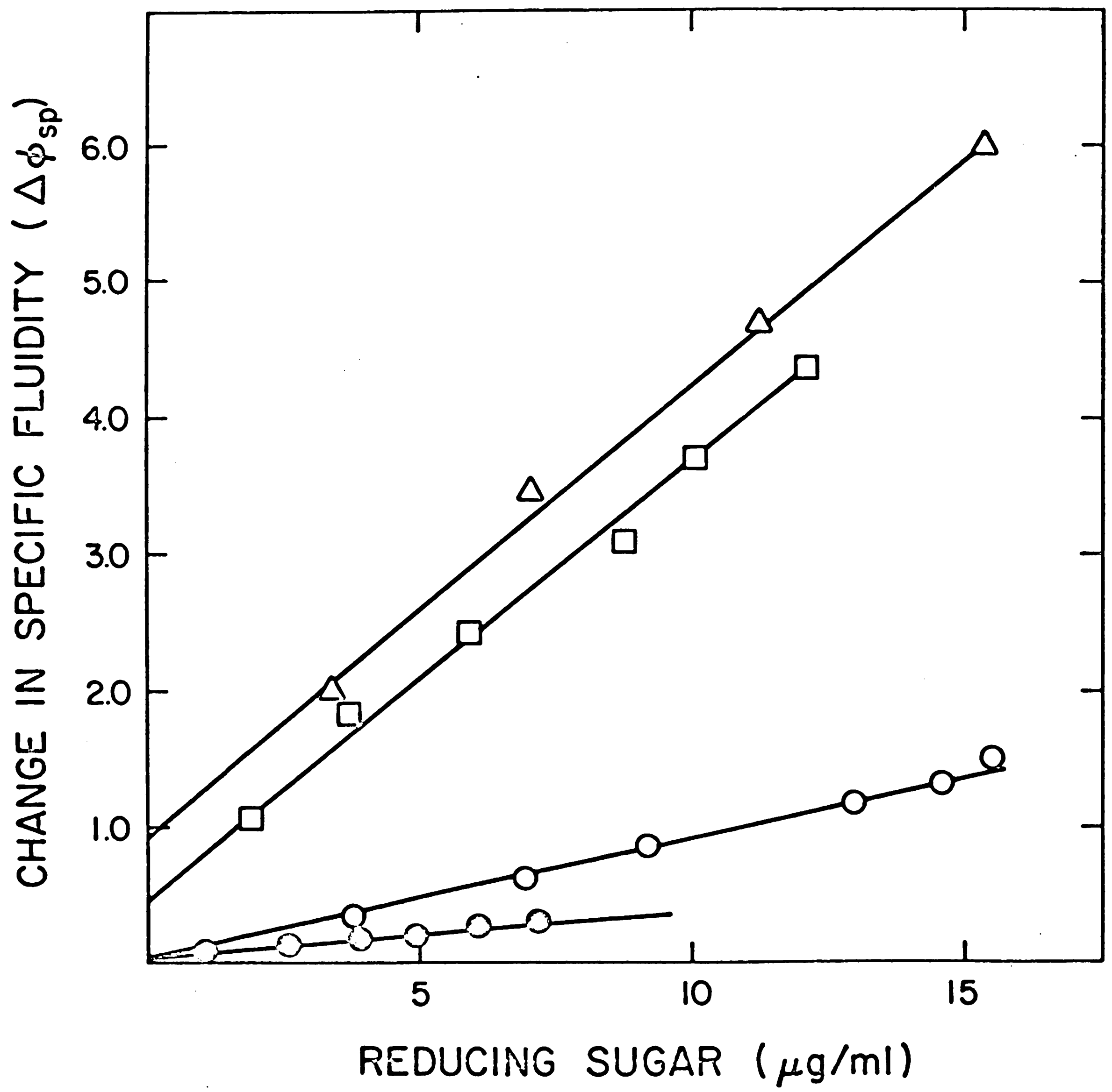
In order to further describe the mode of action of the purified endoglucanases with the CM-cellulose substrate, reducing sugar production was measured, concomitantly with decreases in viscosity after incubation of substrate and enzyme. This method, originally employed by Gilligan and Reese (9), has been discussed in some detail in the Literature Review (cf. p.18). The change in fluidity was plotted versus the corresponding increase in reducing sugar residues after different periods of incubation of enzyme with CM-cellulose at 40°. In each instance the slope of the line formed from such a plot provided information on the type of enzymic mechanism (endo or exo) that was involved (Figure 23).

For this study the enzyme concentrations were chosen to provide equivalent units of activity based on the activity (viscosimetric) of each enzyme on CM-cellulose. Thus the exo-cellobiohydrolase C concentration was ten times that used for the endoglucanases. In addition, Endoglucanases II and IV produced a much greater change in fluidity per reducing

FIGURE 23

Relationship Between Change in Fluidity and Production of Reducing Sugar During the Hydrolysis of CM-Cellulose by Purified 1,4-β-D-Glucanases

Enzyme samples were incubated with CM-cellulose at 40° for different periods of time (5, 10, 15, 20, 25 and 30 minutes) and analyzed for both reducing sugar and fluidity. The values shown for Endoglucanases II (Δ), III (o) 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.


CHANGE IN SPECIFIC FLUIDITY ($\Delta\phi_{sp}$)
6.0
5.0
4.0
3.0
2.0
1.0
5
10
15
REDUCING SUGAR (μg/ml)

sugar released and a lower enzyme concentration (0.006 μg/ml and 0.021 μg/ml, respectively) had to be used for the viscosimetric assay in order to give reliable drain times. The resulting change in fluidity values were multiplied by the appropriate factors (20 and 9, respectively) to permit comparison of these values at enzyme concentrations equivalent to those used for the reducing sugar determinations (0.119 μg/ml and 0.19 μg/ml, respectively).

The values obtained for the slope, determined in this manner, reflected the type of enzymic action with CM-cellulose; a more endo-type action being related to a larger positive slope. As expected the exo-cellobiohydrolase C produced a very small slope (0.05) indicative of its exo-action from chain ends. Endoglucanases II and IV exhibited nearly identical slopes (0.32 and 0.31, respectively) reflecting a similar endo-mode of action on this substrate. This similarity would lend support for a multi-chain type of attack mechanism (one cleavage per encounter) for Endoglucanase IV like that already suggested for Endoglucanase II. However, possibly because of its greater affinity for cellulose, it is less effective than Endoglucanase II in producing both a decrease in viscosity and an increase in reducing sugars at a given enzyme concentration.

A value in slope intermediate to that of Endoglucanases II and IV and the exo-cellobiohydrolase C was obtained with Endoglucanase III (0.10). This enzyme appears to have a mode of action different from the other endoglucanases. It possesses sufficient viscosimetric activity with CM-cellulose to be named an endoglucanase, but is more "exo" in its mode of action. Endoglucanase III appears to exert its action on internal linkages in the substrate with subsequent multiple cleavages on this single

cellulose chain (i.e., more than one cleavage per encounter). In this single chain attack mechanism the enzyme binds near its site of action and cleaves repeatedly while remaining in that productive complex.

Okada (19) has presented a similar study of reducing sugar versus change in fluidity on endoglucanases II-A and II-B. He concluded from the different patterns obtained for these endoglucanases that II-A, which gave a steeper slope, was more "random" in its attack mechanism than II-B. Comparison with his results revealed that identical lines were obtained for endoglucanase II-A and Endoglucanase IV and that the slope for the endoglucanase II-B line was closely related to that for Endoglucanase III. Thus endoglucanases II-A and II-B could be the same as Endoglucanases IV and III, respectively. However, since structural characterization has not been reported for endoglucanases II-A and II-B it is impossible to confirm the identities of these enzymes, or rule out the possibility of endoglucanase II-A being Endoglucanase II.

Low-Molecular-Weight Substrates - Non-reduced and reduced cellooligosaccharides and *p*-nitrophenyl glycosides were employed as model substrates in studies on the action patterns of endo-1,4-β-D-glucanases. The course of enzymic reactions with cellooligosaccharide substrates was followed using high pressure liquid chromatography. This highly sensitive method allowed the determination of initial rates and products of "multi-step" enzymic reactions. Using one percent (w/v) solutions in water of each of the purified cellooligosaccharides, cellobiose through cellohexaose, initial rates of reaction with Endoglucanases II, III and IV were measured at 30° as described in Experimental Procedures (cf. p.

47). The concentration of cellohexaose was less than one percent (w/v) since the solution was filtered before use to remove minor amounts of undissolved cellohexaose.

Results of this study are shown in Table IX. As expected for endoglucanases, the initial reaction rate, in terms of specific activity of Endoglucanases II, III and IV, increased with substrate chain-length. This trend was much more prominent with Endoglucanases II and IV for which a marked increase in rate was found with each successively higher chain-length cellooligosaccharide. The rates of reaction with Endoglucanase III, on the other hand, tended to level off at oligosaccharides larger than cellotetraose. In addition, Endoglucanase III was differentiated from the other two endoglucanases by its relatively high activity with cellotriose.

The absolute values for the specific activities obtained in this study should be interpreted with caution since reactions were run at only one substrate concentration, which could have been either near or below the K_m for that substrate or high enough to be inhibitory to the enzyme. Furthermore, the reaction mixtures were not buffered near the optimum pH (4.5) found for enzymic activity in this preliminary study because of possible interference with the HPLC method. However, it can be noted using this method that none of the endoglucanases demonstrated any activity on one percent cellobiose after 48 hours of incubation, that Endoglucanase II had a higher specific activity than Endoglucanase IV on all these soluble substrates (as with CM-cellulose), and that Endoglucanases II and III were nearly equal in their ability to cleave cellohexaose.

TABLE IX

Specific Activities of Endo-1,4-β-D-glucanases with Cellooligosaccharide Substrates

SUBSTRATE[b]	μmole/min/mg protein[a]		
	II	III	IV
Cellohexaose	22.52	19.0	8.5
Cellopentaose	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

[a]Determined as described in Expermental Procedures.

[b]Substrate concentration was one percent (w/v).

In order to examine these endoglucanase activities more completely with cellooligosaccharide substrates an extensive kinetic study was conducted. The highly sensitive and specific glucose oxidase method for determination of glucose concentration was employed to follow production of glucose from hydrolytic enzyme reaction at 40° with cellobiose or cellotriose substrates. For substrates of higher chain-length, an HPLC method was developed as described in Experimental Procedures (cf. p.53). In this study the enzyme solutions were made with pH 4.5, 0.01 mM sodium acetate buffer.

The resulting Lineweaver-Burk plots for Endoglucanases II, III and IV are shown in Figures 24, 25 and 26, respectively. In each case, excellent linearity was achieved. Unfortunately, for Endoglucanase II action on cellotetraose, and each of the endoglucanases on cellopentaose, the K_m values were so low that the necessary substrate concentration range necessary for kinetic analysis was below the sensitivity limit of the HPLC method (1.0 mM) and the kinetic constants could not be determined.

A summary of the kinetic parameters derived by least squares regression analysis from the double reciprocal plots is shown in Table X. In this study, the sensitive glucose oxidase method permitted demonstration of endoglucanase activity with cellobiose as substrate. The K_m and V_{max} values obtained are consistent with the rates observed when one percent (w/v) cellooligosaccharides were used as substrates. The similarity of Endoglucanases II and IV are reflected in the close correspondence of the kinetic constants observed when each acted on cellobiose and cellotriose. In each case the concentration at which the reaction velocity

FIGURE 24

Lineweaver-Burk Plots of Cellobiose and Cellotriose Hydrolysis Catalyzed by Endoglucanase II

Enzyme concentrations were 18.7 μg/ml and 14.0 μg/ml with cellobiose (A) and cellotriose (B) substrates, respectively. Reaction conditions were as described in Experimental Procedures (cf. p.53). Velocity, v, was determined using the glucose oxidase assay by the production of glucose per minute and expressed per milligram of protein.

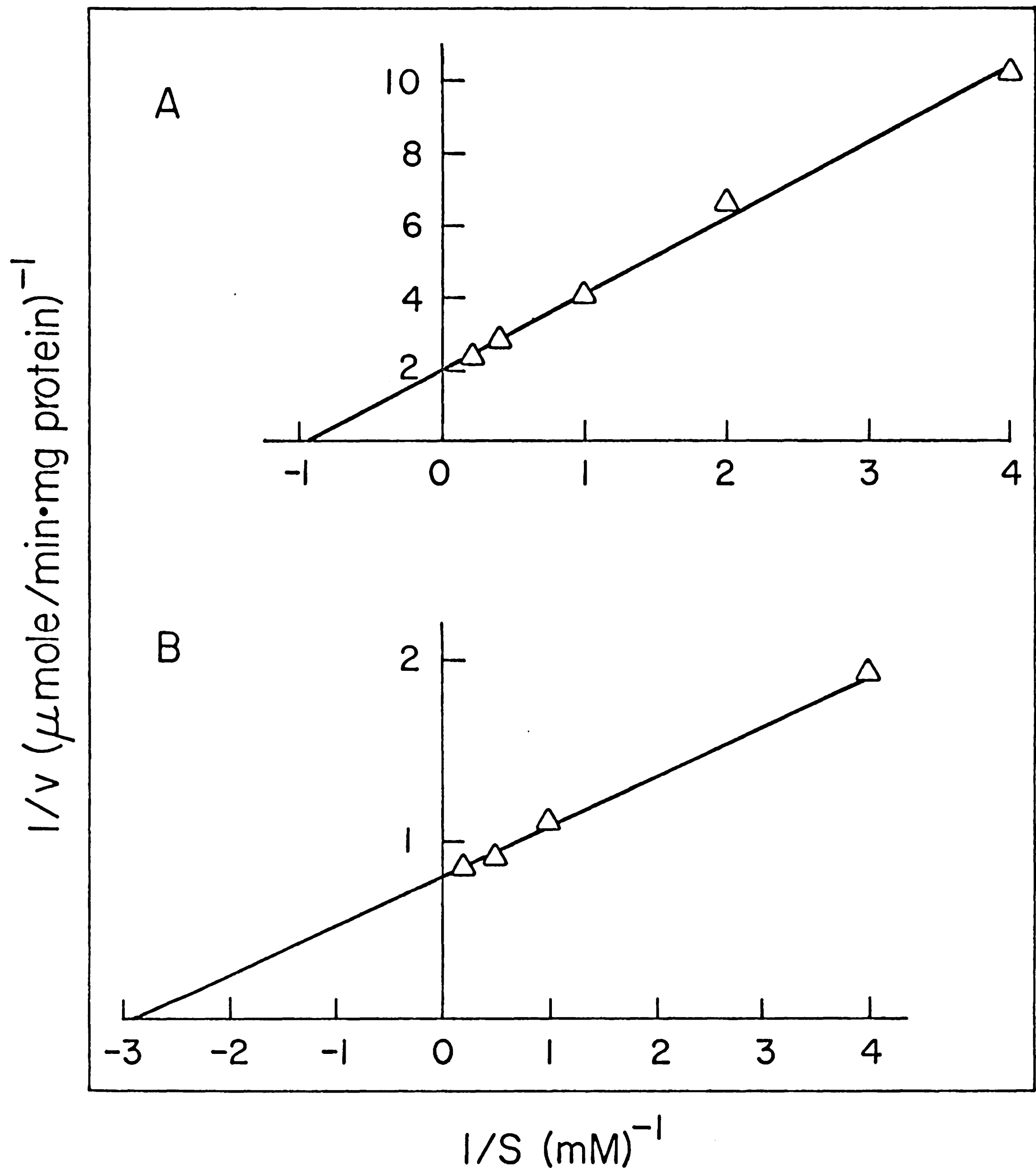

A
B
10
8
6
4
2
1
-3
-2
-1
0
1
2
3
4
1/v (μmole/min•mg protein)$^{-1}$
1/S (mM)$^{-1}$

FIGURE 25

Lineweaver-Burk Plots of Cellobiose, Cellotriose and Cellotetraose Hydrolysis Catalyzed by Endoglucanase III

Enzyme concentrations were 16.6 µg/ml, 1.5 µg/ml and 0.5 µg/ml with cellobiose (A), cellotriose (B) and cellotetraose (C), respectively. Reaction conditions were as described in Experimental Procedures (cf. p. 53). Velocity, v, was determined by one of two methods. With cellobiose and cellotriose, it was measured as described in Figure 24. With cellotetraose as substrate, velocity was measured by the decrease in substrate with time under initial conditions using an HPLC method.

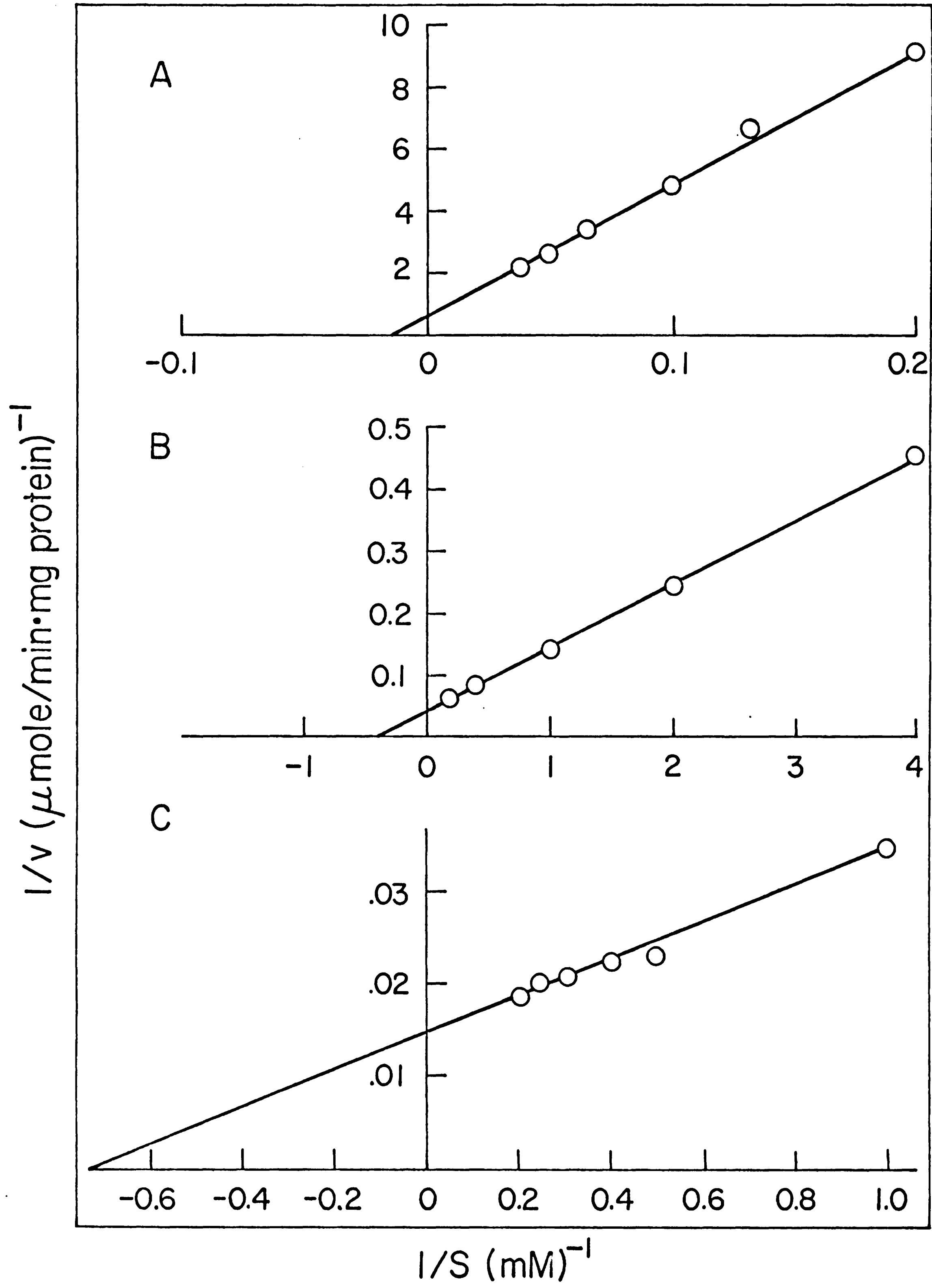

A
10
8
6
4
2
-0.1
0
0.1
0.2
B
0.5
0.4
0.3
0.2
0.1
-1
0
1
2
3
4
C
.03
.02
.01
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
1/v (μmole/min·mg protein)$^{-1}$
1/S (mM)$^{-1}$

FIGURE 26

Lineweaver-Burk Plots of Cellobiose, Cellotriose and Cellotetraose Hydrolysis Catalyzed by Endoglucanase IV

Enzyme concentrations were 21.2 μg/ml, 17.0 μg/ml and 2.6 μg/ml with cellobiose (A), cellotriose (B) and cellotetraose (C), respectively. Reaction conditions were as described in Experimental Procedures (cf. p. 53). Velocity, v, was determined in the same manner as described in Figure 25.

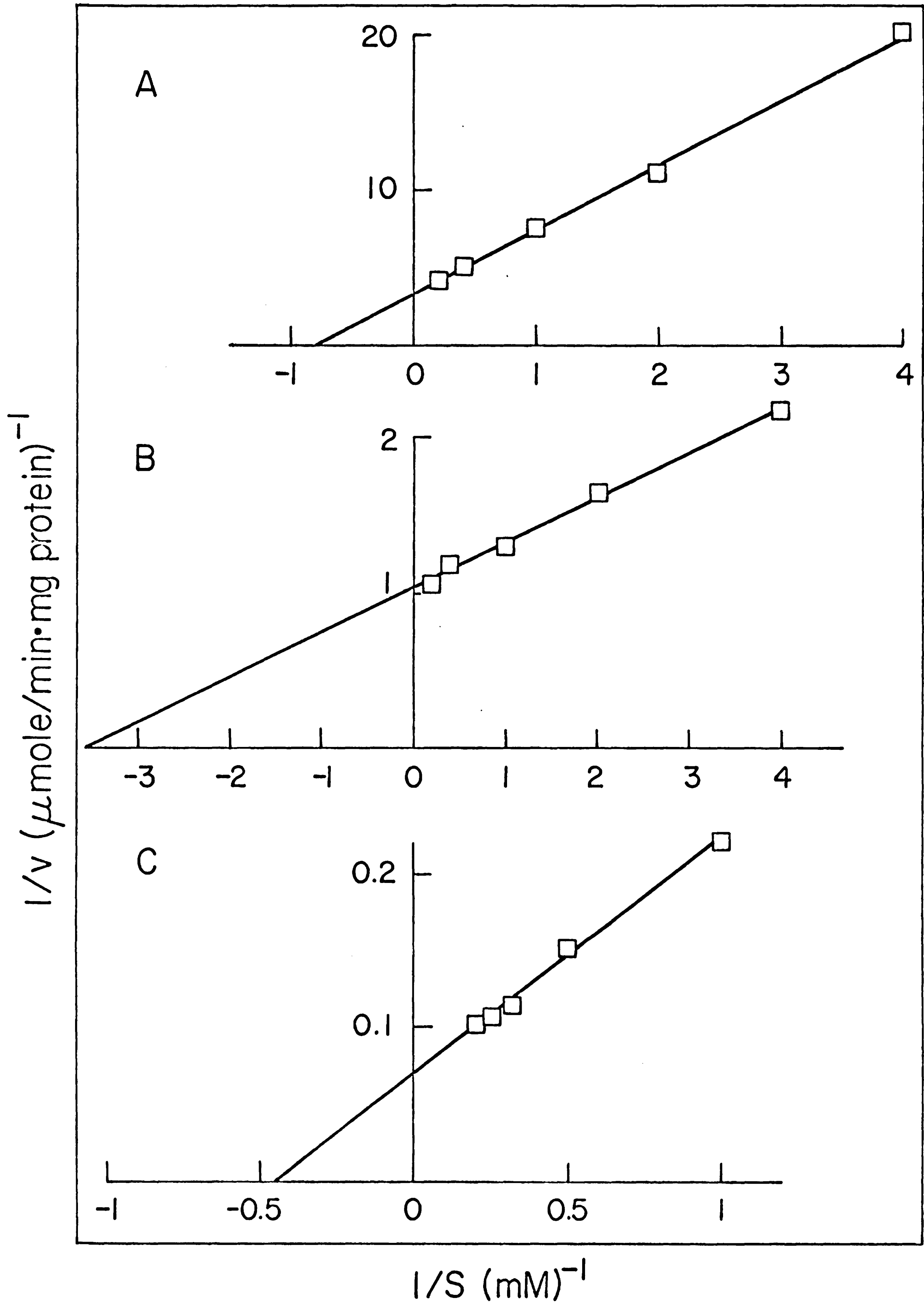

A
20
10
-1
0
1
2
3
4
B
2
1
-3
-2
-1
0
1
2
3
4
C
0.2
0.1
-1
-0.5
0
0.5
1
1/v (μmole/min•mg protein)⁻¹
1/S (mM)⁻¹

TABLE X

Kinetic Constants of Endo-1,4-β-D-glucanases with Cellobiose, Cellotriose and Cellotetraose Substrates[a,b,c]

SUBSTRATE	II	III	IV
Cellobiose			
K_m	1.03 ± 0.07	162.0 ± 39	1.26 ± 0.09
V_{max}	0.498 ± 0.031	3.65 ± 0.88	0.301 ± 0.021
Cellotriose			
K_m	0.339 ± 0.006	2.49 ± 0.18	0.279 ± 0.17
V_{max}	1.22 ± 0.012	24.4 ± 1.79	0.957 ± 0.029
Cellotetraose			
K_m	-	1.33 ± 0.11	2.13 ± 0.12
V_{max}	-	66.7 ± 3.1	14.1 ± 0.6

[a] K_m is given in terms of millimolar concentration.

[b] V_{max} is given as specific activity, μmole/min/mg protein.

[c] Kinetic constants and corresponding standard deviations.

is half maximal (K_m) decreases and the maximum velocity increases with the larger substrate. The K_m value obtained for Endoglucanase IV with cellotetraose appeared questionable since it did not follow the expected trend (i.e., decrease). Since this value (2.3 mM) is near the lower limits of this detection method (1.0 mM), its accuracy can be confirmed only with a more sensitive experimental method such as radioactively labeled substrates coupled with preparative HPLC.

Endoglucanase III demonstrated unique kinetic patterns with cellobiose as substrate. Its extraordinarily high K_m value, which can only be estimated because it is greater than any cellobiose concentration that could be used (25 mM) in the HPLC study, reflects the enzyme's apparently low affinity for cellobiose. Yet at high concentrations, this endoglucanase has significant activity with cellobiose; seven to twelve times more than either Endoglucanase II or IV, respectively. These characteristic properties of Endoglucanase III with cellobiose lend support for its identity with the "low affinity" enzyme Sternberg postulated in his recent paper (32).

Table X illustrates the significantly higher maximum specific activity of Endoglucanase III with each of the substrates tested. As substrate chain-length increased from two to four, Endoglucanase III exhibited the expected trend for an endoglucanase; its K_m value decreased and its V_{max} value increased. In addition, substrate inhibition was observed with all of the endoglucanases at substrate concentrations greater than ten times the K_m value.

A kinetic study of endoglucanases II-A and II-B with cellotriose through cellohexaose substrates was recently reported by Okada and Nisi-

zawa (62). However, the kinetic constants from their investigation must be viewed with caution since they were obtained using a reducing sugar determination. This type of analysis is not sufficient to establish a K_m or V_{max} because it does not differentiate between single and multiple reactions occurring simultaneously. In this type of kinetic study, where the products of one enzymic cleavage can readily become the substrates for the next enzymic attack, it is important to follow only one enzymic reaction. Following an enzymic reaction by release of reducing sugars gives no indication of the number of enzymic reactions occurring. However, brief comparison of the kinetic constants obtained in this questionable manner further suggests the similarity of endoglucanases II-A and II-B to Endoglucanases IV(II) and III, respectively. This is illustrated by the similar K_m and V_{max} trends with cellooligosaccharide substrates, in general, and with cellotriose, in particular.

The products of endoglucanase action with one percent (w/v) cellooligosaccharides were investigated using HPLC in order to further elucidate the patterns of action of these endoglucanases. Endoglucanases II (Figure 27) and IV (Figure 28) demonstrated a similar array of products after limited reaction using cellotetraose or cellopentaose as substrates. Both enzymes not only cleaved cellotetraose yielding two moles of cellobiose, but also cleaved this substrate to form one mole each of glucose and cellotriose. Bond specificity could not be determined using these substrates and the resulting glucose product could have come from either the reducing or non-reducing end of the substrate. In addition to hydrolysis, a small amount of cellopentaose transferase product was detected

FIGURE 27

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase II Reaction with Either Cellotetraose or Cellopentaose

Cellooligosaccharide products from reaction of Endoglucanase II with one percent (w/v) cellotetraose (A) and cellopentaose (B) were separated on a 30 cm μBondapak carbohydrate column. The reaction mixtures contained 46.7 μg/ml of enzyme and were incubated at 30° with cellotetraose and cellopentaose for 45 and 15 minutes, respectively. At this point 57% of cellotetraose and 70% of cellopentaose are remaining. The mixtures were eluted at one milliliter per minute using an acetonitrile:water (6:4) solvent system. Carbohydrate peaks detected by refractive index are G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; and G_5, cellopentaose. The initial peak represents water from the solvent.

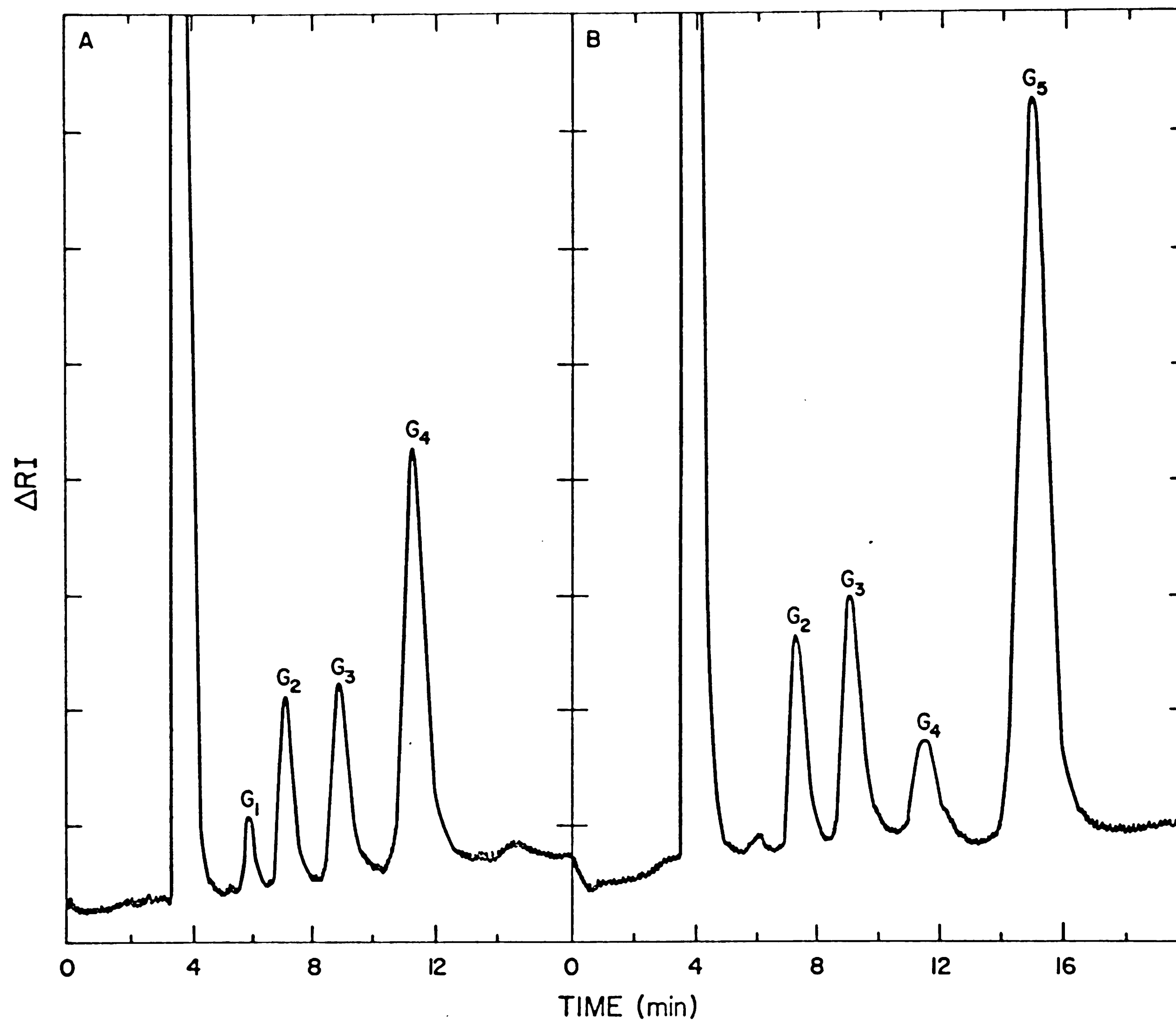
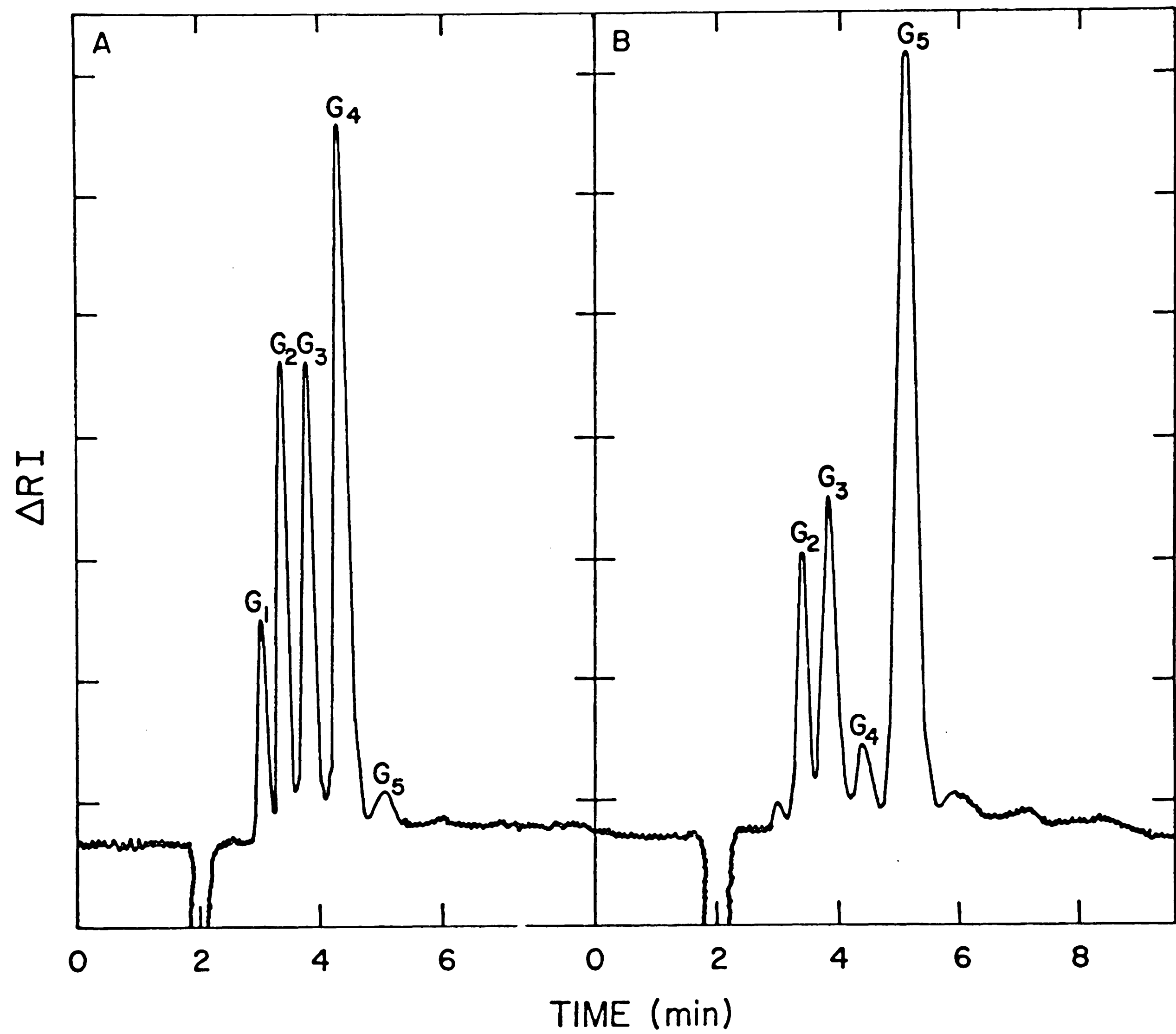

A
B
ΔRI
G1
G2
G3
G4
G2
G3
G4
G5
0
4
8
12
0
4
8
12
16
TIME (min)

FIGURE 28

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase IV Reaction with Either Cellotetraose or Cellopentaose

Cellooligosaccharide products from reactions of Endoglucanase IV with one percent (w/v) cellotetraose (A) and cellopentaose (B) were separated as described in the previous Figure. The reaction mixtures contained 6.5 μg/ml of enzyme and were incubated at 30° with cellotetraose and cellopentaose for 1444 and 300 minutes, respectively. At this point 44% of cellotetraose and 53% of cellopentaose are remaining.


A
B
G_1
G_2
G_3
G_4
G_5
ΔRI
0
2
4
6
8
TIME (min)

with Endoglucanase IV. With cellotetraose as substrate, there was no apparent enzyme preference for internal bonds.

On the other hand, using cellopentaose as a model substrate, resulted in a tendency for cleavage of internal bonds. Endoglucanases II and IV preferentially cleaved cellopentaose to form one mole of cellobiose and cellotriose. Only a relatively small amount of glucose and cellotetraose was formed under initial conditions.

The product of Endoglucanase IV action with cellohexaose after short and extended incubations are shown in Figure 29. This product array illustrates the multiple nature of reactions taking place and the shifts in the product array after short and long enzyme incubations. Under initial conditions, Endoglucanase IV action was directed more toward internal bonds forming two moles of cellotriose or a mole of cellobiose and cellotetraose. After four days of reaction the distinctive products of Endoglucanase IV action were found to be glucose, cellobiose and cellotriose. The presence of cellobiose and cellotriose in addition to glucose is consistent with the low susceptibility to enzymic action found with these substrates.

Not unexpectedly the product array formed from Endoglucanase III action with cellotriose, cellotetraose and cellopentaose was different from those of Endoglucanases II and IV (Figure 30). In the first place, this enzyme exhibits significant transferase activity with all three substrates under initial reaction conditions. This ability to transfer cellobiosyl units in the presence of sufficient acceptor concentration has also been shown with the exo-cellobiohydrolase C (51). In addition, Endoglucanase III preferentially cleaves cellobiosyl units and gives much the

FIGURE 29

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase IV Reaction with Cellohexaose After Different Periods of Incubation

Cellooligosaccharide products from Endoglucanase IV action at 30° on one percent cellohexaose for 298 (A) or 5774 (B) minutes were separated as described in Figure 27. The reaction mixture contained 6.5 μg/ml of enzyme and after 298 minutes, 16% of cellohexaose is remaining. Elution of cellooligosaccharides was as described in Figure 27.

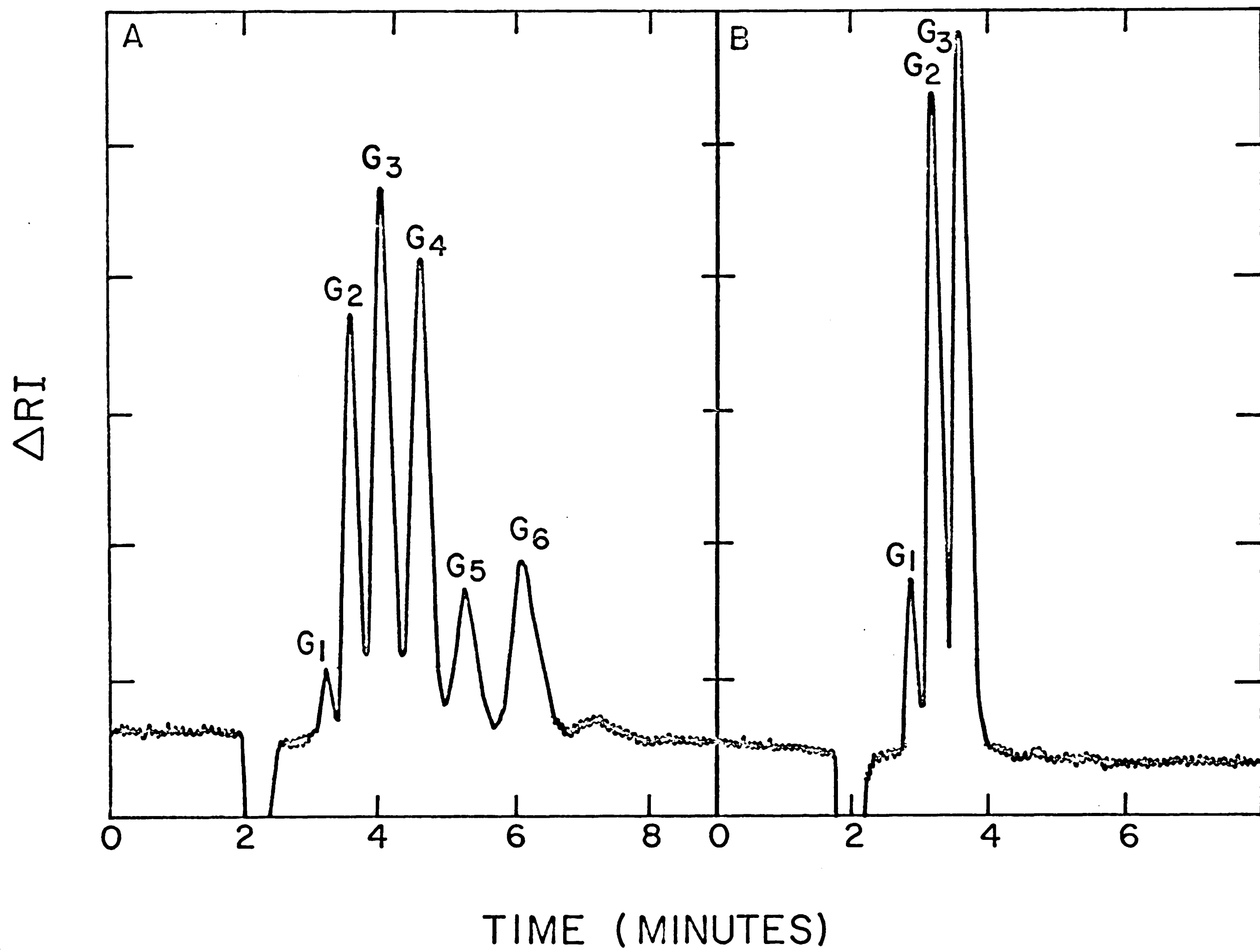
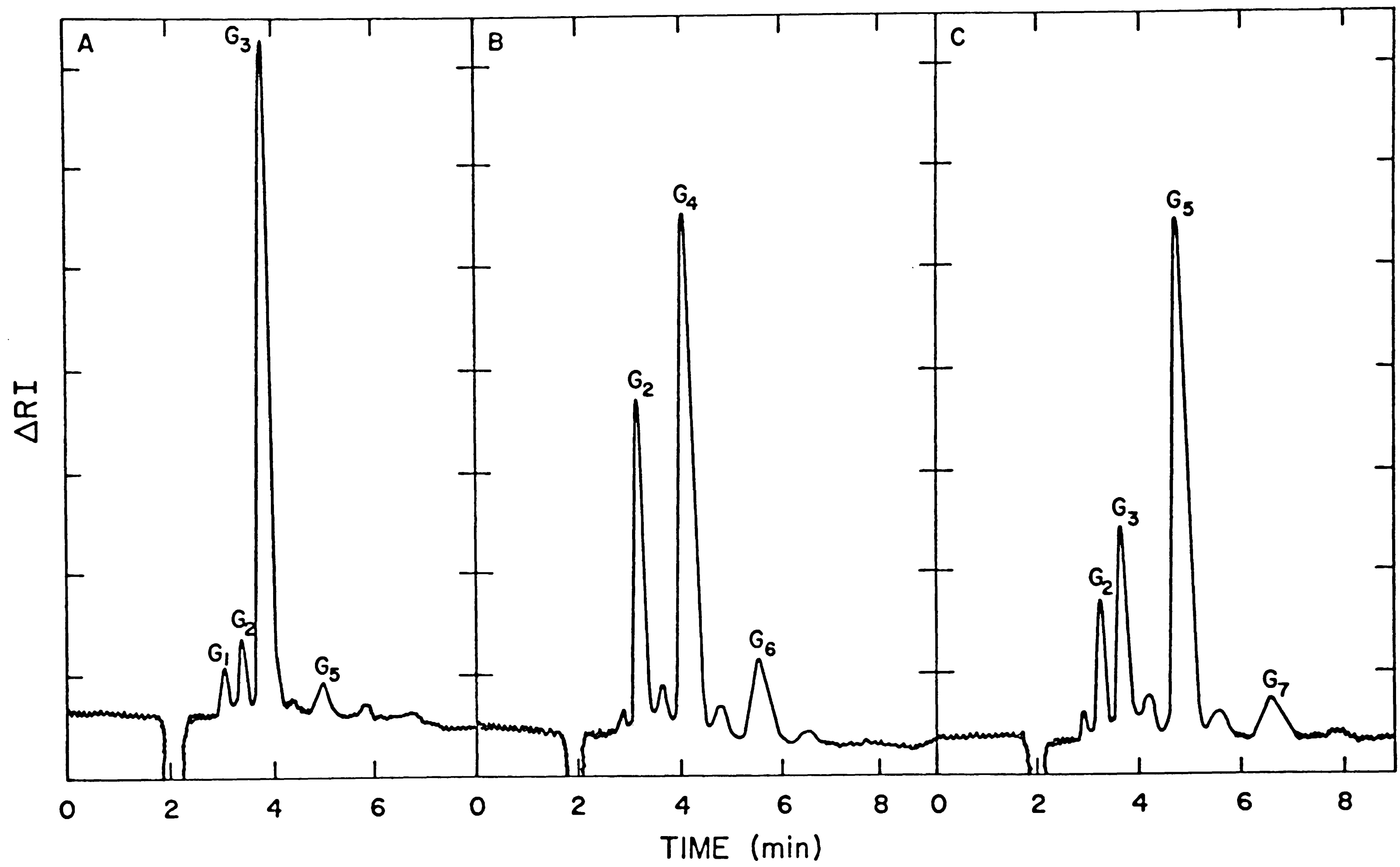

A
B
ΔRI
G1
G2
G3
G4
G5
G6
G1
G2
G3
0
2
4
6
8
0
2
4
6
TIME (MINUTES)

FIGURE 30

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase III Reaction with Cellotriose, Cellotetraose or Cellopentaose

Cellooligosaccharide products from reaction of Endoglucanase III with one percent (w/v) cellotriose (A), cellotetraose (B) and cellopentaose (C) were separated as described in Figure 27. The reaction mixtures contained 6.1 μg/ml of enzyme and were incubated at 30° with cellotriose cellotetraose and cellopentaose for 246, 246 and 256 minutes, respectively. At this point 74% of cellotriose, 45% of cellotetraose and 50% of cellopentaose remained.


A
B
C
G_3
G_1
G_2
G_5
G_4
G_2
G_6
G_5
G_3
G_2
G_7
ΔRI
0 2 4 6 0 2 4 6 8 0 2 4 6 8
TIME (min)

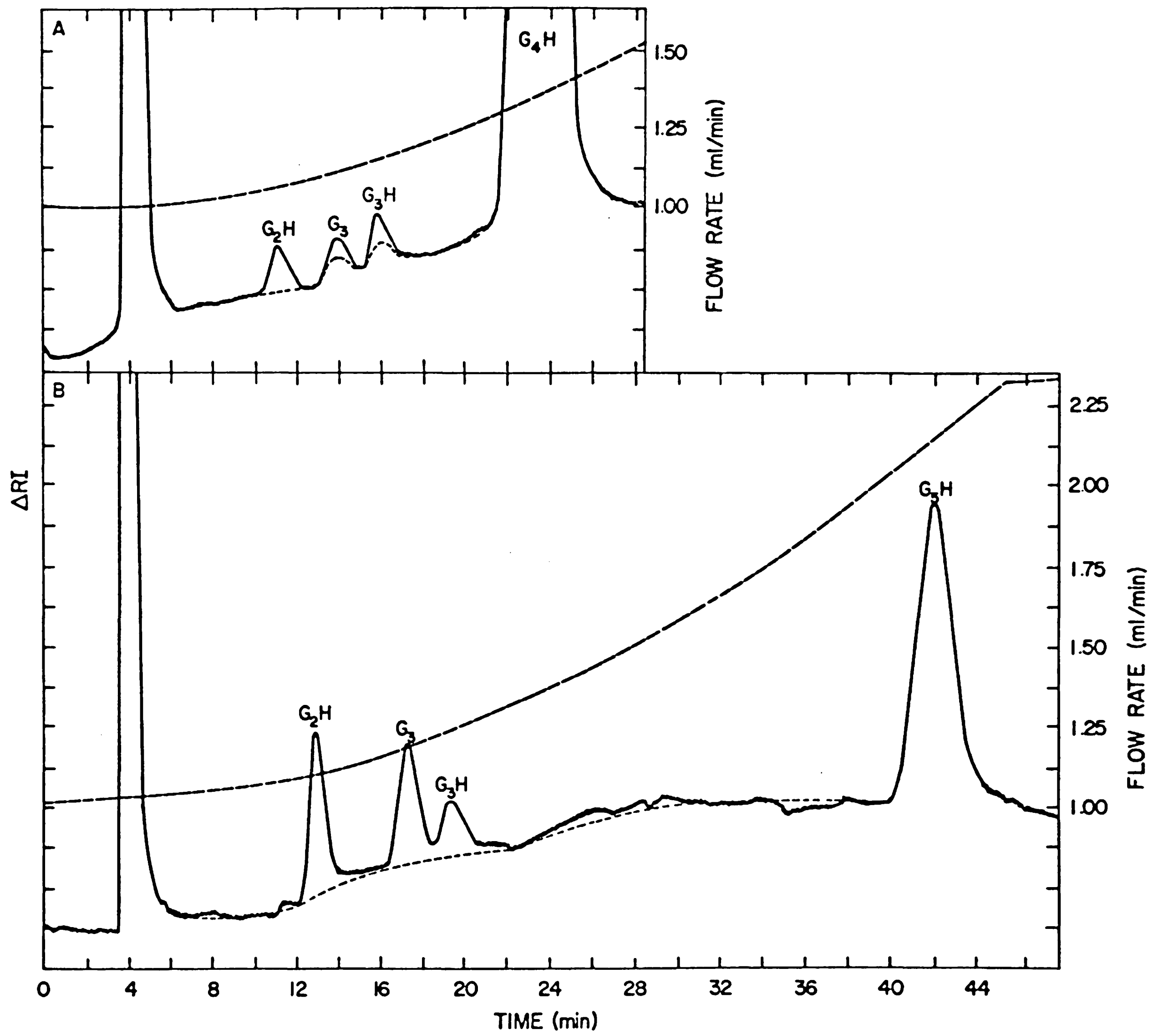
same product distribution found for exo-cellobiohydrolase C (51). The transferase ability of endoglucanases was recently reported by Okada and Nisizawa (62) using cellobiose as substrate. They observed qualitatively with paper chromatography the formation of cellotriose with endoglucanase II-A and cellotriose and cellotetraose with endoglucanase II-B using high concentrations of the substrate, cellobiose (34.2 mg/ml). They noted that this transferase activity was much greater with endoglucanase II-B than II-A.

To permit identification of the glycosidic bonds preferentially cleaved by the endoglucanases, cellopentaitol and cellotetraitol were used as model substrates. The procedure described previously was used to determine the initial products of enzyme reaction. The rate of reaction with cellopentaitol and cellotetraitol was approximately that for the corresponding non-reduced substrates. The product arrays generated by the action of Endoglucanases II and IV are illustrated in Figures 31 and 32, respectively. As expected, each enzyme gave a similar product distribution with the respective substrates. However, interpretation of the origin of the product distribution, especially with cellotetraitol, is only speculative. One possible explanation is that cellotetraitol is cleaved forming cellobiitol and a cellobiosyl-enzyme intermediate. The cellobiosyl unit of the enzyme intermediate is then transferred to an acceptor, cellotetraitol, present in high concentrations. The resulting cellohexaitol is then rapidly cleaved forming equimolar amounts of cellotriose and cellotriitol. Attempts to demonstrate the presence of cellohexaitol using the HPLC method given on page 47 failed, but perhaps the enzyme's high specific activity with this substrate prevents its detec-

FIGURE 31

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase II Reaction with Either Cellotetraitol or Cellopentaitol

Reduced and non-reduced cellooligosaccharide products from reaction of Endoglucanase II with one percent (w/v) cellotetraitol (A) and cellopentaitol (B) were separated using a flow program (---) as described in Experimental Procedures (cf. p.50). 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 3.1 μg/ml of enzyme and were incubated at pH 4.5 (40°) with cellotetraitol and cellopentaitol for 30 and 15 minutes, respectively. At this point, approximately 95% of cellotetraitol and 55% of cellopentaitol are remaining.

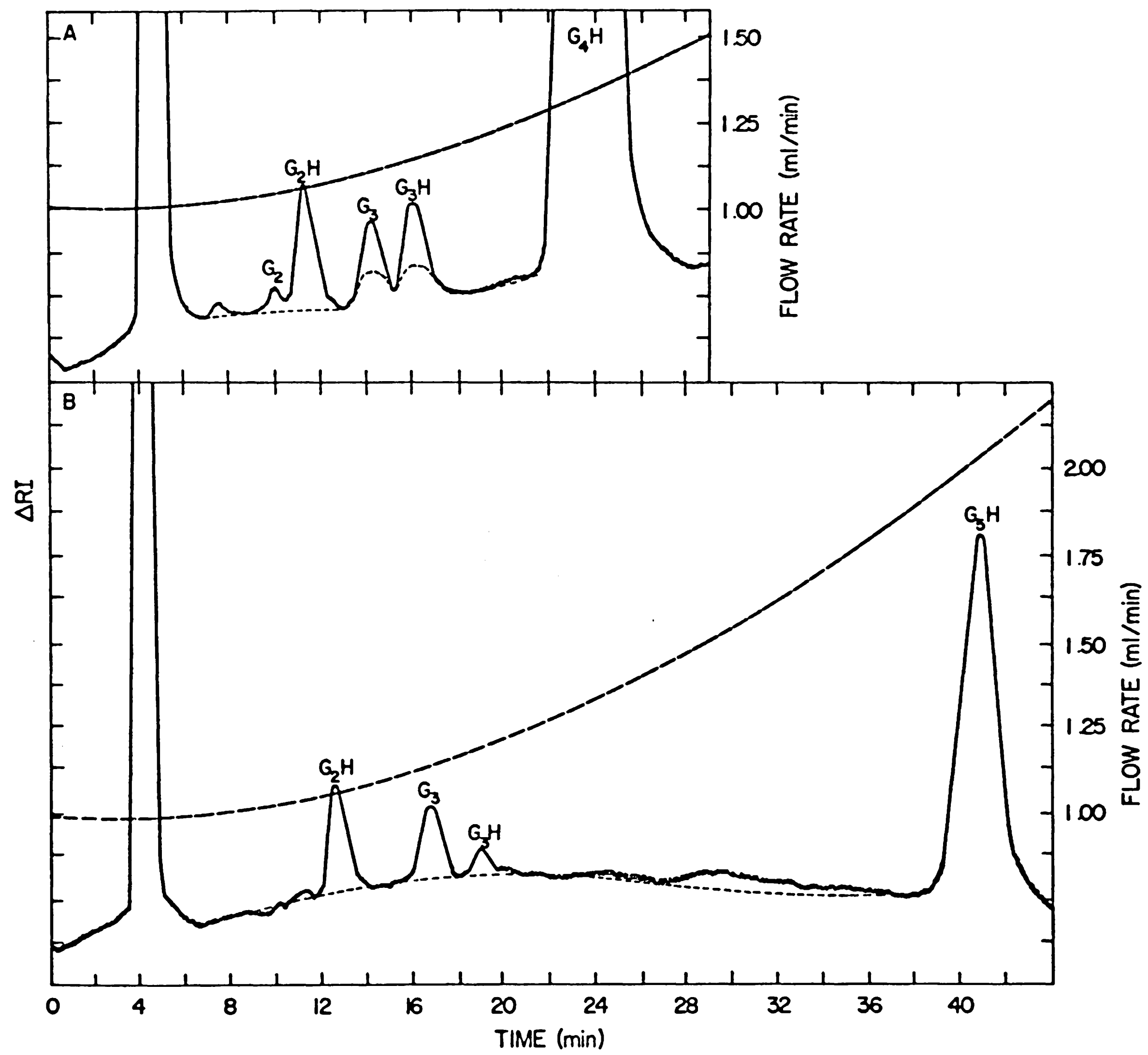


A
G$_4$H
G$_2$H
G$_3$
G$_3$H
1.50
1.25
1.00
FLOW RATE (ml/min)
B
ΔRI
G$_2$H
G$_3$
G$_3$H
G$_5$H
2.25
2.00
1.75
1.50
1.25
1.00
FLOW RATE (ml/min)
0
4
8
12
16
20
24
28
32
36
40
44
TIME (min)

FIGURE 32

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase IV Reaction with Either Cellotetraitol or Cellopentaitol

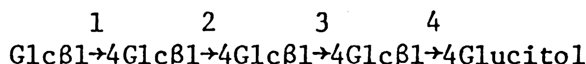
Reduced and non-reduced cellooligosaccharide products from reaction of Endoglucanase IV with one percent (w/v) cellotetraitol (A) and cellopentaitol (B) were separated as previously described. The significance of the solid and dashed lines is the same as that defined for Figure 31. Cellotetraitol was incubated at 40° with 13.0 µg/ml of enzyme for 30 minutes at which time approximately 91% of the substrate was unreacted. Cellopentaitol was incubated at pH 4.5 (40°) with 2.6 µg/ml of enzyme for 60 minutes after which time approximately 75% of substrate was unreacted.



A
B
G2
G2H
G3
G3H
G4H
G5H
ΔRI
FLOW RATE (ml/min)
1.50
1.25
1.00
2.00
1.75
1.50
1.25
1.00
0
4
8
12
16
20
24
28
32
36
40
TIME (min)

tion. In any case, the established occurrence of transferase activity in addition to hydrolytic activity at high acceptor concentrations with glucan-hydrolases and the faster rates of reaction with increased substrate chain-length, must not be overlooked when analyzing the action patterns of the enzymes.

The product patterns produced from Endoglucanases II and IV action on cellopentaitol are more easily interpreted. When referring to the individual glycosidic bonds of these substrates, the following convention, here illustrated for cellopentaitol, will be used:



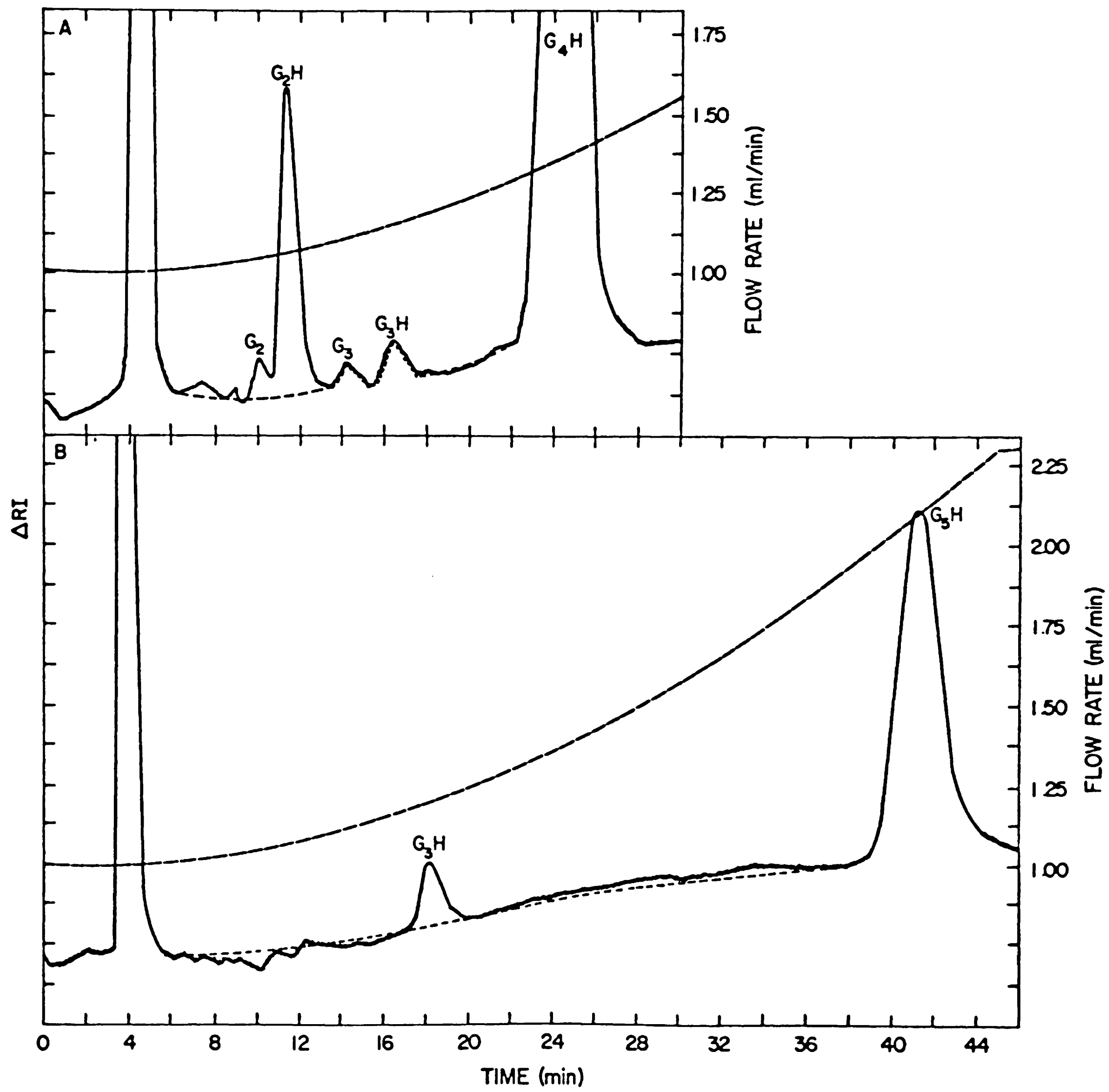
These glucosidic bonds will be numbered consecutively from the non-reducing end of the oligosaccharide chain. Both enzymes preferred the third bond forming as principal products cellobiitol and cellotriose. The presence of cellotriitol appears to be significant, and may have arisen from limited enzymic action at the second bond, such that the resulting cellobiosyl unit is transferred to cellopentaitol. However, this possibility is unsupported by any direct evidence for celloheptaitol.

The distinctive character of Endoglucanase III was demonstrated by its mode of reaction with these reduced substrates (Figure 33). Results with cellotetraitol and cellopentaitol demonstrated an endo-type preference for either the second or third bond, respectively. However, only the corresponding reduced product (cellobiitol or cellotriitol, respectively) was observed. The remaining product appeared to be transferred to the acceptor substrate forming either cellohexaitol from cellotetrai-

FIGURE 33

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase III Reaction with Either Cellotetraitol or Cellopentaitol

Reduced and non-reduced cellooligosaccharide products from reaction of Endoglucanase III with one percent (w/v) cellotetraitol (A) and cellopentaitol (B) were separated as previously described. The significance of the solid and dashed lines is the same as that defined for Figure 31. The reaction mixtures contained 2.0 μg/ml of enzyme and were incubated at pH 4.5 (40°) with these substrates for 30 minutes. Approximately 91 and 85% of cellotetraitol and cellopentaitol, respectively, remained unreacted after this incubation.


A
B
G_2H
G_2
G_3
G_3H
G_4H
G_5H
ΔRI
FLOW RATE (ml/min)
1.00
1.25
1.50
1.75
2.00
2.25
TIME (min)
0
4
8
12
16
20
24
28
32
36
40
44

tol or celloheptaitol from cellopentaitol. This possibility was tested with the enzyme-cellotetraitol mixture using the standard HPLC method (Figure 34). In this case, the presence of the transferase product, cellohexaitol, was confirmed, thus demonstrating the ability of Endoglucanase III to transfer cellobiosyl units.

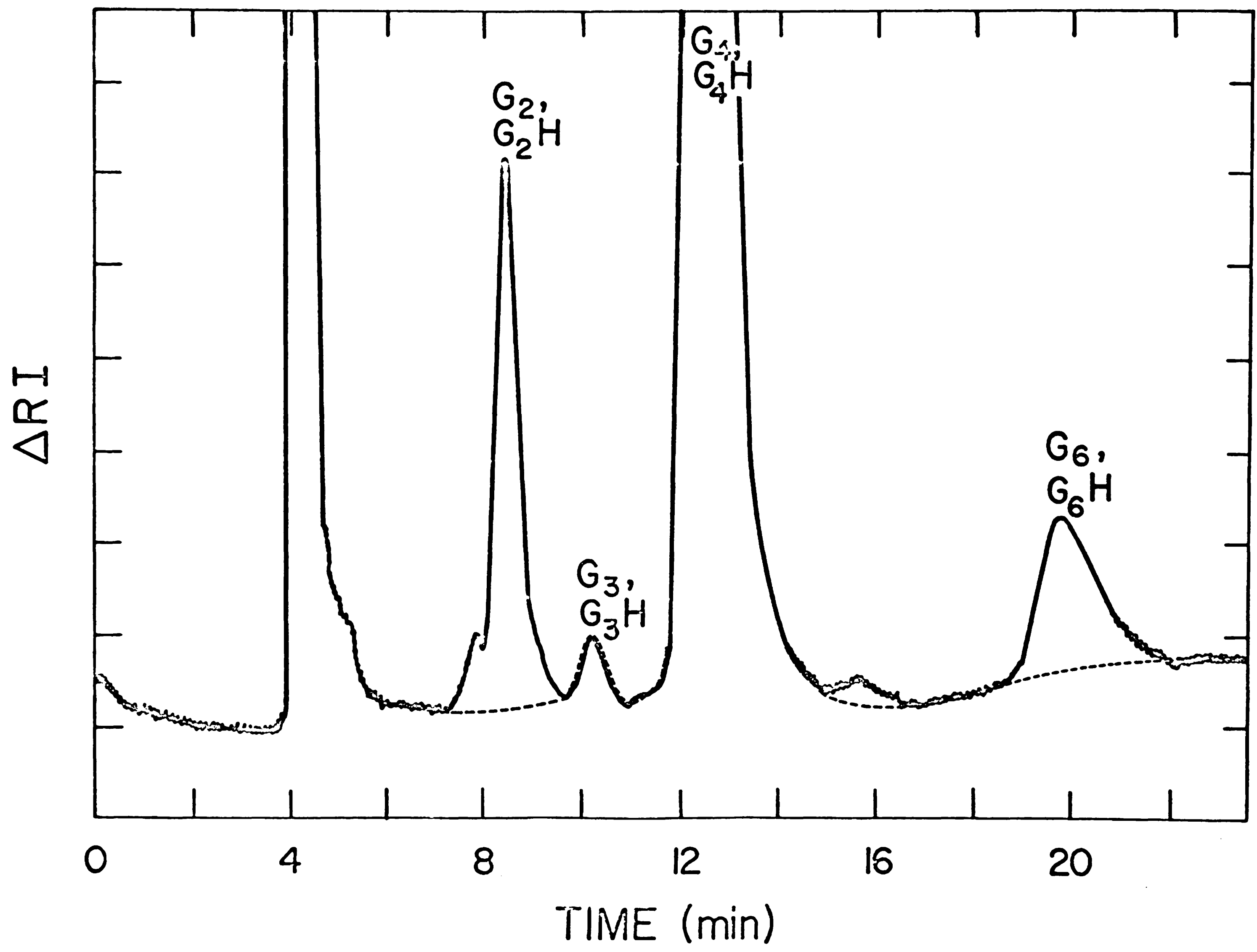
Thus all three endoglucanases illustrated a preference for the second bond in cellotetraitol and either the second (Endoglucanase III) or the third (Endoglucanases II and IV) bond in cellopentaitol. These bond preferences were compared with that of the exo-cellobiohydrolase C. This exoglucanase (Figure 35) demonstrated the same bond preference with both substrates, principally cleaving the second bond of cellotetraitol and cellopentaitol. Thus whereas the exo-cellobiohydrolase C is clearly different from Endoglucanases II and IV with respect to mode of action on oligosaccharides, it is similar to Endoglucanase III in this respect. Of course, Endoglucanase III is quite easily distinquished from exo-cellobiohydrolase C by the high activity of the former on cellooligosaccharides and CM-cellulose.

The close parallels which exist between the modes of action of endoglucanases II-A and II-B (19) and Endoglucanases IV (II) and III, respectively, justify further comparison where possible. In a recent paper, Okada and Nisizawa (62) differentiated endoglucanases II-A and II-B by their respective modes of hydrolysis with p-nitrophenyl cellobioside (NPC). Identification and quantification of the hydrolysis products by paper chromatography demonstrated a preference for the holosidic or glycosidic bond by endoglucanases II-A and II-B, respectively.

FIGURE 34

Separation by High Pressure Liquid Chromatography of Products Formed from Endoglucanase III Reaction with Cellotetraitol

An aliquot of the reaction mixture described in Figure 33 for Endoglucanase III with cellotetraitol was analyzed by the method given in Figure 3. The dashed line traces the relevant segment of the chromatographic pattern obtained with the unreacted substrate.

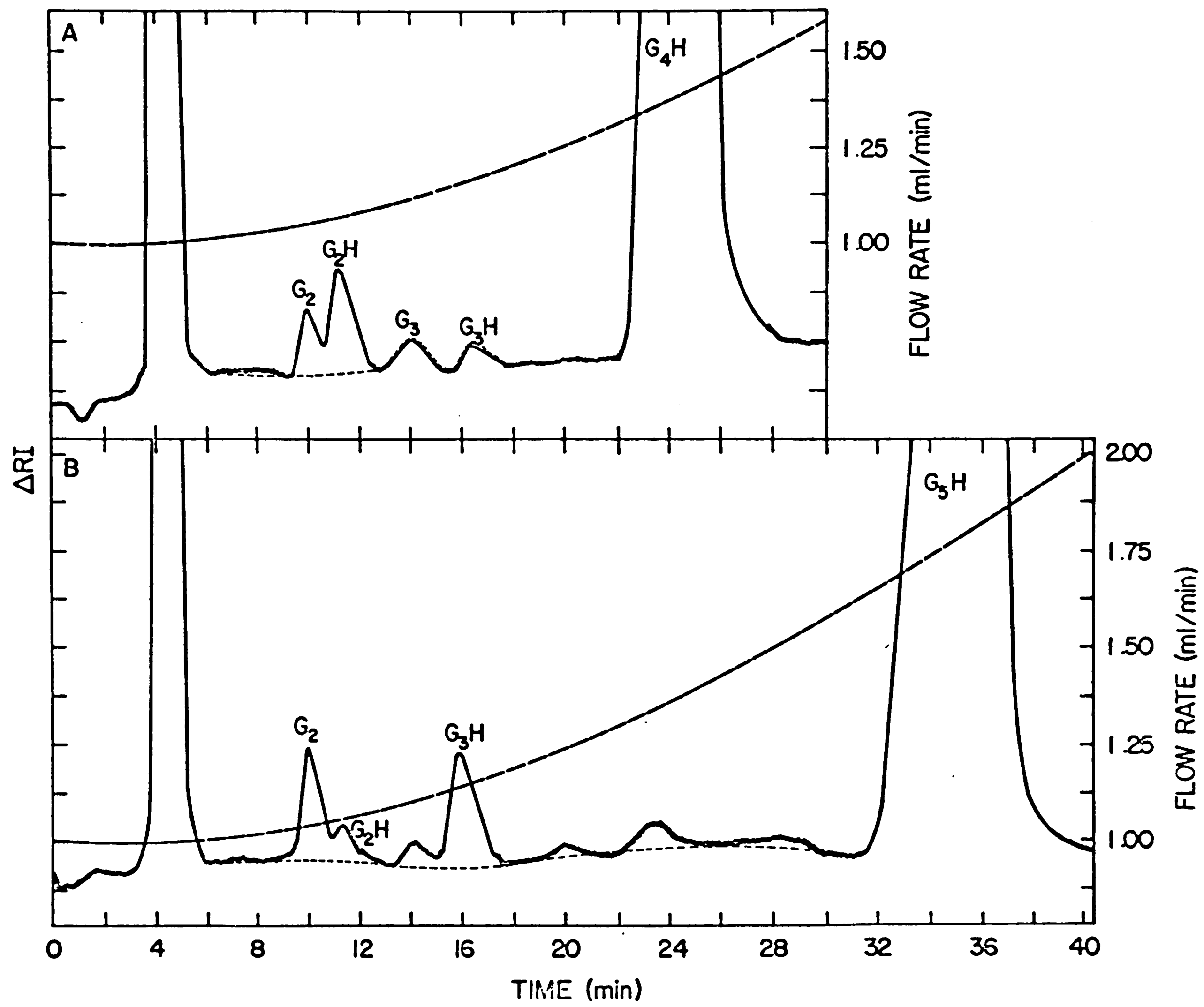


ΔRI
G_2,
G_2H
G_3,
G_3H
G_4,
G_4H
G_6,
G_6H
0
4
8
12
16
20
TIME (min)

FIGURE 35

Separation by High Pressure Liquid Chromatography of Products Formed from Exo-cellobiohydrolase C Reaction with Either Cellotetraitol or Cellopentaitol

Reduced and non-reduced cellooligosaccharide products from reaction of exo-cellobiohydrolase C with one percent (w/v) cellotetraitol (A) and cellopentaitol (B) were separated as previously described. The significance of the solid and dashed lines is the same as that defined for Figure 31. The reaction mixtures contained 21.8 μg/ml of enzyme and were incubated at pH 4.5 (40°) with cellotetraitol and cellopentaitol, respectively, for 60 and 30 minutes, respectively. Approximately 94 and 90% of cellotetraitol and cellopentaitol, respectively, remained unreacted after this incubation.



A
B
G₄H
G$_2$H
G$_2$
G$_3$
G$_3$H
G$_5$H
ΔRI
FLOW RATE (ml/min)
1.50
1.25
1.00
2.00
1.75
0
4
8
12
16
20
24
28
32
36
40
TIME (min)

Stimulated by this report, a study was initiated using p-nitrophenyl glycosides. This investigation was divided into three parts: (a) Determination of specific activities with p-nitrophenyl glucoside (NPG) and NPC; (b) Determination of kinetic constants with NPG; and (c) Identification of the hydrolysis products after prolonged incubation with NPC. The methods employed in this study have been described in Experimental Procedures (cf. pp. 50-56).

The specific activities of Endoglucanases II, III and IV with these model substrates under initial conditions (i.e., <20% substrate reacted) are illustrated in Table XI. Results with NPC must be viewed with caution as the colorimetric detection method was sensitive only to hydrolysis of the glycosidic bond and enzymic action at the holosidic bond would proceed undetected. Endoglucanases II and IV again demonstrated similar patterns of action, readily cleaving the glycosidic bond of NPG but not NPC. On the other hand, Endoglucanase III demonstrated the reverse pattern, cleaving the glycosidic bond of NPC but not of NPG.

Kinetic analysis with NPG revealed similar K_m values for the three endoglucanases but 15- to 20-fold higher V_{max} values for Endoglucanases II and IV than for Endoglucanase III (Table XII). The endoglucanases exhibited nearly identical K_m values (0.17 mM but less than one-hundredth of the maximum velocity found for the β-glucosidase (118 μmole/min/mg protein) (28).

Analysis of products formed after prolonged incubation (23 hours) of Endoglucanases II, IV and III with PNC are shown in Figures 36, 37 and 38, respectively. Glucose and p-nitrophenol (NP) were the sole products

TABLE XI

Specific Activities of Endo-1,4-β-D-glucanases on Aryl-glycosides[a]

	μmole/min/mg protein[a]		
SUBSTRATE	II	III	IV
p-Nitrophenylglucoside	0.642	0.021	0.481
p-Nitrophenylcellobioside	0.025	0.163	0.010

[a]Determined by release of p-nitrophenol.

TABLE XII

Kinetic Constants of Endo-1,4-β-D-glucanases on *p*-Nitrophenylglucoside[a]

	II	III	IV
K_m [b]	0.163 ± 0.011	0.129 ± 0.004	0.143 ± 0.007
V_{max} [c]	1.21 ± 0.03	0.058 ± 0.001	0.855 ± 0.016

[a]Determined by release of *p*-nitrophenol.

[b]Units are mM.

[c]Units are μmoles/min/mg protein.

FIGURE 36

Separation by High Pressure Liquid Chromatography of Hydrolysis Products from Reaction of Endoglucanase II with *p*-Nitrophenyl Cellobioside

Hydrolysis products after a 23-hour incubation of Endoglucanase II with 1.5 mM *p*-nitrophenyl cellobioside at 30° were detected as previously described (cf. p.50). Enzyme concentration was 30 μg/ml.

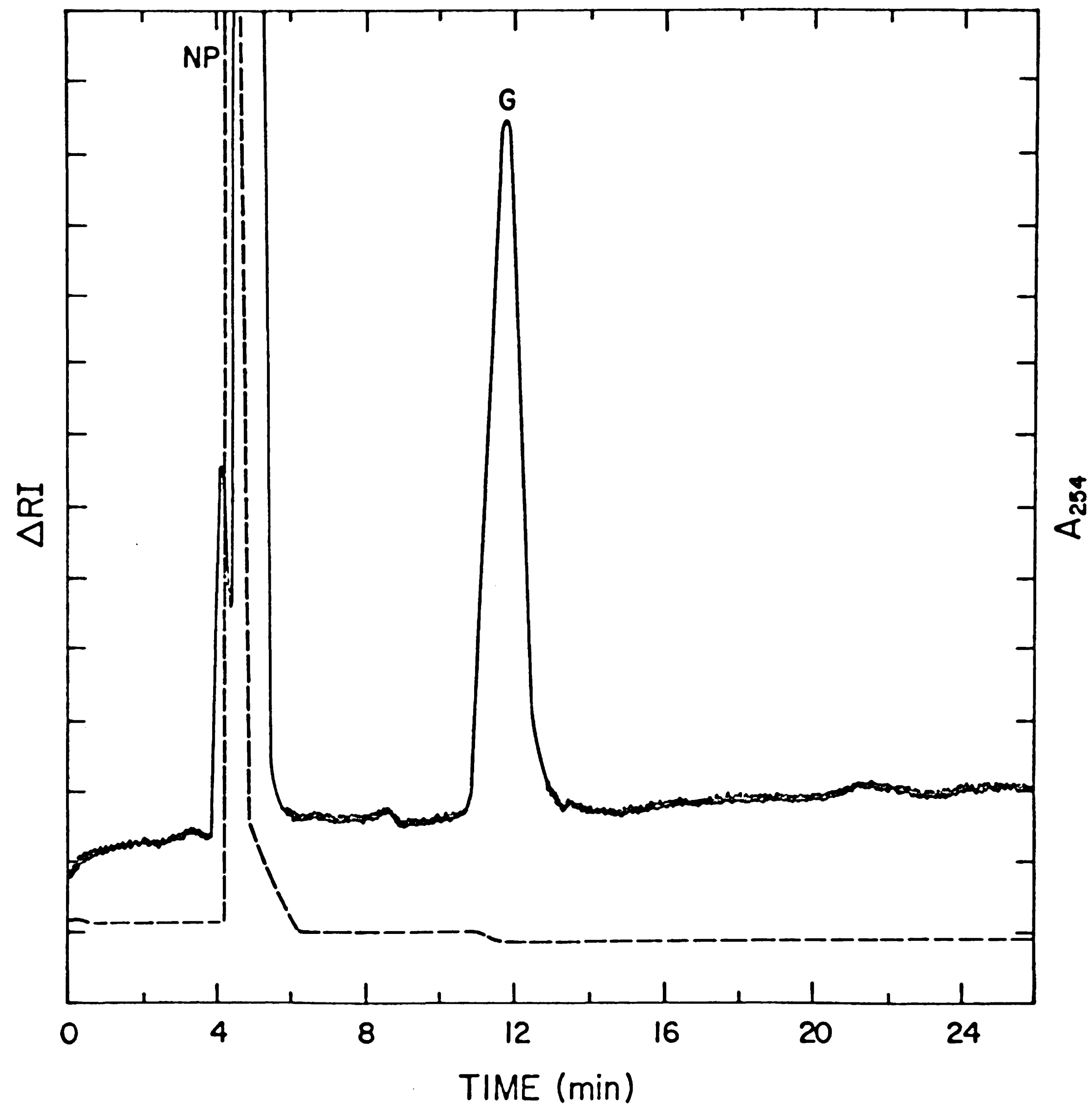
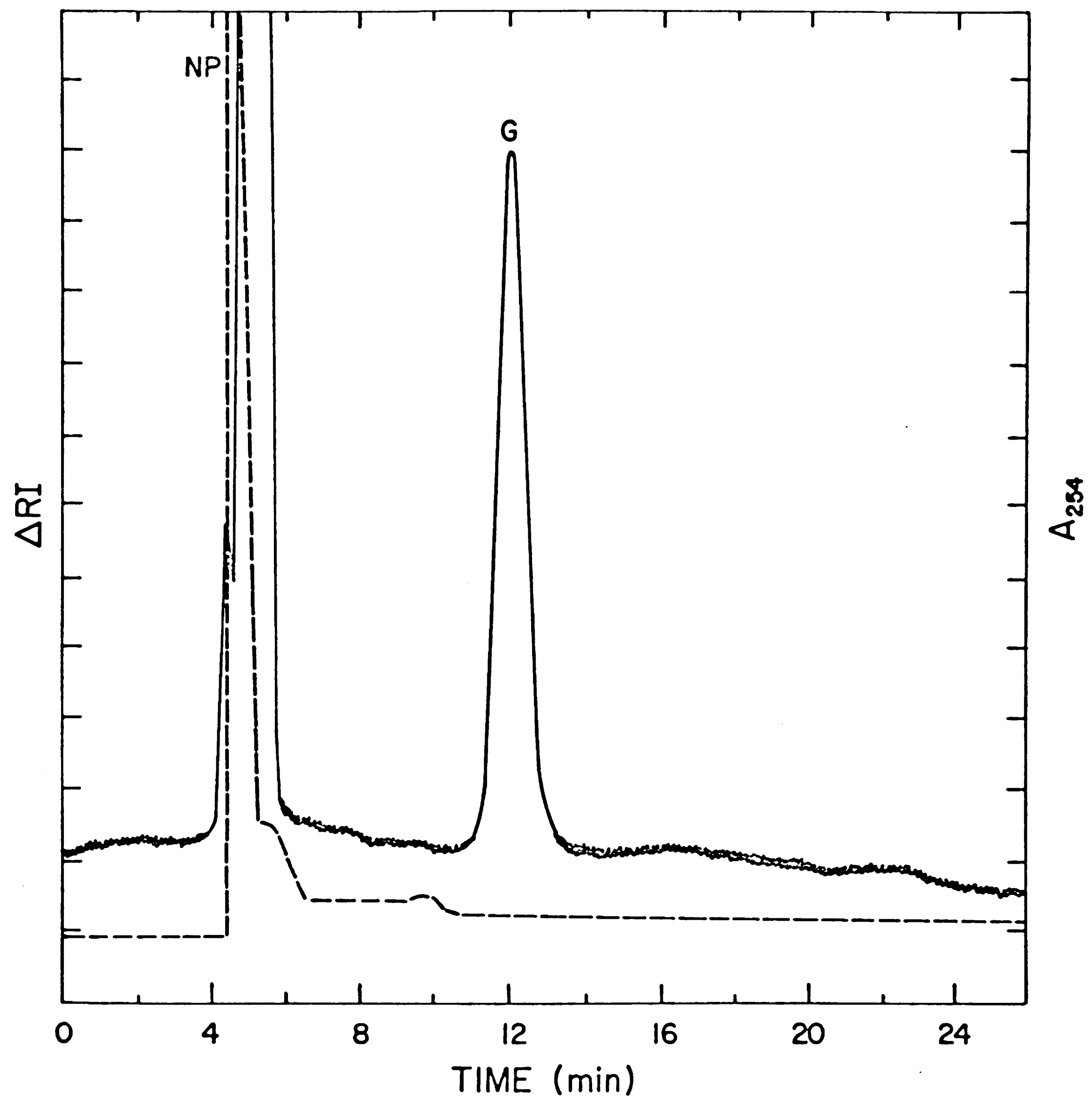

NP
G
ΔRI
A254
0
4
8
12
16
20
24
TIME (min)

FIGURE 37

Separation by High Pressure Liquid Chromatography of Hydrolysis Products from Reaction of Endoglucanase IV with *p*-Nitrophenyl Cellobioside

Hydrolysis products after a 23-hour incubation of Endoglucanase IV with 1.5 mM *p*-nitrophenyl cellobioside at 30° were detected as previously described (cf. p.50). Enzyme concentration was 26 μg/ml.

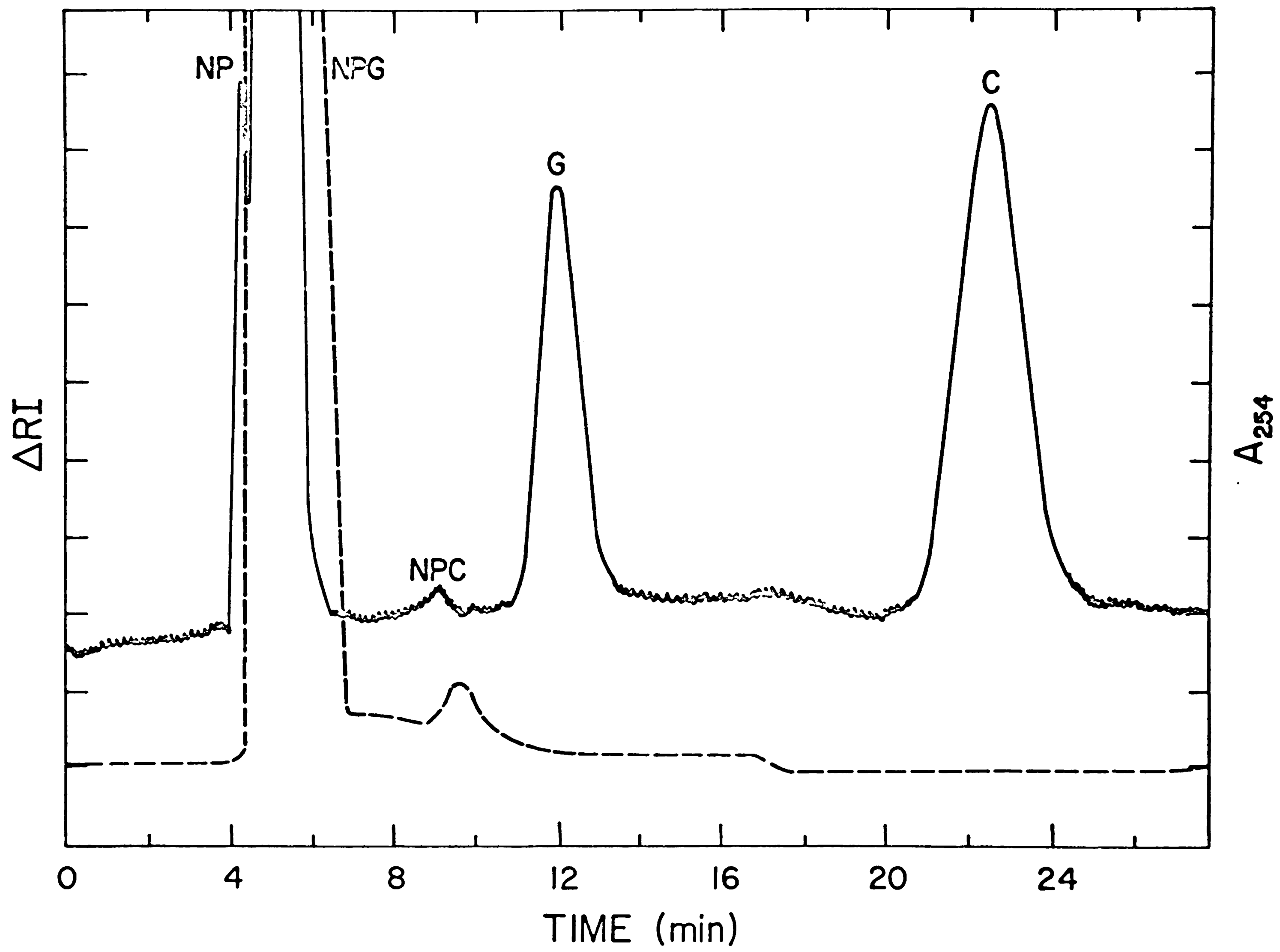


NP
G
ΔRI
A_{254}
0
4
8
12
16
20
24
TIME (min)

FIGURE 38

Separation by High Pressure Liquid Chromatography of Hydrolysis Products from Reaction of Endoglucanase III with *p*-Nitrophenyl Cellobioside

Hydrolysis products after a 23-hour incubation of Endoglucanase III with 1.5 mM *p*-nitrophenyl cellobioside at 30° were detected as previously described (cf. p.50). Enzyme concentration was 13 μg/ml.


NP
NPG
NPC
G
C
ΔRI
A254
0
4
8
12
16
20
24
TIME (min)

found from Endoglucanases II and IV action with PNC. Coupling this finding with previous information suggests that Endoglucanases II and IV preferentially cleave the holosidic bond of NPC forming glucose and NPG as products. The NPG is subsequently cleaved, forming glucose and NP. Thus after an extended incubation NP and glucose would be the only products detected.

Endoglucanase III demonstrated a different product distribution with NPC (Figure 38). In this case, approximately equimolar amounts of glucose and cellobiose were present in addition to NP and NPG. The mode of action of this endoglucanase, consistent with previous rate studies includes cleavage of both the holosidic and the glycosidic bonds of NPC. Further reaction on NPG and cellobiose would be limited as indicated by the kinetic data. Thus Endoglucanases II and IV were again distinguished from Endoglucanase III using these model substrates. The descriptions of endoglucanases II-A and II-B (19) are quite similar to those reported here for Endoglucanases IV(II) and III, respectively.

Polymeric Substrates - Studies on the specificity and action patterns for endo-1,4-β-D-glucanases were extended to the higher-molecular-weight substrates. The 1,4-β-D-glucans are insoluble in aqueous solution and thus possible effects of surface area must be considered in the interpretation of results. Derivatives of these 1,4-β-D-glucans modified to impart solubility are consequently restricted in their utility for evaluation of the mode of action of endoglucanases with the underivatized substrate. Further complicating interpretation of the patterns of soluble products from polymeric substrates are the many alternate paths newly

formed products may take. A product could be transferred to the acceptor-substrate, remain bound or associated with the substrate, be released but remain insoluble, or be released as a soluble product. Only in the latter case, is the product conveniently detected. Moreover, hydrolytic products from one enzymic cleavage can readily serve as substrates for further reactions. Thus initial or extended reactions must be differentiated in evaluation of results.

Linkage and bond specificities of the endoglucanases were determined as previously described (cf. p.39) using a number of one percent (w/v) solutions or suspensions of different polymeric substrates. The results are illustrated in Table XIII. CM-cellulose and amylose were completely soluble, xylan was about 90% soluble, and phosphoric acid-swollen cellulose (PSC) and Avicel were insoluble in pH 4.5, 0.05 M sodium acetate buffer. In addition, Avicel, a highly crystalline cellulose, has a much smaller relative surface area than PSC, an amorphous cellulose. In agreement with the nomenclature of these enzymes, Endoglucanases II, III and IV demonstrated the highest specific activity with CM-cellulose under initial conditions. Furthermore, these enzymes possessed much less ability to produce soluble reducing sugar from Avicel than with the more hydrated and acessible substrate, PSC. Each of the endoglucanases had significant xylanase activity, which has also been shown to be characteristic of an endoglucanase purified from *Irpex lacteus* (61). This xylanase activity coupled with a similar high activity on CM-cellulose, illustrates the lack of a specificity requirement at the sixth carbon atom of the pyranose ring as discussed in the Literature Review (cf. p.16). All three endoglucanases exhibited, as expected, very low amylase activity.

TABLE XIII

Specific Activities of Endo-1,4-β-D-glucanases on Polymeric Substrates

SUBSTRATE	μmole/min/mg protein		
	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
Amylose	0.028	0.068	0.028
Xylan	0.256	0.892	0.248

The individual endoglucanases were distinguished from one another by their specific activities on the polymeric substrates (Table XIII). Endoglucanase II which has demonstrated enzymic properties very similar to Endoglucanase IV is significantly different in its three-fold higher activity with CM-cellulose, as well as its lower activity on Avicel. Endoglucanase II, which has been shown to have a distinctive endoglucanase action pattern, demonstrated the same ability to release reducing sugar residues from CM-cellulose and PSC as Endoglucanase III. However, Endoglucanase III illustrates its unique character in its three-fold higher xylanase activity.

In addition to these substrates, laminarin, a 1,3-β-D-glucan, and pustulan, a 1,6-β-D-glucan, were tested but results with these substrates could not be quantified due to the high absorbance yield with the substrate blanks. However, the endoglucanases indicated significant activity with both of these substrates. Thus these endo-1,4-β-D-glucanases do reflect rather broad specificities.

In order to gain a further understanding of the action patterns of endo-1,4-β-D-glucanases on polymeric 1,4-β-D-glucans, the products formed from endoglucanase action on PSC and Avicel were compared (Figures 39 and 40). After a short (60 minute) incubation at 40° with PSC, Endoglucanases II and IV demonstrated the distinctive products glucose:cellobiose:cellotriose in molar ratios of 3:4:1 and 4:5:3, respectively. However, after a much more extended reaction time (48 and 96 hours, respectively) the product array changes in accord with the slow hydrolysis of cellobiose and cellotriose to give molar ratios of glucose:cellobiose of 8:1 and 3:1, respectively. Endoglucanase III, on the other hand, formed the distinc-

FIGURE 39

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 Endoglucanase II, III or IV (panels A, B and C, respectively) on one percent (w/v) PSC were identified by HPLC (cf. p.47) after incubation at 40° for 60, 60 and 120 minutes, respectively. Concentrations of Endoglucanases II, III and IV were 93, 40 and 53 μg/ml, respectively. Samples for HPLC analysis were deionized with Amberlite MB-3 before injection.

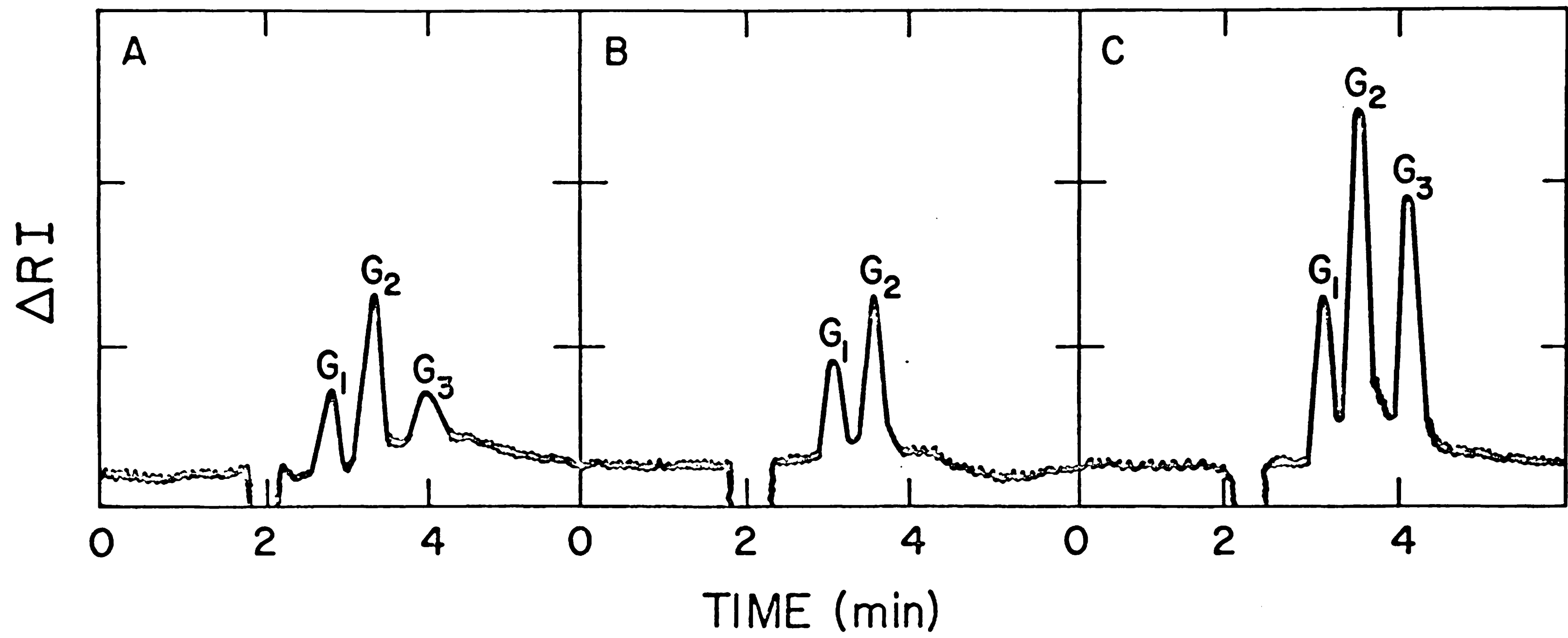
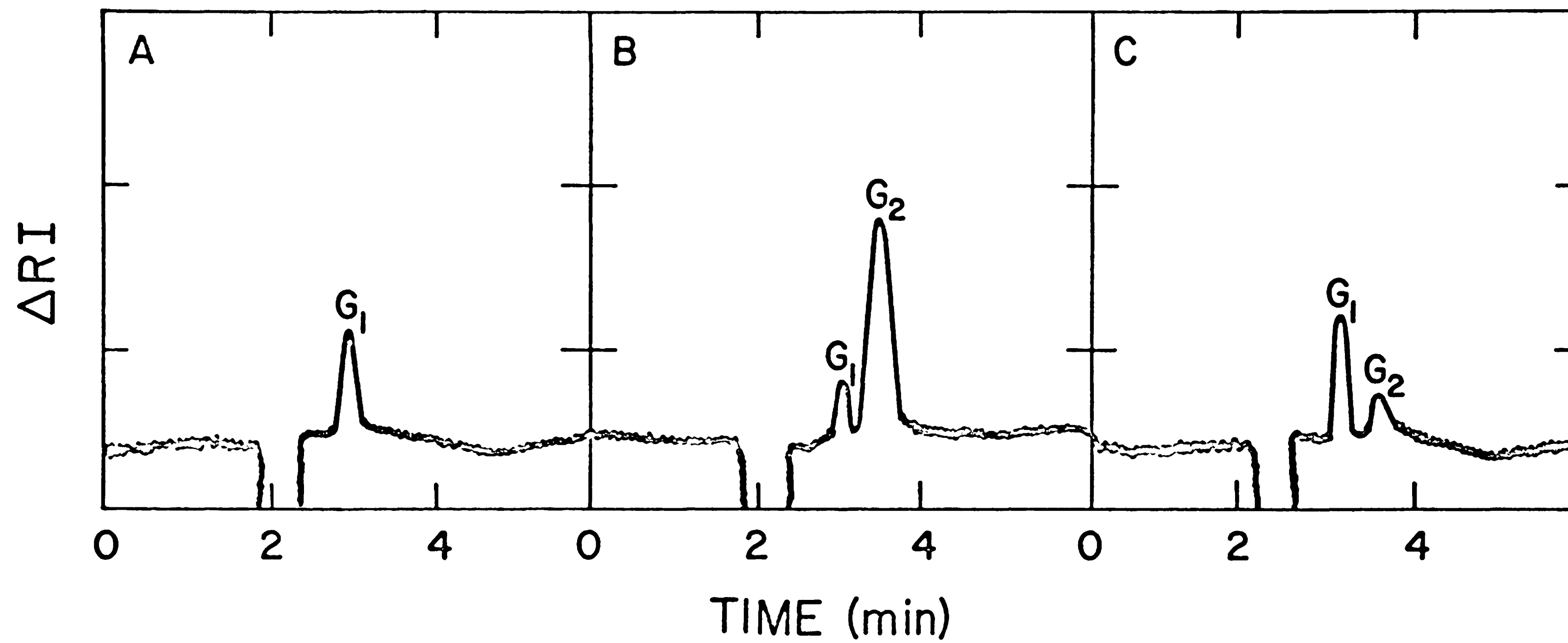

A
B
C
G_1
G_2
G_3
ΔRI
0
2
4
TIME (min)

FIGURE 40

Separation by High Pressure Liquid Chromatography of Soluble Products from Endo-1,4-β-D-glucanase Reaction with Avicel

The soluble products from the action of Endoglucanase II, III or IV (panels A, B and C, respectively) on one per-cent (w/v) Avicel were identified by HPLC (cf. p.47) after incubation at 40° for 95, 144 and 144 hours, respectively. Concentrations of Endoglucanase II, III and IV were 93, 40 and 53 μg/ml, respectively. Samples for HPLC analysis were deionized with Amberlite MB-3 before injection.


A
B
C
G1
G2
G1
G1
G2
ΔRI
0
2
4
0
2
4
0
2
4
TIME (min)

tive products glucose:cellobiose in equimolar amounts after a comparable extent of reaction as Endoglucanase II and IV (Figure 39). In accord with its extremely low activity on cellobiose, the distribution of products slowly shifts toward formation of glucose giving a 2:1 molar ratio of glucose to cellobiose after 144 hours. These results are consistent with the kinetic studies and emphasize the significance of the extent of reaction in determining the resulting product distributions.

The product array formed after extensive incubation of the endoglucanases with Avicel is somewhat different than with PSC (Figure 40). The long incubations were necessary with this substrate in order to obtain detectable amounts of soluble carbohydrates. Endoglucanases III and IV illustrate quite different types of attack on Avicel giving molar ratios of glucose:cellobiose of 1:3 and 3:1, respectively. Furthermore, at no time in this reaction did Endoglucanase II demonstrate any cellobiose as product, which distinguishes it further from Endoglucanase IV in its action on crystalline cellulose. The difference between this array of products compared to those from PSC partially reflects the longer reaction time and possibly reflects different modes of action by each enzyme on these two types of celluloses.

Filter paper (α-cellulose) was used as a substrate recently by Berghem *et al.* (21) to differentiate the activities of the low-molecular-weight (12,500) "endoglucanase I" from the high-molecular-weight (50,000) "endoglucanase II". Both endoglucanases demonstrated short fiber forming activity with filter paper, but "endoglucanase I" was shown to be twice as effective in this respect. The ability of cellulase components to form short fibers from filter paper was studied in depth by Halliwell and Riaz

(13). More recently, this type of activity has been associated with endoglucanase action (21); however, there have been no studies on the molecular basis of this activity. Thus because of its association with endoglucanases and its use in differentiating endoglucanases, this short fiber forming activity was investigated with Endoglucanases II, III and IV.

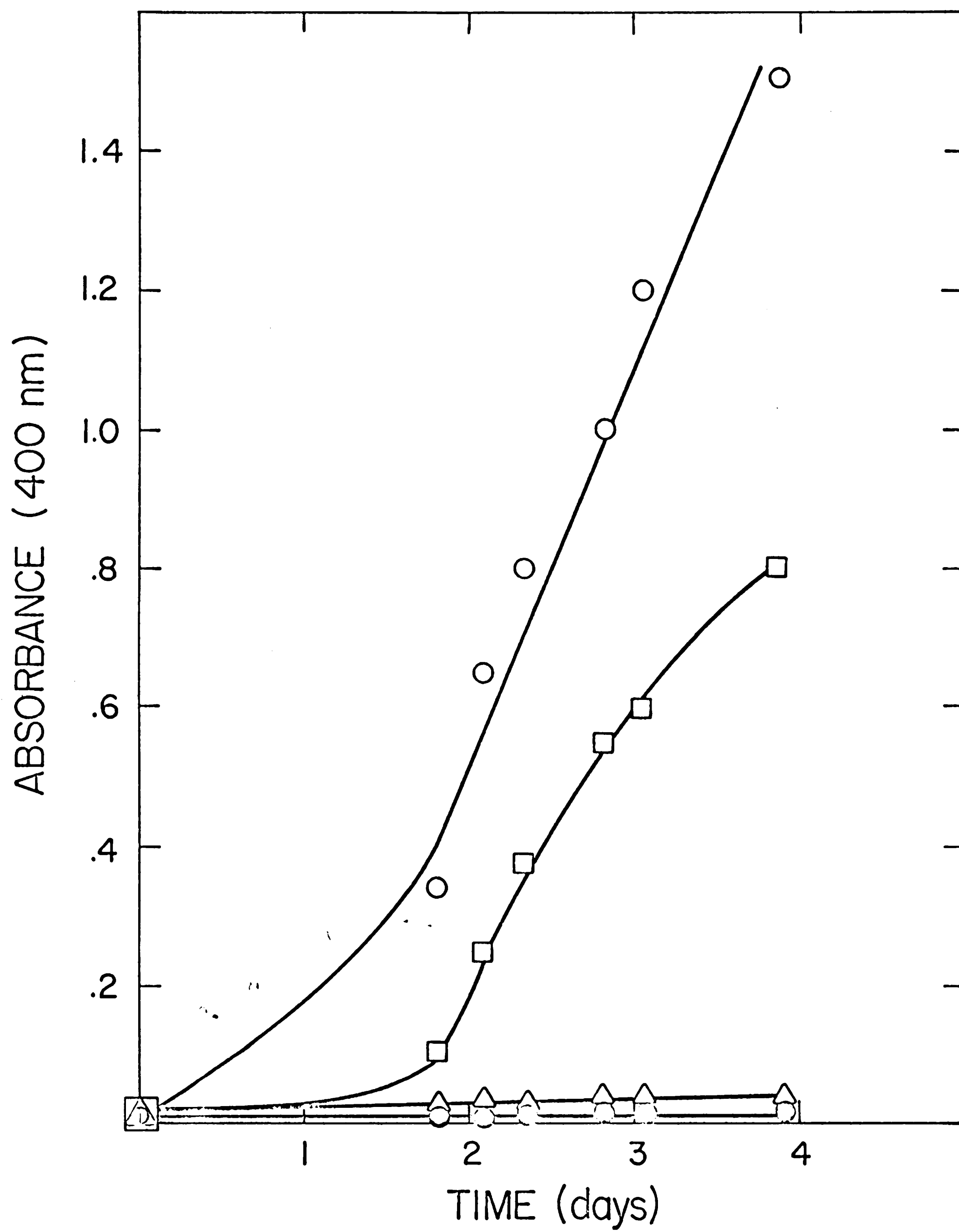
The initial experiment as described in Experimental Procedures (cf. p.56) was designed to duplicate that of Berghem *et al.* (21) using comparable enzyme concentrations. The amount of each enzyme used in the reaction mixtures was chosen to represent an equal capacity to produce reducing sugar from CM-cellulose. Therefore, much higher concentrations of Endoglucanase IV were used than for either Endoglucanase II or III (cf. Table XIII). The results of the spectrophotometric (turbidimetric) measurements used in detection of short fibers are shown in Figure 41. Endoglucanase III was the most effective in releasing short fibers and for two days appeared unique in this regard. However, after two days of incubation at 40°, Endoglucanase IV had demonstrated significant activity. No detectable activity was found with Endoglucanase II even after two weeks of incubation. This short fiber forming activity is illustrated in Figure 42. This experiment suggests that this type of activity is primarily associated with endoglucanases derived from the water fraction (Endoglucanases III and IV) which have the ability to bind to cellulose.

This is not entirely in agreement with Berghem *et al.* since they found that a "buffer fraction" enzyme ("endoglucanase II") had some of this type of activity. However, the tendency of cellulase components to bind to Avicel depends on the conditions of the column (24), and "endo-

FIGURE 41

Short Fiber Forming Activity of Endo-1,4-β-D-glucanases on Filter Paper

The ability of Endoglucanases II (Δ), III (o) and IV (□) to release short fibers was measured at different times by absorbance at 400 nm as described in Experimental Procedures (cf. p.56). A control containing 20 mg filter paper in pH 4.5, 0.05 M sodium acetate-3.0 mM sodium azide buffer is represented by the solid circles (●).

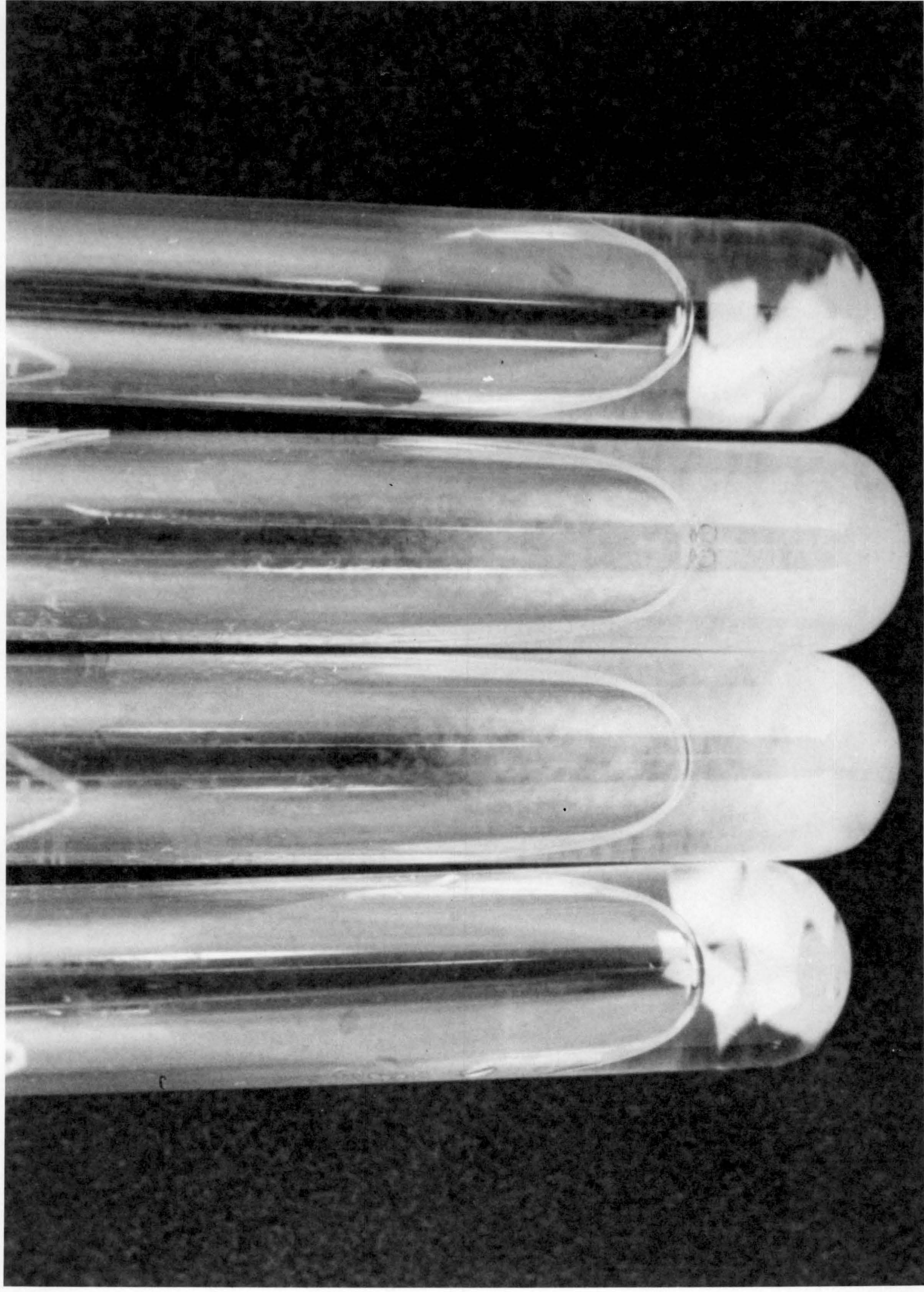


1.4
1.2
1.0
.8
.6
.4
.2
ABSORBANCE (400 nm)
1
2
3
4
TIME (days)

FIGURE 42

Reaction Mixtures Demonstrating Short Fiber Forming Activity of Endo-1,4-β-D-glucanases

Examples of the reaction mixtures after two weeks incubation at 40° with 20 mg of filter paper. They represent from left to right a control without enzyme added, Endoglucanase IV (235 μg/ml), Endoglucanase III (87 μg/ml) Endoglucanase II (75 μg/ml).



glucanase II" might have been associated with the water fraction under different conditions. But direct comparisons of the endoglucanases purified by Berghem *et al.* can not be made, particularly because a similar low-molecular-weight enzyme giving short fiber forming activity has not been found in this laboratory. It can be speculated from the molecular weight and fiber forming activity, that the "endoglucanase II" of Berghem *et al.* (21) is either Endoglucanase III or IV.

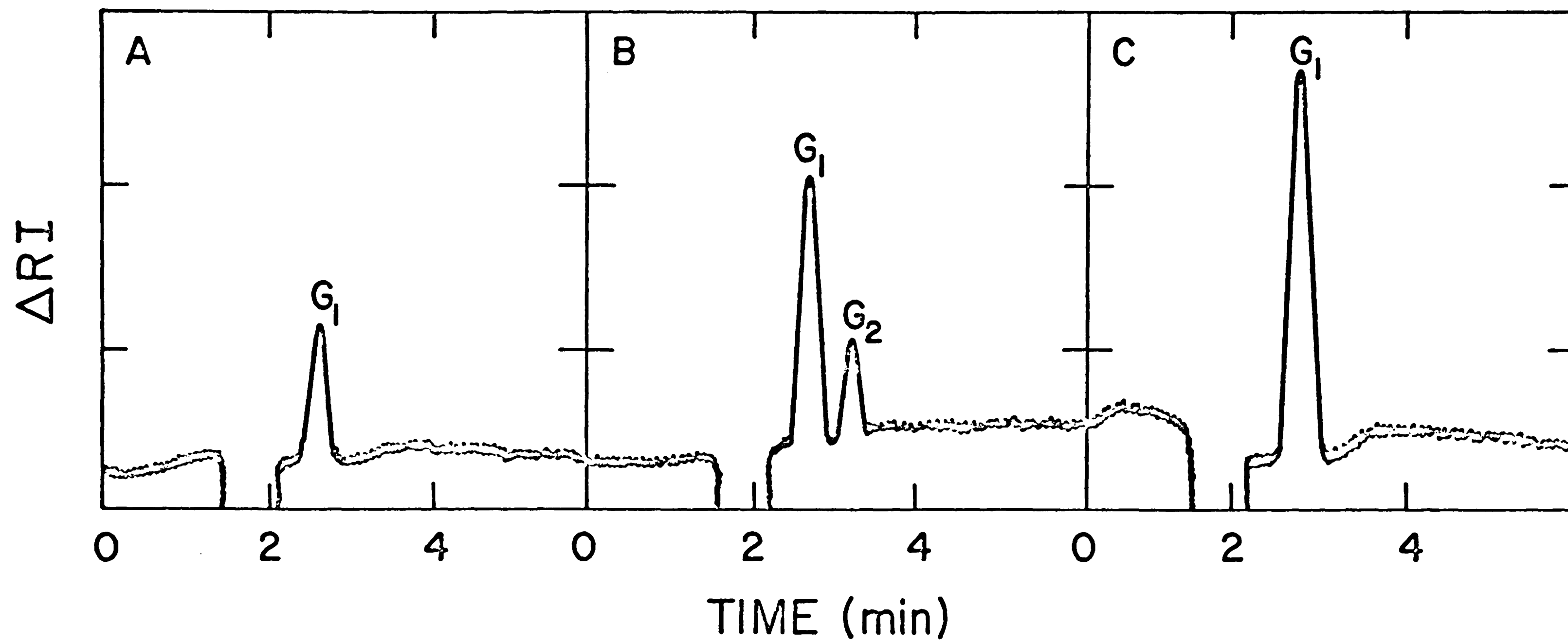
Attempts to further evaluate this type of activity by reducing sugar determination and by identification of soluble products were made using the two week reaction supernatants. Results from the reducing sugar determination corresponded to the results from light scattering measurements giving 30, 244 and 208 μg glucose equivalents for Endoglucanases II, III and IV, respectively. Since the experimental protocol called for different enzyme concentrations these data were further evaluated in terms of moles glucose per mg protein giving 4.5, 31.1 and 9.83, respectively. Examination of results on this basis, clearly demonstrates the three-fold greater ability of Endoglucanase III in releasing short fibers.

Identification by HPLC of the cellooligosaccharide products accompanying short fiber formation is shown in Figure 43. The similarity of the products from Endoglucanases II and IV, which in each case are different from Endoglucanase III products, is consistent with previous enzymic studies. In order to detect any products with Endoglucanase II the sample had to be concentrated forty-fold. The extent of production of glucose or of glucose and cellobiose, neither of which were found in the control, seemed to be related to the short fiber forming activity of these endoglucanases.

FIGURE 43

Separation by High Pressure Liquid Chromatography of Soluble Products from Endo-1,4-β-D-glucanase Reaction with Filter Paper

The soluble products from the action of Endoglucanase II, III or IV (panels A, B and C, respectively) on filter paper were identified by HPLC (cf. p.47) after incubation at 40° for two weeks. Concentrations of Endoglucanases II, III and IV were 75, 87 and 235 μg/ml, respectively. Samples for HPLC analysis were deionized with Amberlite MB-3 before injection. In order to detect products formed by Endoglucanase II action, the 4-ml reaction supernatant was evaporated to dryness and rediluted to 50 μl.


A
B
C
G_1
G_2
ΔRI
0
2
4
TIME (min)

This study which was initially undertaken as a means for comparison of the endoglucanases and extended in attempting to understand the nature of this perplexing activity, left many unanswered questions. One question concerns the role of Endoglucanase II in the cellulase system since it was shown to be the most abundant endoglucanase in crude enzyme preparations. Perhaps this enzyme has a relatively non-hydrolytic function and exerts its action first to "condition" cellulose for further action by other cellulase enzymes.

This possibility was explored in a similar experiment[11] in which short fiber forming activity was measured with exo-cellobiohydrolase C and endoglucanase, alone and together in the reaction mixture. The concentration of exo-cellobiohydrolase C was approximately 200 times greater (1.9 mg/ml) than the endoglucanase (75 μg/ml) in order to give equivalent (reducing sugar) activities with CM-cellulose. Using this high concentration of exo-cellobiohydrolase C, short fiber forming activity was observed. Again none was observed with Endoglucanase II.

This activity as measured by light scattering was significantly higher with the combined exo-cellobiohydrolase C and endoglucanase, than the sum of the activities produced by either enzyme alone. The synergism diminished with the time of incubation but was still evident at longer times; however, a much more detailed investigation with different enzyme concentrations must be performed before this synergism can be confirmed. Results from this initial experiment suggest a possible role for the action of Endoglucanase II on crystalline cellulose. Its low binding affinity for cellulose lends credence to a multi-chain type of attack

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[11] Gum, E. K., Jr. (1976) Personal Communication.

whereby portions of cellulose structure are loosened and/or somehow "conditioned" promoting the further action by other endo- and exoglucanases.

Summary of Activities and Specificity of Endo-1,4-β-D-glucanases - Endo-1,4-β-D-glucanases II, III and IV from *Trichoderma viride* were generally similar, but were distinct in several specific features of their action patterns. In general, these endoglucanases exhibited similar pH optima (4.0-4.5) and stability (labile above pH 7.0). Each was highly active on CM-cellulose and PSC, less active on xylan, and only slightly active on Avicel. Very little activity was observed with the soluble 1,4-α-D-glucan, amylose. Specific activities of the endoglucanases increased with the chain length of the cellooligosaccharide substrates.

The action patterns of these endoglucanases were distinguished by their relative rates on different substrates. In most respects, Endoglucanase III was clearly different from Endoglucanases II and IV. Its similarity to exo-cellobiohydrolase C was demonstrated by reducing sugar versus viscosity plots. Furthermore, products of Endoglucanase III action on reduced or non-reduced cellooligosaccharides indicated predominant cleavage of cellobiosyl units from the non-reducing end. Frequently, under initial conditions, the resulting cellobiosyl units were transferred to the substrate-acceptor. The relatively high activity with cellotriose and low affinity for cellobiose of Endoglucanase III was consistent with its characteristic product distribution from polymeric substrates. Similarly, it exhibited high activity with NPC but low activity with NPG. This endoglucanase was further distinguished by its ability to release rapidly short fibers from filter paper.

Endoglucanases II and IV demonstrated many similar enzymic activities in agreement with their mode of action on CM-cellulose. The distinctive products, glucose, cellobiose and cellotriose, formed from reduced and non-reduced cellooligosaccharides and PSC were usually observed with these enzyme but, after extended reaction, the cellotriose was cleaved to cellobiose and glucose. These enzymes further demonstrated similar linkage specificity in cleaving the second bond in cellotetraitol, the third bond in cellopentaitol and the holosidic bond of NPC. In addition, Endoglucanase II and IV demonstrated significant activity on NPG.

Endoglucanases II and IV, although similar in many respects, were very different in their activities on crystalline cellulose (e.g., Avicel and filter paper). Endoglucanase IV had greater activity on crystalline cellulose and was further distinguished from Endoglucanase II in its ability to form short fibers from filter paper. Endoglucanase II, however, demonstrated greater absolute activities on all substrates except Avicel and filter paper.

This investigation of the endoglucanases in the cellulase system of *Trichoderma viride* will be the framework for future experiments. In addition, the many unidentified protein peaks from the crude cellulase mixture need to be further purified and characterized. *Trichoderma* may synthesize a number of enzymes in order to degrade crystalline cellulose. Yet, in a broad sense it seems that this degradation process occurs with three types of enzymes, exo- and endoglucanases and β-glucosidases. Then why are there so many multiple forms of the exo- and endoglucanases and how is this multiplicity beneficial and economical to the organism? Are

these multiple forms simply mistakes in translation or a result of proteolytic activity? For the endoglucanases, it seems that the water fraction endoglucanases are indeed distinct and are probably synthesized by *Trichoderma* for specific purposes. On the other hand, the origin of the buffer fraction endoglucanase (Endoglucanase II) is questionable since it is similar in many respects to Endoglucanase IV, and perhaps has arisen from proteolytic modification. However, this buffer fraction enzyme's high activities on all substrates except crystalline cellulose, its abundance in crude cellulase preparations and its possible role in crystalline cellulose degradation lend support for it also being a part of the *Trichoderma* enzyme system.

The functional role of these multiple endoglucanases that are apparently synthesized by *Trichoderma viride* still needs to be investigated, both with these enzymes alone and in combination with other cellulase components. It has been only with crystalline cellulose (Avicel) that synergism has been observed with the cellulase components. However, experimental design in this area of research is complicated due to the insoluble, polymorphic properties of cellulose.

The sensitivity of the HPLC techniques developed in this laboratory can be increased by the use of radioactively labeled cellooligosaccharides. This could provide much needed information that would further define and separate the many simultaneously occurring reactions of the endoglucanases on cellooligosaccharide substrates. In addition, it would be beneficial to analyze the products of Endoglucanase III with cellohexaitol in order to examine its bond preferences since it gave a preference for the second bond when cellotetraitol or cellopentaitol served as substrates. Because

of its endoglucanase action, one might expect more-or-less equal preferences for the second and third bonds in cellohexaitol.

A column, which separates components by molecular weight, could be useful in analyzing the type and distribution of the products of endoglucanase action on CM-cellulose. In addition, identification of the transfer products by HPLC of endoglucanase action with cellooligosaccharides would give information toward establishing mechanisms of action. This type of analysis would require, for adequate confirmation of structure, a series of standards of oligosaccharides and derivatives to which the transfer products could be compared.

Thus purification, structural characterization and investigation of enzymic properties of the endoglucanases only sets the stage for the many more specific studies needed in order to establish the individual roles of the cellulase components in the cellulase system of *Trichoderma viride*.

SUMMARY

Four electrophoretically distinct endo-1,4-β-D-glucanases from *Trichoderma viride* have been identified and named as isozymes according to their electrophoretic mobilities on polyacryamide gels. The two most acidic proteins are associated with the buffer fraction (Endoglucanases I and II), whereas the other two are associated with the water fraction (Endoglucanases III and IV) of an Avicel column. The buffer fraction also contains a β-glucosidase and most of the components of the crude cellulase preparation, whereas the water fraction contains proteins, including the exo-cellobiohydrolase C, that binds to crystalline cellulose. Endoglucanases II, III and IV have been purified by both adsorption and repeated anion-exchange chromatography using a viscosimetric technique to monitor activity on CM-cellulose. Disc gel electrophoresis is the principal method used in judging enzyme purity, however slab gel electrophoresis in one percent sodium dodecyl sulfate and ultracentrifugation studies also indicate homogeneous enzymes. The specific activities of purified Endoglucanases II, III and IV on CM-cellulose are 1010, 60 and 250, respectively.

The endoglucanases are high in acidic and hydroxylated amino acids and glycine, but low in basic amino acids. Values of 11.97, 10.32 and 13.12 have been determined for the $E^{1\%}_{280}$ of purified Endoglucanases II, III and IV, respectively. Sedimentation equilibrium analysis has established the molecular weights of Endoglucanases II, III and IV at 37,200, 52,000 and 49,500, respectively.

Carbohydrate composition and analysis of neutral and amino sugars, demonstrate that all three endoglucanases contain mannose, galactose,

glucose and glucosamine. Mannose is the principal neutral sugar in each enzyme. Endoglucanase II differs from the water fraction endoglucanases by its low percentage of total carbohydrate, 4.5% (weight), compared to Endoglucanases III and IV which contain 15.0% and 15.2% carbohydrate, respectively.

These glycoproteins have similar pH optima between pH 4.0 and 4.5 and instability above pH 7.0 and demonstrate rather broad substrate specificity. The mode of action of Endoglucanases II and IV is different from that of Endoglucanase III on CM-cellulose as shown by viscosity versus reducing sugar plots. Such analyses indicate that Endoglucanases II and IV employ a multi-chain attack mechanism, whereas Endoglucanase III may repetitively cleave single chains.

The activities of each of the endoglucanases increases as the chain-length of cellooligosaccharide substrates progresses from two to six. Endoglucanase III is unique in its less rapid increase in activity with substrates larger than cellotetraose and its significantly greater ability to cleave cellotriose. Kinetic analysis of Endoglucanase III confirms its very low affinity for cellobiose.

Product analysis using high pressure liquid chromatography further demonstrates differences between Endoglucanases II(IV) and III. Endoglucanases II and IV display a preference for internal bonds of cellooligosaccharides and, specifically, prefer the second glycosidic bond in cellotetraitol, the third bond in cellopentaitol and the holosidic bond in p-nitrophenyl cellobioside. The charactistic products of Endoglucanases II and IV are glucose, cellobiose and cellotriose, which only after very extended reactions change principally to glucose with some cellobiose.

The singular action of Endoglucanase II with Avicel as substrate yields only glucose as product. This is probably due to its much lower activity on Avicel which permits any cellobiose released to be subsequently cleaved by the time glucose reaches concentrations great enough to be detected. On the other hand, Endoglucanase III demonstrates a preference for cleaving cellobiosyl units which are often, under initial conditions, transferred to the substrate-acceptor. In particular, it prefers the second bond in cellotetraitol and cellopentaitol, and appears to cleave both the holosidic and glucosidic bonds of p-nitrophenyl cellobioside. The characteristic products of Endoglucanase III are principally cellobiose and some glucose.

The endoglucanases may be compared further according to their ability to form short fibers from filter paper. Endoglucanase II is clearly differentiated from III and IV by its apparent absence of any such activity. Endoglucanase III is the most efficient at forming short fibers. This activity is concomitant with release of reducing sugars; after two weeks incubation, Endoglucanase III action yields three times as much reducing sugar as Endoglucanase IV. Product analysis of the soluble carbohydrate released from the filter paper reveals that glucose is the sole product from Endoglucanase IV(II) and that cellobiose and glucose are the products of Endoglucanase III action. The role of Endoglucanase II on crystalline cellulose is unique among the endoglucanases as seen in its lack of affinity and low activity on crystalline cellulose. Preliminary experiments with filter paper as substrate suggests that Endoglucanase II acts, perhaps, non-hydrolytically to "condition" cellulose for further action.

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CHARACTERIZATION OF STRUCTURE AND ENZYMIC ACTIVITY OF ENDO-1,4-β-D-GLUCANASES PURIFIED FROM TRICHODERMA VIRIDE

by

Sharon P. Shoemaker

(ABSTRACT)

Four electrophoretically distinct endo-1,4-β-D-glucanases have been isolated from a commercial *Trichoderma viride* enzyme preparation, Pancellase SS. These endoglucanases were distinguished by their affinity for microcrystalline cellulose; two were adsorbed onto a cellulose column at pH 5.0 (Endoglucanases III and IV) and two were not (Endoglucanases I and II). Endoglucanases II, III and IV have been purified subsequently by ion-exchange chromatography. Enzyme homogeneity has been established using polyacrylamide gel electrophoresis, slab gel eletrophoresis of sodium dodecyl sulfate-protein complexes and ultracentrifugation studies. With CM-cellulose as substrate Endoglucanases II, III and IV have specific viscosimetric activities of 1010, 60 and 250 specific fluidity change minute^{-1} mg protein^{-1}, respectively, and from this substrate release reducing sugars at rates of 29.1, 28.2 and 9.16 μmole minute^{-1} mg protein^{-1}, respectively. The specific activities of these endoglucanases are less when crystalline cellulosic substrates are employed.

Endoglucanases II, III and IV have, respectively, $E_{280}^{1\%}$ values of 11.97, 10.32 and 13.12, and molecular weights of 37,200, 52,000 and 49,000 as determined by sedimentation equilibrium. The three endoglucanases each contain mannose, galactose, glucose and glucosamine. Endoglucanase II contains significantly less carbohydrate (4.5% by weight) than Endoglucanases III and IV which contain 15.0% and 15.2%, respectively.

Plots of viscosity decrease versus reducing sugar production indicate that Endoglucanases II and IV have similar modes of action on CM-cellulose which differ from that of Endoglucanase III. All three endoglucanases have similar pH optima of 4.0-4.5 and lose activity above pH 7.0.

High pressure liquid chromatographic analysis permitted determination of enzymic rates of reaction with purified cellooligosaccharides. Endoglucanases II, III and IV display higher activities as the chain-length of cellooligosaccharide substrates progresses from two to six. Kinetic studies revealed differences in Endoglucanase III as compared to Endoglucanases II and IV; it is distinguished by low affinity for cellobiose (K_m=162 compared to K_m=1.03 and 1.26 mM) and its ten-fold higher activity with cellotriose (V_{max}=2.49 compared to V_{max}=0.339 and 0.279 μmole minute^{-1}mg protein^{-1}). In addition, Endoglucanase III differs from the other endoglucanases in its low activity on p-nitrophenyl glucoside, its preference for the third bond from the non-reducing end of cellopentaitol, and its significant transferase activity in the presence of large amounts of substrate-acceptor oligosaccharides.

Results of high pressure liquid chromatographic analysis of the soluble sugars produced during endoglucanase reaction with cellulose was consistent with kinetic studies using oligosaccharide substrates. From phosphoric acid swollen cellulose, Endoglucanases II and IV yielded glucose, cellobiose and cellotriose as distinctive products, whereas Endoglucanase III action produced only glucose and cellobiose. Endoglucanases III and IV each produce substantial quantities of reducing sugars as well

as short fibers from filter paper, whereas Endoglucanase II does not.

These structural and catalytic properties may be used to describe more precisely the role of each component in the cellulase system.