PURIFICATION AND CHARACTERIZATION OF AN ENDO-1,4-β-D-GLUCANASE AND TWO EXO-1,4-β-D-GLUCANASES FROM THE CELLULASE SYSTEM OF TRICHODERMA REESEI

by

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LIST OF ABBREVIATIONS

CAPS	- Cyclohexylaminopropane sulfonic acid
CBH	- Cellobiohydrolase
$G_1, G_2 \ldots\ldots G_6$	- Glucose, cellobiose ..... cellohexaose
HEPES	- N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPLC	- High performance liquid chromatography
PSA	- Phenol sulfuric acid
PSC	- Phosphoric acid-swollen cellulose
SDS	- Sodium dodcryl sulfate

INTRODUCTION

Cellulose is the world's most abundant renewable resource. It is estimated that approximately 10^{11} tons of cellulose are synthesized by green plants photosynthetically each year. Furthermore, cellulose is a very abundant component of agricultural, industrial and municipal waste materials. Since cellulose is convertible to glucose, which is fermentable to ethanol, a constituent of synthetic fuels, cellulose could serve as a source of food and energy. The possibility of utilizing cellulose as such an alternative energy source has become particularly important recently, with increased awareness of the rapid depletion of conventional, nonrenewable energy sources. In the saccharification of cellulose, the enzymes of the cellulase system are the most valuable tools available, because of their efficiency and specificity. Cellulolytic organisms which produce potent cellulase systems have therefore attracted much attention. Trichoderma reesei strain QM 9414 appears to be the most promising of all the known organisms, due to the unique levels of cellulolytic activity detected in its extracellular culture filtrate. If the potential of this organism is to be properly explored, the properties of the individual enzymic components of its cellulase system must be studied in detail.

LITERATURE REVIEW

The cellulase enzyme system comprises the following types of components which act cooperatively to degrade native, crystalline cellulose to oligosaccharides and glucose: (a) the 1,4-β-D-glucan-4-glucanohydrolases (E.C. 3.2.1.4), which are endoglucanases that produce free glucan chain ends and soluble oligosaccharides by random attack of cellulose chains; (b) the 1,4-β-D-glucan cellobiohydrolases (E.C. 3.2.1.91), which are exoglucanases that cleave cellobiosyl residues from the nonreducing end of cellulose chains and (c) β-glucosidases (E.C. 3.2.1.21), which degrade glucanase products such as cellobiose and oligosaccharides to glucose.

To utilize the full potential of the cellulase system, the properties and mode of action of each enzymatic component of the system must be understood, in order to evaluate its role in the degradation of native cellulose. To elucidate these physico-chemical and catalytic properties requires highly purified enzymes, which are free from contaminating activities associated with the cellulase system. From several microorganisms the component enzymes of the cellulase system have been purified to varying degrees of homogeneity and characterized to different extents. Attention has been focused mostly on the enzymes of true cellulolytic organisms, such as various species of Trichoderma, Sporotrichum pulverulentum and Irpex lacteus, which produce the complete array of enzymes required to degrade native cellulose efficiently to glucose.

Berghem and Pettersson (1) purified to homogeneity a β-1-4-glucan cellobiohydrolase from a commercial cellulase preparation derived from the culture filtrate of Trichoderma viride. This enzyme was characterized by Berghem et al (2), and was found to be a glycoprotein that produces cellobiose from various cellulosic substrates and cellooligosaccharides. Emert et al (3) and Gum and Brown (4) reported the existence of four electrophoretically distinct cellobiohydrolase enzymes, three of which were present in a commercial T. viride cellulase preparation, and one of which was present in the culture filtrate of T. reesei QM 9123 grown on cellulose. These enzymes were purified to homogeneity and characterized; structural evidence and enzymic activities indicated that each represents a differentially glycosylated form of the same or very similar polypeptides. A detailed structural characterization has been reported for one of these glycoprotein enzyme forms (5). The cellobiohydrolase C isolated by Gum and Brown (4), on the basis of its structural and enzymic properties, appears very similar and is likely to be identical to the cellobiohydrolase purified by Berghem (1).

Wood and McCrae (6) also isolated a cellobiohydrolase from Trichoderma koningii, but extensive structural information is not available for this enzyme. Eriksson and Pettersson (7) isolated from S. pulverulentum an extracellular cellobiohydrolase which, according to these authors, is not a glycoprotein. An enzyme which, by its mode of action, appears to be a cellobiohydrolase, was isolated from Irpex lacteus (8); this enzyme was reported to be a glycoprotein containing a small amount of carbohydrate (2.35% by weight).

Several endo-1,4-β-D-glucanases have also been isolated from cellulolytic organisms. Shoemaker and Brown (9) have isolated four such enzymes from a commercial cellulase preparation of Trichoderma viride. All four enzymes were found to be glycoproteins, and three of them were purified to homogeneity and characterized (10). A low molecular weight (ca. 20,000) glycoprotein endoglucanase was also isolated by Gong et al (11) from a different commercial preparation of the same organism (i.e. T. viride); the authors do not state whether this particular enzyme is the only one of its type, or whether it is one of several isozymes in that preparation. This endoglucanase appears to be distinct from the endoglucanases purified by Shoemaker and Brown (9,10). Berghem et al (12) also report the existence of at least two distinct endoglucanases in a commercial preparation of T. viride culture filtrate; a low molecular weight (12,500 daltons) and a high molecular weight (50,000 daltons) enzyme was isolated, and are considered part of a greater array of endoglucanases produced by that organism (12). In agreement with these results, Okada (13,14) has reported the purification, from a commercial T. viride cellulase preparation, of three enzymes, which, by their reported mode of action, are likely to be endoglucanases.

Häkansson et al (15,16) have purified and characterized two endoglucanases from the culture filtrate of Trichoderma reesei QM 9414 grown on cellulose. Both of these enzymes are reportedly not glycoproteins and are stated to have apparently identical roles in degrading cellulose. In a later study by Fägerstam and Pettersson (17) the low molecular weight endoglucanase was reported absent from the culture filtrate of T. reesei QM 9414.

Several other cellulolytic organisms also produce extracellular endo-1,4-β-D-glucanases. According to Wood and McCrae (6) Trichoderma koningii culture filtrates contain five different endoglucanases, each of which degrades cellulose synergistically with the exo-cellobiohydrolase which had been purified from the same organism (6). An array of at least five endoglucanases is produced by the rot fungus S. pulverulentum according to Eriksson et al (18), Almin et al (19) and Streamer et al (20). These enzymes appear to have distinct structural and enzymic properties and thus are considered as isozymes and not derivative forms of the same enzyme.

There are often found to be multiple forms of β-glucosidase produced by cellulolytic organisms. Although only one β-glucosidase has been purified to homogeneity from T. viride (3,21,22), Gong et al report the presence of two other β-glucosidase forms in a commercial enzyme preparation derived from the culture filtrate of this organism (22). Deshpande et al (23) have isolated five β-glucosidase enzymes from S. pulverulentum. These enzymes have been distinguished primarily by means of their different kinetic constants for a variety of β-glucosidase substrates and inhibitors.

In general, there appears to exist in almost all cases a puzzling multiplicity of individual enzymic components within the cellulase system, and especially among the endo-1,4-β-D-glucanases. Although differences in structure and enzymic activity have been detected among multiple components of one type coexisting within the same cellulase system, the significance, mechanistically, of such multiple forms is difficult to understand, as they are often interchangeable in the

degradation of crystalline cellulose. It is not unreasonable to suppose that the organisms do not initially synthesize multiple forms of one enzyme, and that the observed multiplicity is due to degradation of components initially present. Such an assumption would be consistent with the observation of Fägerstam et al (17) that a low molecular weight endoglucanase was found missing from early culture filtrates, and of Nakayama (24), who reported changes in the multiplicity patterns of the extracellular cellulase enzymes of T. viride with age of the culture. Nakayama et al (25) have isolated protease-rich fractions from the extracellular culture filtrate of T. viride, which act on the components of the cellulase system to convert them to other active components with altered substrate specificity. It therefore seemed particularly important to cause elaboration of the cellulase system under such conditions as would ensure minimum modification of the nascent glycopolypeptides originally synthesized by the organism. This might be achieved by allowing the newly synthesized ensymes to remain in the culture medium, in contact with the cells, for only a limited length of time. Rapid production of the enzymes of the cellulase system by various cellulolytic organisms has been stimulated by soluble inducers (26-31), but the majority of these investigations have focused on the carboxymethylcellulase activity of the culture filtrate; only in the case of Trichoderma reesei QM 9414 have the individual enzyme components resulting from such a process been isolated in pure form and identified (29). This organism is specifically stimulated by sophorose (O-β-D-glucopyranosyl (1 → 2)α-D-glucopyranose) to produce the enzymes of the

cellulase system (26). Previous experiments indicated that after a 24-hour incubation of the organism with 1 mM sophorose, there are three major protein components in the culture filtrate. These have been identified as an endoglucanase and two cellobiohydrolases (29) which constitute 95-99% of the total protein released in the extracellular medium under these conditions. β-glucosidase activity is also detectable in the culture filtrate, but the specific activity of partially purified material[1] suggests that it constitutes no more than one percent of the protein in the complete cellulase system.

By contrast, a recent study by Fägerstam and Pettersson (17), of the components present in culture filtrates of T. reesei QM 9414 presents a very complex picture. When a culture filtrate was subjected to isoelectric focusing, 30 different components were detected. The components are subdivided into three groups: two Avicelase groups, and one Carboxymethylcellulase group. Each group is composed of many subcomponents; all of the components within the Carboxymethylcellulase group are reported as crossreacting with antiserum against a previously purified endoglucanase (16). Similarly, the subcomponents in one of the Avicelase groups crossreact with antiserum against a purified cellobiohydrolase within that group, but the components of the second Avicelase group do not crossreact with that antiserum. The authors point out that distribution of components within the T. reesei QM 9414 cellulase system is probably dependent on the culture conditions, since in the

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[1] Gritzali, M. (1979), unpublished results.

enzyme preparation used during this study they were unable to detect the low molecular weight endoglucanase component which they had previously purified to homogeneity (15).

These results render the simple pattern observed during sophorose induction a particularly attractive system for detailed examination. The small number of enzymic components produced under these conditions may represent the primary products of biosynthesis which are essential for the degradation of crystalline cellulose. Understanding the unique contribution of each component to the activity of the induced system required the elucidation of the physical, chemical, and catalytic properties which permit each to function synergistically as part of the cellulase system.

EXPERIMENTAL PROCEDURES

Materials

Enzymes - Crude extracellular enzyme preparation from Trichoderma reesei QM 9414 grown on microcrystalline cellulose (Avicel), was a gift from Gulf Oil Chemicals Company, Merriam, Kansas.

Endoglucanases II, III and IV from Trichoderma viride; prepared by S. P. Shoemaker (April, 1977).

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

Cellobiohydrolases A, B, C and D from Trichoderma; prepared by E. K. Gum (October, 1974).

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

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

Cellooligosaccharides (cellotriose through cellohexaose); prepared according to the method of Miller (32) and purified by recycling chromatography on Biogel P-2 (C. B. Jensen).

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

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

Walseth cellulose (phosphoric acid - swollen cellulose); prepared from Avicel PH 101 by the method of Wood (33).

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

Xylan ($\beta 1 \rightarrow 4$); Pfanstiehl Laboratories, Waukegan, Illinois,

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

Whatman Partisil PXS 10/25 PAC column; Whatman, Inc., Clifton, New Jersey.

DEAE Sephadex A-50 and SP-Sephadex C-50; Pharmacia Pine Chemicals, Piscataway, New Jersey.

OV 225 (cyanopropyl methyl 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.

CHES (2[N-cyclohexylamino] ethane-sulfonic acid, lot #58C-5030; Sigma Chemical Company, St. Louis, Missouri.

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

Glycine (lot 47C-0190); Sigma Chemical Company, St. Louis, Missouri.

HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid), A grade, lot #920371); Calbiochem, Los Angeles, California.

Maleic acid (lot #87C-5056); Sigma Chemical Company, St. Louis, Missouri.

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

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

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

Agarose (electrophoresis grade, lot #16866); Bio-Rad Laboratories, Richmond, California.

Ampholine Carrier Ampholytes (pH 3-6, Batch No. 27); LKB Produkter ABB, Bromma, Sweden.

Basic Fuchsin (lot #45B); Eastman Kodak Co., Rochester, New York.

Biolyte 3/10 (carrier ampholytes for isoelectric focusing, lot #17769); Bio-Rad Laboratories, Richmond, California.

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

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

N,N,N',N' - tetramethylethylene diamine, N,N-methylene-bis-acrylamide, acrylamide (ultrapure), ammonium persulfate, Coomassie Brilliant Blue R250; Miles Laboratories, Inc., Elkhart, Indiana.

Other chemicals were reagent grade.

Methods

Ultrafiltration - Protein solutions were concentrated and dialyzed for application to ion exchange columns using Amicon Ultrafiltration Dia-Flow cells Models 2000, 12, 102 and MMC $4344 (Amicon Scientific Systems, Lexington, Massachusetts). With these cells, noncellulosic membranes with molecular exclusion limits of 10,000 daltons (UM-10) were used.

Protein Determination - Proteins were precipitated with fifteen percent trichloroacetic acid, and the protein content of the pellets was determined according to the method of Lowry et al (34), as described by Leggett Bailey (35). Bovine serum albumin was used as a standard.

Enzyme Assays - Aryl-β-D-glucosidase activity was determined as described previously (29), by following the release of p-nitrophenol from p-nitrophenyl-β-D-glucoside. Units were expressed in terms of μmoles p-nitrophenol released/ min/mg of protein. Endo-1,4-β-D-glucanase activity was measured using the viscosimetric assay as described by Shoemaker (10) and specific activity expressed as the change in specific fluidity/min/mg of protein. Activity on insoluble polymers such as microcrystalline cellulose, phosphoric acid-swollen cellulose, amylose and xylan was determined by the release of reducing sugars from these substrates. All of the latter substrates were used as 1-2% suspensions, and the supernate was assayed for reducing sugars according to the method of Nelson (36) and Somogyi (37) using glucose as a standard. Units were defined as μmoles glucose equivalents/min/mg protein. All

enzyme activities were assayed in 0.05M sodium acetate buffer, pH 5.0, at 40°. A Hitachi 124 Double Beam Spectrophotometer was used to determine absorbance values for aryl-β-D-glucosidase or glucanase assays.

Purification of Cellobiohydrolase I (D) - This method, which is an adaptation of the batch process of Gum (5) to a column procedure, yields homogeneous cellobiohydrolase I (D), as reported previously (29). The enzymes of the cellulase system to be separated on this column were lyophilized proteins from the extracellular culture filtrate of Trichoderma reesei QM 9414 grown on microcrystalline cellulose (Avicel). Five grams of protein were dissolved in 200 ml of 50 mM sodium succinate buffer, pH 6.0, which contained 3 mM sodium azide. This protein solution, which had a specific aryl-β-glucosidase activity of 0.26 units/mg, and a specific endoglucanase activity of 16 units/mg, was applied to a 14 x 33 cm DEAE-Sephadex A-50 column, which had been equilibrated with 50 mM sodium succinate and was eluted at a flow rate of 400 ml/h. All of the aryl-β-D-glucosidase and the endo-1,4-β-D-glucanase activity were eluted isocratically. Pure cellobiohydrolase I was obtained when the pH of the elution buffer was lowered to 3.6, and the ionic strength increased by the addition of 0.5 M NaCl.

Purification of Endoglucanase and Cellobiohydrolase II - The less acidic glucanases were purified using a 4.4 x 55 cm SP-Sephadex C-50 cation exchange column equilibrated with a 2 mM sodium succinate buffer, pH 5.0 and eluted at a flow rate of 160 m//h. The sample to be separated on this column consisted of the pooled fractions 25-65 from the first peak of the DEAE-Sephadex column, which had been

concentrated and dialyzed against 2 mM sodium succinate buffer. This material had a specific endoglucanase activity of 41 units/mg, and a specific aryl-β-D-glucosidase activity of 0.9 units/mg. Protein with high endoglucanase specific activity was eluted with the 2 mM succinate buffer. When the concentration of the succinate elution buffer was increased to 8 mM, cellobiohydrolase II was eluted as the major constituent of two peaks. The second (larger) of these peaks contained pure cellobiohydrolase II. When the ionic strength was further increased by the addition of 0.1 M sodium chloride, tightly bound proteins present as minor components in the starting material were eluted.

All column chromatography was carried out at 4°; the protein content of the fractions was monitored continuously with an ISCO Model UA-4 Absorbance Monitor equipped with a recorder, and the absorbance of each fraction was measured at 280 nm with a Hitachi 124 Double Beam Spectrophotometer.

Polyacrylamide Disc Gel Electrophoresis - Proteins were separated electrophoretically in cylindrical polyacrylamide gels (0.5 x 8.5 cm) at 4°, using the discontinuous buffer system No. 1 described by Maurer (28). A Hoefer PS 1200 DC Power supply was used (Hoefer Scientific Instruments, San Francisco, California) with a Canalco electrophoresis chamber (Miles Laboratories, Inc., Elkhart, Indiana). After electrophoresis, proteins were precipitated by immersing the gels in 12% trichloroacetic acid for 30 min, and stained with 0.1% Coomassie Brilliant Blue R250 in a water-acetic acid-methanol (45:10:45) solution for 1 h. Carbohydrates were stained with the periodic acid-Schiff (PAS)

reagent, using the following method which has been described by Lang (39): after precipitating the protein bands on the gels, as described above, the gels were incubated for one hour in 0.5% (w/v) aqueous periodic acid. At the end of this period, excess periodic acid was removed from the gels by three successive (20 min) washes in fresh 7% acetic acid. The gels were then placed in tubes containing Schiff reagent (1 g Basic Fuchsin and 1.9 g sodium metabisulfite dissolved in 100 ml of 0.15 N HCl), after which a purple color developed within 40 min. Background was destained using 0.1% aqueous sodium metabisulfite at 40° for two days.

Isoelectric Focusing - A Dual Vertical Slab Gel Electrophoresis Unit - Model 220 (Bio-Rad Laboratories, Richmond, California) was used to cast a polyacrylamide thin layer gel and a Hoefer PS 1200 DC Power Supply (Hoefer Scientific Instruments, San Francisco, California) used for electrofocusing of the purified glucanases on a Savant Flat Platen (FP22A, Savant Instruments, Hicksville, New York) cooled to 4°. Gel composition and running conditions were according to the instructions given by Bio-Rad Laboratories except that 1.0 ml of Biolyte 3/10 and 1.5 ml of pH 3-6 Ampholine ampholytes were included per 50 ml of gel formulation. A pH range of 3.88 to 9.05 was established after 3.5 h at a constant power supply of 4.1 watts. A strip of the gel 1 cm in width (the length of which was parallel to the imposed electrical field) was cut into 0.5 cm segments. Each segment was added to one ml of deionized water and mixed for 10 sec using a Vortex mixer. The pH of each segment was determined immediately using a Corning General Purpose

Digital 109 pH meter Model S8220 (Scientific Products, McGraw Park, Illinois) equipped with a semi-micro combination electrode (Catalog Number 476050, Corning Scientific Instruments, Medfield, Massachusetts).

Ultracentrifugation Analysis - Sedimentation equilibrium studies were performed on the purified glucanases according to the method of Chervenka (40). From multiple runs of three different concentrations for each enzyme, data were obtained from which molecular weights were determined using partial specific volumes calculated from amino acid and carbohydrate composition by the method of Cohn and Edsall (41).

Amino Acid Analysis - The amino acid composition of the purified glucanases was determined using a Model 121 Automatic Amino Acid Analyzer and a Beckman System AA computing integrator (Beckman Instruments, Inc., Palo Alto, California) according to the method of Spackman, Stein and Moore (42) as outlined in the Model 121 Instruction Manual. Samples containing approximately 1.0 mg of enzyme in solution were hydrolyzed in constant-boiling HCl at 110° for 24, 48 and 72 h. To correct for destruction of serine or threonine, and tyrosine where necessary, values for these amino acids were extropolated to zero hydrolysis time. To accurately determine the sulfur amino acid content, a separate sample was oxidized with performic acid by the method of Hirs (43) to convert cystine to cystatic acid and methionine to methionine sulfone. These stable oxidized forms were determined after a 24 h hydrolysis. Tryptophan was calculated from its molar ratio to tyrosine, which was obtained using the spectrophotometric method of Bencze and Schmid (44).

Carbohydrate Determination - After hydrolysis of each glycoprotein at 100° for 6 h in 1N HCl, identification and quantitation of neutral monosaccharides was accomplished by gas-liquid chromatographic separation of the peracetylated aldononitrile derivatives, using the procedure described by Seymour et al (45). The separation of the peracetylated aldononitrile derivatives of xylose (used as an internal standard), mannose, glucose and galactose on a 10 ft OV-225 column, at 218°, is illustrated in Fig. 1. The identity of the peracetylated aldononitrile derivatives of the neutral sugars was confirmed by use of a Varian MAT-112 Gas Chromatograph-Mass Spectrometer equipped with a Varian 620/L Computer. Total neutral carbohydrate was also determined by the phenol-sulfuric acid method of Dubois et al (46), for which mannose serviced as the standard.

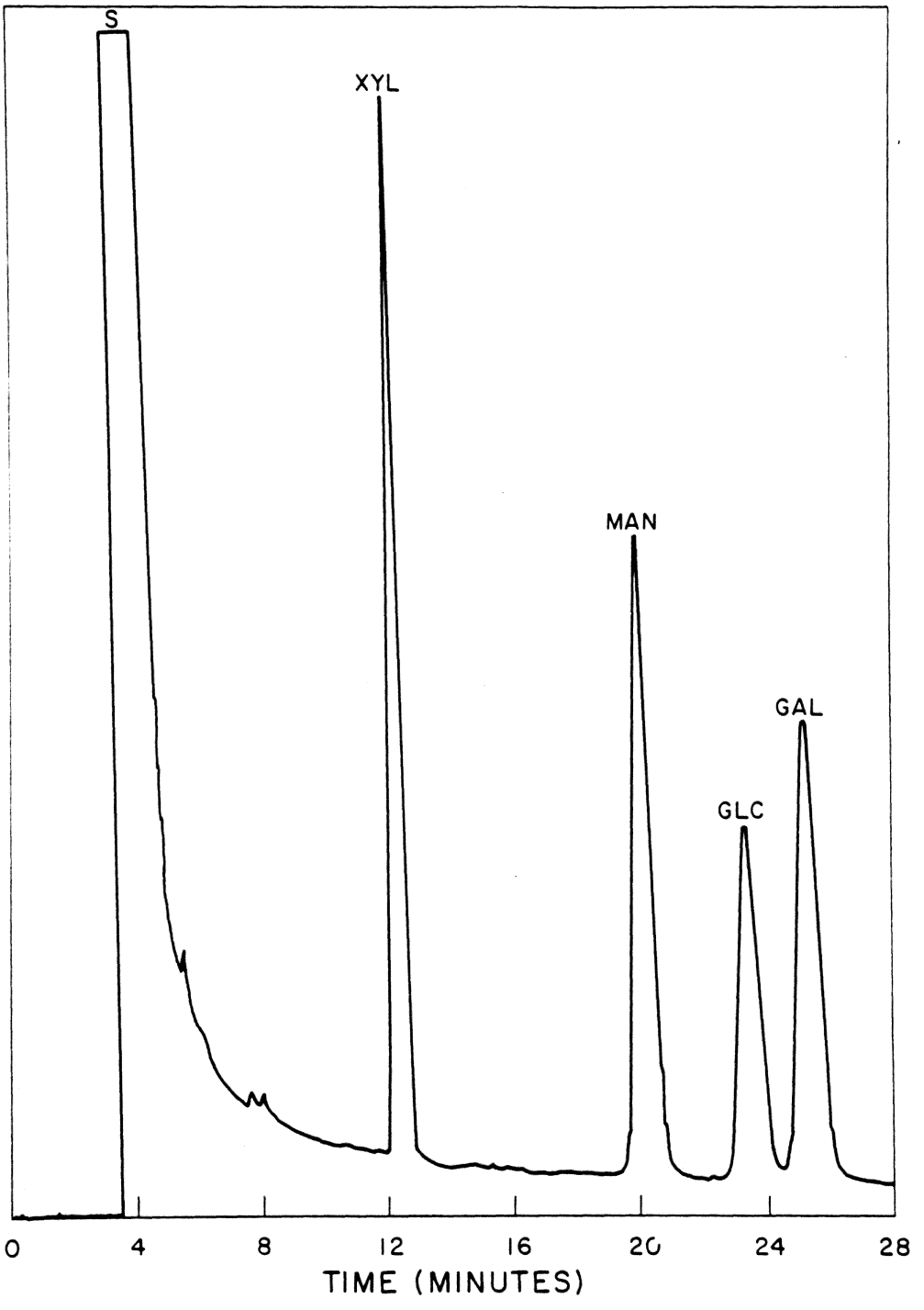
Amino sugar content was determined by the method of Walborg (47) which was adapted by Gum (48) for use with the Beckman Model 121 Automatic Amino Acid Analyzer.

Determination of pH Optima for Enzyme Activities - The dependence on hydrogen ion concentration of each glucanase activity was determined by the reducing sugar assay using phosphoric acid-swollen cellulose as substrate. Swollen cellulose suspensions in 0.2 M maleate: 0.2 M succinate buffer were adjusted to different pH values by varying the relative amounts of these two acids, and incubated with enzyme at 40° for 30 min. Control samples containing no enzyme, and a series of glucose standards were determined at each pH value, to accurately evaluate experimental results.

FIGURE 1

Separation of peracetylated aldononitrile derivatives of monosaccharides by gas chromatography.

Separation was accomplished as described in Experimental Procedures. Peaks are Xyl, Xylose; Man, Mannose; Glc, Glucose and Gal, Galactose.


S
XYL
MAN
GLC
GAL
0
4
8
12
16
20
24
28
TIME (MINUTES)

Alkali Stability - The stability of the purified glucanases to alkaline conditions was examined using buffer solutions at 0.05 M HEPES (pKa_1 = 7.55), CHES (pKa_1 = 9.3) and CAPS (pKa_1 = 10.4). Each enzyme was preincubated in alkaline buffered solutions at 0.2 mg protein/ml for various lengths of time, and the effect of exposure to low hydrogen ion concentrations on the stability was measured by the reducing sugar assay at pH 5.0, with phosphoric acid-swollen cellulose as the substrate. Incubation with at least two different buffers at the same pH should reveal any specific buffer effects.

Thermal Stability - To determine the thermal stability of the purified enzymes, solutions of each were preincubated for various lengths of time at selected high temperatures. At several points during preincubation of each enzyme, residual activity was measured by the reducing sugar assay, using phosphoric acid-swollen cellulose as the substrate. For comparison, the viscosimetric assay was also used to evaluate the effect of heating on this property of the purified endoglucanase.

High Performance Liquid Chromatography - This technique has been used successfully for the separation and quantitation of oligosaccharides produced or degraded by enzymic action (49). A Waters Associates Model ALC 202/401 Liquid Chromatograph was used, supplemented with a Model 6000 Solvent Delivery System (Waters Associates, Inc., Milford, Massachusetts). The instrument was equipped with a differential refractometer to monitor column effluents, and a Spectra-Physics Autolab System I computing integrator (Spectra-Physics, Santa Clara, California) which was used to compute relative areas of chromatographic peaks.

Separation of oligosaccharides was accomplished using a Whatman Partisil PXS 10/25 PAC column, with acetonitrile:water solvent systems. In this fashion, the products of enzymic action with various cellulosic substrates were identified, and reaction rates were obtained with soluble cellooligosaccharides as substrates, with which it is impossible to use the reducing sugar assay. The latter studies with cellotetraose, cellopentaose and cellohexaose, were performed as described previously (50).

The Effect of Ethanol on Glucanase Activity - The effect of ethanol on the activity of each purified glucanase was investigated using phosphoric acid-swollen cellulose as the substrate and assaying the supernate for reducing sugar production. Appropriate volumes of a 24% (w/w) ethanol solution were added to the cellulose suspension, prior to addition of enzyme, to produce ethanol concentrations of either 4 percent or 8 percent. Incubation of the tightly capped tubes took place at 40° for 30 min, after which samples were withdrawn for reducing sugar analysis. Similar experiments were performed, during which the products of swollen cellulose hydrolysis in the presence of ethanol were subjected to HPLC analysis.

Short Fiber Forming Activity - Each purified glucanase was tested for its ability to form short fibers from filter paper (12). To a test tube containing 10 mg of Whatman No. 3 MM filter paper cut into 10 pieces of 0.25 cm^2 each, and suspended in 50 mM sodium acetate buffer, pH 5.0, containing 3 mM sodium azide, was added an appropriate amount of enzyme solution, and the reaction mixtures were incubated at 40°.

After 48 h the reaction mixtures were refrigerated and the short fiber formation recorded photographically.

Immunodiffusion - Immunodiffusion was carried out on lantern slide cover glasses (8.3 x 10.2 cm), which had been coated with 20 ml of 1% electrophoresis grade agarose (Bio-Rad Laboratories, Richmond, California) in water. The volume of both antiserum (in the center well) and antigens (in the peripheral wells) was 25 µl, and the distance between wells was 0.5 cm. Experiments were carried out at room temperature, and precipitin lines formed were recorded photographically.

RESULTS AND DISCUSSION

The principal enzymic components of the cellulase system which are produced when resting mycelia of T. reesei QM 9414 are exposed to sophorose were isolated previously in small quantities (29); it was established that these are also present as the major components of the enzyme system produced by the same organism during growth on cellulose. In order to understand the role of each enzyme in this cellulase system, it was decided to use culture filtrates of cellulose-grown cells as a source, in order to purify in quantities adequate for structural work those enzymic components, which are common to both sophorose and cellulose-derived systems, and which are likely to represent the primary products of biosynthesis.

It had been found previously (29) that all of the enzymes of interest were glycoproteins; therefore attempts at purification by affinity chromatography using a lectin such as Concanavalin A, would probably not yield satisfactory results. Similarly, preliminary experiments indicated that Avicel affinity colums (51) could not be used, as all of the enzymes appeared to bind to cellulose. It was desirable to avoid methods such as preparative isoelectric focusing and preparative disc gel electrophoresis, which are complex and possibly deleterious to enzymic activity; the purification process should ensure a minimum number of purification steps, involve mild conditions, and have possibilities for adaptation to large scale purification. Since the enzymes appeared to differ principally with respect to their isoelectric points rather than molecular weights, ion exchange

chromatography was the method of choice. Therefore, a two-step process was developed, involving sequential anion and cation exchange.

Purification of Glucanases

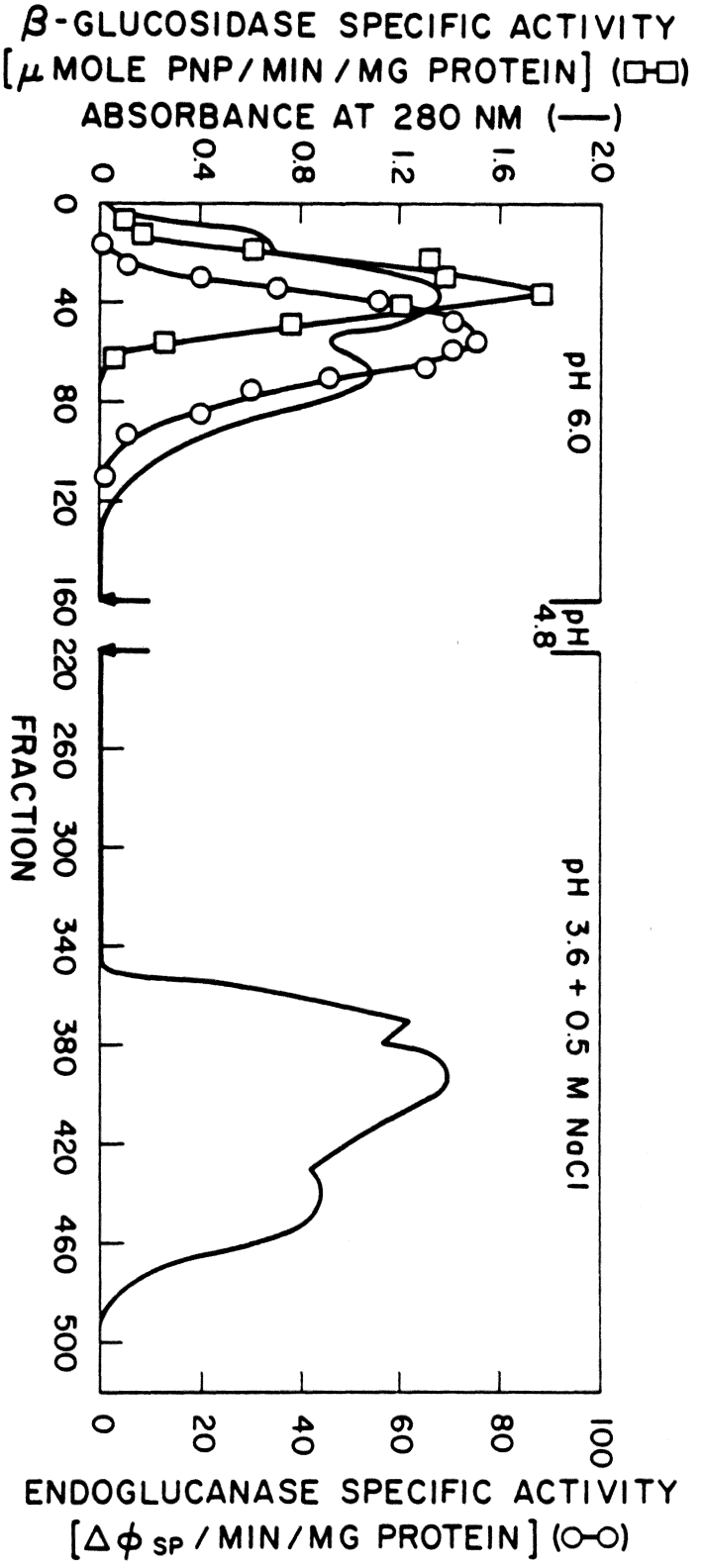
Purification of cellobiohydrolase I (D) - The crude enzyme preparation derived from the culture filtrate of Trichoderma reesei QM 9414 grown on Avicel, was chromatographed on a DEAE-Sephadex ion exchange column, which was developed as described in Experimental Procedures. The elution profile obtained from this column is shown in Fig. 2. Two protein peaks were obtained: the majority of the protein components in the starting material, including those responsible for all of the endo-1,4-β-D-glucanase and aryl-β-D-glucosidase activity did not adsorb to the column under these conditions but were eluted isocratically, whereas cellobiohydrolase I (D), which is the most acidic protein in the mixture, bound tightly to the anion exchanger at the initial ionic strength (which was calculated to be approximately 0.095 M). Pure cellobiohydrolase I (D) was obtained when the pH of the elution buffer was lowered to 3.6 and the ionic strength increased by the addition of 0.5 M NaCl. An intermediate step during which the pH of the elution buffer was decreased to 4.8 (Fig. 2) did not result in elution of cellobiohydrolase I (D) from this column; this is in agreement with information (which will be discussed later), indicating that this protein has an isoelectric point less than 3.8.

Separation of proteins across ion exchange column peaks was followed by disc gel electrophoresis and, whenever possible, enzyme

FIGURE 2

Elution pattern from DEAE-Sephadex column chromatography of the extracellular proteins produced by T. reesei QM 9414 grown on cellulose.

Column dimensions and elution conditions were described in Experimental Procedures. Each fraction contained 24 ml.


β-GLUCOSIDASE SPECIFIC ACTIVITY
[μ MOLE PNP/MIN/MG PROTEIN] (□-□)
ABSORBANCE AT 280 NM (—)
0
0.4
0.8
1.2
1.6
2.0
0
40
80
120
160
220
260
300
340
380
420
460
500
FRACTION
pH 6.0
pH 4.8
pH 3.6 + 0.5 M NaCl
0
20
40
60
80
100
ENDOGLUCANASE SPECIFIC ACTIVITY
[Δφ SP / MIN/MG PROTEIN] (○-○)

assays. Fig. 3 illustrates the disc gel electrophoretic pattern of successive protein fractions which did not adsorb to the DEAE-Sephadex column. The two protein bands which apparently constitute the two major components in this group of proteins represent an endoglucanase (the more acidic of the two proteins) and cellobiohydrolase II.

Fig. 4 represents the patterns obtained by electrophoresis of successive protein fractions which eluted from the column when the pH was lowered and the ionic strength increased (Fig. 2). These fractions contain homogeneous cellobiohydrolase I (D).

Of the 4.9 g of protein applied to this column, 2.09 g were recovered in the fractions eluted with the starting buffer, and 2.76 g were recovered as cellobiohydrolase I (D). These results represent nearly 99% recovery of protein, of which 56.9% is cellobiohydrolase I (D).

Purification of cellobiohydrolase II and endoglucanase - Since cellobiohydrolase II cannot be assayed in the presence of the other enzymes of the cellulase system, it was necessary to rely exclusively on protein electrophoretic patterns as a criterion of enrichment of protein fractions in that enzyme. On this basis, fractions 25-65 from the DEAE-Sephadex column (Fig. 2) were combined; the resulting material was simultaneously enriched in endoglucanase activity. Although a significant fraction (ca. 36%) of the total endoglucanase activity resided in fractions 66-90, these were not included because of their low cellobiohydrolase II content, and because they would add a new type of contaminant, which, due to its electrophoretic proximity to the

FIGURE 3

Polyacrylamide disc gel electrophoresis of successive protein fractions eluted isocratically from a DEAE-Sephadex column during chromatography of T. reesei enzymes.

These gels represent, from left to right, electrophoretic protein patterns of the culture filtrate proteins, and of fractions 25, 35, 45, 55, 65, 75, 85, 95, 105, and 115, which were eluted by 50 mM sodium succinate, pH 6.0, during DEAE-Sephadex column chromatography of T. reesei extracellular enzymes. The gel on the far left contains 150 µg of protein, while all others contain 80 ± 5 µg of protein.

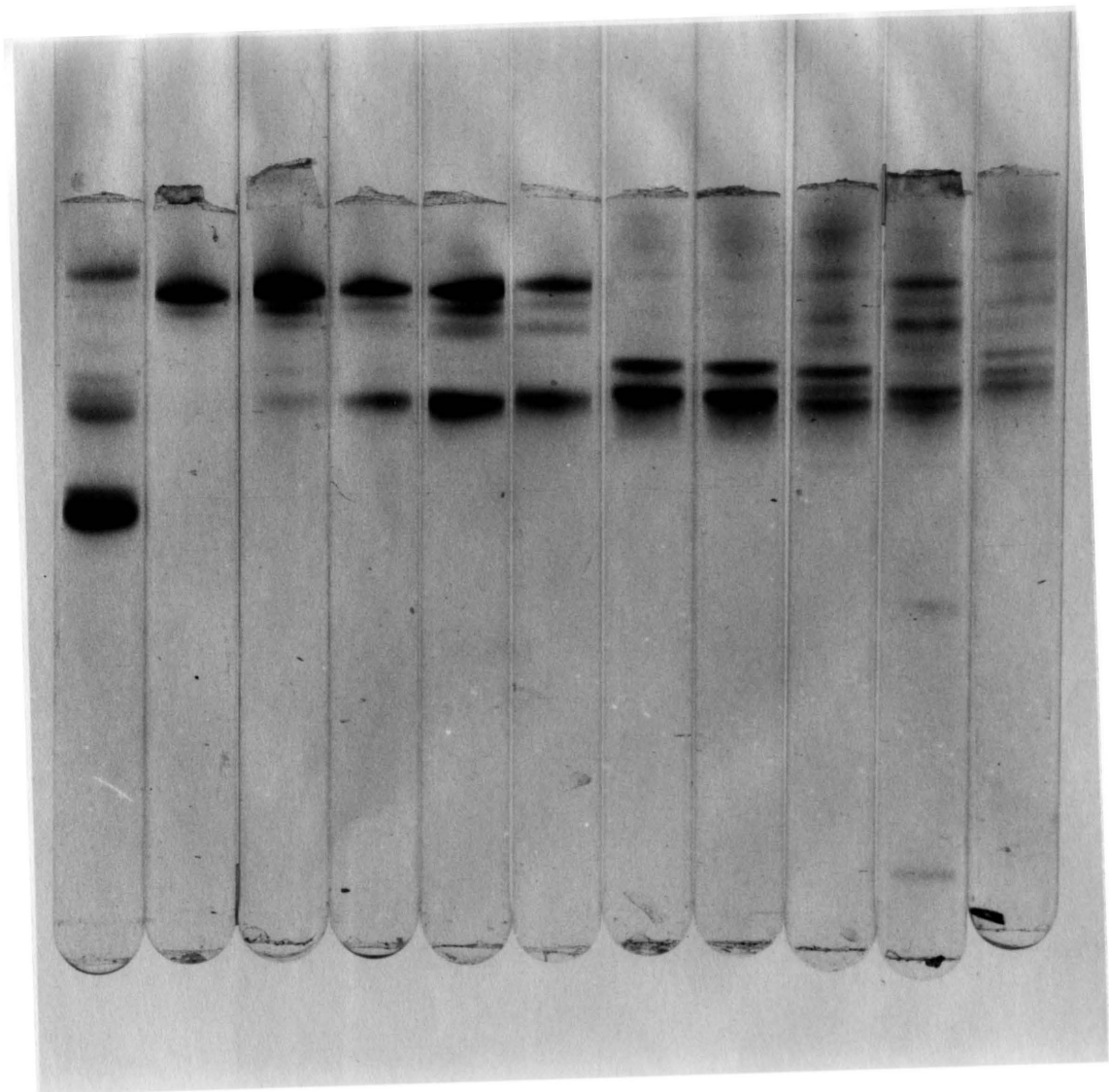
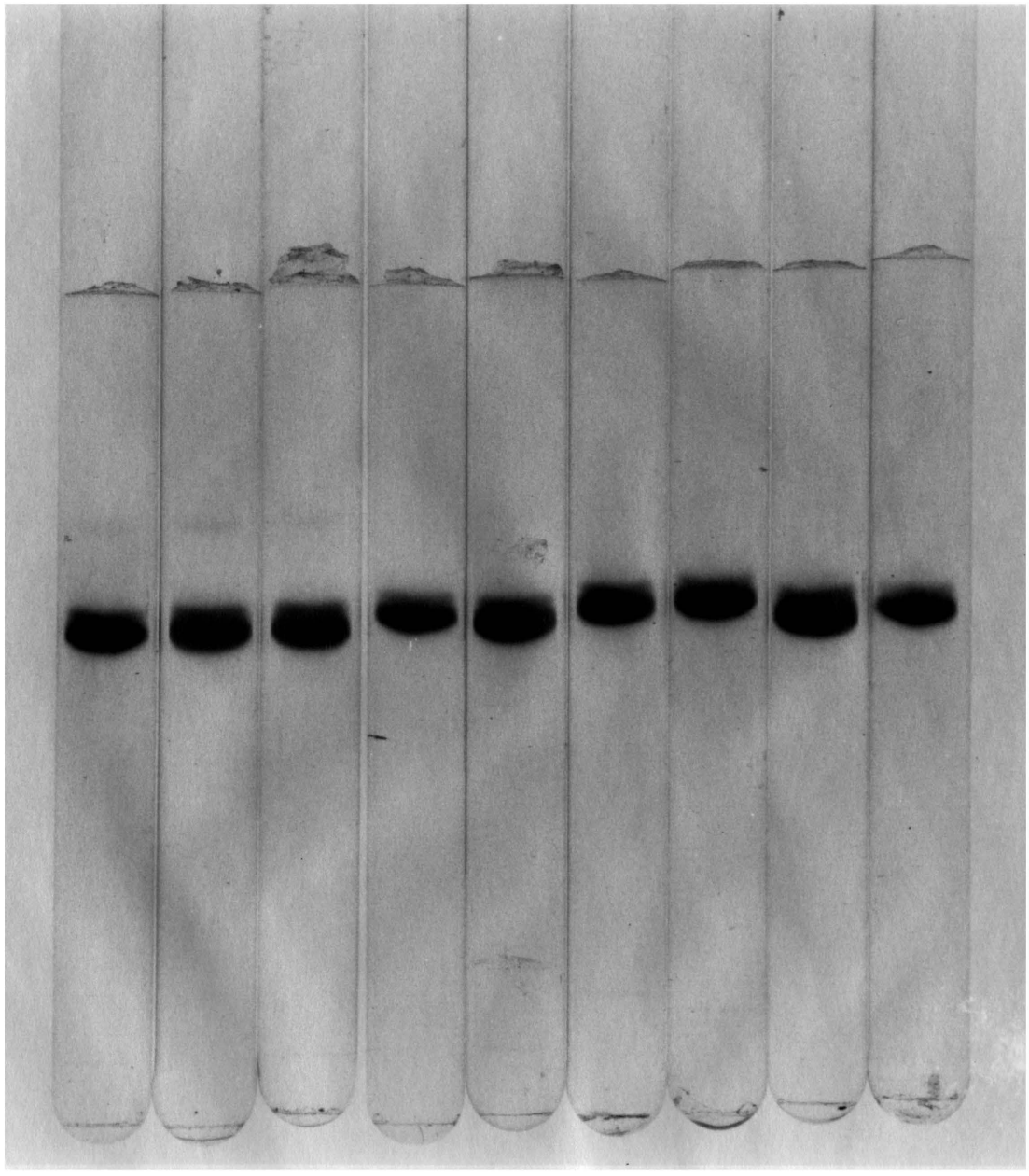


FIGURE 4

Polyacrylamide disc gel electrophoresis of successive fractions of cellobiohydrolase I (D) obtained during chromatography of T. reesei enzymes on a DEAE-Sephadex column.

These gels represent, from left to right, patterns obtained by electrophoresis of approximately 60 µg of protein from fractions 360, 370, 380, 390, 400, 410, 420, 430 and 440, respectively. These fractions were eluted during DEAE-Sephadex column chromatography of T. reesei extracellular enzymes, after the pH of the succinate elution buffer was lowered to 3.6, and the ionic strength increased by the addition of 0.5 M NaCl.



endoglucanase, might have complicated purification of the latter. Of the two main protein components present in fractions 66-90 (as evidenced by their electrophoretic patterns), one has an electrophoretic mobility identical to that of the endoglucanase present in fractions 25-65, whereas the second component does not migrate as far toward the anode; a small amount of the latter component was obtained in pure form in fractions 232-235 of this cation exchange column, and it was found that it exhibits no activity toward carboxymethyl cellulose. It was concluded that the endoglucanase activity in fractions 25-65 and 66-90 is probably due to the same protein.

After concentration and dialysis, fractions 25-65 were applied to an SP-Sephadex C-50 column, which was eluted as described in Experimental Procedures. The elution profile from this column is shown in Fig. 5. A single sharp endoglucanase peak eluted with the starting buffer of 2 mM sodium succinate. Successive fractions from this protein peak, together with the starting material are shown in Fig. 6. This endoglucanase appears homogeneous by the criterion of polyacrylamide disc gel electrophoresis. When the concentration of the succinate elution buffer was increased to 8.0 mM, two protein peaks were eluted; electrophoretic patterns of the proteins in these peaks are shown in Fig. 7. The first, minor peak, consisted primarily of cellobiohydrolase II and a minor unidentified contaminant (Fig. 7); the second, major peak contained homogeneous cellobiohydrolase II. When the ionic strength of the elution buffer was further increased with 0.1 M NaCl, a group of strongly adsorbed proteins were eluted from the column. Addition of

FIGURE 5

Elution pattern from SP-Sephadex column chromatography of the combined fractions 25-65 which had been eluted from a DEAE-Sephadex column.

Column dimensions and elution conditions were described in Experimental Procedures. Each fraction contained 8 ml.

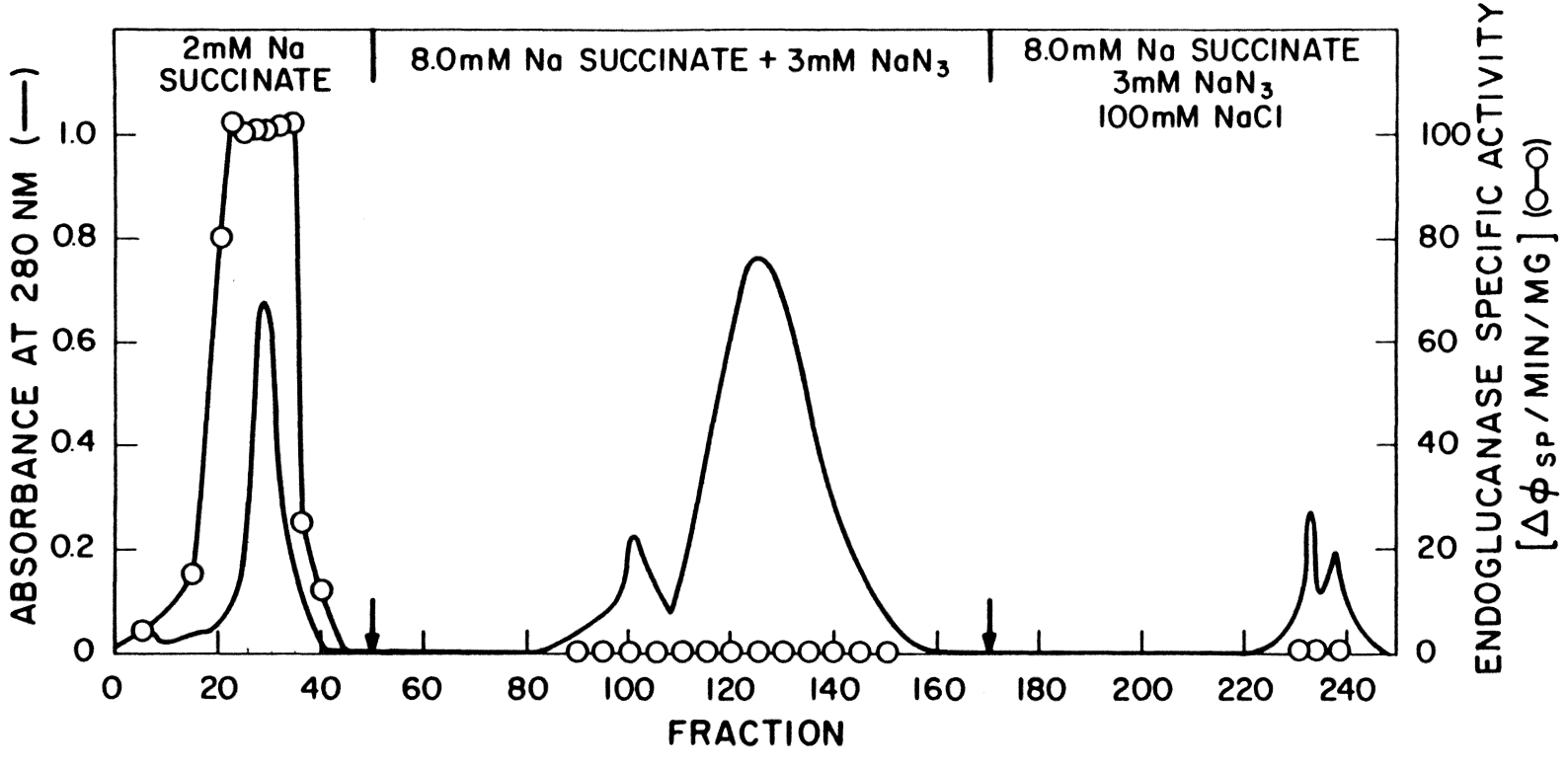

2mM Na SUCCINATE
8.0mM Na SUCCINATE + 3mM NaN3
8.0mM Na SUCCINATE
3mM NaN3
100mM NaCl
ABSORBANCE AT 280 NM (—)
ENDOGLUCANASE SPECIFIC ACTIVITY
[Δφ SP / MIN / MG] (O—O)
FRACTION
0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
0
20
40
60
80
100
120
140
160
180
200
220
240

FIGURE 6

Polyacrylamide disc gel electrophoresis of successive fractions containing endo-1,4-β-D-glucanase obtained during chromatography on SP-Sephadex of the proteins in the combined fractions 25-65 previously eluted from DEAE-Sephadex column.

Fractions 25-65, which had been eluted from a DEAE-Sephadex column, were combined, concentrated and further separated on an SP-Sephadex column. These gels represent, from left to right, proteins in the starting material, and in fractions 23, 30, 35 and 40, respectively, which had been eluted with 2 mM sodium succinate, pH 5.0. The gel on the far left contains 80 µg of protein, while all others contain approximately 46 µg of protein each.

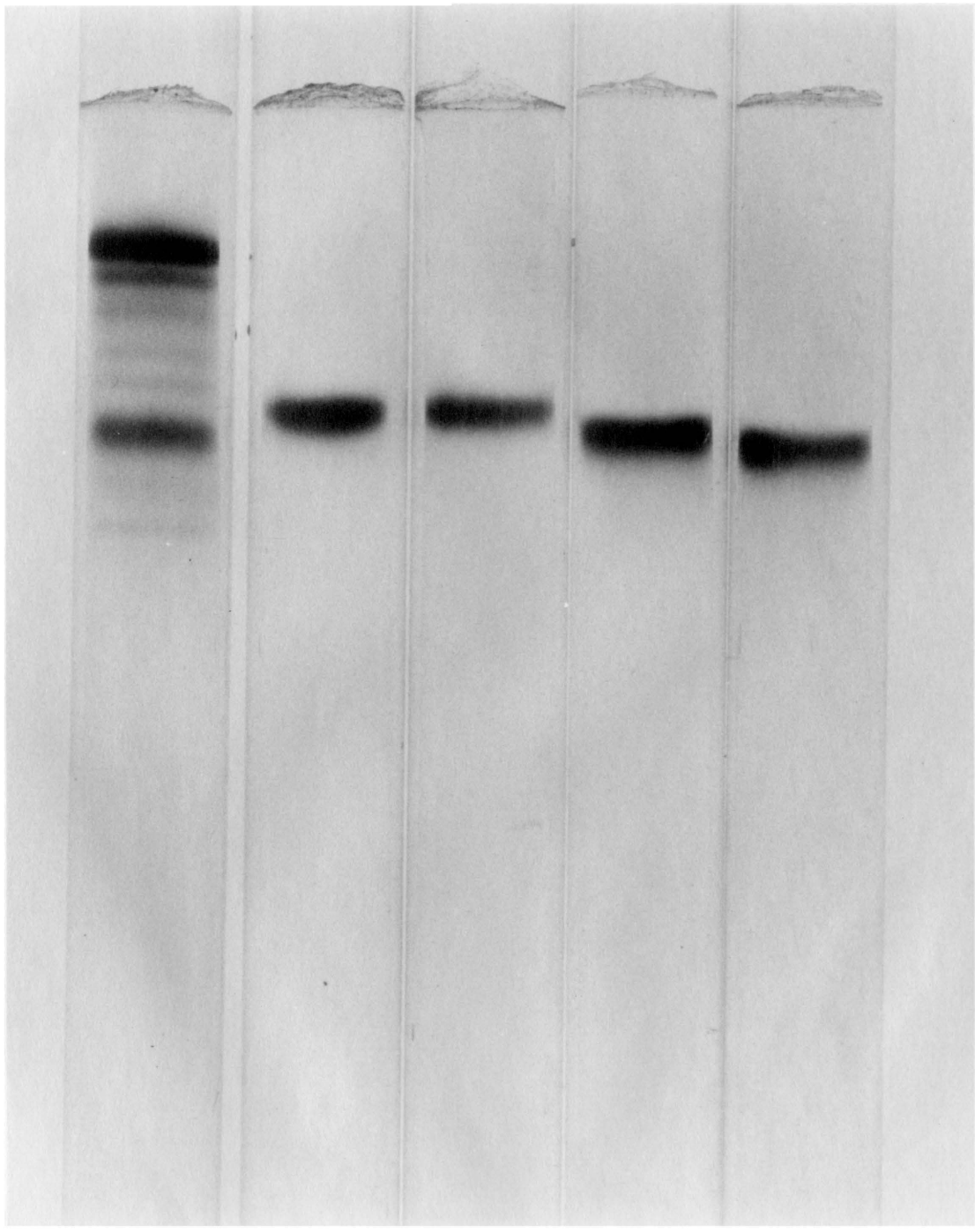
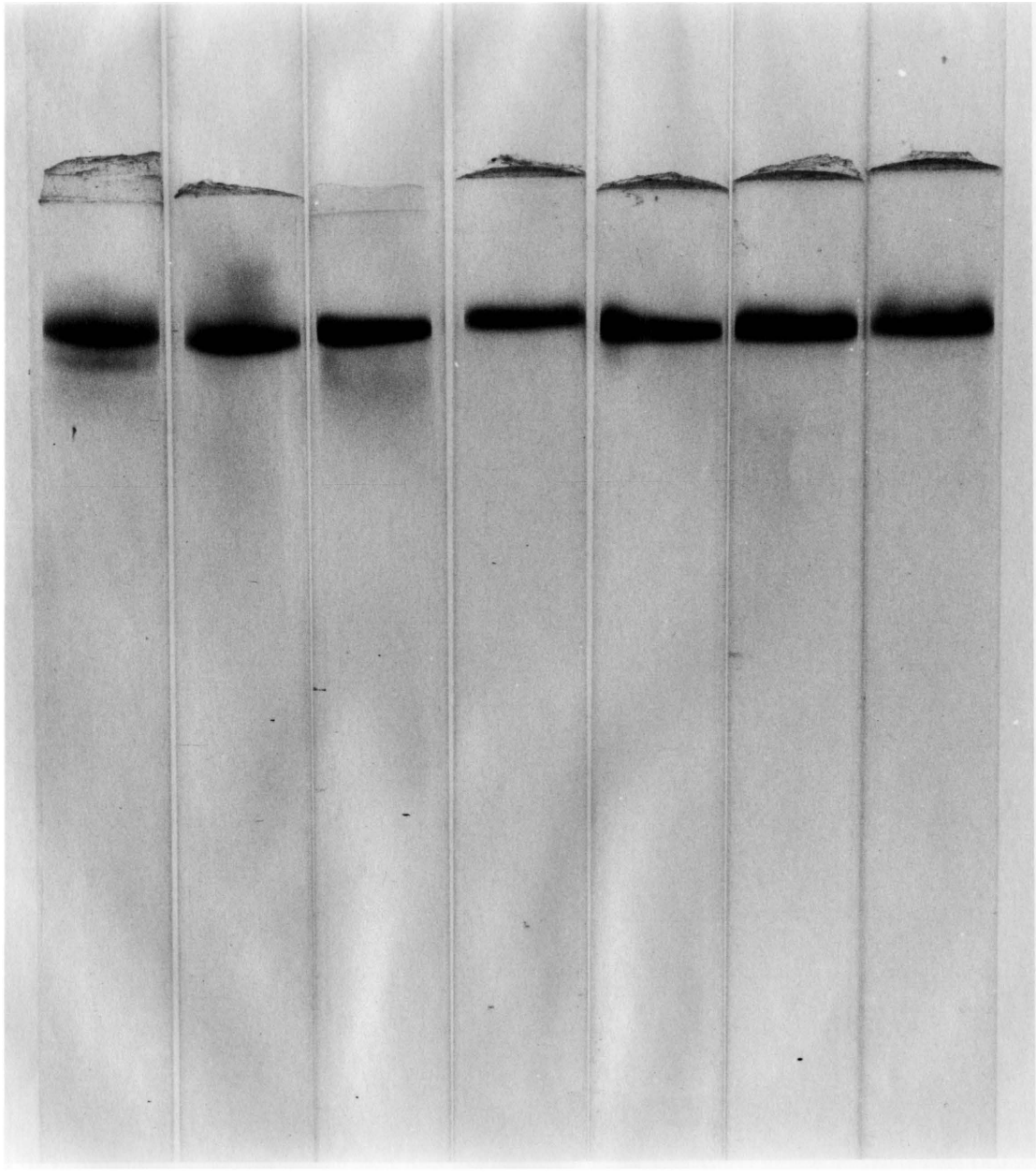


FIGURE 7

Polyacrylamide disc gel electrophoresis of successive fractions containing cellobiohydrolase II obtained during chromatography on SP-Sephadex of the proteins in the combined fractions 25-65 previously eluted from a DEAE-Sephadex column.

These gels, to each of which had been applied approximately 60 µg of protein, represent patterns obtained by electrophoresis of the proteins in fractions 103, 115, 120, 125, 130, 135 and 140 which had been eluted from the SP-Sephadex column with 8.0 mM sodium succinate buffer containing 3.0 mM sodium azide.



higher concentrations of NaCl to the elution buffer (up to 1.0 M), did not result in the recovery of more protein from the column.

Recovery of protein from this column was 97%; quantification of protein and enzymic activity in each of the steps involved in the purification of the endoglucanase is presented in Table I. A 97.6% recovery of endoglucanase activity, nearly all of which resided in the Peak I fractions, was achieved during the DEAE-Sephadex chromatography step. A fraction of that protein, containing 53.5% of the endoglucanase activity, was applied to the SP-Sephadex column. During cation exchange chromatography, 80% of the endoglucanase activity applied to the column was recovered in Peak I, and was due to a single protein. The net recovery of endoglucanase activity was therefore 43%. A 7.7 fold purification of this endoglucanase was achieved.

During three applications of the cation-exchange chromatography step, for which fractions 25-65 of the DEAE-Sephadex column had served as the starting material, 590 mg in total of homogeneous cellobiohydrolase II were obtained. An additional 190 µg were obtained from fractions 1-24 by the same procedure. This represents approximately 17% of the total protein in the original culture filtrate.

Electrophoretic evidence for the purity of the endo-1,4-β-D-glucanase and exo-cellobiohydrolases I (D) and II - Evidence for homogeneity of the proteins purified by the methods described above is presented in Fig. 8, in which are shown the polyacrylamide disc gel electrophoretic patterns of the pure glucanases together with the starting material. A single protein band was obtained for each

TABLE I

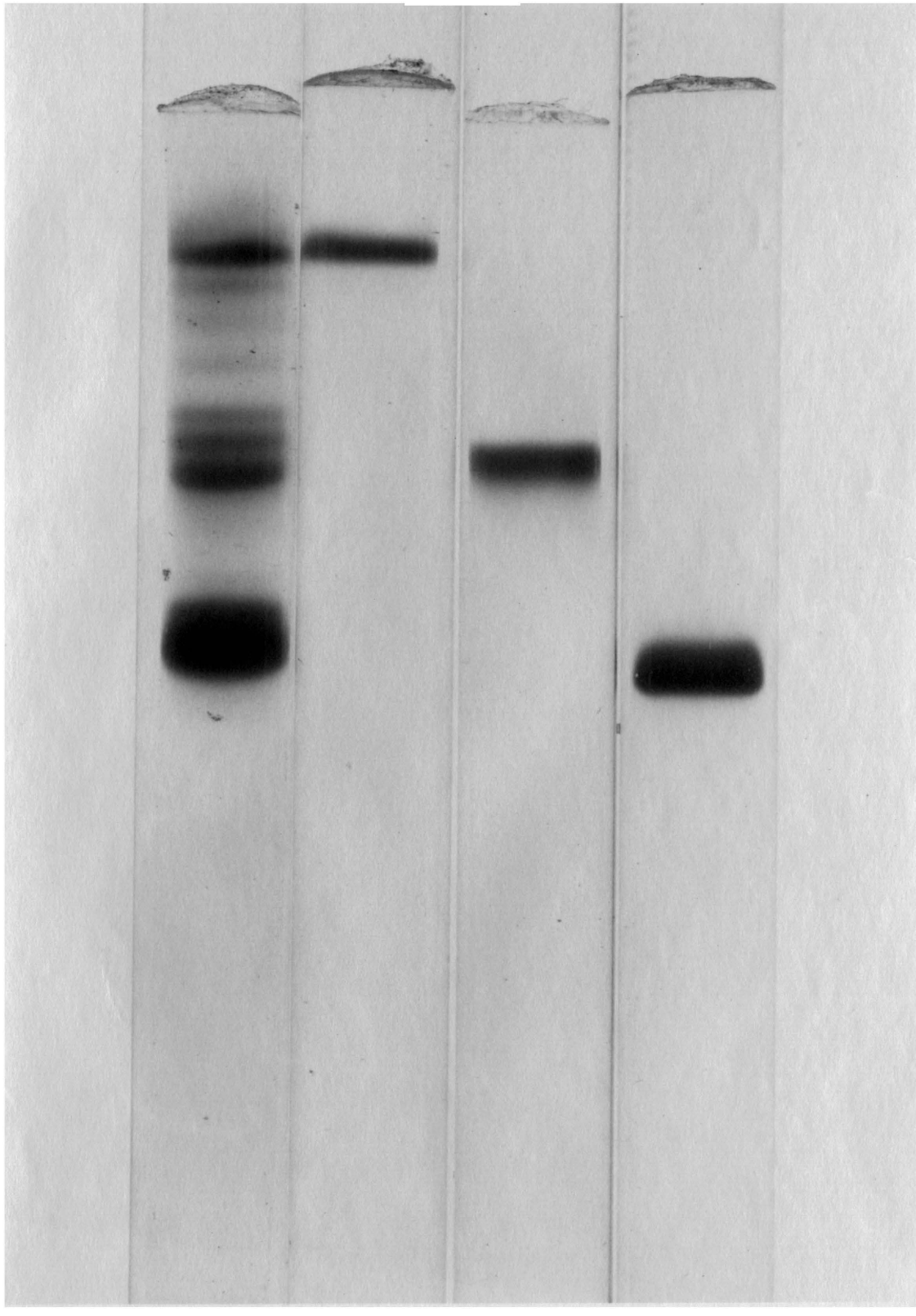
Summary of purification of endoglucanase

	Fraction number	Protein (mg)	Total activity (units)	Specific activity (units/mg)
DEAE SEPHADEX CHROMATOGRAPHY OF DIALYZED CULTURE FILTRATE				
(Sample)		4900	73,500	15
Peak I	1- 24	204	2,400	12
	25- 65	935	38,335	41
	66- 90	665	25,270	38
	91-125	295	5,015	17
Peak II	352-480	2760	690	0.25
SP-SEPHADEX CHROMATOGRAPHY OF FRACTIONS 25-65 FROM DEAE-SEPHADEX COLUMN				
(Sample)		360	14,760	41
Peak I	1- 24	9	666	74
	25- 35	82	9,860	116
	36- 40	12	1,032	86
Peak II	85-115	19	3.4	0.18
	116-155	208	24.9	0.12
Peak III	225-235	12	48	4
	236-245	9	23.4	2.6

FIGURE 8

Polyacrylamide disc gel electrophoretic patterns of three β-glucanases purified from Trichoderma reesei (Coomassie Blue Stain).

These gels contain, from left to right, 175 µg of culture filtrate proteins; 55 µg of cellobiohydrolase II; 48 µg of endoglucanase; and 55 µg of cellobiohydrolase I (D)



glucanase under the conditions of these gels. A single band was also obtained when, in a similar experiment, polyacrylamide gels to which had been applied a suitable quantity of each glucanase, were stained for carbohydrate. This result is illustrated in Fig. 9. All three enzymes are glycoproteins.

Disc gel electrophoresis of the glucanases following treatment with 0.1% sodium dodecyl sulfate at 40° for 1 h, also yielded a single protein band upon staining with Coomassie Brilliant Blue (data not shown). This indicated lack of protein heterogeneity, and lack of subunits. To further confirm homogeneity, and to determine the isoelectric point for each purified glucanase, electrofocusing was carried out in a polyacrylamide slab gel, as described in Experimental Procedures. Isoelectric pH values of 4.7 and 5.6 were obtained for the endo-1,4-β-D-glucanase and cellobiohydrolase II, respectively (Fig. 10). Each protein yielded a single band when stained with Coomassie Brilliant Blue. Although a cellobiohydrolase I (D) sample was also applied to the gel, no protein band could be detected for that enzyme after termination of electrofocusing and staining; this result is not particularly surprising, as the isoelectric point of this protein is probably less than the lower limit of the pH gradient used during this experiment. Berghem *et al* (2) had determined an isoelectric point of 3.6 for a cellobiohydrolase which they had isolated from *T. viride*, and which probably represents cellobiohydrolase I (C) (5), a differentially glycosylated form of cellobiohydrolase I (D).

Cellobiohydrolase II has not been purified and described previously. Of the two endoglucanases isolated from *T. reesei* QM 9414 by

FIGURE 9

Polyacrylamide disc gel electrophoretic patterns of three β-glucanases purified from Trichoderma reesei (Periodic Acid-Schiff reagent stain).

Protein samples applied to these gels were, from left to right, 70 µg of cellobiohydrolase II; 65 µg of endoglucanase; 80 µg of cellobiohydrolase I (D).

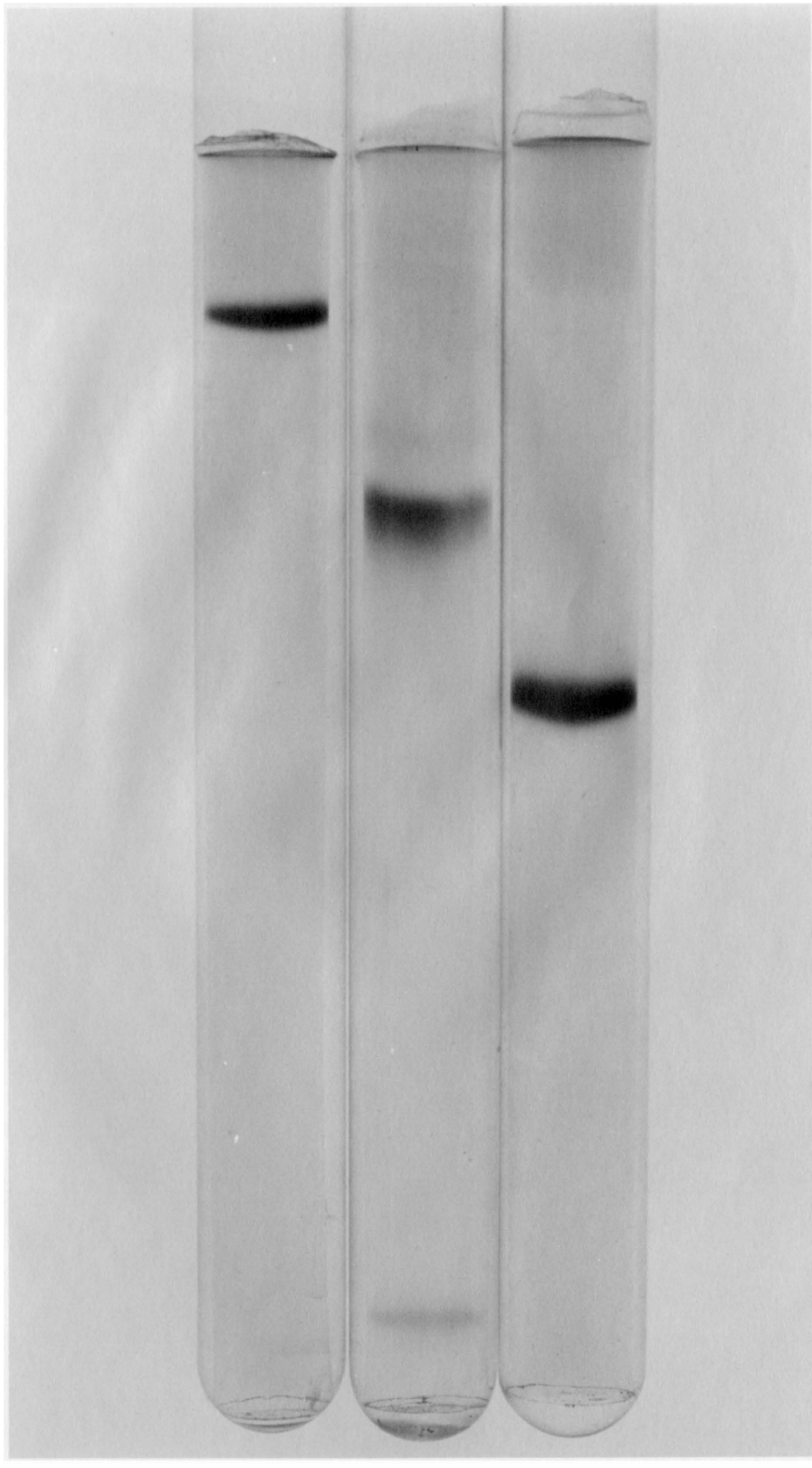
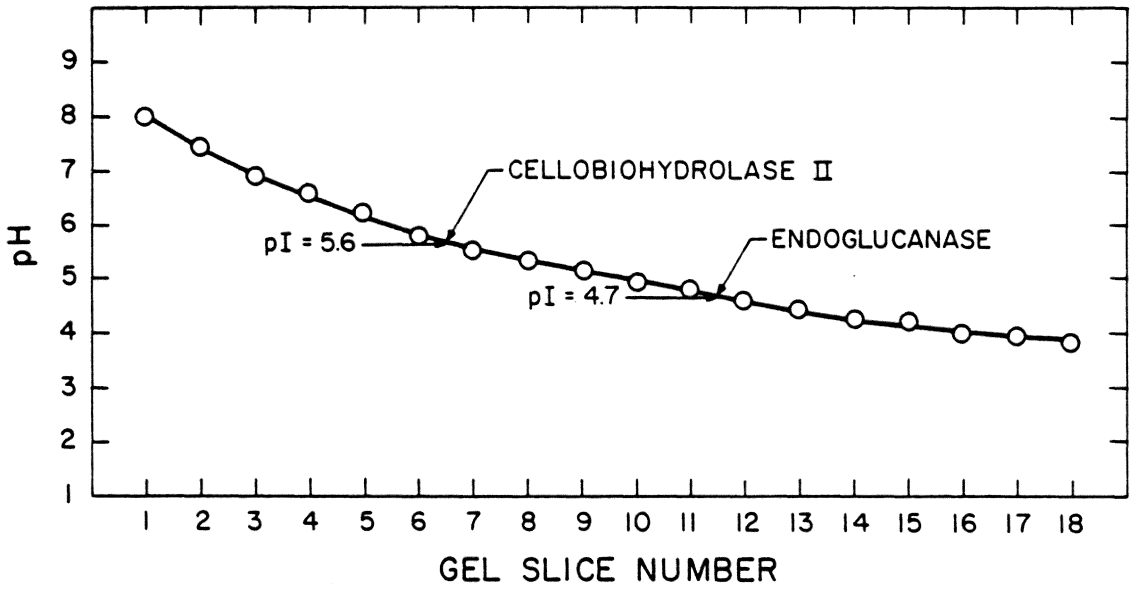


FIGURE 10

Slab gel isoelectric focusing of purified glucanases.

This plot indicates the positions to which the endoglucanase and cellobiohydrolase II had migrated in the pH gradient established after 3.5 of electrofocusing as described in Experimental Procedures.


pH
9
8
7
6
5
4
3
2
1
CELLOBIOHYDROLASE II
pI = 5.6
ENDOGLUCANASE
pI = 4.7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
GEL SLICE NUMBER

Häkansson et al (15,16), one reportedly has an isoelectric point of 4.66, which is very similar to that determined for the endoglucanase isolated during this investigation. However, a number of other structural properties of that endoglucanase (16) clearly distinguish it from the enzyme described in this report.

Structural Characterization of Glucanases

Ultraviolet Absorption Spectra - The ultraviolet absorption spectra of the purified glucanases are shown in Fig. 11. Cellobiohydrolase I (D) has an absorbance maximum at 277.5 nm, and a minimum at 250 nm. A slight shoulder is visible at 290 nm. These results are in agreement with previous results obtained for cellobiohydrolase I (D) isolated from T. reesei QM 9414 cells which had been exposed to sophorose (29). Cellobiohydrolase II and the endoglucanase have absorbance maxima at 279.5 and 289 nm, respectively; they each have a pronounced shoulder at 291 nm and 292 nm, respectively, which is consistent with results (discussed later) indicating that each of these proteins contains a higher proportion of tryptophan than cellobiohydrolase I (D).

Ultracentrifugation Studies - Sedimentation equilibrium experiments were conducted using purified β-glucanases, in order to determine their molecular weight and state of aggregation. Concentrations of 0.33, 0.50 and 1.0 mg protein/ml for each enzyme were used during these experiments. A straight line was obtained for each glucanase when the logarithm of fringe displacement was plotted vs. the square of the distance from the center of the rotor (Fig. 12). This relationship

FIGURE 11

Ultraviolet absorbance spectra of purified glucanases.

The concentrations of the endoglucanase (-··-) and cellobiohydrolases I (D) (-) and II (--) were 0.56 mg/ml, 0.56 mg/ml and 0.51 mg/ml, respectively, in deionized distilled water. Each spectrum was determined using a Beckman Model ACTA-MVI recording spectrophotometer.

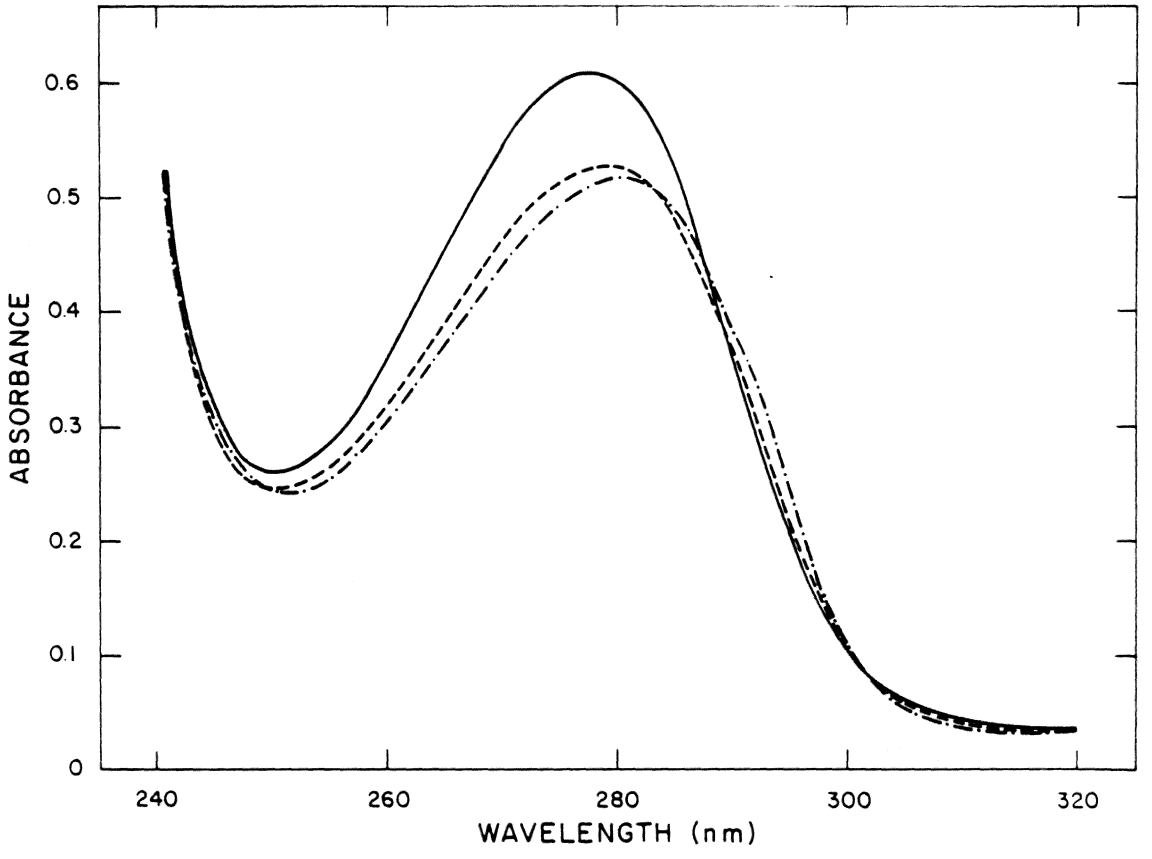
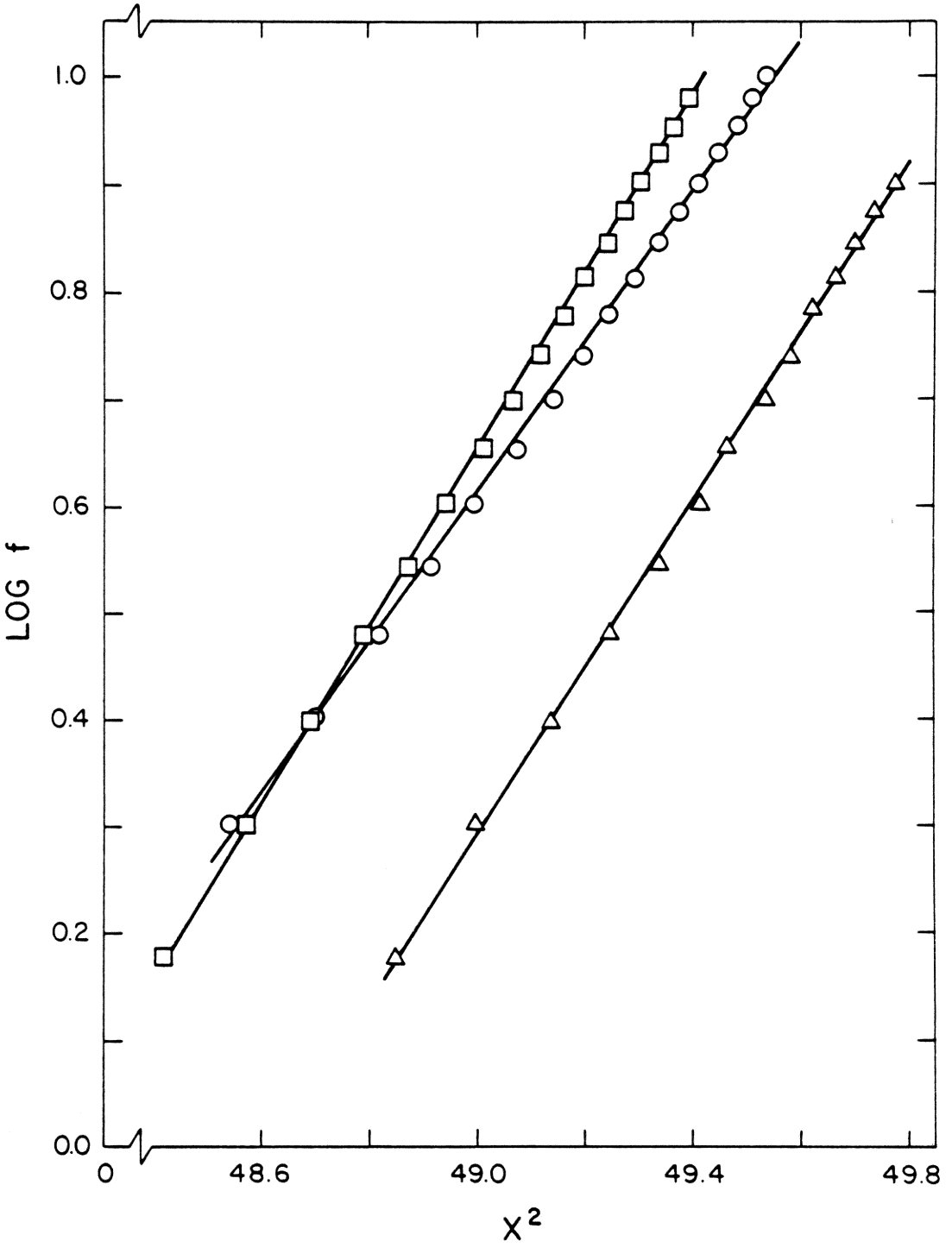

ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0
240
260
280
300
320
WAVELENGTH (nm)

FIGURE 12

Sedimentation equilibrium analysis of purified glucanases.

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 lines represent cellobiohydrolase I (D) (□), the endoglucanase (0) and cellobiohydrolase II (Δ).


1.0
0.8
0.6
0.4
0.2
0.0
LOG f
0
48.6
49.0
49.4
49.8
X²

indicates homogeneous, aggregate-free enzyme solutions. A weight-average molecular weight was calculated for each glucanase using the long-column meniscus depletion technique of Yphantis as modified by Chervenka (41). The partial specific volumes for each glucanase were calculated from amino acid and carbohydrate compositions by the method of Cohn and Edsall (42), and found to be 0.697, 0.702 and 0.691 for the endo-1,4-β-D-glucanase, and cellobiohydrolases I (D) and II, respectively. Computer analysis yielded weight-average molecular weights of 45,215 ± 1483, 53,220 ± 1479 and 54,682 ± 2683 for the endoglucanase, and for cellobiohydrolases I (D) and II, respectively. These molecular weights are in good agreement with those calculated for each enzyme from its composition.

The molecular weight obtained for cellobiohydrolase I (D) is in good agreement with that previously reported by Gum and Brown (4) for the same enzyme. The endoglucanase isolated during the present investigation has a lower molecular weight than that of the enzyme isolated by Häkansson et al (16) from the same strain of Trichoderma. The latter enzyme had a molecular weight of 51,000, as determined by gel filtration; this method, however, has often been found to yield erroneous results with glycoproteins.

Composition of glucanases - The composition of the purified glucanases is shown in Table II. All three are glycoproteins, containing covalently bound carbohydrate. With respect to amino acid composition, the most pronounced difference among the three glucanases is evidenced in the alanine content of cellobiohydrolase II, which is

COMPOSITION OF B-GLUCANASES FROM THE CELLULASE SYSTEM OF TRICHODERMA REESEI

(MOLES / MOLE PROTEIN)

	CBH I		CBH II		ENDOGLUCANASE	
LYSINE	12		9		5	
HISTIDINE	5		3		4	
ARGININE	10		9		7	
ASPARTIC ACID (AND AMIDE)	51		44		45	
THREONINE	52		29		43	
SERINE	50		40		42	
GLUTAMIC ACID (AND AMIDE)	41		25		28	
PROLINE	30		25		17	
GLYCINE	61		35		40	
ALANINE	28		48		25	
HALF CYSTINE	18		8		10	
VALINE	22		23		19	
METHIONINE	8		4		3	
ISOLEUCINE	10		11		17	
LEUCINE	24		24		18	
TYROSINE	22		16		13	
PHENYLALANINE	14		10		11	
TRYPTOPHAN	16		20		23	
TOTAL AMINO ACID RESIDUES	474	(50,492)a	383	(42,443)a	370	(40,074)a
MANNOSE	13		51		32	
GLUCOSE	4		20		9	
N-ACETYL GLUCOSAMINE	4		1		0.4	
TOTAL GLYCOSYL RESIDUES	21	(3,475)b	72	(11,694)b	41	(6,659)b
MOLECULAR WEIGHT BY COMPOSITION		53,967		54,136		46,733
MOLECULAR WEIGHT BY SEDIMENTATION EQUILIBRIUM		53,220 ± 1479		54,682 ± 2683		45,215 ± 1483

a POLYPEPTIDE
b CARBOHYDRATE

twice that of the other two enzymes on a mole percent basis. All three glucanases are high in glycine, serine, threonine and acidic amino acids but low in basic amino acids. The amino acid compositions of cellobiohydrolases I (D) and II when expressed as mole percent of constituent amino acids are quite different. In all but six amino acids (lysine, histidine, arginine, serine, tyrosine, and phenylalanine cellobiohydrolases I (D) and II differ by more than 0.6 mole percent. This was considered adequate reason to name these enzymes as isozymes rather than forms of the same enzyme.

Attempts to identify the N-terminal amino acid of each enzyme using the Edman degradation procedure[2] (52) were unsuccessful; a blocked N-terminus was indicated in each case.

It had been previously shown (29) that cellobiohydrolase I (D) isolated from resting cells of T. reesei QM 9414 incubated in the presence of sophorose was very similar, if not identical, in its amino acid composition to the cellobiohydrolase I (D) which had been isolated by Gum (4), from the culture filtrate of T. reesei QM 9123. It was of interest to examine whether the observed small differences in amino acid composition were significant, or were due to experimental error. Table III compares the amino acid compositions of these three cellobiohydrolases I (D); these have been adjusted to account for the fact that the amount of tryptophan had not been estimated for the T. reesei QM 9123 enzyme. Although the respective

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[2]Hermondson, M. (1979), Personal communication.

TABLE III

Amino acid composition of cellobiohydrolases I (D)

Amino acid	*T. reesei* QM 9414 (induced)	*T. reesei* QM 9414 (cellulose culture)	*T. reesei* QM 9123[a] (cellulose culture)
	Mole percent		
Lysine	2.7	2.6	2.7
Histidine	1.1	1.1	1.1
Arginine	1.7	2.2	2.1
Aspartic acid	11.8	11.1	11.2
Threonine	11.2[b]	11.4[b]	11.4
Serine	11.0[b]	10.9[b]	11.0
Glutamic acid	9.1	9.0	9.1
Proline	6.2	6.6	6.6
Glycine	13.0	13.3	13.5
Alanine	6.5	6.1	6.2
Half cystine	4.0[c]	3.9[c]	3.8[c]
Valine	5.2[d]	4.8[d]	4.8
Methionine	1.4[c]	1.7[c]	1.8[c]
Isoleucine	2.4	2.2	2.3
Leucine	5.4	5.2	5.3
Tyrosine	4.0	4.8	4.8
Phenylalanine	3.4	3.1	3.1

[a]Composition of 24-h hydrolysate (2).

[b]Values extrapolated to zero-time hydrolysis to correct for destruction of serine and threonine.

[c]Determined after performic acid oxidation.

[d]Based on 72-h hydrolysate.

compositions are very similar as expected, the two cellobiohydrolases I (D) which were derived from cellulose-grown T. reesei QM 9123 and QM 9414, respectively, have more nearly identical amino acid compositions than either with respect to the enzyme derived from cells exposed to sophorose. This is particularly evident with respect to arginine, aspartic acid, threonine, proline, alanine, valine, methionine, tyrosine and phenylalanine content.

It was important to compare the amino acid composition of the endoglucanase isolated during this study, to the "high molecular weight" endoglucanase isolated by Häkansson et al (16). The latter enzyme appears significantly different, in that it contains three times the amount of methionine, more than twice the amount of tyrosine and half-cystine, and only 25% the amount of tryptophan present in the endoglucanase isolated during this investigation; it is particularly amazing that the enzyme appears to contain no histidine (16). The endoglucanase of the present report appears similar in its composition to the endoglucanases isolated from T. viride by Shoemaker and Brown (10), and especially to endoglucanase IV. However, the T. reesei enzyme may be distinguished from those endoglucanases as well by its remarkably high tryptophan content, which amounts to 2.5 fold that of the T. viride endoglucanases.

The carbohydrate content of the three glucanases is presented in Table II in terms of moles of sugar per mole of protein, and in Table IV as weight percent. Mannose is the predominant neutral sugar in these enzymes, of which cellobiohydrolase II is the most heavily

TABLE IV

Carbohydrate composition (weight percent) of glucanases[a]

	CBH I	CBH II	Endoglucanase
Total carbohydrate (PSA)	4.26	19.5	10.6
Amino sugar			
N-acetyl glucosamine	1.4	0.40	0.18
N-acetyl galactosamine	<0.7	<0.10	<0.03
Neutral carbohydrate			
Total	5.04	21.23	14.07
Mannose	3.90	15.23	11.04
Glucose	1.14	6.00	3.03

[a]Determined as described in Experimental Procedures.

glycosylated, while cellobiohydrolase I (D) contains the least carbohydrate. Cellobiohydrolase II contains the highest proportion of glucose, whereas cellobiohydrolase I (D) contains the highest amount of glucosamine (presumably present as the N-acetylated form). The endo-1,4-β-D-glucanase isolated during this investigation contains a considerable amount of carbohydrate, by contrast to the enzyme isolated by Häkansson et al from T. reesei QM 9414, which reportedly contains no carbohydrate. This endoglucanase is also different from the T. viride endoglucanases isolated by Shoemaker and Brown (10), in that it contains no galactose.

Previous work by Gum and Brown (5) on the structure of the carbohydrate portion of cellobiohydrolase I, form C (5) indicates that oligosaccharides are bound to the protein via O-mannosyl bonds to serine and threonine residues. Similar linkages are very likely to exist not only in cellobiohydrolase I (D), which represents a differentially glycosylated form of a similar peptide, but also in cellobiohydrolase II and in the endo-1,4-β-D-glucanase. The particularly low N-acetylglucosamine content of the latter two enzymes suggests that it is very unlikely that the carbohydrate moiety is linked to the protein via N-glycosyl bonds to asparagine residues.

Thermal Stability - It has been suggested that the carbohydrate portion of glycoproteins serves to impart stability to the tertiary structure of the protein moiety (53,54). Chu et al (55) found that removal of 90% of the carbohydrate portion of yeast invertase (a glycoprotein the mass of which is 50% carbohydrate) resulted in an

enzyme of unaffected catalytic properties, but of markedly lower stability to multiple freeze-thaw treatment and to incubation at 50°. Furthermore, the carbohydrate-depleted enzyme exhibited less ability to recover its initial activity after exposure to denaturants and subsequent removal of the latter.

The effect of enzyme preincubation at elevated temperatures on the hydrolysis of swollen cellulose by the purified glucanases is illustrated in Fig. 13. Cellobiohydrolase I (D), which contains the least carbohydrate among the three enzymes, is least stable to preincubations at 60°, undergoing loss of 46% of its initial activity after twenty min. Cellobiohydrolase II, which is more heavily glycosylated, exhibits greater stability to identical treatment, losing 23% of its initial activity. The endoglucanase, although intermediate in carbohydrate content between cellobiohydrolase I (D) and cellobiohydrolase II, exhibits greater stability than the latter enzyme, retaining 100% of its initial activity after 20 min of preincubation at 60°. After preincubation at 70° for the same length of time, this endoglucanase still retains 72% of its initial activity (Fig. 13). A similar experiment was performed with the endoglucanase, using the viscosimetric assay to evaluate inactivation. The course of inactivation followed a pattern identical to that presented in Fig. 13.

Alkali Stability - The stability of the purified glucanases to preincubation under alkaline conditions was investigated. Fig. 14 illustrates the effect of preincubation of cellobiohydrolases I (D) and II at pH 9 and 40° for various lengths of time. Cellobiohydrolase I (D)

FIGURE 13

Thermal inactivation of purified glucanases. Enzyme samples were preincubated at elevated temperatures for various lengths of time; aliquots were withdrawn at several points and assayed as described in Experimental Procedures. The curves in Panels A, B and C represent residual activity of the endoglucanase, cellobiohydrolase II, and cellobiohydrolase I (D), respectively.

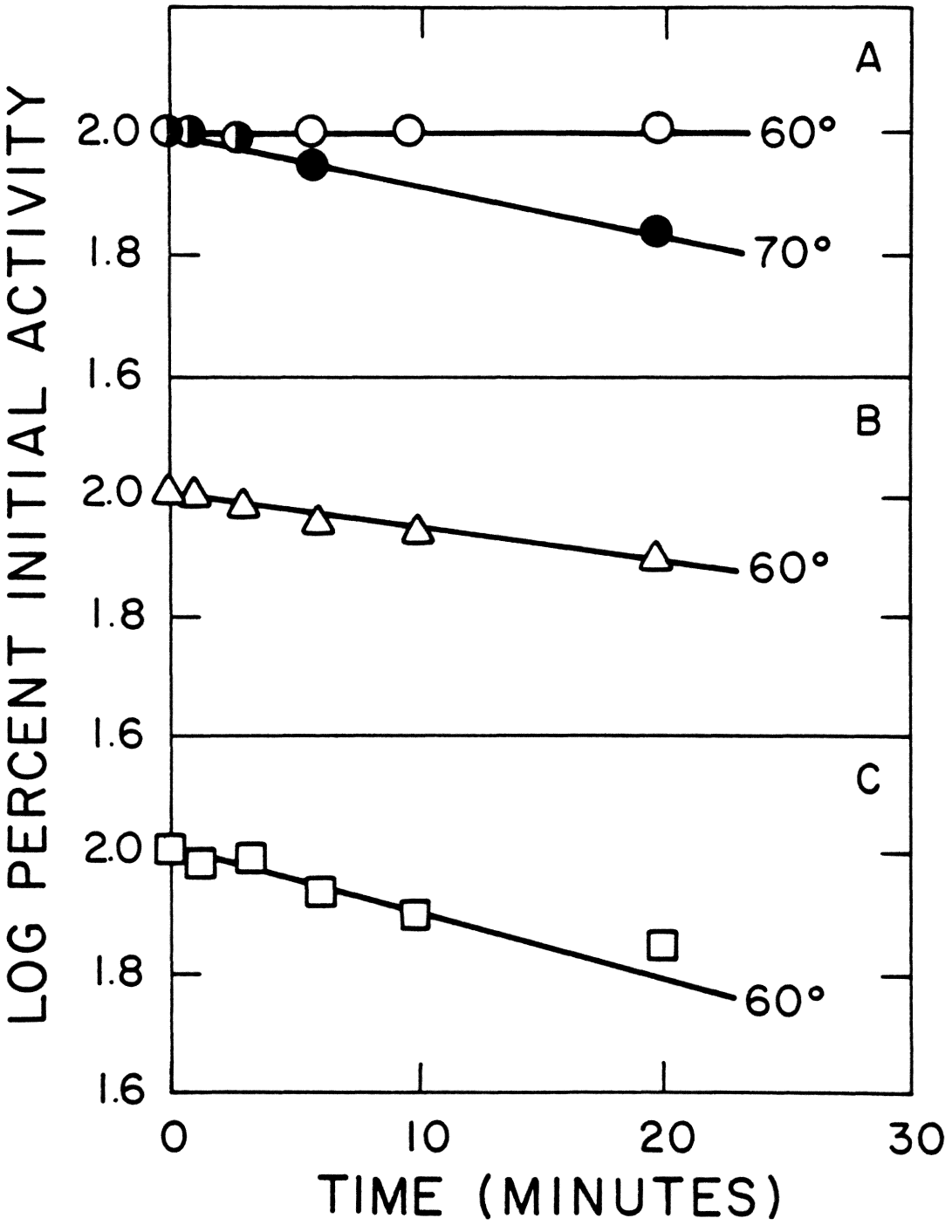
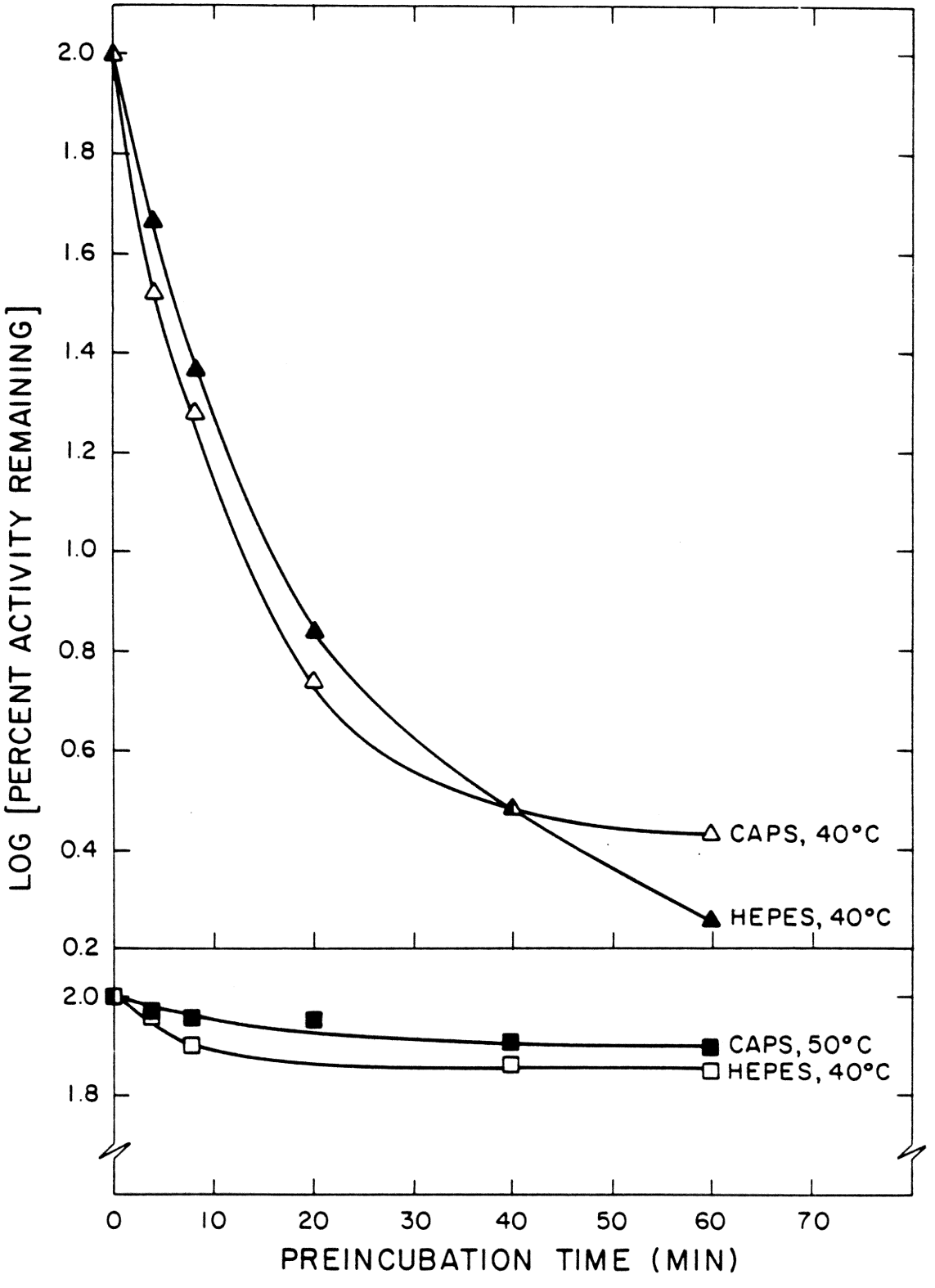

A
B
C
60°
70°
60°
60°
2.0
1.8
1.6
2.0
1.8
1.6
2.0
1.8
1.6
LOG PERCENT INITIAL ACTIVITY
0
10
20
30
TIME (MINUTES)

FIGURE 14

Alkali inactivation of cellobiohydrolases I (D) and II.

Preincubation of cellobiohydrolases I (D) and II took place in CAPS or HEPES buffers at pH 9, as described in Experimental Procedures. The curves represent cellobiohydrolase II (Δ) and cellobiohydrolase I (D) (□).


LOG [PERCENT ACTIVITY REMAINING]
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2.0
1.8
CAPS, 40°C
HEPES, 40°C
CAPS, 50°C
HEPES, 40°C
0
10
20
30
40
50
60
70
PREINCUBATION TIME (MIN)

exhibits much greater stability to alkali preincubation than cellobiohydrolase II, which is almost completely inactivated after 60 min of preincubation. For each enzyme, the course of inactivation is virtually identical with either HEPES or CAPS buffers, indicating the absence of any specific buffer effects. The greater susceptibility of cellobiohydrolase II to alkali is not entirely unexpected, as this enzyme contains more carbohydrate than cellobiohydrolase I, and therefore potentially a greater number of alkali labile O-glycosyl linkage sites.

In Fig. 15 and 16 are shown the results of endoglucanase preincubation at pH 8 and 9, respectively, on the activity of this enzyme on swollen cellulose. A very rapid initial inactivation of the enzyme was observed after only 2 min of preincubation in the presence of CHES buffer at pH 8 (Fig. 15); surprisingly, extending the length of preincubation to 20 min brings about no further loss of activity. Activity loss during preincubation of the endoglucanase in CHES buffer indicated a specific effect of this buffer on the inactivation of this enzyme. An increased loss of activity occurred at higher preincubation temperatures. The results of endoglucanase preincubation at pH 9 are shown in Fig. 16. A pattern similar to that of Fig. 15 is observed; very rapid inactivation takes place during the initial 2-5 min of preincubation, after which no further loss of enzymatic activity occurs, even after 50 min of additional preincubation. Nearly identical patterns are observed with HEPES, CAPS, and CHES buffers, although CAPS or CHES had a greater effect at 45° than did HEPES.

FIGURE 15

Inactivation of the endo-1,4-β-D-glucanase during preincubation at pH 8.

Preincubation took place in CHES buffer at pH 8, and at the indicated temperatures.

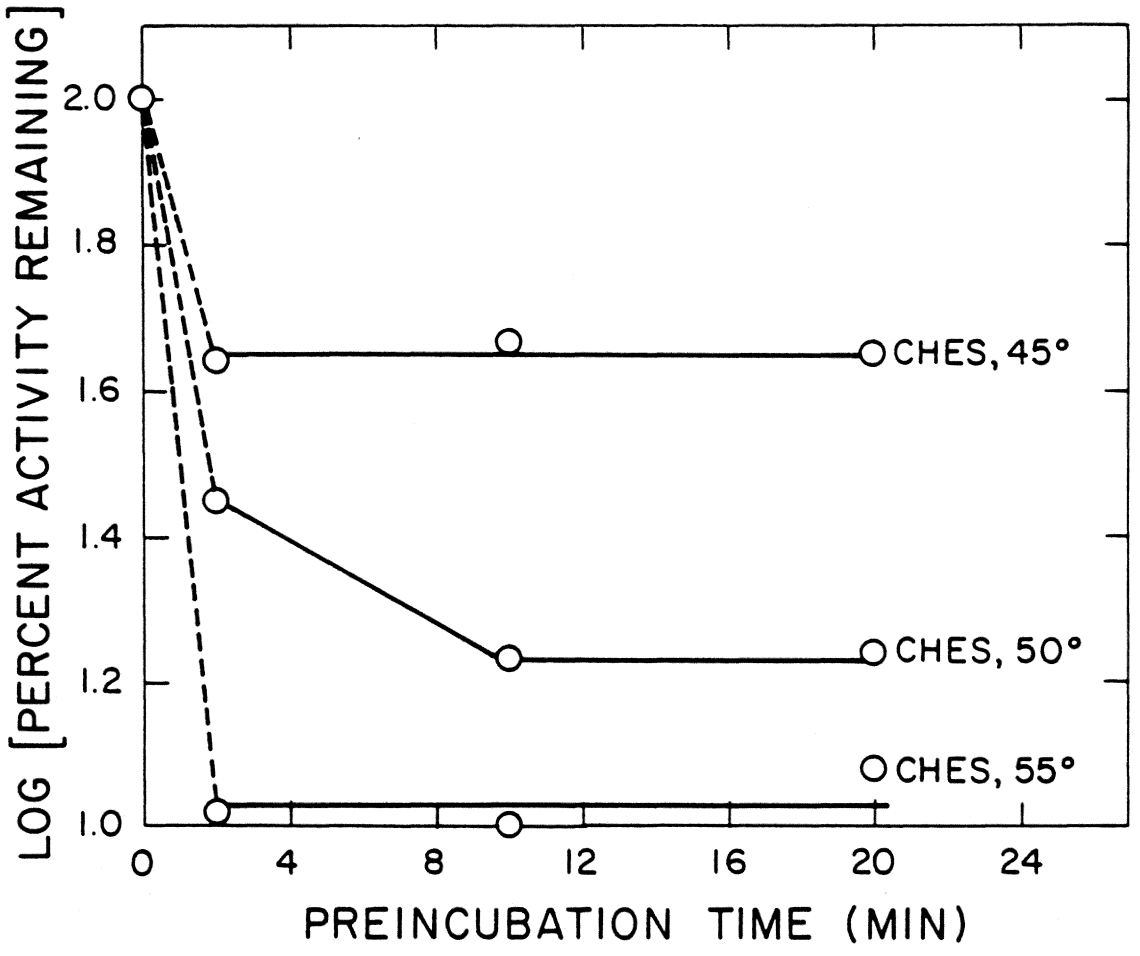
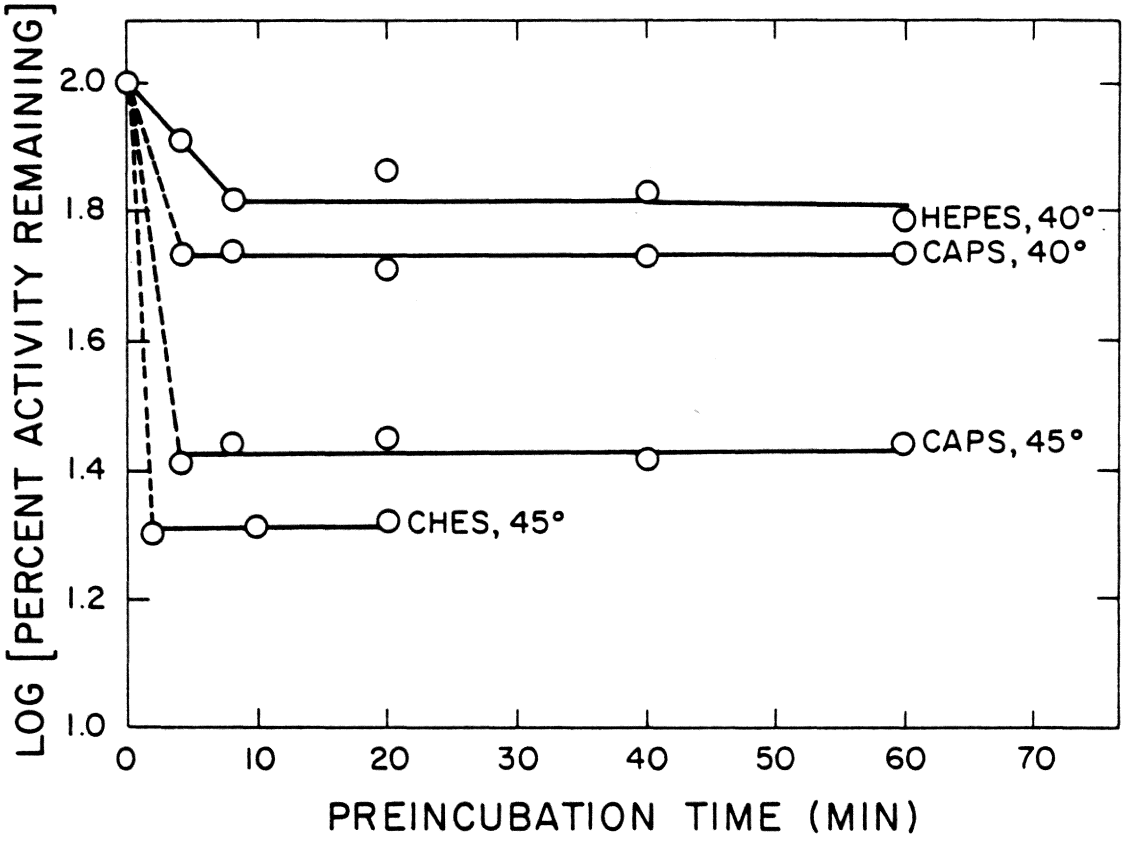

LOG [PERCENT ACTIVITY REMAINING]
2.0
1.8
1.6
1.4
1.2
1.0
0
4
8
12
16
20
24
PREINCUBATION TIME (MIN)
CHES, 45°
CHES, 50°
CHES, 55°

FIGURE 16

Inactivation of the endo-1,4-β-D-glucanase during preincubation at pH 9.

Preincubation took place in HEPES, CAPS, or CHES buffers at pH 9 and at the indicated temperatures.


LOG [PERCENT ACTIVITY REMAINING]
2.0
1.8
1.6
1.4
1.2
1.0
0
10
20
30
40
50
60
70
PREINCUBATION TIME (MIN)
HEPES, 40°
CAPS, 40°
CAPS, 45°
CHES, 45°

The unusual results obtained with the endoglucanase during these experiments may be explained as follows: there may be a small number (1-3) of O-glycosyl linkage sites on the protein moiety of the enzyme, which immediately undergo β-elimination upon exposure to alkali, while an alkali-resistant portion of the molecule (the polypeptide) still retains some activity. Alternately, each one of the buffers utilized in these experiments may bring about modification of the structure of the protein or result in a conformational change with concomitant loss of activity; upon completion of this hypothetical change, a new, lower level of activity is observed for the modified enzyme. Detailed structural work is necessary in order to establish the validity of either of the above suggestions.

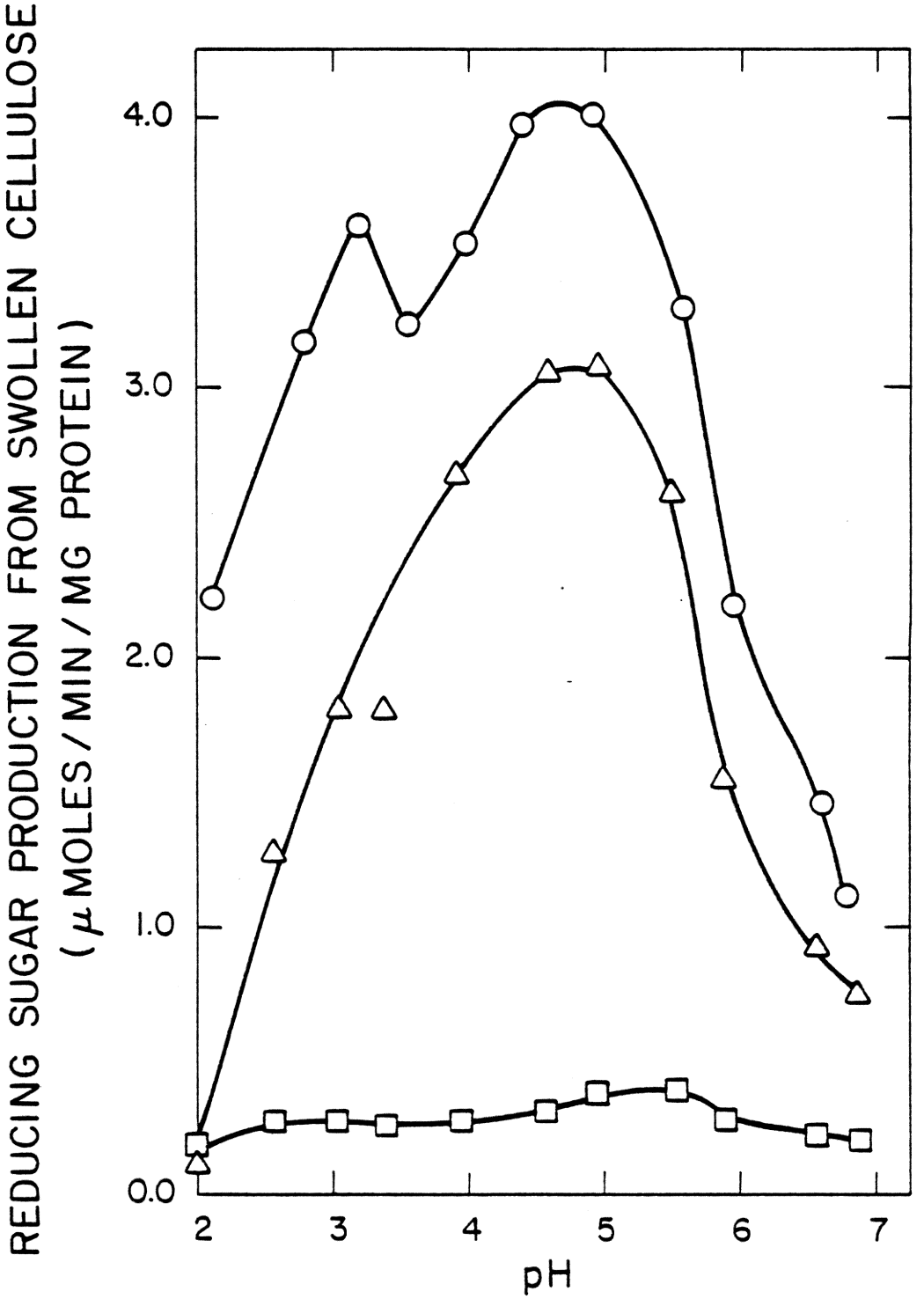
pH Optimum - The optimum pH for enzymatic activity of each glucanase was investigated as described in Experimental Procedures, and the results are shown in Fig. 17. Both cellobiohydrolase I (D) and the endoglucanase exhibit a rather broad pH range for optimum activity, from pH 4.8 to pH 5.6 and from pH 4.2 to pH 4.8, respectively. A second peak of optimum activity is observed at pH 3.3 and pH 3.2 for cellobiohydrolase I (D) and the endoglucanase (Fig. 17); at the latter pH values, these enzymes exhibited 80% and 90% of their maximum activity, respectively. Both enzymes are able to exhibit approximately half of their maximum activity at pH 2.0.

Cellobiohydrolase II presents a simpler pattern with a pH value for optimum activity of 4.9. A plot of the logarithm of specific activity versus pH yielded pK values of 3.35 and 5.8 for the catalytic

FIGURE 17

pH Optima for purified endoglucanase and for cellobiohydrolases I (D) and II

Enzyme samples were incubated with phosphoric acid-swollen cellulose at different pH values, as described in Experimental Procedures. The curves represent the activities of the endoglucanase (O), cellobiohydrolase I (D) (□), and cellobiohydrolase II (Δ).


REDUCING SUGAR PRODUCTION FROM SWOLLEN CELLULOSE
(μ MOLES / MIN / MG PROTEIN)
4.0
3.0
2.0
1.0
0.0
2
3
4
5
6
7
pH

groups of this enzyme.

Enzymic Activity and Specificity

The mode of action of the two exoglucanases (cellobiohydrolases I (D) and II), and the endo-1,4-β-D-glucanase purified during this investigation was studied by examining initial rates and action patterns of the three enzymes on various cellulosic substrates. High Performance Liquid Chromatography was employed to follow the course of enzymic reactions and to examine the array of products arising from these substrates as a result of enzymic action.

Cellooligosaccharide substrates - To study the action patterns of the β-1,4-glucanases, cellotetraose, cellopentaose and cellohexaose were each allowed to react with appropriate amounts of each purified enzyme at 40°. Substrate concentrations were not identical due to the decreasing solubility of the higher oligomers. Initial rates were estimated by measuring the disappearance of substrate. The results of this study for cellobiohydrolases I (D) and II, and for the endoglucanase are summarized in Table V. Cellobiohydrolases I (D) and II exhibit an approximately twofold increase in rate with cellohexaose, as compared to cellopentaose and cellotetraose. Similarly, the rate of the endoglucanase on cellopentaose is higher than its rate on cellotraose, but, surprisingly, the same is not observed with cellohexaose as substrate. This result is difficult to interpret, as it is generally expected that the rate of an endoglucanase should increase with

TABLE V

Specific activities of glucanases on oligosaccharides

	(μmole substrate degraded/min/mg protein)		
Substrate	CBH I (D)	CBH II	Endoglucanase
Cellotriose (50 mM)	0.2	<0.01	<0.01
Cellotetraose (14 mM)	0.5	5	8
Cellopentaose (10 mM)	0.4	5	14
Cellohexaose (3 mM)	1	9	11

increasing substrate chain length. However, the values presented in Table V should be interpreted with caution, as reactions took place using 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. At lower endoglucanase concentrations higher initial specific activities were achieved, but reaction nearly ceased at 20-30% degradation. It can be noted from this study that cellobiohydrolase II cleaves oligosaccharides far more rapidly than cellobiohydrolase I (D); this tendency was observed also with polymeric substrates.

The products of glucanase action with cellooligosaccharide substrates were investigated in order to elucidate further the action pattern of each enzyme. Fig. 18, 19 and 20 illustrate the array of products arising from the action of cellobiohydrolase I (D), cellobiohydrolase II and the endoglucanase, respectively, after various lengths of incubation with cellotetraose. As expected for cellobiohydrolases, the predominant reaction which occurs as inferred from the products, is cleavage of cellotetraose to yield two moles of cellobiose. Cellobiohydrolase II exhibits this action pattern exclusively, even after more than 95% of the substrate had been degraded (Fig. 18). An apparent loss of specificity is observed with cellobiohydrolase I (D), which, in addition to cellobiose, yields glucose and cellotriose from cellotetraose (Fig. 18). A similar array of products is observed with the endoglucanase, indicating that it does not only cleave the "middle" bond of cellotetraose, but also one or

FIGURE 18

Separation by high performance liquid chromatography of products formed from the reaction of cellobiohydrolase I (D) with cellotetraose.

The reaction mixture consisted of 0.9% cellotetraose, 0.25% α-methyl-D-glucoside (used as internal standard), and 50 μg of cellobiohydrolase I (D) in a total assay volume of 225 μl. Panel A represents the chromatographic pattern of the resulting mixture at the time of addition of the enzyme (0 time). Panels B and C show the components present after 20 and 220 min of reaction, respectively, at which times 25% (Panel B) and 86% (Panel C) of the substrate had been degraded. Under the conditions of this experiment, (75: 25, (w/w) acetonitrile: water as the solvent for chromatography at a flow rate of 1.5 ml/min) retention times for the oligosaccharides were: α-methyl-D-glucoside 3.8 min; G_1, 4.6 min; G_2, 6.2 min; G_3, 8.4 min; G_4, 12 min.

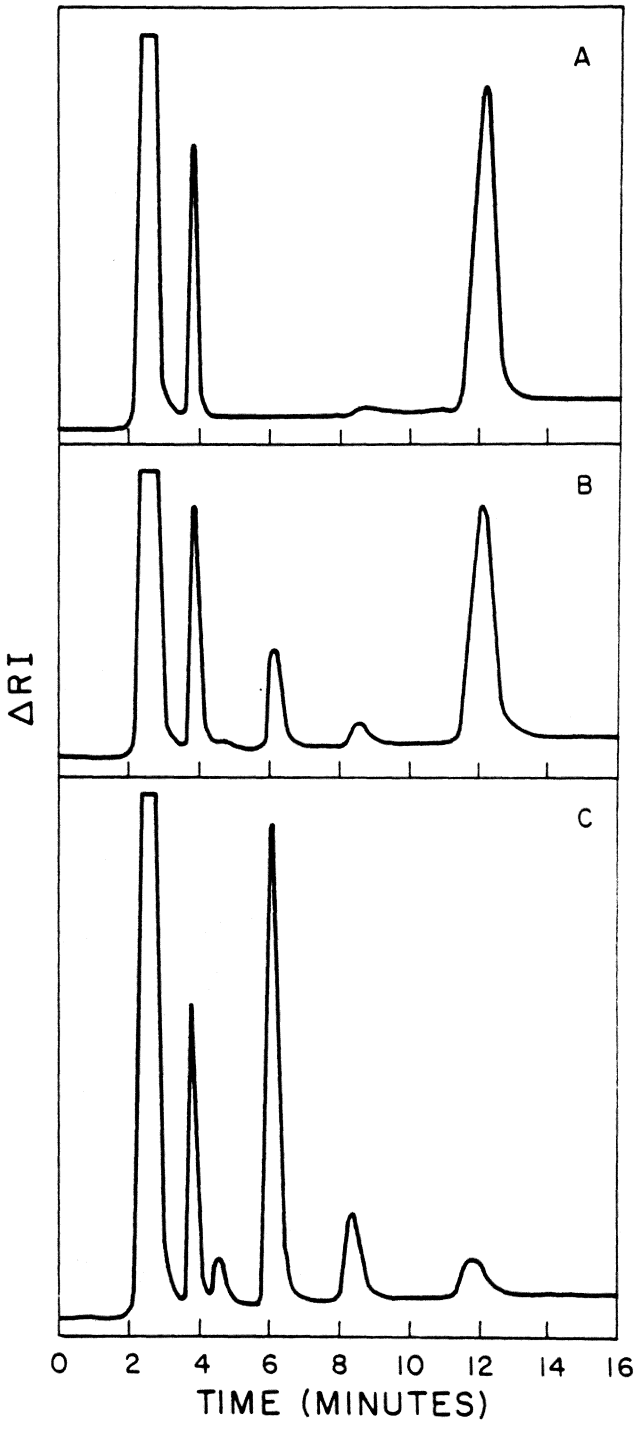

A
B
C
ΔRI
0
2
4
6
8
10
12
14
16
TIME (MINUTES)

FIGURE 19

Separation by high performance liquid chromatography of products formed from the reaction of cellobiohydrolase II with cellotetraose.

The reaction mixture consisted of 1% cellotetraose, 1% α-methyl-D-glucoside (used as internal standard) and 5.34 μg of cellobiohydrolase II in a total assay volume of 203 μl. Panel A represents the components of the incubation mixture at time zero. Panels B, C and D show the components present after 16, 46, and 136 min of reaction, respectively, representing 12.7% (Panel B), 70% Panel (C) and 95.3% (Panel D) degradation of the substrate. Retention times for the oligosaccharides during this experiment (68:32, (w/w) acetonitrile: H_2O as the solvent for chromatography at a flow rate of 1.5 ml/min) were: α-methyl-D-glucoside, 3.8 min; G_2, 5.6 min; G_3, 7.3 min; G_4, 9.8 min.

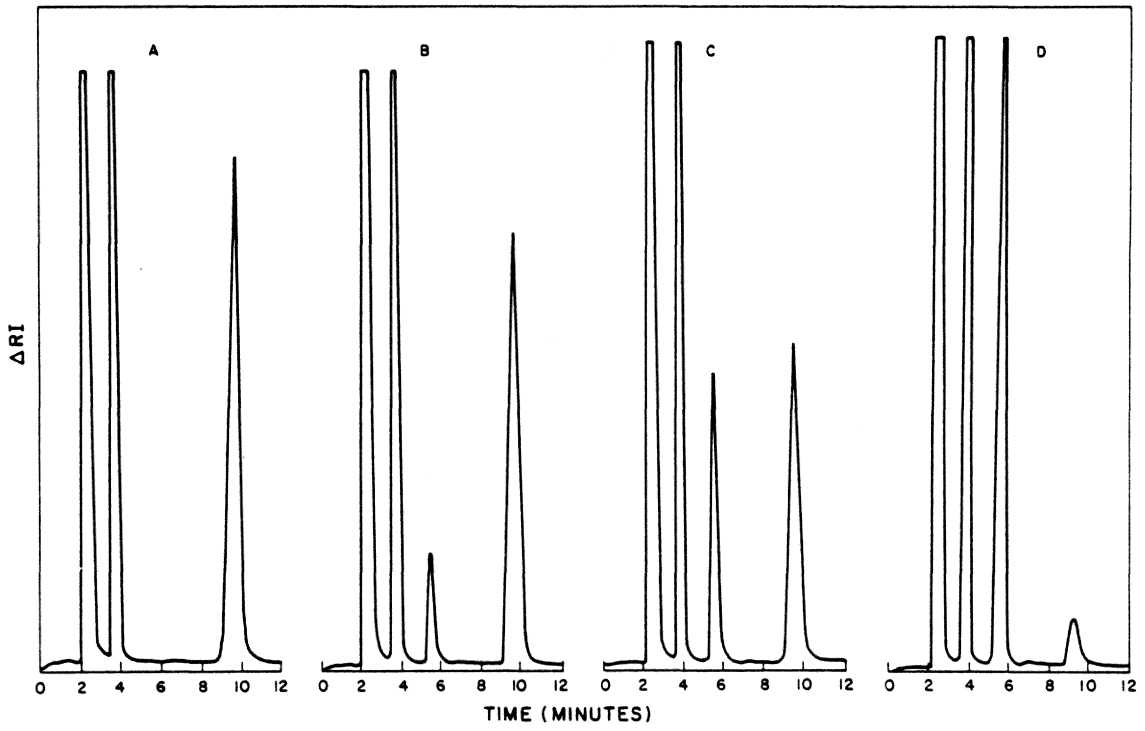
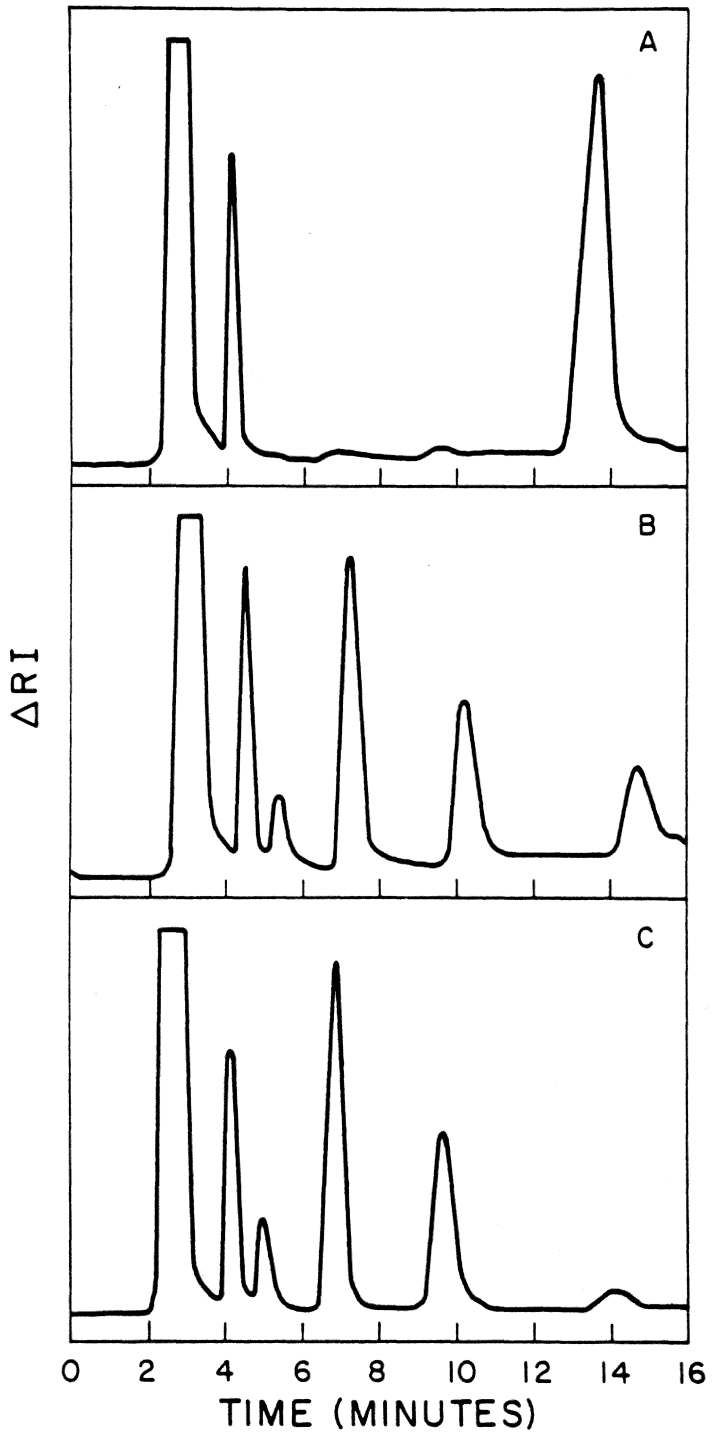

A
B
C
D
ΔRI
0 2 4 6 8 10 12
TIME (MINUTES)

FIGURE 20

Separation by high performance liquid chromatography of products formed from the reaction of the endo-1,4-β-D-glucanase with cellotetraose.

To a 1% solution of cellotetraose containing 0.25% α-methyl-D-glucoside as internal standard, 25 µg of endoglucanase were added. The total assay volume was 416 µl. In Panels A, B and C are shown the separated components of the incubation mixture at 0, 25 and 40 min, respectively, at which times 0, 74% and 93% of the substrate had been degraded. With the chromatographic solvent system used in this experiment (75:25 (w/w) acetonitrile: H_2O at a flow rate of 1.5 ml/min) oligosaccharide retention times were as follows: α-Me-Glc, 4.3 min; G_1, 5.1 min; G_2, 7.0 min; G_3, 9.9 min; G_4, 14.0 min.


A
B
C
ΔRI
0
2
4
6
8
10
12
14
16
TIME (MINUTES)

both of the terminal bonds. Bond specificity could not be determined using the normal cellotetraose (nonreduced), and the resulting glucose could have arisen from either the reducing or the nonreducing end of the substrate. The fact that a significant amount of cellotriose remains among the products of both cellobiohydrolase I (D) and the endoglucanase even after most of the substrate had been depleted, indicates that each of these enzymes exhibits very little activity toward cellotriose as it exists at low concentrations. This could be confirmed by rate studies with these enzymes, using cellotriose at several substrate concentrations. The disproportionately large amount of trisaccharide relative to glucose indicates that transglycosylation is catalyzed by the endoglucanase.

Fig. 21, 22 and 23 illustrate the action patterns of cellobiohydrolases I and II, and the endoglucanase, respectively, with cellopentaose as the substrate. Cellobiohydrolase II cleaves cellopentaose to yield cellobiose and cellotriose only (Fig. 22); cellotriose is not further cleaved by this enzyme as evidenced by the complete absence of glucose among the products. Cellobiohydrolase I (D) cleaves cellopentaose to yield predominantly cellobiose and cellotriose (Fig. 21); however, cleavage of one or both of the terminal bonds of the substrate must also take place, although at lower frequency, as indicated by the presence of some glucose, as well as cellotetraose, in the products. Although the most abundant products from endoglucanase action on cellopentaose are cellobiose and cellotriose (Fig. 23), a small amount of glucose and substantial amount of cellotetraose are also observed after complete disappearance of cellopentaose has taken place. The persistence

FIGURE 21

Separation by high performance liquid chromatography of products formed from the reaction of cellobiohydrolase I (D) with cellopentaose.

This incubation mixture was composed of 0.9% cellopentaose, 0.25% α-methyl-D-glucoside (internal standard) and 50 μg of cellobiohydrolase I (D) in a total incubation volume of 225 μl. Chromatograms of this mixture are shown at the time of initiation of the reaction by addition of the enzyme (Panel A) and after 25 min (Panel B) and 175 min (Panel C) of reaction, respectively; 23% and 85% of the substrate had been degraded at 25 min and 175 min, respectively. Separation was accomplished by using a 75:25, (w/w) acetonitrile: water chromatographic solvent system at a flow rate of 1.5 ml/min. Oligosaccharide retention times were as follows: α-methyl-glucoside 4.1 min; G_1, 4.8 min; G_2, 6.5 min; G_3, 9.1 min; G_4, 12.9 min and G_5, 18.8 min.

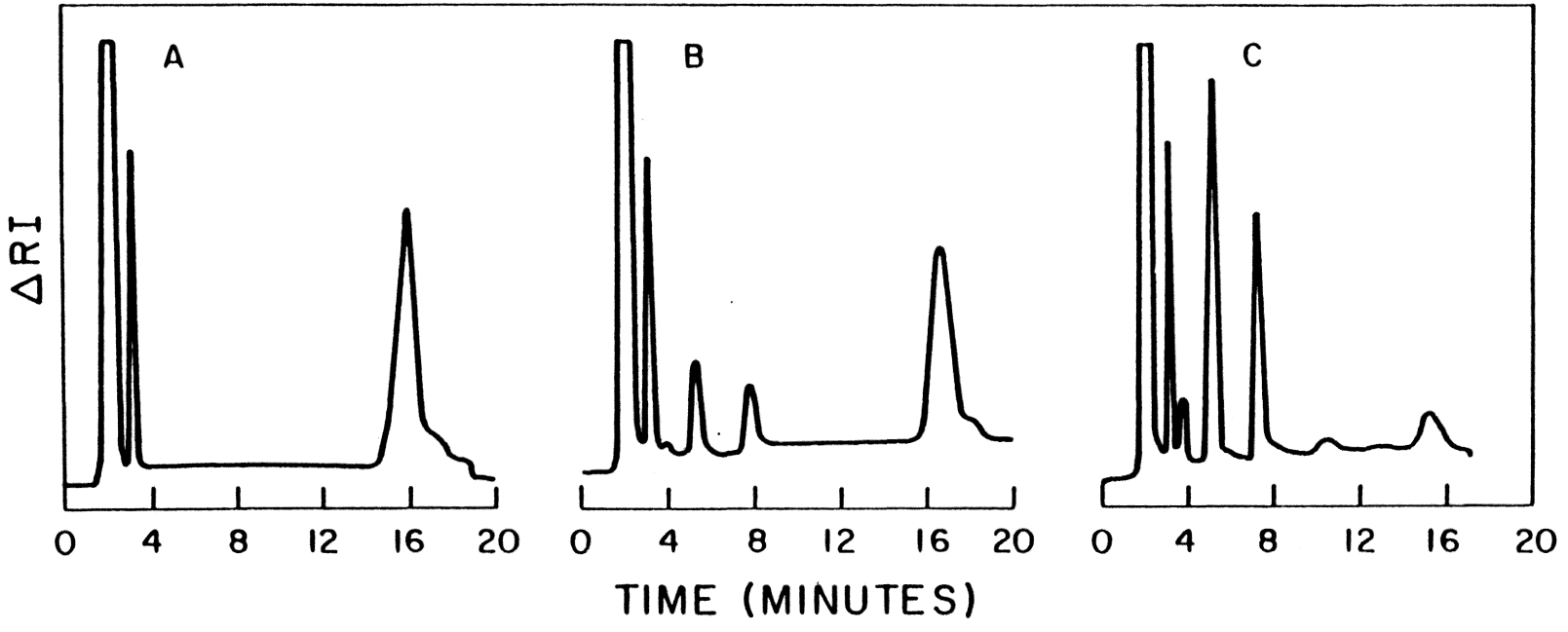

A
B
C
ΔRI
0
4
8
12
16
20
TIME (MINUTES)

FIGURE 22

Separation by high performance liquid chromatography of products formed from the reaction of cellobiohydrolase II with cellopentaose.

To a 0.5% solution of cellopentaose containing 0.5% α-methyl-D-glucoside as an internal standard, 7.1 μg of cellobiohydrolase II were added to initiate the reaction. Total reaction volume was 404 ml. Immediately after enzyme addition, an aliquot of the incubation mixture was injected into the liquid chromatograph, and gave the pattern shown in Panel A. Panels B and C are chromatograms of the same after 20 and 100 min of reaction, respectively, and represent 21.2% and 94.9% of the substrate. Separation was accomplished using a 78:28 (w/w) acetonitrile; water solvent system at a flow rate of 1.5 ml/min, and yielded the following oligosaccharide retention times: α-methylglucoside, 3.8 min; G_1, 4.4 min; G_2, 5.7 min; G_3, 7.5 min; G_4, 12.4 min, and G_5, 13.7 min.

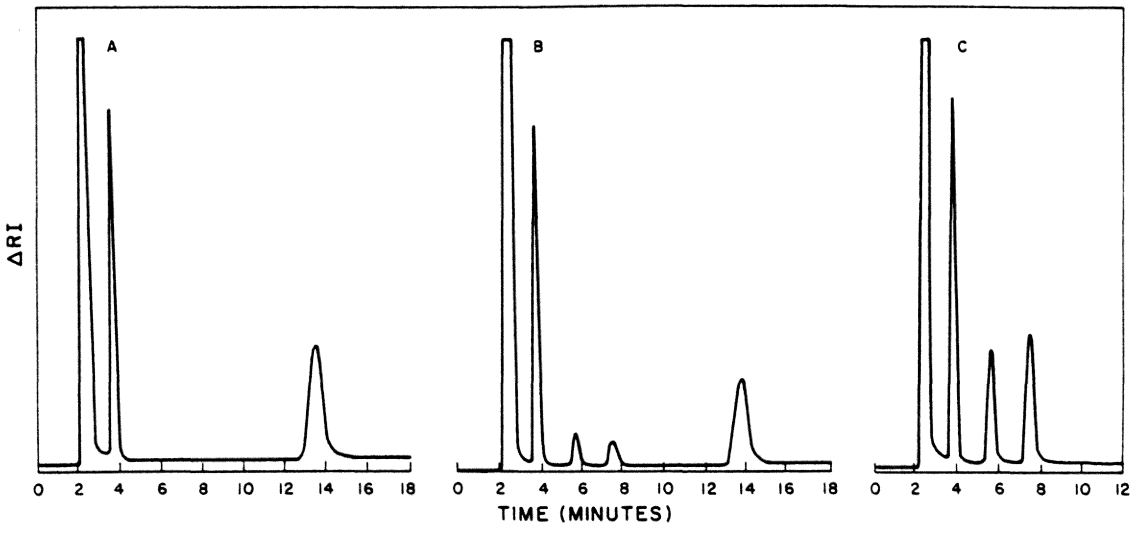
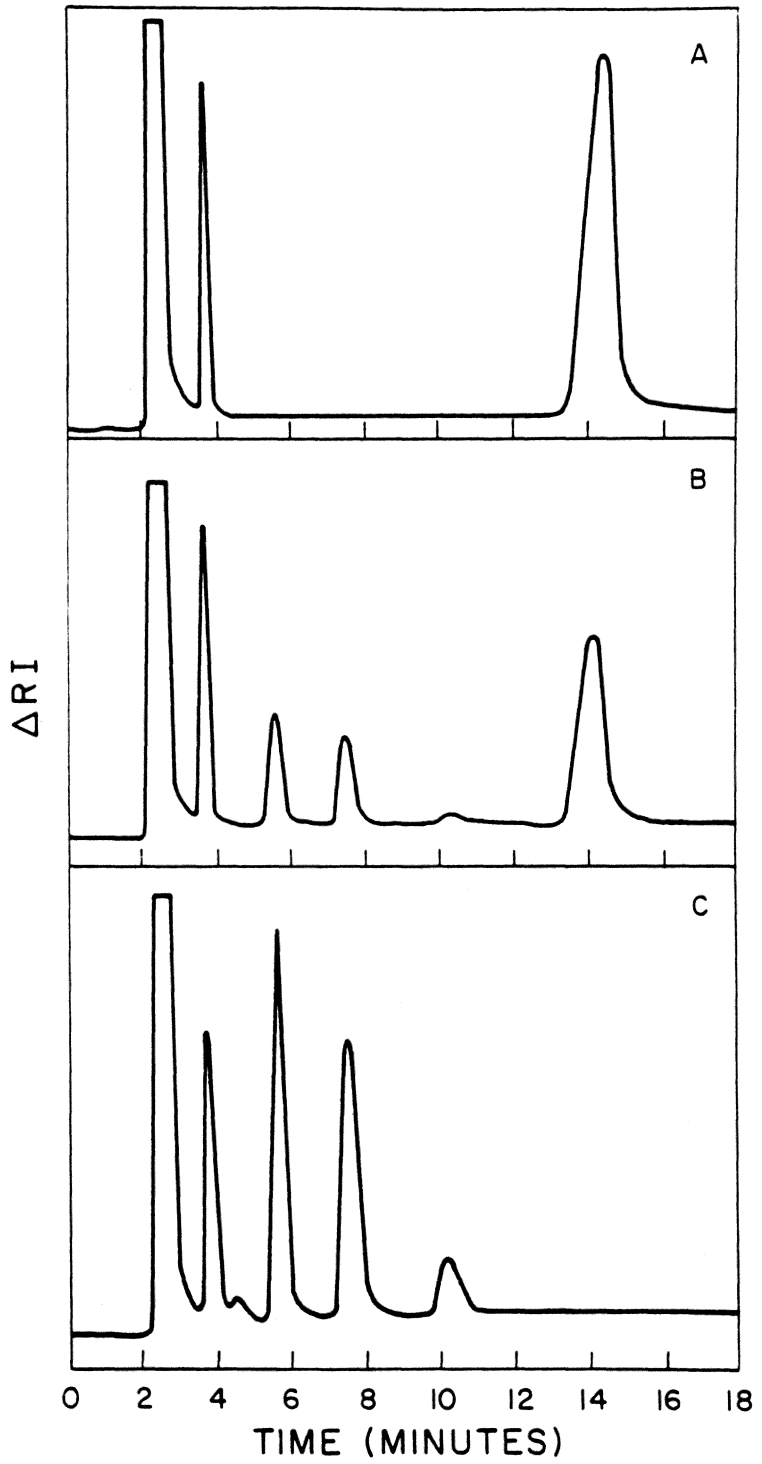

A
B
C
ΔRI
0 2 4 6 8 10 12 14 16 18
0 2 4 6 8 10 12 14 16 18
0 2 4 6 8 10 12
TIME (MINUTES)

FIGURE 23

Separation by high performance liquid chromatography of products formed from the reaction of the endo-1,4-β-D-glucanase with cellopentaose.

A 1% solution of cellopentaose containing 0.25% α-methyl-D-glucoside as internal standard was incubated with 6.2 µg of the endoglucanase. The total incubation volume was 404 µl. Panel A is a chromatogram of the incubation mixture at the time of addition of the enzyme (0 time), whereas Panels B and C are chromatograms of this mixture after 20 and 100 min of incubation, respectively; at those times, 44% (Panel B) and 100% (Panel C) degradation of the substrate had taken place. Separation was accomplished by means of a 75:25 (w/w), acetonitrile: water solvent system at a flow rate of 1.5 ml/min. Oligosaccharide retention times were as follows: α-Me-Glc, 3.9 min; G_1, 4.5 min; G_2, 5.7 min; G_3, 7.6 min; G_4, 10.3 min; G_5, 14.2 min.


A
B
C
ΔRI
0
2
4
6
8
10
12
14
16
18
TIME (MINUTES)

of cellotriose in the products observed again after the original substrate has been depleted entirely, lends further support to the idea that this endoglucanase is not very, if at all, active, towards cellotriose. The cellotetraose which is present in the products may have arisen principally from transglycosylation as discussed below.

By comparison of the molar ratios of products from the action of the glucanases on several oligosaccharide substrates (Table VI), one may better describe differences between the modes of action of these enzymes and permit inference of the manner in which polymers are attacked. Cellobiohydrolase II produces only cellobiose, or a mixture of cellobiose and cellotriose from cellotetraose, cellopentaose or cellohexaose. These product mixtures can arise from hydrolysis of interior bonds in the substrates, with no production of glucose from terminal residues (Tables VII-IX), and without involving transglycosylation. By contrast, cellobiohydrolase I (D) yields increasing proportions of glucose and cellotriose as the chain length of the oligosaccharide substrate is increased. This may be due to a tendency for this enzyme to cleave internal linkages, and to produce glucose and cellobiose from the resultant cellotriose (Tables VII-IX). This was found to be the only glucanase (of the three described in this report) which could convert cellotriose to glucose and cellobiose. Both cellobiohydrolases formed cellobiose from cellohexaose without commensurate production of cellotetraose, indicating a processivity mechanism favoring sequential cleavage and release of cellobiosyl units.

TABLE VI

OBSERVED MOLAR RATIOS AMONG PRODUCTS FROM
ENZYMIC CLEAVAGE OF CELLOOLIGOSACCHARIDES

SUBSTRATE AND ENZYME	PENTASACCHARIDE(S)	TETRASACCHARIDE(S)	TRISACCHARIDE(S)	DISACCHARIDE(S)	GLUCOSE
CELLOTETRAOSE					
CELLOBIOHYDROLASE I (D)	-	-	0.17	1	0.16
CELLOBIOHYDROLASE II	-	-	0	1	0
ENDOGLUCANASE	-	-	0.60	1	0.17
CELLOPENTAOSE					
CELLOBIOHYDROLASE I (D)	-	0.06	0.54	1	0.25
CELLOBIOHYDROLASE II	-	0	0.91	1	-
ENDOGLUCANASE	-	0.15	0.67	1	0.01
CELLOHEXAOSE					
CELLOBIOHYDROLASE I (D)	0.02	0	0.61	1	0.37
CELLOBIOHYDROLASE II	0	0	0.63	1	0
ENDOGLUCANASE	0.19	0.88	1.34	1	0

TABLE VII

MECHANISMS BY WHICH OBSERVED MOLAR PROPORTIONS OF REACTION PRODUCTS MAY BE FORMED FROM CELLOTETRAOSE

ENZYME			MOLAR RATIOS (OBSERVED) G_3	G_2	G_1
CELLOBIOHYDROLASE I (D)	G-G↑G-G	G-G-G-G ↑ ↑ OR	17	100	17
	0.75	0.25	(17)	(100)	(16)
CELLOBIOHYDROLASE II	G-G↑G-G		0	100	0
	1.0		(0)	(100)	(0)
ENDOGLUCANASE	G-G↑G-G	G-G-G-G ↑ ↑ OR	60	100	20
	0.6	0.4	(60)	(100)	(17)

G-G↑G → G-G-G
0.2

TABLE VIII

MECHANISMS BY WHICH OBSERVED MOLAR PROPORTIONS OF REACTION PRODUCTS MAY BE FORMED FROM CELLOPENTAOSE

ENZYME				MOLAR RATIOS (OBSERVED) G_4	G_3	G_2	G_1
CELLOBIOHYDROLASE I (D)	G-G↑-G↑-G-G	G-G↑-G-G↑-G	G-G↑-G-G↑	6	55	100	28
	OR 0.66	OR 0.07	AND 0.27	(6)	(54)	(100)	(25)
CELLOBIOHYDROLASE II	G-G↑-G↑-G-G			0	100	100	0
	OR 1.0			(0)	(91)	(100)	(0)
ENDOGLUCANASE	G-G↑-G↑-G-G	G-G↑-G-G↑-G	G-G↑-G-G-G	12	65	100	1
	OR 0.87	AND 0.13	0.01	(15)	(67)	(100)	(1)

G-G-G+G ⟶ G-G-G-G
0.13

TABLE IX

MECHANISMS BY WHICH OBSERVED MOLAR PROPORTIONS OF REACTION PRODUCTS MAY BE FORMED FROM CELLOHEXAOSE

ENZYME				MOLAR RATIOS (OBSERVED) G_5	G_4	G_3	G_2	G_1
CELLOBIOHYDROLASE I (D)	G-G-G-G-G-G ↑ ↑ AND 0.29	G-G-G-G-G-G ↑ 0.16	G-G-G-G-G-G ↑ ↑ AND 0.55	0	0	61	100	39
				(2)	(0)	(61)	(100)	(37)
CELLOBIOHYDROLASE II	G-G-G-G-G-G ↑ ↑ AND 0.5	G-G-G-G-G-G ↑ 0.5		0	0	66	100	0
				(0)	(0)	(63)	(100)	(0)
ENDOGLUCANASE	G-G-G-G-G-G ↑ ↑ OR 0.56	G-G-G-G-G-G ↑ 0.38	G-G-G-G-G-G ↑ 0.05	18	91	136	100	0
				(19)	(88)	(134)	(100)	(0)

G-G-G-G+G ⟶ G-G-G-G-G
0.05

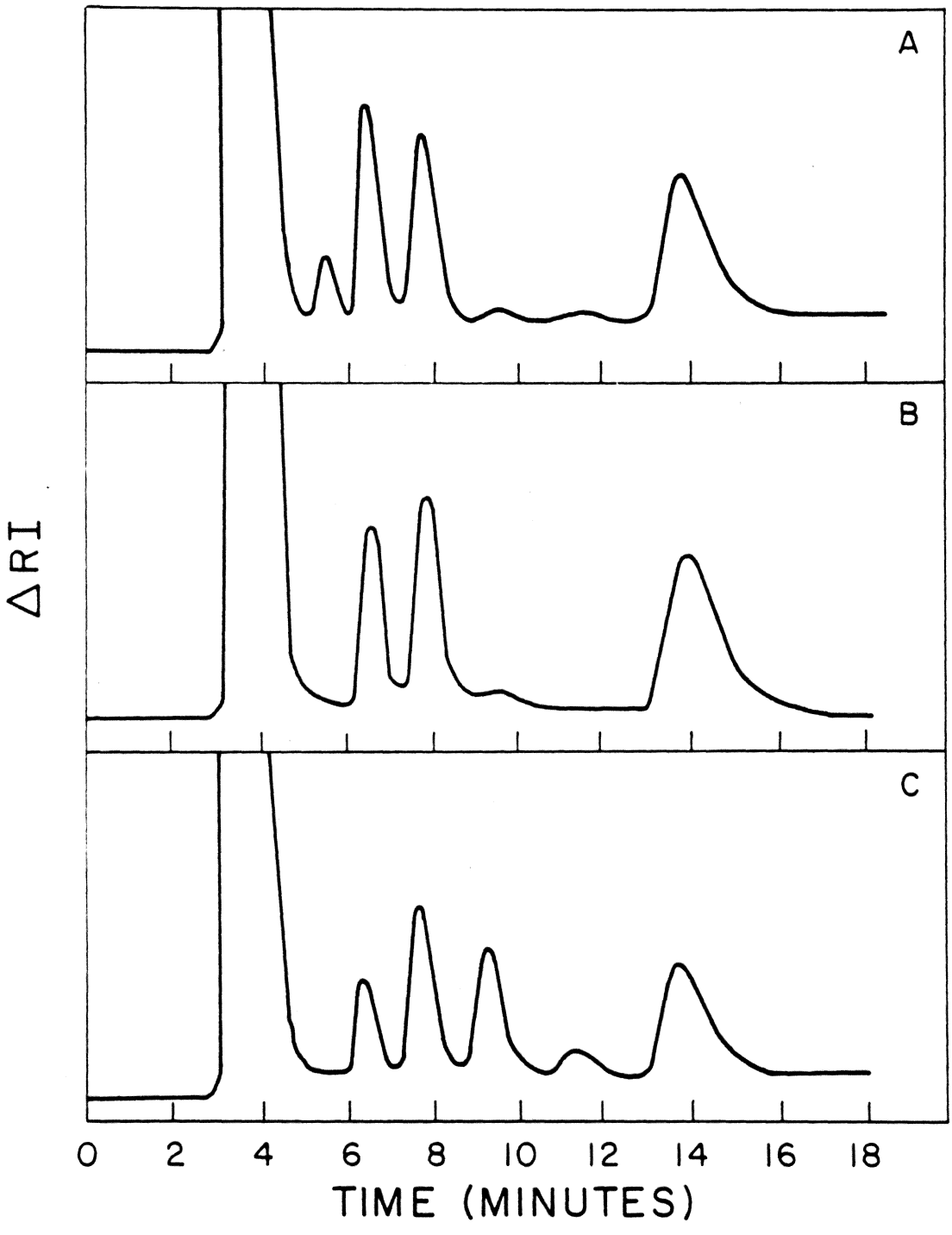
The pattern of products resulting from reaction of the endoglucanase with cellooligosaccharides (Table VI) required a transglucosylation step to account for the ratios observed (Tables VII-IX). Such transfer had been shown (Fig. 30) to occur in the degradation of phosphoric acid-swollen cellulose by endoglucanase in the presence of ethanol, resulting in ethyl glucoside formation. No evidence for the transfer of longer units was seen, a further similarity of the T. reesei endoglucanase to the endoglucanase IV of T. viride (9). The acceptor of choice for transglycosylation seemed to be the oligosaccharide which was two glucosyl units less than the original substrate, although transfer to form substrate would not have been detected. The products of transglucosylation are termed oligo- rather than cello-oligosaccharides as the position and configuration of the linkage formed has not been determined. Further clarification of the origin of each product could be gained by using sodium borohydride reduced cellooligosaccharide substrates. However, the presence of a sorbitol residue at the former reducing end may alter affinity for an enzyme, rate of reaction and proportions of products.

In Fig. 24 are shown the arrays of products obtained from each glucanase with cellohexaose as the substrate. Cellobiohydrolase I (D) produces primarily glucose, cellobiose and cellotriose, although small amounts of cellotetraose and cellopentaose can also be detected. This complex pattern is probably the result of multiple, sequential reactions, during which the product of one enzymic reaction becomes the substrate for another. Thus, cellobiose could have arisen from

FIGURE 24

Separation by high performance liquid chromatography of products formed from the reaction of the pure glucanases with cellohexaose.

Panels A, B and C represent the products which are formed after 40 min of reaction of the endoglucanase, cellobiohydrolase I (D) and cellobiohydrolase II, respectively, with 0.4% cellohexaose. Retention times for G_1, G_2, G_3, G_4, G_5, and G_6 under these conditions (75:25 (w/w), acetonitrile: water as the chromatographic solvent, at a flow rate of 1.5 ml/min) were, respectively, 5.6, 6.6, 7.8, 9.3, 11.3 and 13.8 min.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ΔRI
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8
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12
14
16
18
TIME (MINUTES)

cellohexaose by cleavage of either of the penultimate bonds, which would yield cellotetraose as the second product; cellotetraose could be cleaved to yield two moles of cellobiose, or glucose and cellotriose; cellotriose could alternately have arisen from cleavage of the middle bond in the original substrate, cellohexaose. The small amounts of both cellotetraose and cellopentaose among the products can be considered evidence that these oligomers are fairly good substrates for this enzyme. The production of substantial levels of glucose relative to cellobiose or cellotriose indicates that cellobiohydrolase I (D) not only cleaves terminal glucosyl units, but catalyzes little or no transglycosylation.

The action pattern presented by cellobiohydrolase II appears simpler. Cellobiose and cellotriose are the sole products from the reaction of this enzyme with cellohexaose (Fig. 24). This result could be interpreted as indicating two types of reaction: (a) cleavage of the middle bond of cellohexaose to yield two moles of cellotriose, and (b) cleavage of the penultimate bond of cellohexaose (whether from the reducing or nonreducing end) to yield cellobiose and cellotetraose; then subsequent cleavage of cellotetraose to yield two moles of cellobiose. As in the case of cellobiohydrolase I (D), the failure to accumulate cellotetraose indicates that cellotetraose arising from hydrolysis of a penultimate bond is quickly processed to two cellobiose units.

Fig. 24 also shows that the main products of cellohexaose degradation by the endoglucanase are cellobiose, cellotriose and cellotetraose; some cellopentaose is also present, although no

glucose can be detected in the products. This pattern indicates a random attack at internal glycosidic bonds with little tendency to cleave the cellotetraose produced. The obvious absence of glucose is consistent with its formation from the nonreducing end of a substrate with subsequent transfer to an acceptor oligosaccharide.

It should be noted at this point that when each of the three glucanases was tested for activity toward p-nitrophenyl-β-D-glucoside using substantial amounts of protein (30 μg), no release of p-nitrophenol could be detected (as little of 0.02 μg of β-glucosidase protein releases easily detectable amounts of p-nitrophenol). Therefore, glucose production during any of the reactions discussed earlier is not likely to be due to contaminating β-glucosidase activity.

Polymeric Substrates - Investigation of the model of action of the pure glucanases was extended to polymeric substrates. In Table X are shown the specific activities of each glucanase on several polymers chosen to provide information on the linkage and bond specificity of the glucanases. These polymers were insoluble in aqueous solution, with the exception of carboxymethylcellulose. As expected, the β-D-glucanases exhibited no activity toward amylose. However, all three, and especially CBH I (D) and the endoglucanase, demonstrated some ability to degrade xylan, which may indicate the lack of a specificity requirement for a hydroxymethyl group on the pyranose ring. Only the endoglucanase has the ability to reduce significantly the viscosity of a solution of carboxymethylcellulose,

TABLE X

Specific activities of glucanases on polymeric substrates

	(μmole reducing sugar/min/mg/protein)		
Substrate	CBH I (D)	CBH II	Endoglucanase
Phosphoric acid-swollen cellulose	0.30	3.20	4.04
Microcrystalline cellulose (Avicel)	0.07	0.13	0.12
Amylose	0.002	0.004	0.008
Xylan	0.036	0.046	0.066
Carboxymethyl cellulose	0.25[a]	0.12[a]	116[a]

[a]Reported as change in specific fluidity of carboxymethyl cellulose per min per mg protein.

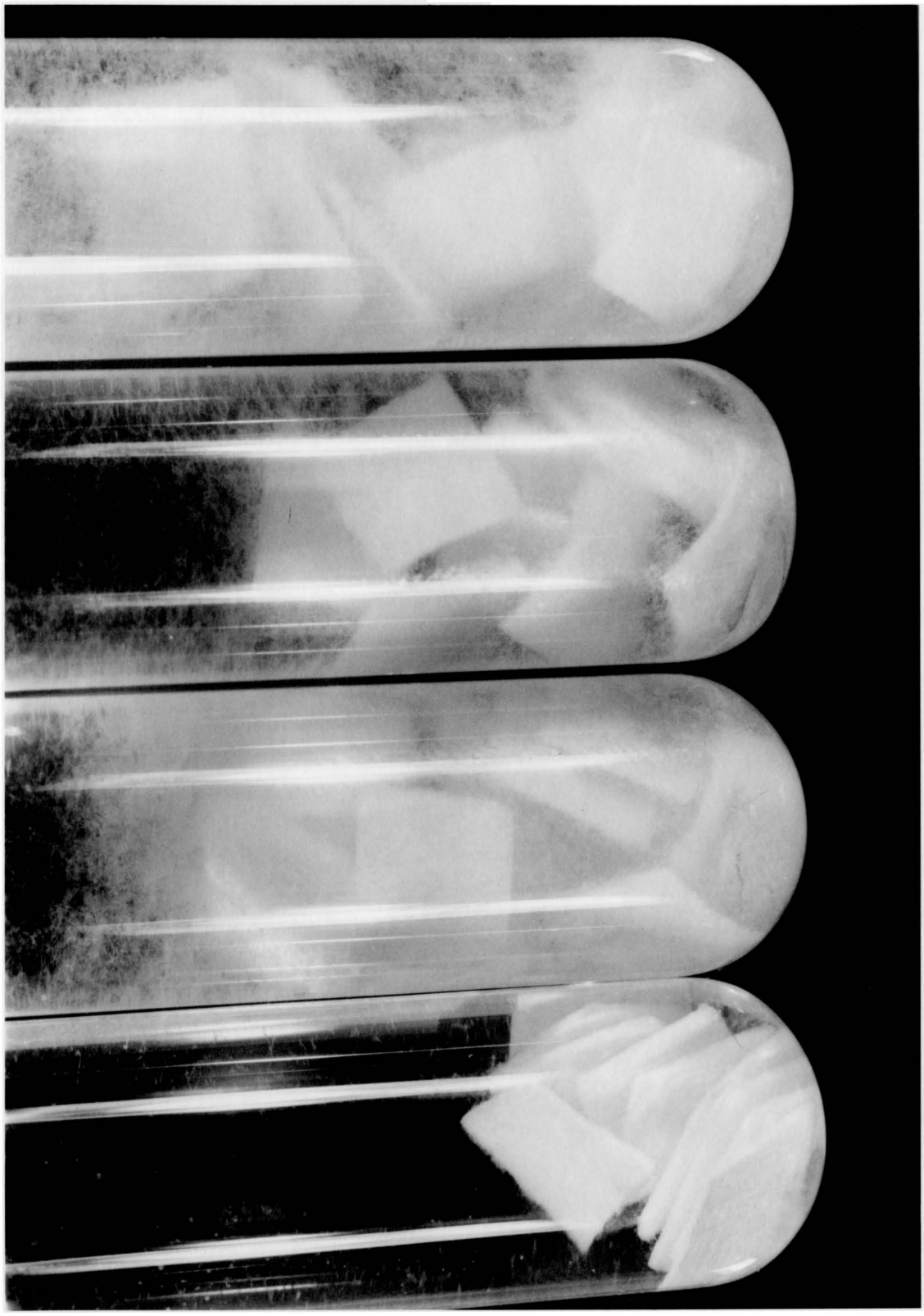
as expected from the operational definition for such an enzyme. All three glucanases have significantly higher activity on phosphoric acid-swollen cellulose, a hydrated, more accessible substrate, than on microcrystalline cellulose. Cellobiohydrolase I (D) appears to possess approximately one-tenth or less the activity of either cellobiohydrolase II or the endoglucanase to hydrolyze swollen cellulose, and one-half the activity of either of the latter enzymes toward Avicel as a substrate.

Filter paper was also used as a substrate with the purified glucanases in order to test their ability to release short fibers from that substrate. The results of this experiment are shown in Fig. 25. Although quantitation of the short fiber forming activity was not attempted, it appears that cellobiohydrolase I (D) is least effective among the three glucanases in releasing short fibers from filter paper. This is consistent with its apparently lower activity on the other polymeric substrates. Similar experiments testing the short fiber forming activity of purified T. viride endoglucanases have been reported by Berghem et al (12) and Shoemaker and Brown (9). The results were used to differentiate endoglucanase isozymes.

It should be emphasized that reaction rates with and product patterns from insoluble substrates cannot be completely relied upon to elucidate the mode of enzymic action on those substrates. This is due to the fact that only reactions which result in the release of soluble products can be detected, whereas reactions whose products remain bound to or associated with the substrate, or are transferred to it,

FIGURE 25

Short fiber information by pure glucanases. The reaction mixtures represent, from left to right, a control (containing filter paper squares without enzyme), endoglucanase (173 µg/ml), cellobiohydrolase I (D) (182 µg/ml) and cellobiohydrolase II (179 µg/ml). The tubes were incubated at 40° for 48 h.



remain "invisible". The action patterns of the purified glucanases on phosphoric acid-swollen and microcrystalline cellulose were investigated by High Performance Liquid Chromatographic analysis of the soluble hydrolysis products arising from these substrates as a result of enzymic action. Fig. 26 illustrates the soluble products formed initially by cellobiohydrolases I (D) and II from phosphoric acid-swollen cellulose after only 1 h of reaction; as expected, cellobiose is the main product of PSC hydrolysis by these cellobiohydrolases. A barely detectable amount of glucose is also formed, especially by cellobiohydrolase I (D). A similar experiment, which was performed using the purified endoglucanase is illustrated in Fig. 27; a significant amount of glucose is produced by the action of this endoglucanase on swollen cellulose, together with cellobiose and cellotriose. In Fig. 28 are shown the products formed by the three glucanases after prolonged incubation with the substrate (PSC); a large amount of glucose and cellobiose are the products of endoglucanase action under these conditions. No cellotriose could be detected among these products. A substantial amount of glucose, besides cellobiose, is also produced by cellobiohydrolase I (D), whereas the products of cellobiohydrolase II are primarily cellobiose and cellotriose; a negligible amount of glucose is also present. It is suggested by these results that cellobiohydrolase II possesses the lowest activity toward cellotriose among the three glucanases.

The products of prolonged incubation of each enzyme with microcrystalline cellulose (Avicel) are shown in Fig. 29. Cellobiohydrolase II is again distinguished both from its isozyme, cellobiohydrolase I

FIGURE 26

Separation by high performance liquid chromatography of products formed after one hour of reaction of cellobiohydrolase I (D) or II with phosphoric acid-swollen cellulose (PSC)

The reaction mixture consisted of a 1% suspension of PSC, which contained 0.25% α-methyl-D-glucoside (used as an internal standard). Panel A is a chromatogram of the components present in the incubation mixture, which had a total volume of 1 ml, after one hour of reaction with 165 μg of cellobiohydrolase I (D), at which time 6.1% degradation of the substrate had occurred. Panel B, is a similar chromatogram of the products of cellobiohydrolase II reaction with PSC; enzyme concentration was 62.8 μg/ml, and the substrate was degraded by 12%. The solvent system used for chromatographic separation was a mixture of 84:16, (w/w) acetonitrile: water, and the flow rate was 1.5 ml/min. Retention times for α-methyl-glucoside G_1, G_2 and G_3 were 4.9, 6.6, 11.3 and 21.3 min, respectively.

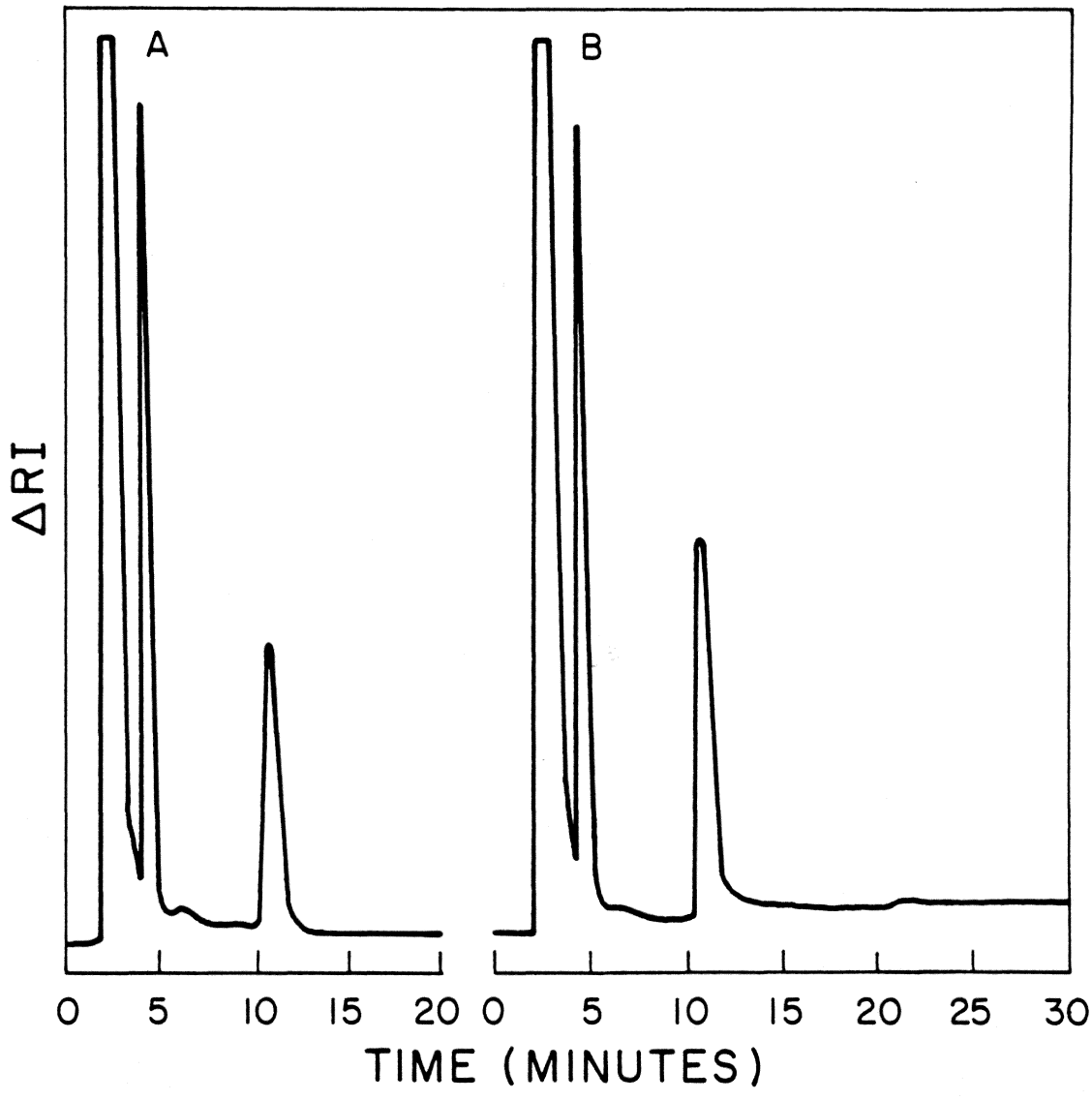
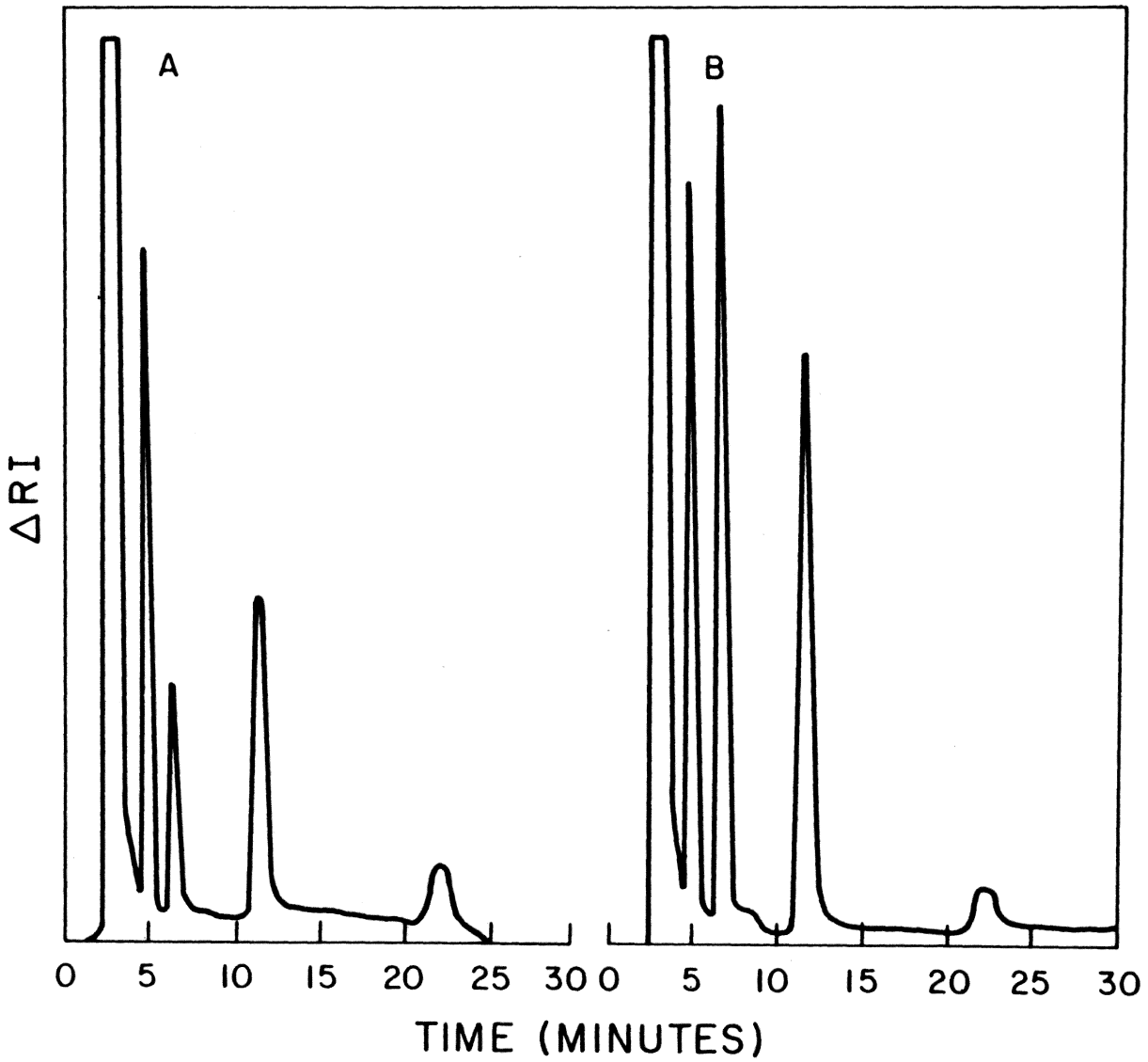

A
B
ΔRI
0
5
10
15
20
0
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 27

Separation by high performance liquid chromatography of products formed from the reaction of the endo-1,4-β-D-glucanase with phosphoric acid-swollen cellulose (PSC)

The incubation mixture consisted of one milliliter of a 2% suspension of PSC containing 0.25% α-methylglucoside (used as internal standard) and 320 μg of purified endoglucanase. Panels A and B show, respectively, the compositions of this incubation mixture after one and five h of reaction at which times the substrate had been degraded by 30% and 42.5%, respectively. Chromatographic separations were accomplished with a 84:16, (w/w) acetonitrile: water solvent system; the flow rate was 1.5 ml/min. Retention times of celloligosaccharides were: α-methyl-D-glucoside 4.9 min; G_1, 6.6 min; G_2, 11.6 min and G_4, 22.3 min.


A
B
ΔRI
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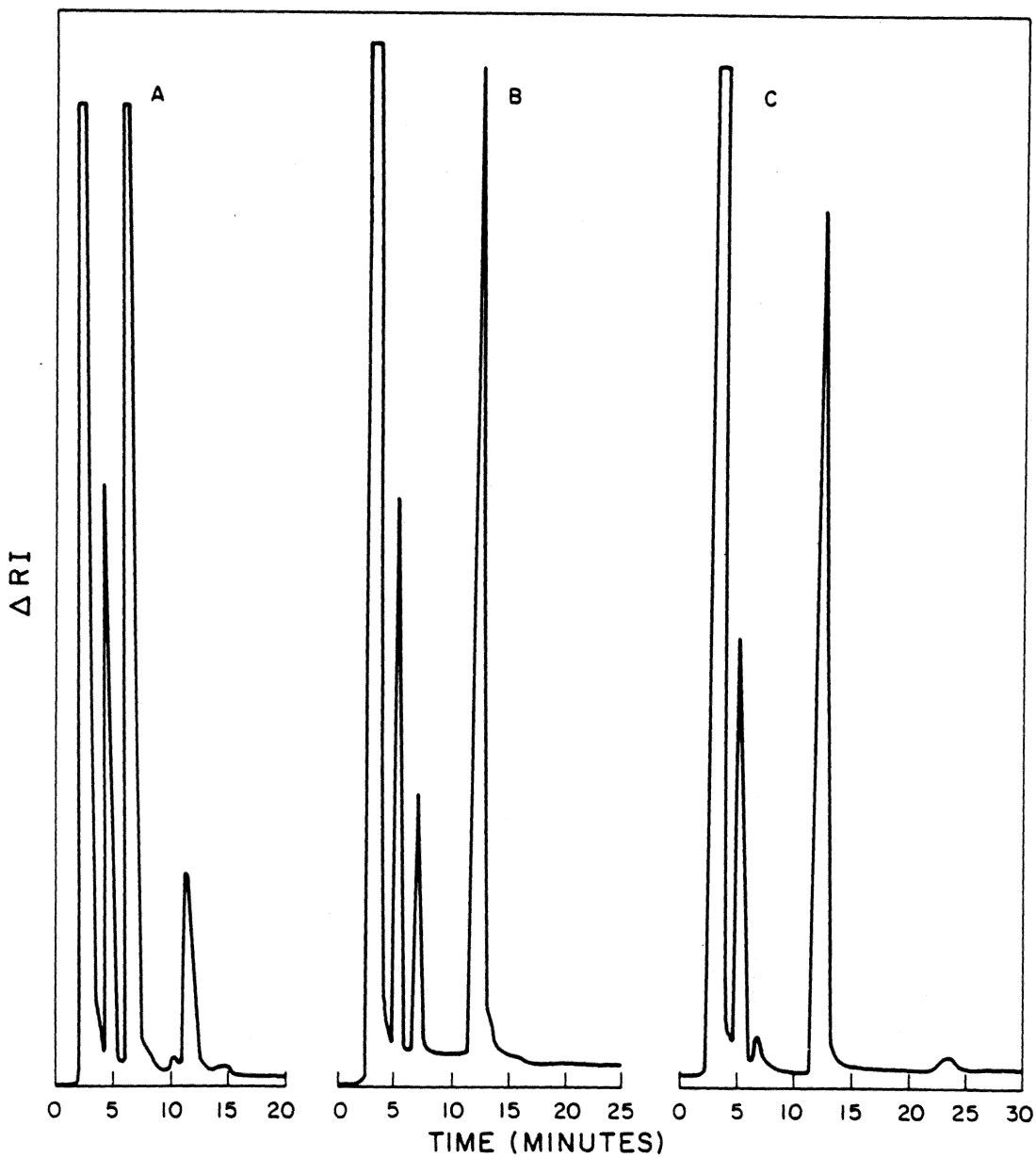
5
10
15
20
25
30
0
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 28

Separation by high performance liquid chromatography of products formed from the reaction of the purified glucanases with phosphoric acid-swollen cellulose (PSC).

The substrate was a 1% suspension of swollen cellulose which contained 0.25% α-methylglucoside as internal standard. The total incubation volume was 1 ml. In Panel A are shown the soluble hydrolysis products present in the mixture after 96 h of reaction with 3.11 μg of endoglucanase, at which time the substrate had been degraded by 60%; Panels B and C show the soluble products formed from PSC after 20 h of reaction with cellobiohydrolases I (828 μg) and II (312 μg), respectively; the substrate had at that time been degraded by 55% (Panel B), and 53% (Panel C). Separation was accomplished with a 84:16, (w/w) acetonitrile: water chromatographic solvent system. Retention times for α-methyl-D-glucoside, G_1, G_2, and G_3 were 4.9, 6.6, 11.6 and 22.3 min.


A
B
C
ΔRI
0 5 10 15 20
0 5 10 15 20 25
0 5 10 15 20 25 30
TIME (MINUTES)

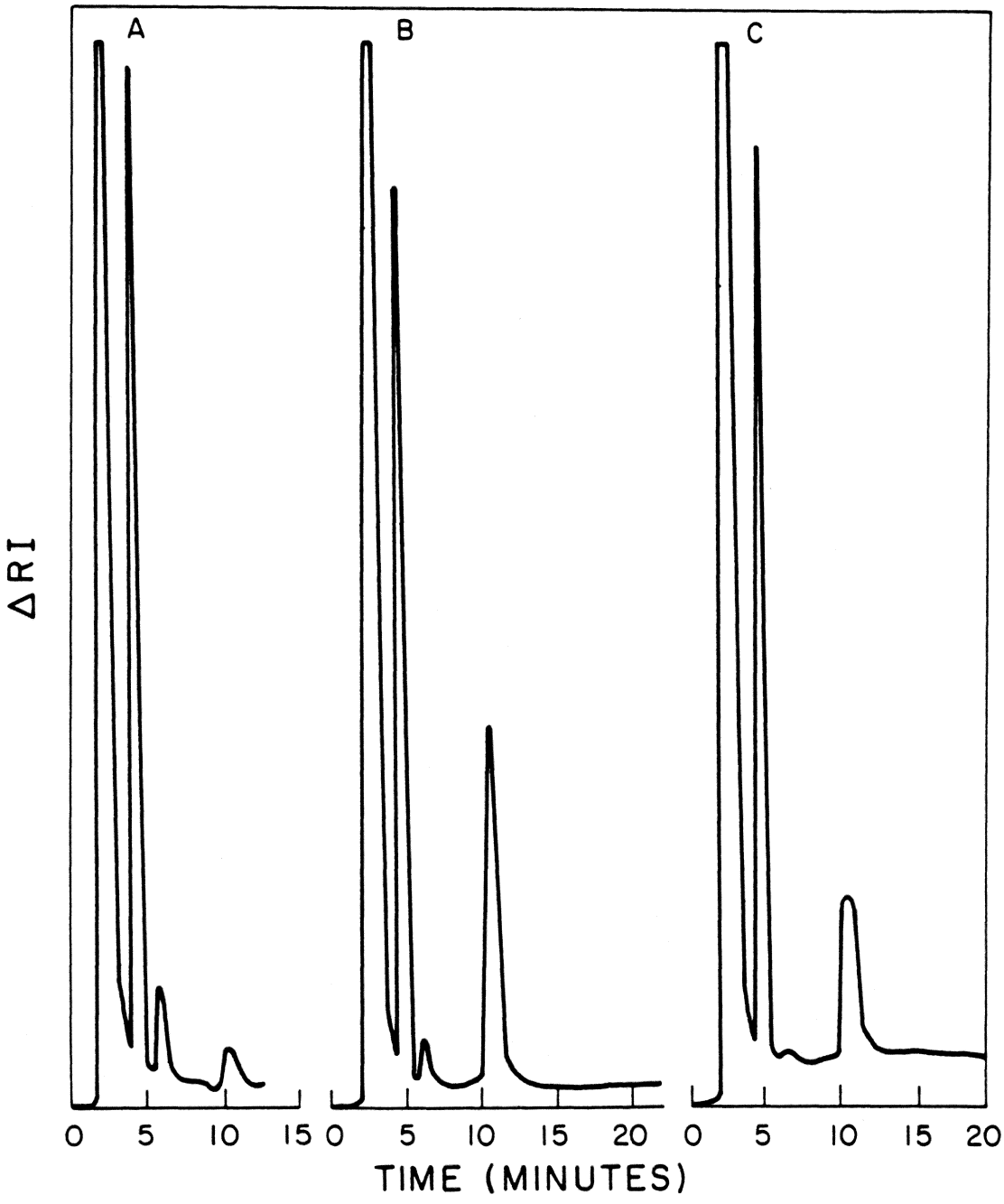
(D), and from the endo-1,4-β-D-glucanase by the absence of substantial amounts of glucose from its Avicel hydrolysis products. Both cellobiohydrolase I (D) and the endoglucanase produce significant amounts of glucose, as well as cellobiose.

One of the most promising methods of increasing efficiency of utilization of the cellulase system involves a simultaneous saccharification-fermentation process, during which the glucose produced from cellulose by the saccharifying enzymes (cellulases) is simultaneously fermented to ethanol. This process has been studied by Blotkamp et al (56). Pemberton et al (57) observed ethanol inhibation of aryl-β-D-glucosidase activity in culture filtrates of T. reesei QM 9414. It was of interest to examine the effect of ethanol on the β-glucanases purified during this investigation. The results of this experiment, using phosphoric acid-swollen cellulose as the substrate, are shown in Table XI. Although cellobiohydrolase I (D) and the endoglucanase are inhibited to a similar extent by ethanol, a peculiar stimulation of cellobiohydrolase II is observed under the same conditions. After prolonged incubation with each enzyme, the products of swollen-cellulose degradation in the presence of ethanol were separated by High Performance Liquid Chromatography, and are shown in Fig. 30. The product arrays arising from the action of cellobiohydrolases I (D) or II on swollen cellulose, appear identical in the absence and presence of ethanol (cf Fig. 28). A more unusual product array is observed with the endoglucanase which, in addition to the expected products, glucose, cellobiose, and some cellotriose, forms a previously unobserved

FIGURE 29

Separation by high performance liquid chromatography of products formed from the reaction of the purified glucanases with microcrystalline cellulose (Avicel).

The reaction mixture consisted of a 1% suspension of Avicel containing 0.25% α-methyl-D-glucoside as an internal standard. Quantities of enzymes added were 156 μg of endo-1,4-β-D-glucanase, 414 μg of cellobiohydrolase I (D), and 156 μg of cellobiohydrolase II. The total volume of the assay was 0.5 ml. Panels A, B and C show the products of hydrolysis, after 30 h of reaction, of the endo-1,4-β-D-glucanase (at 2.2% degradation of the substrate), cellobiohydrolase I (D) (at 11.1% degradation) and cellobiohydrolase II (at 5% degradation). Separation was accomplished with a 84:16, (w/w) acetonitrile: H_2O chromatographic solvent system at a flow rate of 1.5 ml/min. Retention times for α-methylglucoside, G_1 and G_2 were 5.0, 6.9 and 11.6 min.


A
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C
ΔRI
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10
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15
20
0
5
10
15
20
TIME (MINUTES)

TABLE XI

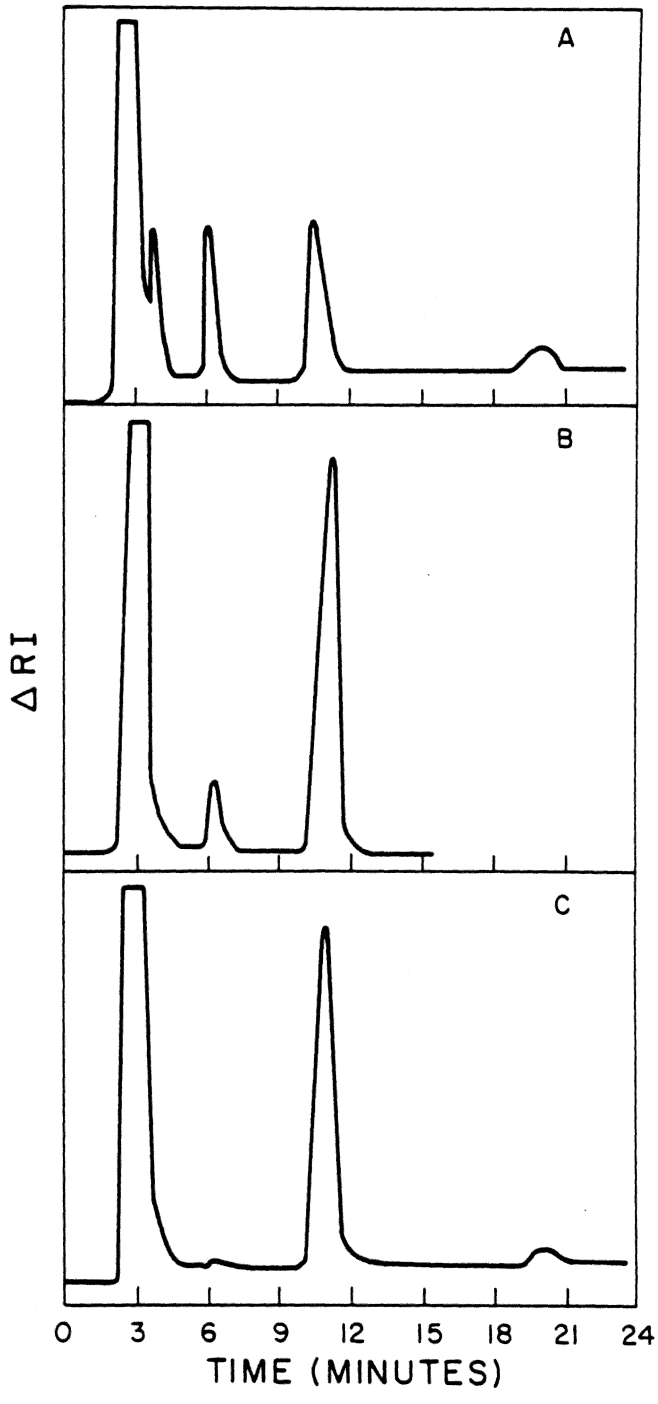
Effect of ethanol on swollen cellulose hydrolysis by glucanases

	Ethanol (wt %)		
	0	4	8
Cellobiohydrolase I	100	92.0	69.7
Cellobiohydrolase II	100	109	110.3
Endoglucanase	100	88.7	70

FIGURE 30

Separation by high performance liquid chromatography of products formed from the reaction of the purified glucanases with phosphoric acid-swollen cellulose (PSC) in the presence of ethanol.

The incubation mixture consisted of a 0.7% suspension of swollen cellulose which contained 8% (w/w) ethanol. The total incubation volume was 1.5 ml. Products are shown after 96 h of reaction with 30 µg of pure endoglucanase, (Panel A), 240 µg of CBH I (D) (Panel B), and 30 µg of CBH II (Panel C). Separations were accomplished with a 84:16, (w/w) acetonitrile water chromatographic solvent system, and a column flow rate of 1.5 ml/min. Retention times for G_1, G_2 and G_3 were 6.5, 11.0 and 19.9, min, respectively.


A
B
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ΔRI
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3
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12
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18
21
24
TIME (MINUTES)

product, which appears in Fig. 30 as a rapidly eluting peak, not quite resolved from the solvent peak. The identity of this compound was later confirmed as ethyl glucoside, probably formed under the conditions of this experiment via transfer of a glucosyl unit to ethanol. The ability of endoglucanases to carry out transglycosylation reactions has been reported previously by Okada (14) and by Shoemaker and Brown (9). It is probable that the observed inhibition of endoglucanase activity in the presence of ethanol is at least in part due to increased transglycosylation activity of this enzyme under these conditions.

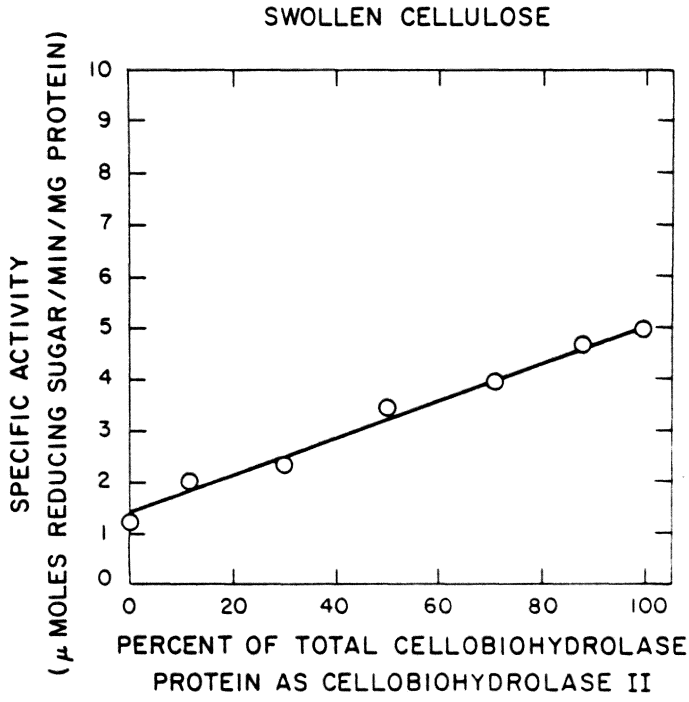
Role of each glucanase in the cellulase system - During this investigation, the assumption was made that <95% of the extracellular protein found in culture filtrates of T. reesei QM 9414 could be accounted for by the enzymes of the cellulase system. On the basis of protein recovery from the DEAE-Sephadex column, it was concluded that cellobiohydrolase I (D) constitutes (60 ± 5%) by weight of the total protein in the cellulase enzyme system. It was further assumed that all, or nearly all, of the endoglucanase activity in the extracellular culture filtrate was due to the enzyme reported here (information supporting the validity of this assumption is provided during discussion of the purification); comparison of the specific activity of the pure enzyme with that of the culture filtrate led to the conclusion that this endoglucanase constitutes approximately (15 ± 2%) by weight of the total protein in the cellulase mixture. The remaining (25 ± 5%) is principally cellobiohydrolase II, as indicated by protein recovery data and estimates of the amount of unrecovered cellobiohydrolase II. If the above estimates are correct, then recombination of the purified

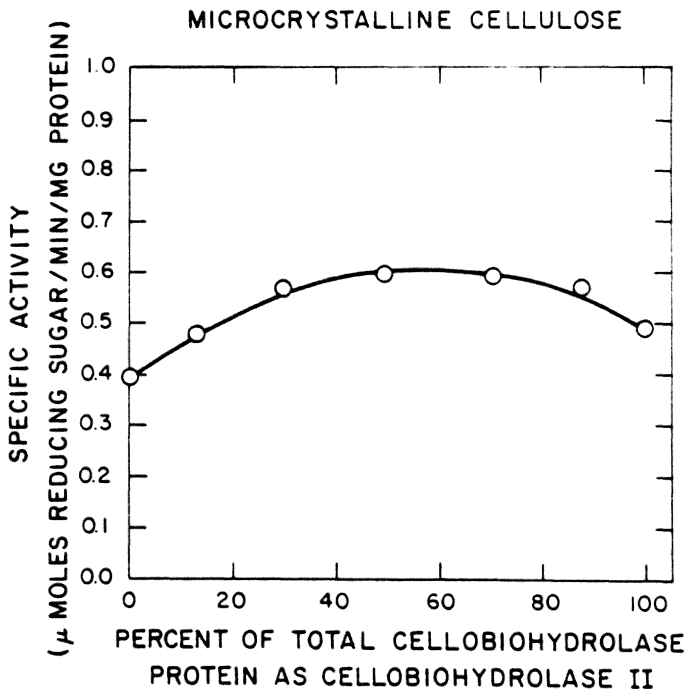
glucanases in their respective assumed proportions should result in an enzyme system that has activity on cellulose equal to that of the crude enzyme preparation.

To further assess the role of each glucanase in the cellulase system, the pure component glucanases were combined in various proportions, and the specific activity of each resulting mixture was determined using both microcrystalline cellulose (Avicel) and phosphoric acid-swollen cellulose. In combinations of the pure components, endoglucanase concentration was kept constant, at 15% by weight of the total glucanase protein. The remaining 85% of the glucanase protein was provided as cellobiohydrolase protein, and comprised varying proportions of cellobiohydrolases I (D) and II. To provide a complete cellulase system, purified β-glucosidase from T. viride was added as 0.6% by weight of the total protein; this concentration resulted in a specific aryl-β-glucosidase activity for the recombination mixtures of 0.25 units/mg, which is equal to that of the native enzyme preparation. The results of these experiments with microcrystalline or swollen cellulose are illustrated in Fig. 31. When swollen cellulose is used as the substrate, the specific activity of the "artificial" cellulase systems increases with cellobiohydrolase II concentration; this is not surprising, in view of the fact that cellobiohydrolase II exhibits a much higher activity on swollen cellulose than cellobiohydrolase I (D) (Table X). The mixture which contained the glucanases in the putatively correct proportions, 60:25:15 by weight of CBH I (D): CBH II:endoglucanase, exhibited an activity identical to that of the native system (as

FIGURE 31

Specific activity of the cellulase system reconstituted from purified glucanases as a function of cellobiohydrolase II concentration.


SWOLLEN CELLULOSE
SPECIFIC ACTIVITY
(μ MOLES REDUCING SUGAR/MIN/MG PROTEIN)
0
1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100
PERCENT OF TOTAL CELLOBIOHYDROLASE
PROTEIN AS CELLOBIOHYDROLASE II


MICROCRYSTALLINE CELLULOSE
SPECIFIC ACTIVITY
(μ MOLES REDUCING SUGAR/MIN/MG PROTEIN)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
20
40
60
80
100
PERCENT OF TOTAL CELLOBIOHYDROLASE
PROTEIN AS CELLOBIOHYDROLASE II

represented by the crude enzyme preparation) under these conditions. When all of the cellobiohydrolase protein in the mixture is present as cellobiohydrolase II, the observed specific rate on swollen cellulose is twice that of the native mixture (Fig. 31).

Results of a different nature are observed with microcrystalline cellulose as the substrate. In this case, increasing cellobiohydrolase II concentration does not result in a linear increase in the specific rate of reducing sugar production from this substrate. On the contrary, a decrease in specific activity of the system is observed at cellobiohydrolase II concentrations exceeding 70% of the total cellobiohydrolase protein, indicating the cellobiohydrolase I (D), although apparently acting more slowly (Table X) is essential and irreplaceable in the overall mechanism of cellulose degration. From Fig. 31 it appears that the system which would most effectively degrade crystalline cellulose should contain cellobiohydrolase I (D), cellobiohydrolase II and endoglucanase in the proportions of 42.5:42.5:15 (w/w). As previously, the combination which was intended to reproduce the native system, yielded an activity identical to that of the crude enzyme preparation. Thus marked synergism is observed among the three glucanases in the degradation of crystalline cellulose. The observed increase in the specific rate of degradation of this substrate by the complete system is greater than 4-fold when compared with that exhibited by any single glucanase (Table X).

Immunochemical Properties

To examine further relationships among the purified glucanases and to the enzymes purified previously (9,10) from culture filtrates of Trichoderma viride, antisera prepared to each purified glucanase were tested for their ability to crossreact with a variety of enzymes purified from the cellulase system of Trichoderma. Antiserum to β-glucosidase was prepared by using the enzyme purified from T. viride (2), since pure β-glucosidase from T. reesei QM 9414 was not available. Antiserum which had been prepared previously (4) to cellobiohydrolase form C was also used during these studies.

Antisera to cellobiohydrolases I (D) and II specifically crossreact with homologous antigens (Fig. 32), but with none of the other glucanases. Similar behavior was observed with the antiserum to the β-glucosidase from T. viride. When antiserum to the purified endo-1,4-β-D-glucanase was employed, a precipitin line was formed not only with homologous antigen but also with cellobiohydrolase II. The broad and possibly double line which is observed with cellobiohydrolase I (D) and the endoglucanase (Fig. 32) and their respective antisera cannot be easily interpreted; it is unlikely that this phenomenon is due to the presence of an additional crossreacting component in the antigen solutions, as these appeared homogeneous by a variety of methods (cf. p. 39). It should be noted that single precipitin lines have been obtained occasionally from these reactions by allowing immunodiffusion to take place in 0.05 M sodium acetate buffer pH 5.0, containing 3 mM sodium azide (an example of a single, though broad precipitin line formed between the T. reesei QM 9414

FIGURE 32

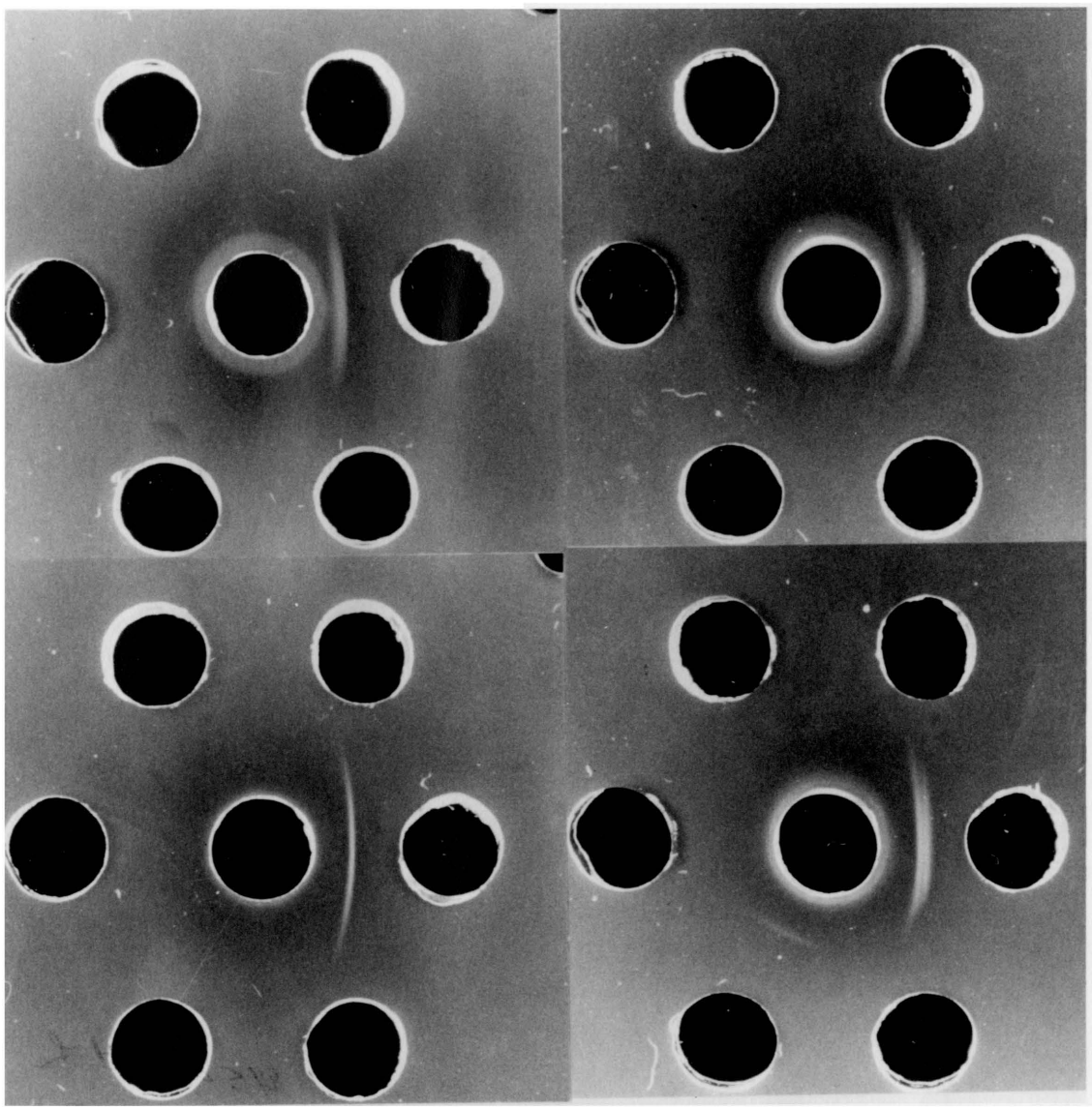
Immunological relationships among Trichoderma glucanases.

Upper left panel: Center well contains antiserum to cellobiohydrolase II. The peripheral wells contain (clockwise from marker well) 25 ml of distilled water; 5 µg of CBH II; 4 µg of endoglucanase (T. reesei QM 9414); 5 µg of CBH I (D); 4.2 µg of β-glucosidase (T. viride); and 25 ml of distilled water, respectively.

Upper right panel: Center well contains antiserum to cellobiohydrolase I (D). The peripheral wells contain (as above), 25 ml of distilled water; 5 µg of CBH I (D); 4 µg of endoglucanase (T. reesei QM 9414); 5 µg of CBH II; 4.2 µg of β-glucosidase (T. viride); and 25 ml of distilled water, respectively.

Lower left panel: Center well contains antiserum to β-glucosidase (T. viride). The peripheral wells contain (as above), 25 ml of distilled water; 4.2 g of β-glucosidase (T. viride); 4 µg of endoglucanase (T. reesei QM 9414); 5 µg of CBH I (D); 5 µg of CBH II; and 25 ml of distilled water, respectively.

Lower right panel: Center well contains antiserum to endoglucanase (T. reesei QM 9414). The peripheral wells contain (as above), 25 ml of distilled water; 4 µg of endoglucanase (T. reesei QM 9414); 5 µg of CBH I (D); 5 µg of CBH II; and 25 ml of distilled water, respectively.



endoglucanase and its antiserum is provided in Fig. 33). It is therefore likely that ionic strength of the medium influences diffusion and equilibria governing formation of antigen-antibody complexes and thereby the appearance of the precipitin lines.

The immunochemical relationship of enzymes isolated previously from commercial Trichoderma cellulase preparations to the T. reesei QM 9414 glucanases was examined (Fig. 33). The endoglucanases IV and II purified from T. viride crossreact weakly with antiserum to the endoglucanase from T. reesei QM 9414. This is consistent with the notion that endoglucanase IV is structurally and enzymically related to the T. reesei QM 9414 endoglucanase, and with the suggestion of Shoemaker and Brown (9) that endoglucanase II may have been derived from endoglucanase IV by proteolytic cleavage.

When antiserum prepared previously to cellohydrolase I form C was employed, all of the cellobiohydrolases from Trichoderma, with the exception of cellobiohydrolase II, exhibited crossreactivity. None of the forms of cellobiohydrolase I crossreacted to antiserum to cellobiohydrolase II. These results support the idea that cellobiohydrolase II constitutes a distinct isozyme, rather than a fifth form of cellobiohydrolase I.

To examine the relationship of the T. reesei QM 9414 β-glucosidase to the enzyme isolated from T. viride (2), both the homologous antigen and a 100-fold enriched preparation of the T. reesei enzyme were allowed to diffuse toward antiserum prepared to the T. viride β-glucosidase. The perfect hexagon of Fig. 34 indicates that these two β-glucosidases are very closely related.

FIGURE 33

Immunological relationships among T. reesei and T. viride glucanases.

Upper panel: The center well contains antiserum to purified endoglucanase from T. reesei QM 9414. The peripheral wells contain (clockwise from marker well) 4 µg of endoglucanase (T. reesei); 4 µg of endoglucanase II (T. viride; 4 µg of endoglucanase III (T. viride); 4 µg of endoglucanase IV (T. viride); 25 ml of distilled water; and 8 µg of endoglucanase (T. reesei).

Center panel: Center well contains antiserum to cellobiohydrolase I (C), purified from a T. viride cellulase preparation. The peripheral wells contain (as above), 20 µg of CBH II; 20 µg of CBH I (D) (T. reesei QM 9414); 20 µg of CBH I (A); 15 µg of CBH I (B); 20 µg of CBH I (D) (T. reesei QM9123); and 20 µg of CBH I (C).

Lower panel: Center well contains antiserum to cellobiohydrolase II. The peripheral wells contain (as above), 5 µg of CBH I (D) (T. reesei QM 9414); 5 µg of CBH II; 5 µg of CBH I (A); 5 µg of CBH I (B); and 5 µg of CBH I (C).

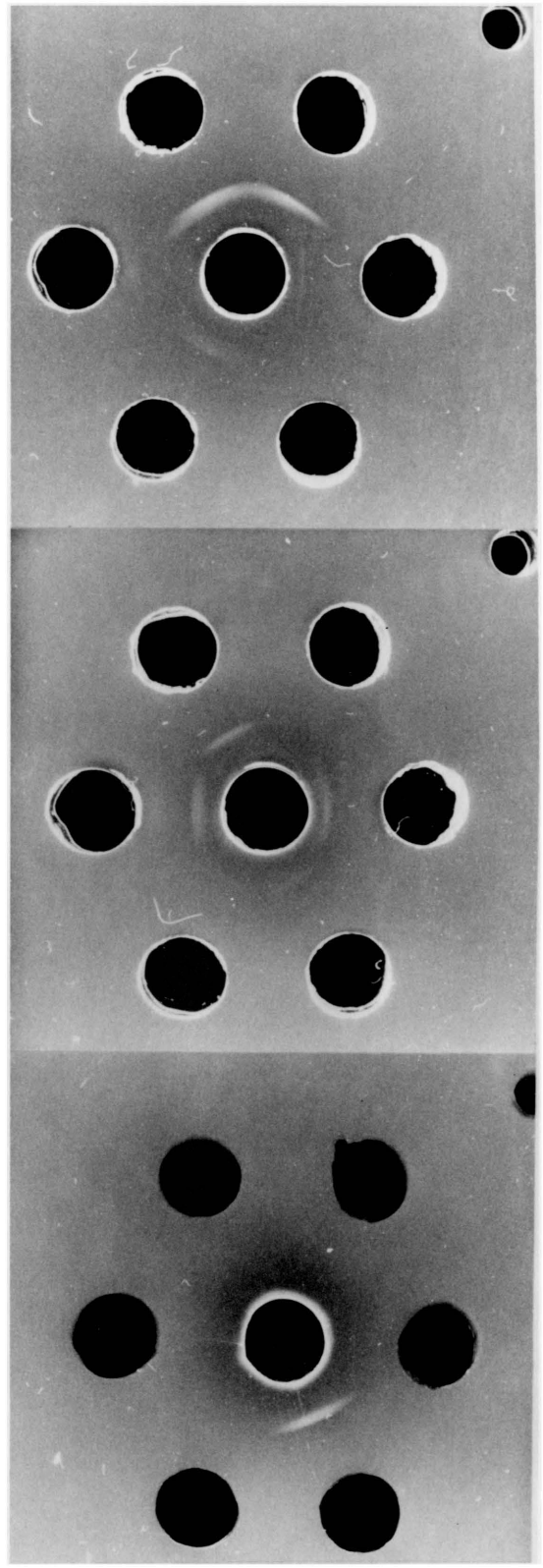
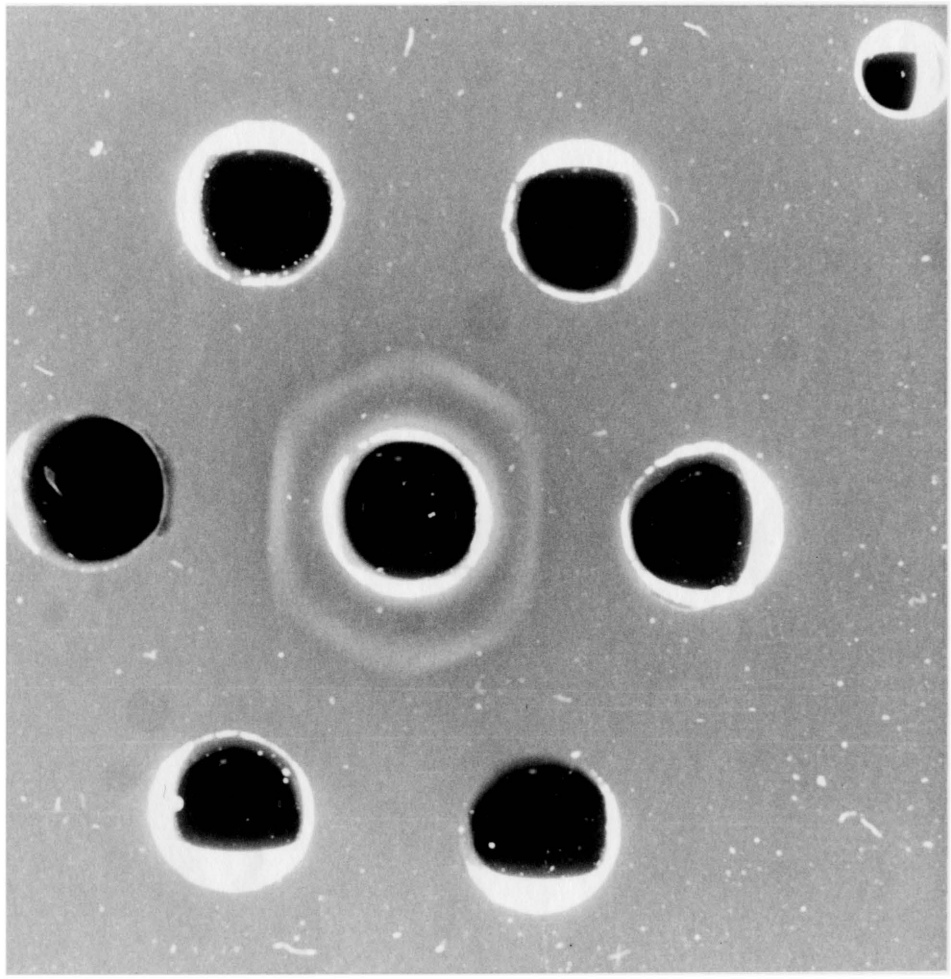


FIGURE 34

Immunological relationship between the β-glucosidases of T. viride and T. reesei.

The center well contains antiserum to the β-glucosidase purified from a commercial T. viride cellulase preparation. The peripheral wells contain, alternately, and clockwise from marker well, 4.25 µg of β-glucosidase (T. viride), and 11 µg of an enriched β-glucosidase preparation (T. reesei QM 9414).



SUMMARY

The major glycoenzyme components of the cellulase system produced by the fungus Trichoderma reesei QM 9414 have been purified to electrophoretic homogeneity by sequential anion and cation exchange chromatography and characterized with respect to a number of structural and enzymic properties. There are two cellobiohydrolases, named I (D) and II, respectively, and an endo-1,4-β-D-glucanase. These enzymes are elaborated by the organism under resting conditions in the presence of the soluble inducer sophorose, or during growth on cellulose. Cellobiohydrolase I (D), which is one of four differentially glycosylated forms of the same polypeptide is identical on the basis of its amino acid composition to the enzyme isolated previously by Gum and Brown (4) from the culture filtrate of Trichoderma reesei QM 9123. Cellobiohydrolase II constitutes a distinct isozyme, significantly different from cellobiohydrolase I (D). The endoglucanase exhibits a specific activity on carboxymethylcellulose of 116 and bears a structural and enzymic similarity to Endoglucanase IV, an enzyme isolated previously by Shoemaker and Brown (9,10) from a T. viride cellulase preparation. From the limited structural information provided by Häkansson et al (16) on an endoglucanase recently purified by these authors from the same organism, it appears that the endoglucanase of the present report is not the same enzyme. Furthermore, no "low molecular weight" endoglucanase was identified in our preparation, by contrast to Häkansson et al (15); the results of the present investigation indicate that there is only one endoglucanase in the T. reesei system.

Structurally, all three glucanases were high in acidic and hydroxylated amino acids and low in basic amino acids; they ranged in carbohydrate content from 5.0% (CBH I (D)) to 21.2% (CBH II); the predominant neutral sugar in all cases was mannose. Cellobiohydrolase II was distinct in that it contained twice the amount of alanine when compared to either of the other glucanases, and the lowest mannose:glucose ratio. It was the most alkali labile enzyme (probably due to its high content of O-glycosyl linkage sites) and exhibited a sharp optimum pH for activity at 4.9. Cellobiohydrolase I (D) and the endoglucanase had a broad pH range for optimum activity, ranging from 4.8 to 5.6 and 4.2 to 4.8, respectively.

Enzymic activity and specificity was tested using both cellooligosaccharide and polymeric substrates. Cellobiohydrolase II and the endoglucanase exhibited twofold (on Avicel) to tenfold (on oligosaccharides) higher activity than cellobiohydrolase I (D) on all substrates tested. Cellobiohydrolase I (D) was unique among the three glucanases in its ability to cleave cellotriose. The endoglucanase was the only enzyme of the three which exhibited significant transglycosylation activity. Cellobiohydrolase II was the enzyme with the most uncomplicated mode of action cleaving cellobiosyl units only from both oligosaccharide and polymeric substrates. The transglycosylation properties of the endoglucanase may be very important in promoting biosynthesis of these enzymes (29).

Immunochemical crossreactivity among these glucanases and other cellulase components purified from Trichoderma, was tested employing

appropriate antisera. Cellobiohydrolases I and II were mutually not crossreactive, justifying their designation as isozymes. Cellobiohydrolase II weakly crossreacts to antiserum prepared against the endoglucanase, which suggests the probability of common antigenic determinants between these two enzymes; this is not unexpected, as they both contain a substantial amount of mannose.

Purification results indicated that the relative proportions (w/w) of cellobiohydrolase I (D): cellobiohydrolase II: endoglucanase in the crude enzyme preparation containing the complete system are 60 $\pm$ 5: 25 $\pm$ 5: 15 $\pm$ 2, respectively. To confirm this prediction and to elucidate the role of each glucanase within the cellulase system, experiments were carried out during which the pure glucanases were combined in varying proportions, and their activity compared to that of the crude enzyme preparation. It was found that (a) the activity of the recombined mixtures in their assumed respective proportions reproduced that of the native system; (b) all three components are necessary for optimum activity on crystalline cellulose; (c) the most effective enzyme system would include, besides the endoglucanase, both cellobiohydrolases I (D) and II in a 50:50 (w/w) ratio.

The fact that it was possible to reproduce full activity of the mixture by recombining purified hydrolytic components renders the concept of a nonhydrolytic enzyme, a hydrogen bondase, very unlikely (59).

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PURIFICATION AND CHARACTERIZATION OF AN ENDO-1,4-β-D-GLUCANASE AND TWO EXO-1,4-β-D-GLUCANASES FROM THE CELLULASE SYSTEM OF TRICHODERMA REESEI

by

Mikelina Gritzali

(ABSTRACT)

An endo-1,4-β-D-glucanase (E.C. 3.2.1.4) and two exo-1,4-β-D-glucanases (exo-cellobiohydrolases I (D) and II, E.C. 3.2.1.91) have been purified to electrophoretic homogeneity from the extracellular culture filtrate of the imperfect fungus Trichoderma reesei QM 9414 grown on microcrystalline cellulose (Avicel). These three glycoprotein enzymes are the principal components of the cellulase system and constitute <95% of the extracellular protein produced by this organism when grown on cellulose or when incubated in the presence of sophorose (O-β-D-glucopyranosyl (1→2) α-D-glucopyranose). The glucanases have been characterized with respect to a number of structural and enzymic properties. Neutral carbohydrate, predominantly mannose with some glucose, contributes 5, 21.2 and 14.1 per cent of the weight of cellobiohydrolase I (D), cellobiohydrolase II and the endoglucanase, respectively. All three enzymes have a high proportion of acidic and hydroxylated amino acids, but much less of basic amino acids. Sedimentation equilibrium studies yielded the following molecular weights for the glucanases: cellobiohydrolase I (D) 53,220±1479; cellobiohydrolase II 54682±2683; endoglucanase 45,215±1483.

Cellobiohydrolase I (D) is the most acidic (pH_I <3.8) of the

three enzymes, whereas the endoglucanase and cellobiohydrolase II have isoelectric points of 4.7 and 5.6, respectively. All three lose activity when exposed to alkaline conditions, and the extent of alkali lability is directly related to carbohydrate content. This instability, as well as the low amino sugar content of the enzymes, indicates that the carbohydrate may be linked to the polypeptide via O-glycosyl bonds to serine or threonine residues. Among the three enzymes, the endoglucanase is most resistant to thermal inactivation retaining 70% of its initial activity on swollen cellulose after a 20 min preincubation at 70°. The optimum pH for activity is ca.4.9 for the endoglucanase and cellobiohydrolase II, whereas cellobiohydrolase I (D) has a broader pH range for activity, between pH 5.2 to 5.6.

The mechanism of action of each glucanase was investigated by quantitative high performance liquid chromatographic analysis of the products arising from various cellulosic substrates as a result of enzymic action. Cellobiohydrolases I (D) and II produce predominantly (>90%) cellobiose from either oligosaccharides or cellulose. Cellobiohydrolase I (D) is unique among the three glucanases in its ability to cleave cellotriose. The endoglucanase reduces the viscosity of carboxymethylcellulose solutions with a specific activity of 116 (expressed as the change in specific fluidity/min/mg protein). This enzyme also possesses significant transglycosylation activity.

When the three glucanases are combined in the proportion 60:25:15, cellobiohydrolase I (D): cellobiohydrolase II: endoglucanase (w/w) respectively, the resulting mixture exhibits activity identical

to that of the crude enzyme preparation, with either swollen or crystalline cellulose as the substrate. All three glucanases are essential for the degradation of crystalline cellulose.

Cellobiohydrolase I (D) crossreacts immunologically with cellobiohydrolases I forms A, B and C, but not with cellobiohydrolase II, justifying the designation of CBH I and II as isozymes rather than forms of the same enzyme. An enriched β-glucosidase preparation from the culture filtrate of Trichoderma reesei QM 9414 formed a single precipitin line when allowed to react with antiserum to the β-glucosidase previously purified from a commercial T. viride cellulase preparation. The absence of any spurs of nonidentity indicates that these two enzymes are structurally very similar. Lack of crossreactivity is observed between cellobiohydrolase I (D) and the endoglucanase purified during this investigation, but the latter crossreacts weakly with antiserum to cellobiohydrolase II.