PURIFICATION AND PROPERTIES OF A β-1,4-GLUCAN 4-GLUCANOHYDROLASE FROM <u>TRICHODERMA</u> <u>VIRIDE</u>

by

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I. INTRODUCTION

"Cellulose and its related polysaccharides", according to King (1), "are particularly conspicuous in the chemical cycling of carbon on earth from a quantitative standpoint, because through them passes a greater weight of carbon per unit time than through any other closely related family of compounds. As with any other natural event, control of cellulose degradation would be suspected of being a very practical accomplishment, but to date this has not been achieved".

Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of cellulose, but the process has been studied only cursorily until the last decade. Unlike a variety of chemical modifications of cellulose, the enzymatic process has only been adapted to useful industrial purposes during the past few years primarily in Japan.

In the enzymatic degradation of cellulose the nomenclature has been confusing. In 1950, Reese et al. (2) created the terms "C_1" for the enzymes responsible for the cleavage of the crystalline portion of native cellulose and "C_x" for the enzymes responsible for the hydrolysis of the amorphous portion of native cellulose and of some modified celluloses such as CMC. In 1961, the Commission on Enzymes of the International Union of Biochemistry (3) classified cellulase among the glycoside hydrolases, gave the systematic name β-1,4-glucan 4-glucanohydrolase, and assigned a code number of 3.2.1.4. The first part of the systematic name indicates substrate, in this case β-1,4-polymers of glucose.

The second part of the name designates the reaction of that substrate being catalyzed by the enzyme. Whitaker (4) however, pointed out that the systematic name did not confine the hydrolysis to specific linkage at the ends of chains, and he suggested that the systematic name was only suitable for the enzymes hydrolyzing cellulose in a random fashion with oligoglucosides as end products. Whitaker also suggested that "β-1,4-glucan 4-glucohydrolases" and "β-1,4-glucan 4-cellobiohydrolases" might be designated for the enzymes splitting off glucose or cellobiose units exclusively. If an enzyme degraded not only oligoglucosides but also highly polymeric β-glucans in this manner, he predicted that the term "cellulase" would probably follow the course of the term "amylase" and aquire prefixes.

Terms used throughout this thesis are purified "β-1,4-glucan 4-glucanohydrolase" or "3.2.1.4." for the purified enzyme isolated from Trichoderma viride which is able to hydrolyze amorphous cellulose and CMC in a rather random fashion with oligoglucosides as principal end products. This corresponds to the terms C_x-cellulase and CMCase. The name "hydrocellulase" is used for the enzyme capable of decomposing hydrocellulose or crystalline portions of native cellulose corresponding to the term "C_1" given by Reese et al. (2). The name "aryl-β-D-glucosidase" or "β-D-1,4-exoglucanase" is used for the enzyme which can break down aryl-β-D-glucosides. Such enzymes may or may not hydrolyze cellulodextrins of DP less than five completely to glucose.

With regard to the nature of cellulose decomposition by enzymes, two concepts dominate the theoretical field, namely attack by a single enzyme and attack by a multiple enzyme system. The concept of a single enzyme capable of hydrolyzing native cellulose to glucose was developed by Whitaker (5) in 1953 after obtaining electrophoretic and ultracentrifugal data on a purified cellulase from Myrothecium verrucaria. The concept of the multiple nature of cellulase systems which was put forth by Reese, Siu, and Levinson (2) in 1950 has been supported by partial evidence obtained by many workers (6, 7, 8, 9) and by direct proof obtained from Aspergillus niger by Li and King (10) and King and Smibert (11).

Unlike the cellulase system of A. niger, the very active cellulase derived from T. viride possesses the complete system for decomposing native cellulose to glucose. In addition, it is produced on an industrial scale as described by Toyama (12). Therefore, the cellulase system from T. viride has been chosen as the subject in this study in attempting to isolate a highly purified enzyme by means of various fractionation techniques and to determine its chemical and physical properties as well as its mode of action with regard to site of attack. The role of this purified enzyme in relation to other cellulases isolated from T. viride can then be understood and differentiated with the cooperation of other workers from this laboratory.

II. THE REVIEW OF LITERATURE

Trichoderma viride is a cellulolytic microorganism classified among the fungi imperfecti. Prior to 1950, studies on the cellulase system of this fungus attracted little attention and little or no purification work had been done. In 1952, Reese and Levinson (13) did a comparative study on the breakdown of cellulose by a great many microorganisms and pointed out that T. viride was a very potent cellulolytic organism, producing both hydrocellulase (C_1) and C_x-cellulase, which could reduce tensile strength of cotton duck. They considered that β-glucosidase was absent extracellularly when T. viride as well as most other organisms attacked cellulose. Two months later, Reese, Gilligan, and Norkrans (14) examined the effect of cellobiose on the enzymatic hydrolysis of cellulose and its derivatives and found that cellobiose might be an end product inhibitor for both hydrocellulase and C_x activities in culture filtrates of T. viride. Glucose, however, did not inhibit the hydrolysis. C_x activity was determined viscosimetrically in this study. In the same year, Toyama (15) started to work on cellulose decomposition by fungi. He tested 24 species of microorganisms and found that T. viride had the strongest cellulose decomposing power when it was grown on pieces of filter paper in synthetic media.

Concerning the cultural conditions for cellulose decomposition by this organism, Ishimam and Toyama (16) have reported that the optimum conditions included pH 6.0 and 25°C. Also, 0.5 - 1.0% ammonium sulfate appeared to be best among the many nitrogen sources used. Toyama (17) in 1953 indicated that the cellulolytic enzyme activity of culture filtrates was inactivated at 55°C. for 10 minutes, while optimum pH and optimum temperature were 5.0 and 40°C. respectively. The weight of hydrolyzed cellulose was found to agree closely with the accumulation of glucose which appeared to be the main product of hydrolysis of cellulose. Studying the influence of different cultural conditions such as shaken cultures and stationary cultures in different volumes on fungal cellulase production, Norkrans (18) has found that only stationary cultures with a shallow liquid layer provided with an adequate oxygen supply were cellulolytically active. This applied to culture filtrates of T. viride as well as to those of the other fungi tested.

Cellulase is an adaptive enzyme in T. viride as well as in many other fungi as indicated by Reese and Levinson (13) in 1952. In 1953, Toyama (19) reported that cellulolytic enzyme was only liberated in synthetic media with cellulose, expecially cottons, whereas other sources of carbon such as glucose and starch appeared to have no inducing effect. He also noticed that cellulase activity was stronger in surface cultures than in submerged ones. In 1957, Mandels and Reese (20) reinvestigated the induction of cellulase in T. viride as influenced by carbon sources and metals. They found that cellulase can be produced on cellulose, lactose, glucose, and cellobiose, but not on a wide variety of other substances. Since a rather high initial glucose concentration was required for the production of cellulase and the cellulase activity did not appear until the glucose had been removed from the medium, they thought that glucose might not be the true inducer of cellulase rather being metabolized to an inducer, possibly a β-glucoside. Calcium and certain trace elements were required for cellulase production, although the growth of this fungus (except on pure cellulose) was not affected by their addition to the medium. The trace element requirement could be met by cobalt alone, but best cellulase production occurred if iron or manganese, and zinc or cobalt were also added. The level of cellulase activity was closely correlated with the amount of protein in the culture filtrate. In the following year, Toyama (21, 22) obtained the best results in inducing the cellulolytic enzymes from *Trichoderma* spp. by setting up a liquid medium containing inorganic salts and cotton. The other carbon sources, such as regenerated cellulose, filter paper, and sodium carboxymethylcellulose appeared to be weak inducers. Of various strains which secrete cellulase, carboxymethylcellulase (CMCase), amylase, maltase, protease and saccharase, only one strain (*T. viride* type) was found to be a weak producer of cellobiase. Furthermore, Toyama found both cellulase and CMCase produced in this synthetic medium were thermostable. Heat treatment of the culture filtrate at 70°C. for ten minutes followed by precipitation with acetone resulted in an enzyme preparation which had the activities of cellulases and CMCase exclusively.

It was generally believed that to serve as an inducer, a substance must reach the site of enzyme production. The relatively poor inducing ability of the soluble cellulose derivatives, which offer much better contact than does insoluble cellulose, however, led Mandels and Reese to think that contact with the outer cell surface was not enough. They introduced an idea about the natural inducers of cellulase being soluble products of cellulose hydrolysis. Since cellobiose is a soluble product commonly found during enzymatic hydrolysis of cellulose, their efforts had been directed to it. In 1960, they (23) demonstrated that cellobiose did act as an inducer of cellulase in many fungi including T. viride. Cellulase production on cellobiose, however, was much less than that on cellulose. According to them, the reason for this was mainly due to the inhibitory and inactivating factors produced during rapid growth on soluble sugar. When these were overcome as by slowing down the growth rate, the yields of cellulase on cellobiose could be as great as those on cellulose. They believed that cellobiose and related oligosaccharides were the true inducers of cellulase in this fungal culture. Cellulose was an inducer only by virtue of its being hydrolyzed to these by cellulase. Other active carbohydrates such as lactose, cellobiose octaacetate, and salicin functioned as inducers probably owing to their similarity to cellobiose. In 1962, Mandels, Parrish, and Reese (24) further found that sophorose (2-O-β-D-glucopyranosyl-D-glucose), an impurity in glucose, was responsible for C_x-cellulase induction in T. viride. It appeared to be an inducer of markedly high activity and specificity, since sophorose was 2,500 times as active as cellobiose and was specific for T. viride. Modifications of sophorose, such as reduction or glucoside formation, destroyed its inducing ability. This result might explain why glucose showed inducing ability as reported earlier.

Work concerning the purification of cellulase from T. viride did not actually appear until 1954. During that year, Gilligan and Reese (7) obtained the first evidence in support of the concept of the multiple nature of cellulase systems which had been put forth by Reese, Siu, and Levinson (2) in 1950. By means of various fractionation techniques, such as paper and column chromatography, several cellulolytic components were partially separated from the extracellular filtrates of _T. viride_ and _Myrothecium verrucaria_. These components differed in their relative activities on native cotton, swollen cellulose, and CMC. A synergistic effect was observed when these components were recombined.

In 1955, Toyama (25, 26, 27) tried to isolate a purified enzyme from extracts of wheat bran-sawdust cultures of _T. viride_ by means of precipitations with lead acetate, ammonium sulfate, rivanol, ethanol, and acetone applied in that order. In spite of the above purification procedures, the cellulase preparation still possessed the activities of CMCase, cellobiase, and amylase in addition to cellulase. In the following year, Toyama (28) attempted further purification on cellulase of this fungus by means of paper chromatography but extensive purification was still not possible. Cellulase activity lay at the bottom and CMCase extended from the bottom to the center of his chromatograms, whereas amylase spread over all the paper. In the meantime, Toyama (29) tried to distinguish cellulase, CMCase, cellobiase and amylase from one another by examing the inhibitory action of various metallic salts on them. Cellulase and CMCase gave no discrimination between their activities, but Mn^{++} promoted reducing sugar formation from cuprammonium rayon, and filter paper a great deal. The limiting temperatures of thermostability over 10 minutes were 60°C. for cellulase, 70°C. for CMCase, 80°C. for cellobiase and amylase. The optimum pH's for CMCase, cellobiase, and amylase were 4.0 to 5.2, 3.0 to 4.5, and

4.5 to 5.5 respectively, while the optimum pH for cellulase varied with the substrate being decomposed. Approximately pH 4.0 was optimum for cuprammonium rayon, and 4.0 to 6.0 for filter paper.

By employing ammonium sulfate and ethanol precipitation from surface culture filtrates of T. viride, Toyama (30) again obtained in 1959 an enzyme preparation which possessed both cellulase and xylanase with little cellobiase. A thermal denaturation study at 70°C. for ten minutes, indicated that cellulase and xylanase had more or less the same stability. This led Toyama to think that the hydrolysis of cellulose and xylan might be carried out by the same enzyme. By means of paper chromatography, he also established that hydrolysis of filter paper, rayon, and cotton yield mostly cellobiose and a trace of glucose. Hydrolysis of CMC yielded glucose, cellobiose, and cellotriose, while xylose, xylobiose, and a mixture of xylooligosaccharides were the hydrolytic products of xylan. He interpreted these results to reflect a random attack by these enzymes. Later, Toyama (31, 12) tried to differentiate cellulase from xylanase by means of ethanol fractionation and storage at 40°C. and pH 2.4. Cellulase was partially separated from xylanase, indicating that these two enzymes were likely to be different.

Recently, Toyama and Shibata (32) demonstrated the difference between cellulase and CMCase. They employed 80% saturated ammonium sulfate precipitated enzyme preparations from extracts of wheat bran-sawdust cultures and filtrates of the surface cultures grown on synthetic medium containing absorbant cotton. When both enzyme preparations were subjected to zone electrophoresis using starch gel as supporting medium in veronal acetic acid buffer at pH 8.5 and at a μ-value of 0.045, only one peak was detected. On the other hand, partial separation of the two enzymes was achieved by filter paper electrophoresis under the same conditions. One peak which stayed at the origin attacked filter paper more rapidly than CMC, whereas the second peak which moved toward the cathode, hydrolyzed CMC faster than filter paper. Both peaks possessed CMCase and cellulase but differed in their relative activities, therefore, Toyama suspected that there were at least two kinds of cellulase produced by _T. viride_.

Prior to the present study, attempts to isolate highly purified hydrocellulase, C_x-cellulase, or β-glucosidase from _T. viride_ had not been successful.

III. EXPERIMENTAL PROCEDURES

Materials

The crude enzyme, obtained from Meiji Seika Kaisha, Ltd., Tokyo, Japan, was prepared as follows. Wheat bran-sawdust tray cultures of *Trichoderma viride* were extracted with water and then precipitated with ammonium sulfate. After that the precipitated enzyme was redissolved, desalted with ion exchange resin, and precipitated with isopropyl alcohol. The powder-form of the enzyme was made by spray drying (33). This material is referred to hereafter as "crude enzyme".

Substrates used included carboxymethylcellulose (CMC), para-nitrophenyl-β-D-glucoside, water soluble cellulodextrins, amorphous cellulose, and hydrocellulose.

CMC-70-L (Hercules Powder Co., Wilmington, Del.) with a degree of polymerization (DP) of 125 and a degree of substitution (DS) of 0.88 was used as substrate in reducing sugar determinations, and CMC-70-H with a DP of 200 and a DS of 0.82 was used in the viscosimetric determinations.

P-nitrophenyl-β-D-glucoside (California Biochemical Corporation, Los Angeles, Calif.) was employed for the assay of the aryl-β-D-glucosidase activity.

Water soluble cellulodextrins, namely cellotriose through cellohexaose (Abbreviated as G_3, G_4, G_5, and G_6) were obtained chromatographically on a charcoal-celite-1% stearic acid according to the procedures of Miller *et al*. (34). Cellobiose (G_2) was obtained from Eastman Organic Chemicals, Rochester, N. Y.

The reduced cellulodextrins (Abbreviated as G_2H - G_6H) were prepared by reducing the corresponding cellulodextrins with sodium borohydride in aqueous solution by the procedures of Storvick, Cole, and King (35) and of Cole and King (36).

Amorphous cellulose, used for assaying β-1,4-glucan 4-glucanohydrolase or C_x-cellulase activity, was prepared from Avicel (A microcrystalline cellulose manufactured by American Viscose Corporation, Marcus Hook, Penn.) as follows. 15 g of Avicel were made to a slurry with 100 ml of concentrated HCl, added to 400 ml of supercooled (-20°C.) concentrated HCl, mixed well, and then filtered through glass wool into about 3 liters of distilled water. After washing out all Cl^- (Negative test with silver nitrate) the material was blended in the Waring blendor for 5 minutes.

The hydrocellulose employed for the hydrocellulase or C_1 assay was prepared from cotton linters (Hercules Powder Co., Hopewell, Va.) in the following way (37). Cotton linters were packed into a flask containing 270 ml of HCl and diluted to 370 ml so all the free liquid was taken up. After being kept at room temperature for 24 hours with occasional agitation, the slurry was poured into a large volume of water, washed several times by decantation, and then neutralized with sodium bicarbonate. Exhaustive dialysis against water was continued until a negative test for the presence of Cl^- was received. The material was then agitated briefly in a Waring blendor, adjusted to about 2 mg of hydrocellulose per ml, and stored under refrigeration as a stock suspension.

For column chromatography, Avicel used for the separation of C_x-cellulase from hydrocellulase, was prepared by suspending the material in water, and then decanting the supernatant after most of the material had been settled in order to remove the very fine particles. After repeating this process several times a suspension was made with 0.05 M, pH 4.8 sodium citrate buffer.

Alkali swollen cellulose was prepared as described by Hash and King (38) by soaking Whatman No. 1 ashless chromatographic powder (Standard grade, Fisher 5-720 W & R Balston, Ltd., England) in 35% NaOH (6 ml per g of powder) for about 30 minutes until the suspension turned an orange-red color. It was then filtered and washed thoroughly with water till the filtrate became neutral. After the removal of water, a suspension was made in 0.05 M, pH 5.0 potassium phosphate buffer.

Sephadex G-25 and G-200 (Pharmacia Inc., Uppsala, Sweden) were used for dialysis of enzymes.

A charcoal-celite column coated with stearic acid for quantitative analysis of oligosaccharide polymer mixtures was prepared in the following manner. 400 g of charcoal (Darco G-60) and the same amount of celite 545 were mixed with 3.2 liters of 2.5% stearic acid in absolute ethanol for 30 minutes with continuous stirring. The slurry was filtered to dryness, resuspended in 3.2 liters of 50% ethanol saturated with stearic acid and filtered again. Finally, the column packing was resuspended in two volumes of 50% ethanol saturated with stearic acid and stored tightly stoppered.

Methods

Two independent determinations for protein were used in most of the purification studies; the colorimetric procedure of Lowry et al. (39), and the spectrophotometric procedure of Kalckar and Shafran (40). Since the dry weight of the purified β-1,4-glucan 4-glucanohydrolase or C_x-cellulase was relatively close to the protein value obtained from the Folin-Lowry method, data concerning purification were based on protein values calculated from the colorimetric determination.

For analysis of the amino acid composition of the purified enzyme, the approximately 4 mg of the lyophilized preparation was hydrolyzed in 5.0 ml of 6 N HCl by flushing with nitrogen and autoclaving in a sealed tube at 121°C. for 18 hours. After this, the hydrolyzate was dried in vaccuo, redissolved in 0.5 ml of H_2O and redried. After repeating this for 2 or 3 times, the hydrolyzate was dissolved in 2.5 ml of pH 2.2 filling buffer (41). One ml portions of this solution were then used for analysis in a Beckman-Spinco amino acid analyzer as described by Spackman et al. (41). An internal nor-leucine standard was used routinely.

Three assays for β-1,4-glucan 4-glucanohydrolase were used; a reducing sugar assay, a viscosimetric assay, and a turbidometric assay.

The reducing sugar assay was carried out by the procedure of Hash and King (38). One ml of enzyme solution mixed with 5 ml of 3.33% CMC-70-L in 0.05M, pH 5.0 acetate buffer was incubated at 40°C. for one hour. The enzyme action was then terminated by mixing 1 ml of this reaction system with 2.0 ml of the Somogyi copper reagent (42). On addition of 2.0 ml of Nelson's arsenomolybadate reagent (43) the unhydrolyzed substrate precipitates from solution. The supernatant was diluted to 10 ml and an aliquat of the supernatant solution was used for determination of optical density at 505 mμ. One unit of enzyme has been defined as the amount of enzyme liberating reducing sugars equivalent to 10 μg of glucose per ml of reaction system in 1 hour.

For the viscosimetric assay, 0.1 ml of enzyme solution was added to 3.9 ml of 0.2% CMC-70-H in 0.05 M potassium phosphate buffer, pH 5.0 in an Ostwald-type viscosimeter. The initial drain time and that after incubation at 40°C. for 1 hour were recorded. Specific fluidities were calculated as described by Thomas (44). Details of the assay were described by King (45).

The turbidometric assay was performed as follows: to 3.0 ml of 0.05 M sodium citrate buffer at pH 4.0 were added 4.4 mg of the very fine suspension of amorphous cellulose described earlier. Enzyme and water were added to make a final volume of 6.0 ml and the initial turbidity was read in a Klett-Summerson colorimeter using red filter. Control tubes containing no enzyme were included in each assay series. After incubation at 40°C. for a time which varied according to the activity of the sample, the amorphous cellulose was resuspended, and the turbidity was read again. The change in turbidity was interpreted as a measurement of relative β-1,4-glucan 4-glucanohydrolase activity, but no attempt to develop a rigidly quantitative assay was made.

Hydrocellulase assays were also conducted turbidometrically using a highly crystalline cellulose as substrate under the conditions described by Li, Flora, and King (46).

The aryl-β-D-glucosidase activity was determined by the method of Hash and King (47). To 2 ml of substrate (1.42 gm of p-nitrophenyl-β-Dglucopyranoside in 1 liter of 0.05 M acetate buffer, pH 5.35. The substrate concentration was 0.005 M), was added 1 ml of enzyme solution, and the tubes were incubated at 40°C. for one hour. The enzyme activity was then stopped by the addition of 5 ml of 25% K_2HPO_4. The concentration of p-nitrophenol liberated from the β-D-glucoside by the enzyme was determined from the optical density at 400 mμ. One unit of aryl-β-D-glucosidase has been defined as the amount of enzyme yielding 0.01 μM of p-nitrophenol per ml of reaction system in one hour.

Glucose produced from hydrolysis of cellulose and its derivatives was determined enzymatically using a modification of the procedure of Saifer and Gerstenfeld (48). One ml of reaction mixture was incubated with 2 ml of notatin reagent at 40°C. for 20 minutes, then the color was stabilized by the addition of 2 ml of 0.5 M HCl, and the optical density was read at 420 mμ against an enzyme blank. Glucose standards of up to 100 μg were analyzed simultaneously.

For the study of the mode of action of the purified enzyme on reduced cellulodextrins, the products were determined quantitatively by the procedures of Cole and King (36), modified from Miller (49). In this analysis the charcoal-celite column coated with stearic acid adsorbs oligosaccharides quantitatively from water and the sugars can then be eluted in increasing order of DP by selective desorption with gradually increased concentrations of ethanol in water. The fractions were dried in an oven at 105° - 110°C. The amount of sugar in each fraction was determined by the phenol-sulfuric acid procedure described by Timell (50). To each dried fraction was added 3 ml of 1% phenol in water and 5.0 ml of concentrated sulfuric acid with thorough mixing. After standing for 10 minutes at room temperature, the tubes were cooled in water bath and the optical density was measured at 480 mμ. Glucose standards ranging from 10 to 200 μg were included with each series of unknowns.

IV. RESULTS

Purification

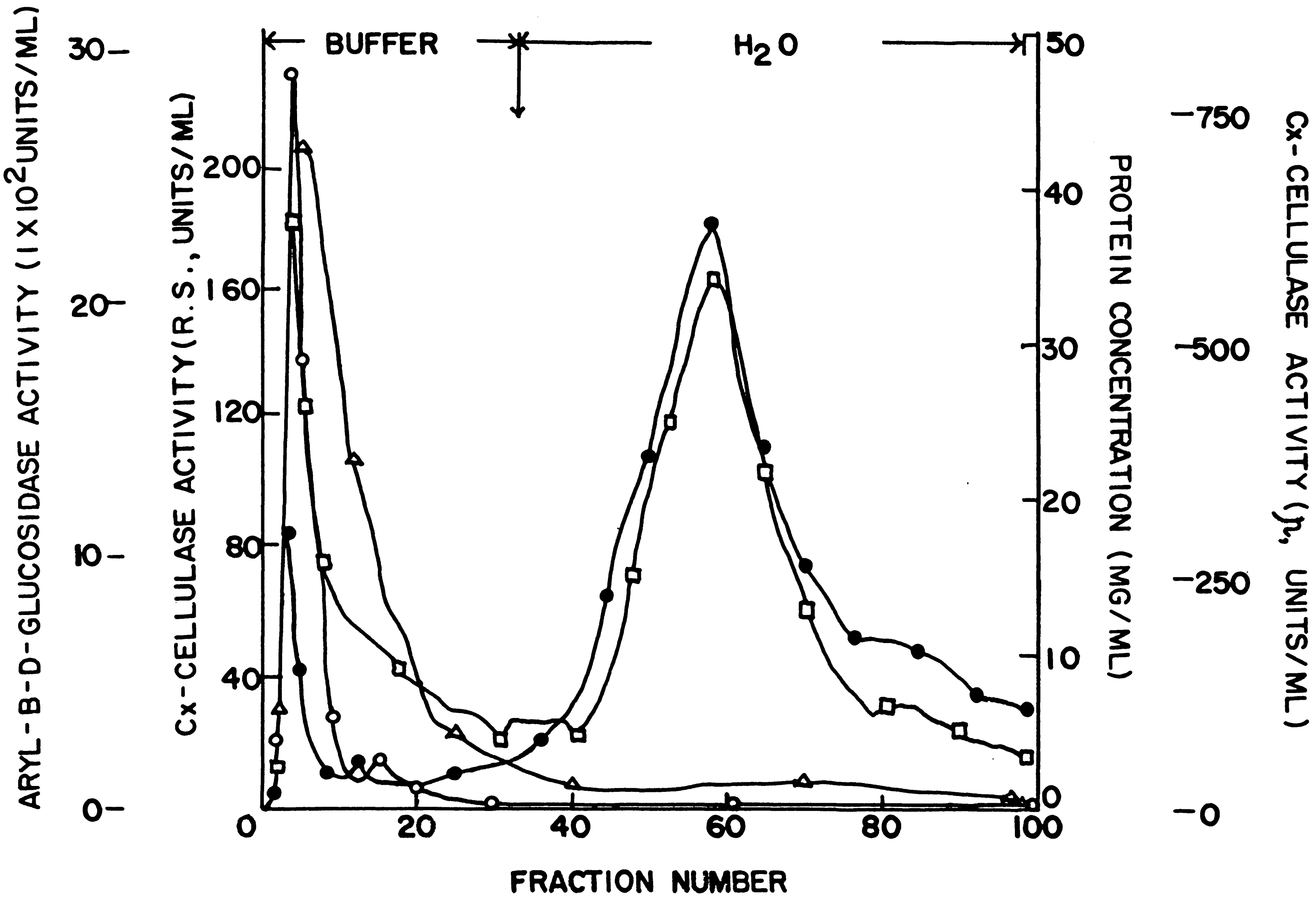
Several attempts at fractionation of the crude enzyme from *Trichoderma viride*, such as column chromatography on DEAE-Sephadex A-25, and Sephadexes G-25 and G-200 showed little or no promise.

The first success in fractionation was obtained by applying 10% crude enzyme solution in 0.05 M sodium citrate buffer, pH 4.8 to an Avicel column (enzyme : column packing = 1 ; 1, v/v). The hydrocellulase was strongly adsorbed on the column, while C_x-cellulase was readily eluted by 0.05 M sodium citrate buffer at pH 4.8 with about 95% recovery of enzyme activity.

The buffer effluent from the Avicel column was then subjected to adsorption on alkali swollen cellulose by column chromatography (enzyme : column packing = 1 : 6, v/v). Two peaks were resolved by eluting with 0.05 M potassium phosphate buffer at pH 5.5 followed by integrating the solvent with water. The first peak, coming out with buffer solution, contained a large amount of protein with most of the aryl-β-D-glucosidase activity and some C_x-cellulase measured by the reducing sugar assay on CMC. The second peak with low protein content came out with water, and was composed mainly of C_x-cellulase activity and little aryl-β-D-glucosidase activity. A typical fractionation pattern is shown in Fig. 1.

After pooling the fractions of the second peak, C_x-cellulase was concentrated by precipitation at 80% saturation of ammonium sulfate. The redissolved precipitate contained almost all of the C_x-cellulase

Fig. 1. Fractionation of crude cellulase from Trichoderma viride by adsorption chromatography using alkali swollen cellulose. (□) C_x-cellulase activity in units per ml determined by reducing sugar assay on CMC, (●) C_x-cellulase activity in units per ml determined by viscosimetric assay on CMC, (○) protein concentration in mg per ml determined by spectrophotometric method, (△) aryl-β-D-glucosidase activity in units per ml. In this experiment the following conditions were used: column dimensions 1.8 cm x 40 cm; 15 ml of Avicel effluent; fraction size approximately 12 ml; flow rate approximately 10 ml per minute; temperature 4^{o}C.; solvents 0.05 M potassium phosphate buffer at pH 5.5 followed by integration with H_2O.



ARYL-B-D-GLUCOSIDASE ACTIVITY (1 X 10^2 UNITS/ML)
30
20
10
0
Cx-CELLULASE ACTIVITY (R.S., UNITS/ML)
200
160
120
80
40
BUFFER
H_2O
50
40
30
20
10
0
PROTEIN CONCENTRATION (MG/ML)
750
500
250
0
Cx-CELLULASE ACTIVITY (η, UNITS/ML)
0
20
40
60
80
100
FRACTION NUMBER

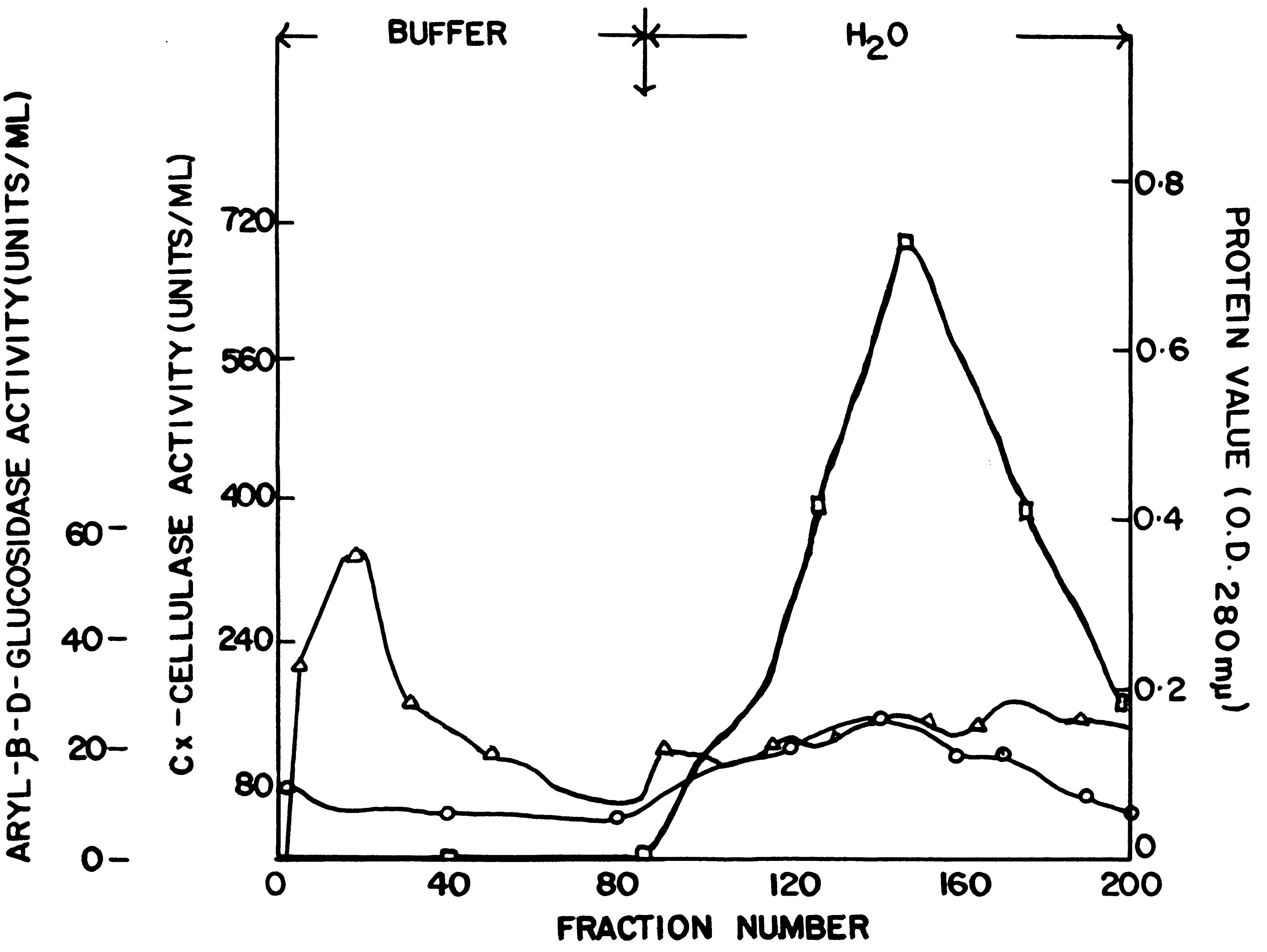
activity but only 62% of protein was recovered.

The concentrated enzyme was reapplied to the alkali swollen cellulose column (enzyme : column packing = 1 : 20, v/v), and again two peaks were resolved. The first one contained only aryl-β-D-glucosidase and the second peak had mainly C_x-cellulase activity still contaminated with a trace of aryl-β-D-glucosidase activity. The results are shown in Fig. 2.

In order to establish whether the C_x-cellulase and the aryl-β-D-glucosidase from the second peak were two different enzymes or a single enzyme possessing both activities, a thermal denaturation experiment was performed at 70°C. The results, shown in Table 1 and Fig. 3, indicated that they were likely to be different enzymes, since C_x-cellulase had a much higher thermal stability than aryl-β-D-glucosidase.

After pooling the fractions of the second peak from the second alkali swollen cellulose column containing relatively high C_x-cellulase activity, the enzyme was concentrated by lyophilization and subjected to electrophoresis using glass paper as supporting medium in an attempt to separate C_x-cellulase from aryl-β-D-glucosidase. Among many conditions tried, only pH 4.0 acetate buffer with low ion strength (< 0.01) under a rather high voltage for a short period gave satisfactory results. A partial separation was obtained by the use of either pH 4.0 or pH 8.3 buffer solution with an ion strength of 0.1, and the loss of enzyme activity was increased as the running time increased, even under a low voltage, especially in the case of using pH 8.3 buffer solution. Eventually, C_x-cellulase was completely separated from aryl-β-D-glucosidase

Fig. 2. Purification of C_x-cellulase from Trichoderma viride cellulases by rechromatography using alkali swollen cellulose. (□) C_x-cellulase activity in units per ml determined by reducing sugar assay on CMC, (△) aryl-β-D-glucosidase activity in units per ml, (○) protein value expressed by optical density at 280 mμ. In this experiment, the following conditions were used: column size 1,000 ml; 50 ml of ammonium sulfate concentrated enzyme (enzyme : column packing = 1 : 20, v/v); fraction size approximately 25 ml; temperature 4°C.; solvents 0.05 M potassium phosphate buffer at pH 5.0 followed by integration with H_2O.

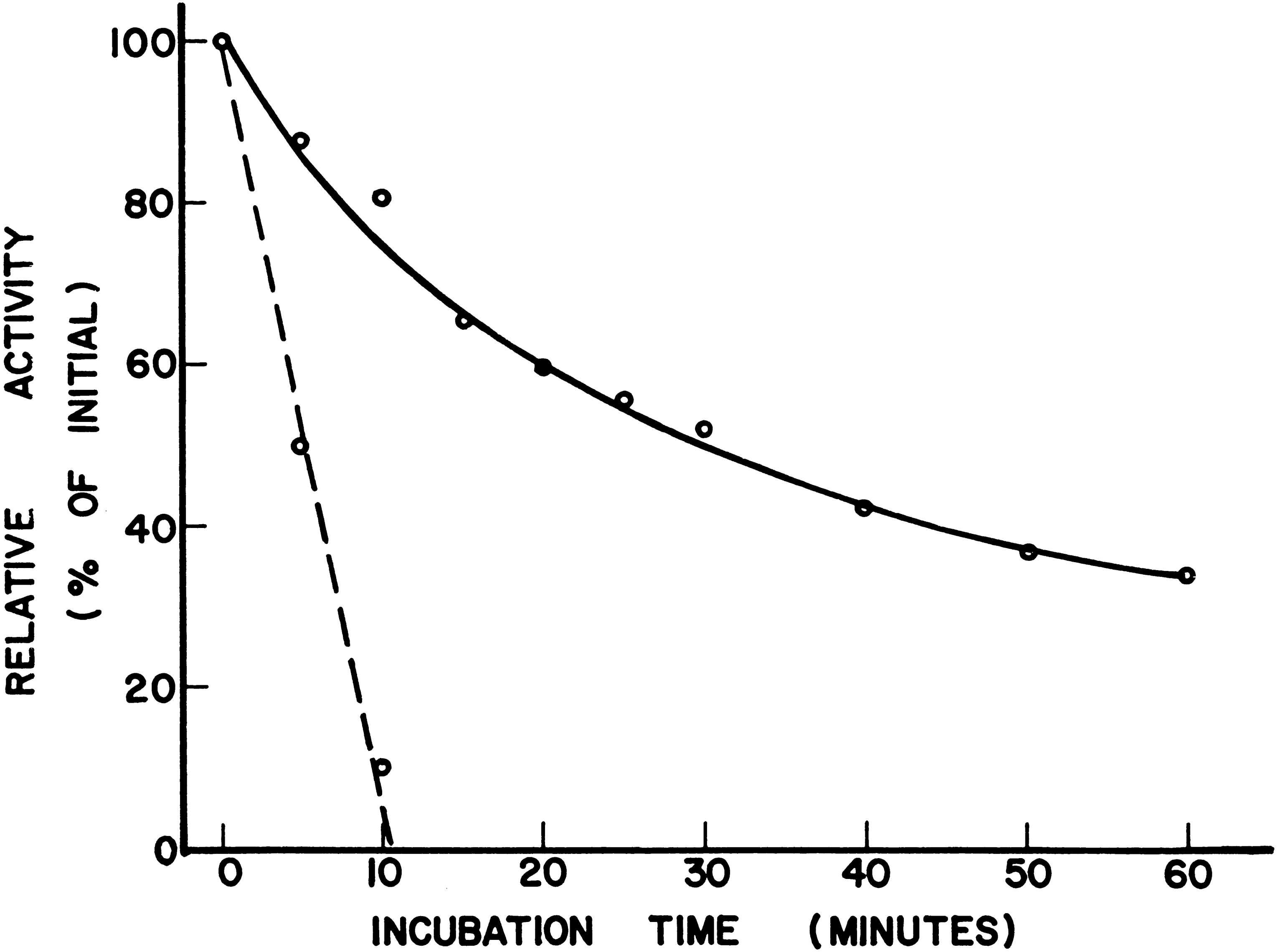


BUFFER
H_2O
ARYL-β-D-GLUCOSIDASE ACTIVITY (UNITS/ML)
0
20
40
60
Cx-CELLULASE ACTIVITY (UNITS/ML)
80
240
400
560
720
PROTEIN VALUE (O.D. 280mμ)
0
0·2
0·4
0·6
0·8
0
40
80
120
160
200
FRACTION NUMBER

Table 1. Thermal denaturation of partially purified C_x-cellulase after second alkali swollen cellulose column chromatography. Enzyme was incubated at 70°C., and the zero time sample was taken as soon as the enzyme solution reached that temperature. In this experiment the following conditions were used: 0.35 ml of second alkali swollen cellulose effluent for aryl-β-D-glucosidase assay using p-nitrophenyl-β-D-glucoside as substrate, and 10 μl effluent for C_x-cellulase using CMC as substrate for reducing sugar assay.

	Relative activity	
Heat treatment time	Aryl-β-D-glucosidase	C_x-cellulase
Minutes	%	%
0	100.0	100.0
5	50.0	87.5
10	10.0	80.7
15	0	65.1
20	0	59.4
25	0	55.3
30	0	52.0
40	0	42.2
50	0	36.5
60	0	33.8

Fig. 3. Thermal denaturation at 70°C. of partially purified C_x-cellulase from second alkali swollen column chromatography. ————, C_x-cellulase activity determined by reducing sugar assay on CMC, – – – – – –, aryl-β-D-glucosidase activity. In this experiment, the following conditions were used: 0.35 ml of second alkali swollen cellulose effluent for aryl-β-D-glucosidase assay using p-nitrophenyl-β-D-glucoside as substrate, and 10 μl effluent for C_x-cellulase using CMC as substrate for reducing sugar assay.


RELATIVE ACTIVITY
(% OF INITIAL)
100
80
60
40
20
0
0
10
20
30
40
50
60
INCUBATION TIME (MINUTES)

by electrophoresis using glass-paper as supporting medium under the following conditions; in pH 4.0 sodium acetate buffer with an ion strength of 0.01 at 4°C. and constant current (4.5 Ma corresponding to an initial potential of 500 volts) for 2 hours. The paper strip was then cut into 1.5 cm sections and extracted with 1 ml of water at 4°C. overnight. The enzyme activity of the extracts was determined with the results shown in Fig. 4. Electroendosmotic flow was measured using parallel strips spotted with ionic and non-ionic dyes (Gelman Instrument Co., Ann Arbor, Mich.).

Having gone through the above procedures, the C_x-cellulase with a 39-fold purification was completely free of hydrocellulase as well as aryl-β-D-glucosidase and is referred to hereafter as a "purified β-1,4-glucan 4-glucanohydrolase" or "purified 3.2.1.4. enzyme," since it seems to fit the conventions under which the name has been given by the Commission on Enzymes of the International Union of Biochemistry (3). The over-all purification procedures are shown in Fig. 5, and the over-all purification data including percentage of recovery, specific activity (units of enzyme per mg of protein), and extent of purification are summarized in Table 2.

Properties

Since the recovery of β-1,4-glucan 4-glucanohydrolase or 3.2.1.4. enzyme activity for each step of purification was over 90%, removal of cofactors or activators was not likely. Enzyme-time studies on purified 3.2.1.4. as well as on the crude enzyme were carried out, and the data

Fig. 4. Electrophoresis of partially purified C_x-cellulase after second alkali swollen cellulose column chromatography. Using glass-paper as supporting medium, 10 µl of concentrated enzyme was applied on the paper strips in pH 4.0 sodium acetate buffer of 0.01 ion strength, at 4°C. and constant current (4.5 ma corresponding to the initial potential of 500 volts) for 2 hours. ————, C_x-cellulase activity in units per ml of extract determined by reducing sugar assay on CMC, ----------, aryl-β-D-glucosidase activity in units per ml of extract. The paper strip (3 cm x 27 cm) was cut into 18 sections of 1.5 cm each, and extracted with 1.0 ml of H_2O overnight. The reference dyes were used for indicating electroendosmotic flow, the yellow dye being non ionic, and both red and blue dyes being ionic. In this case blue was anionic, and red was cationic.

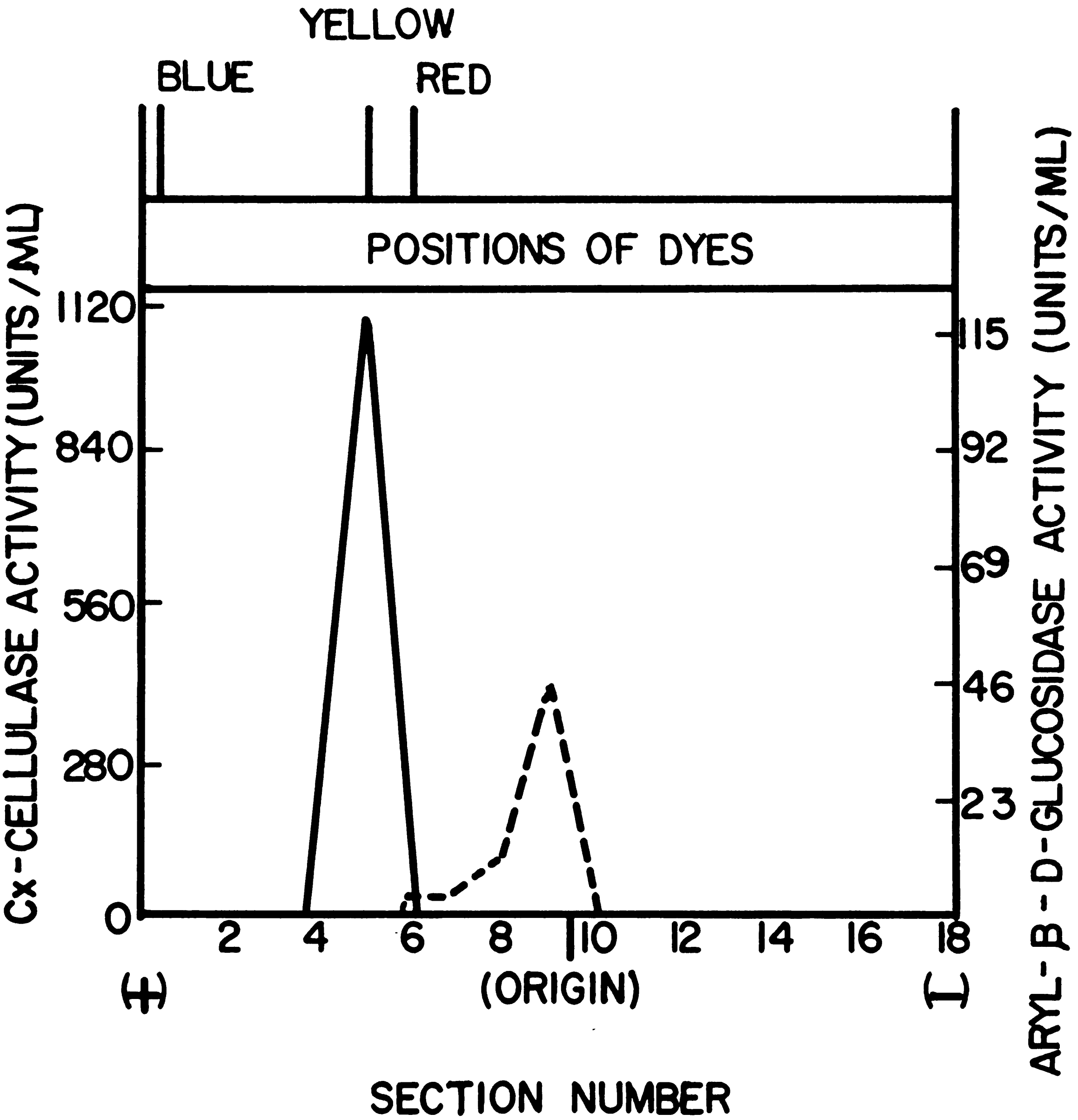

YELLOW
BLUE
RED
POSITIONS OF DYES
Cx-CELLULASE ACTIVITY (UNITS/ML)
1120
840
560
280
0
ARYL-β-D-GLUCOSIDASE ACTIVITY (UNITS/ML)
115
92
69
46
23
2
4
6
8
10
12
14
16
18
(+)
(ORIGIN)
(−)
SECTION NUMBER

Fig. 5. Scheme of over-all purification procedures for β-1,4-glucan 4-glucanohydrolase from Trichoderma viride.

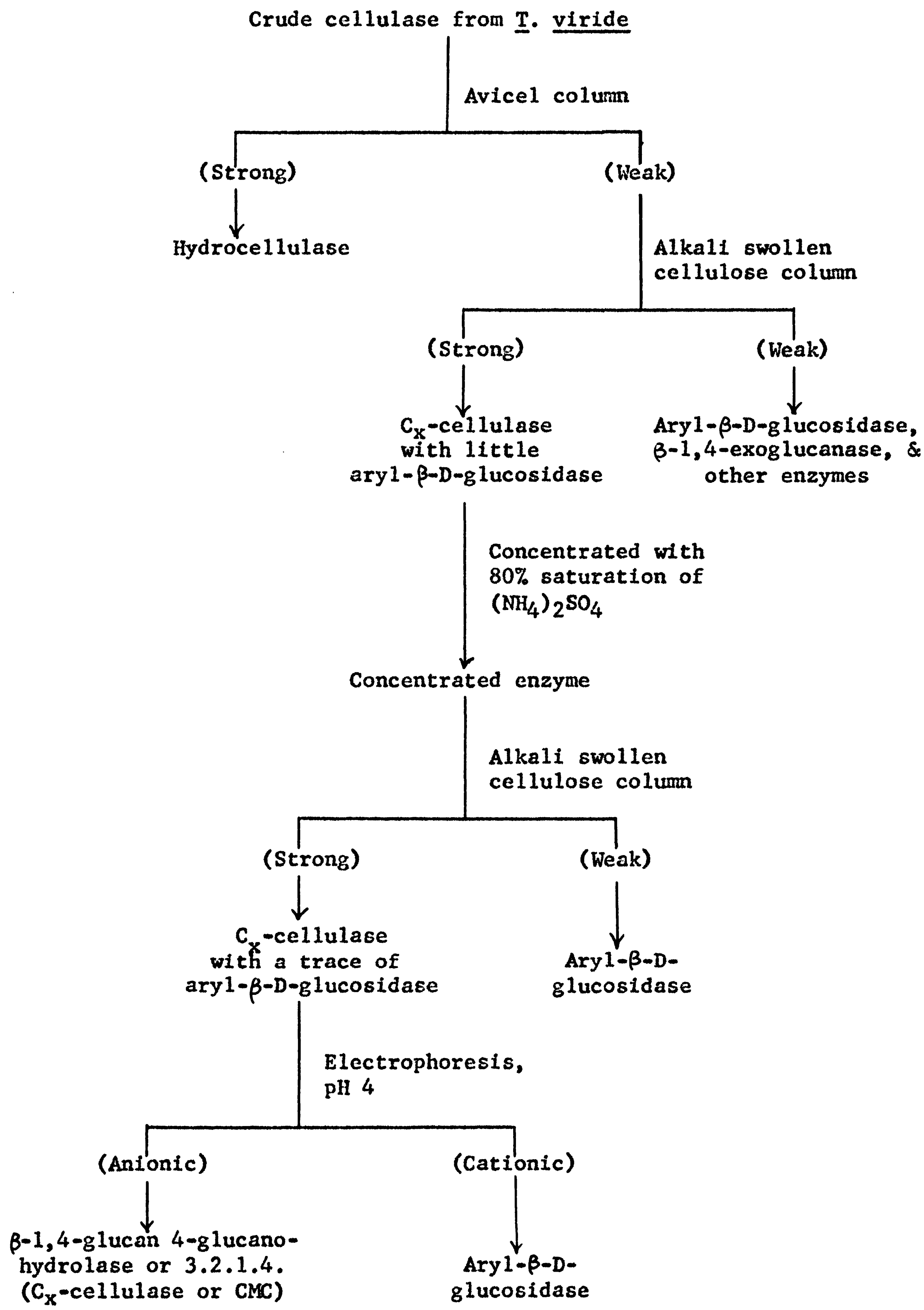

Crude cellulase from T. viride
Avicel column
(Strong)
Hydrocellulase
(Weak)
Alkali swollen cellulose column
(Strong)
C_x-cellulase with little aryl-β-D-glucosidase
(Weak)
Aryl-β-D-glucosidase, β-1,4-exoglucanase, & other enzymes
Concentrated with 80% saturation of $(NH_4)_2SO_4$
Concentrated enzyme
Alkali swollen cellulose column
(Strong)
C_x-cellulase with a trace of aryl-β-D-glucosidase
(Weak)
Aryl-β-D-glucosidase
Electrophoresis, pH 4
(Anionic)
β-1,4-glucan 4-glucanohydrolase or 3.2.1.4. (C_x-cellulase or CMC)
(Cationic)
Aryl-β-D-glucosidase

Table 2. Purification of β-1,4-glucan 4-glucanohydrolase from <u>Trichoderma viride</u>. Protein value was determined colorimetrically by the procedure of Lowry <u>et al</u>. (39).

Stage of purification	β-1,4-glucan 4-glucanohydrolase (reducing sugar assay on CMC)		
	Recovery	Specific activity	Purification
	%	units/mg of protein	over crude
Crude enzyme	100.0	259	1.0
Avicel column	95.0	355	1.4
Alkali swollen cellulose column	85.5	3,910	15.0
Concentrated with $(NH_4)_2SO_4$	84.0	6,670	26.0
2nd alkali swollen cellulose column	75.5	8,670	34.0
Paper electrophoresis	-	10,300	39.0

shown in Table 3 and Fig. 6, clearly indicated that neither inhibitor nor activator was involved in the action of the purified 3.2.1.4. Some inhibitors in the crude enzyme appeared to have been removed during the purification. From this study, there was also a possibility of activators being involved in the crude enzyme preparation; however, no real activator has been found from a preliminary test carried out by incubating the crude enzyme with a concentration of 1×10^{-3} M of various metallic salts and of other chemicals, such as $Mo_4^{=}$, $So_4^{=}$, Ba^{++}, Cd^{++}, Ca^{++}, Ce^{++}, Cr^{++}, Co^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, Pb^{++}, Mg^{++}, Li^{++}, Mn^{++}, Hg^{++}, Ni^{++}, K^{+}, Ag^{+}, Na^{+}, Zn^{++}, Sn^{++}, and $UO_2(NO_3)_2$. Among these chemicals, only Mn^{++} and Hg^{++} had a high degree of inhibition. Cu^{++} and Ce^{++} showed some inhibitory effect.

Although CMC of standard grade and high solubility in water was used as substrate for the reducing sugar assay of the β-1,4-glucan 4-glucanohydrolase for the most part of this study, it certainly is not the natural substrate for this enzyme because of its ionic characteristic. Non-ionic substrates such as amorphous cellulose and water soluble cellulodextrins should also be hydrolyzed by this purified enzyme if the enzyme was considered to be a true β-1,4-glucan 4-glucanohydrolase or C_x-cellulase. In fact, it did attack amorphous cellulose as well as water soluble cellulodextrins. The optimum pH's for the action of this purified enzyme were found to be about 5.0 for CMC and 4.2 for amorphous cellulose. The data are summarized in Table 4 and Fig. 7.

The effect of temperature on the action of this purified enzyme was

Table 3. Enzyme-time studies on both crude enzyme and purified β-1,4-glucan 4-glucanohydrolase by means of reducing sugar assay on CMC. Five percent crude cellulase was diluted ten fold with H_2O. The reducing sugar assay on CMC was used for assaying both crude and purified enzyme activity and incubated at 40^oC. for a given time.

	Crude cellulase		β-1,4-glucan 4-glucanohydrolase	
Incubation time	Enzyme used	Relative activity	Enzyme used	Relative activity
Minutes	μl	%	μl	%
6.0	100	81.0	-	-
7.5	-	-	800	97.7
12.0	50	84.4	-	-
15.0	40	100.0	400	97.7
20.0	30	86.3	-	-
30.0	20	89.2	200	100.0
60.0	10	70.0	100	99.7
120.0	5	69.3	50	97.1
240.0	-	-	25	96.8

Fig. 6. Enzyme-time studies on both crude cellulase and purified β-1,4-glucan 4-glucanohydrolase by means of reducing sugar assay on CMC. (o) crude cellulase, (●) purified β-1,4-glucan 4-glucanohydrolase. The enzymes were incubated at 40°C. for a given time.

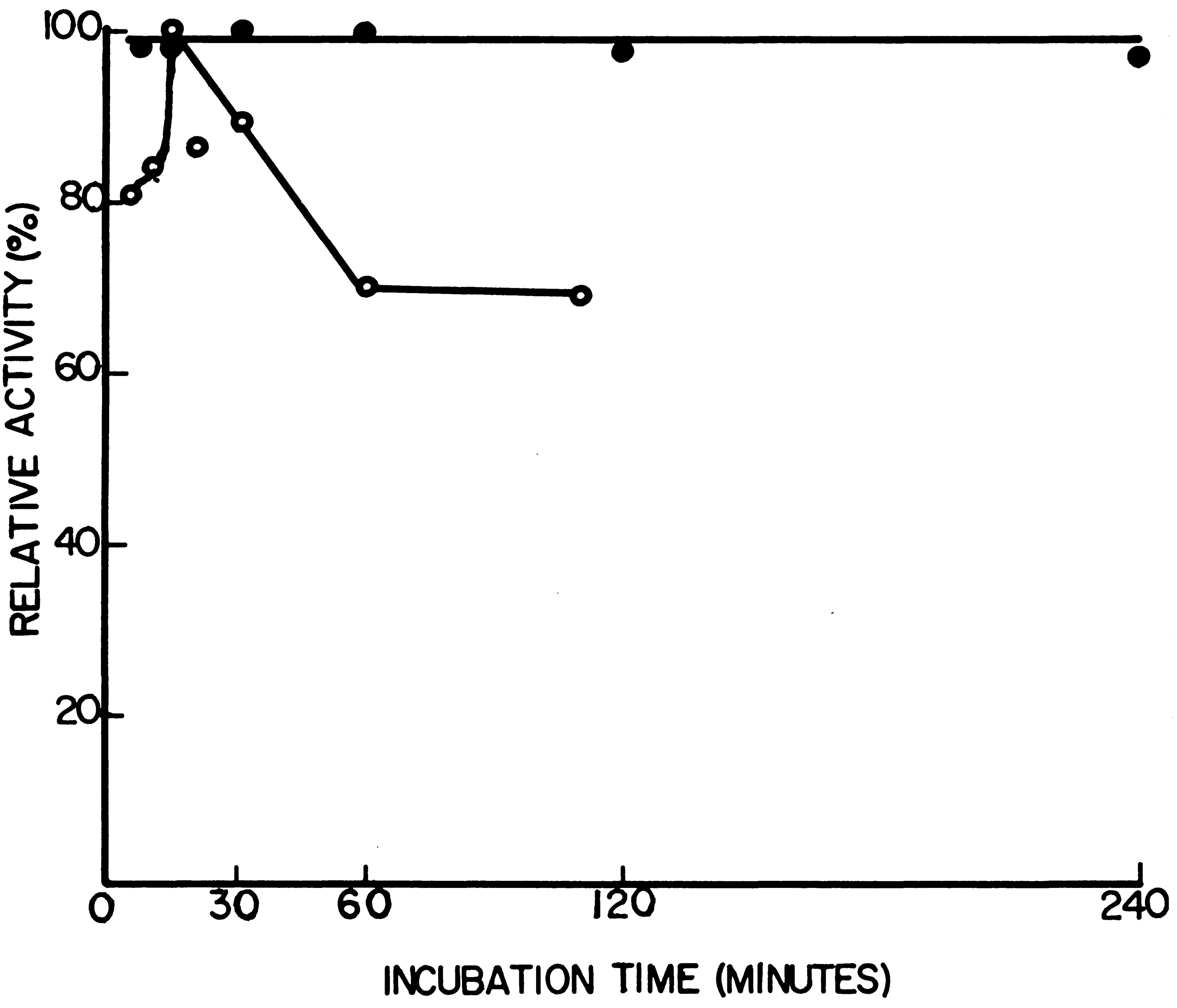
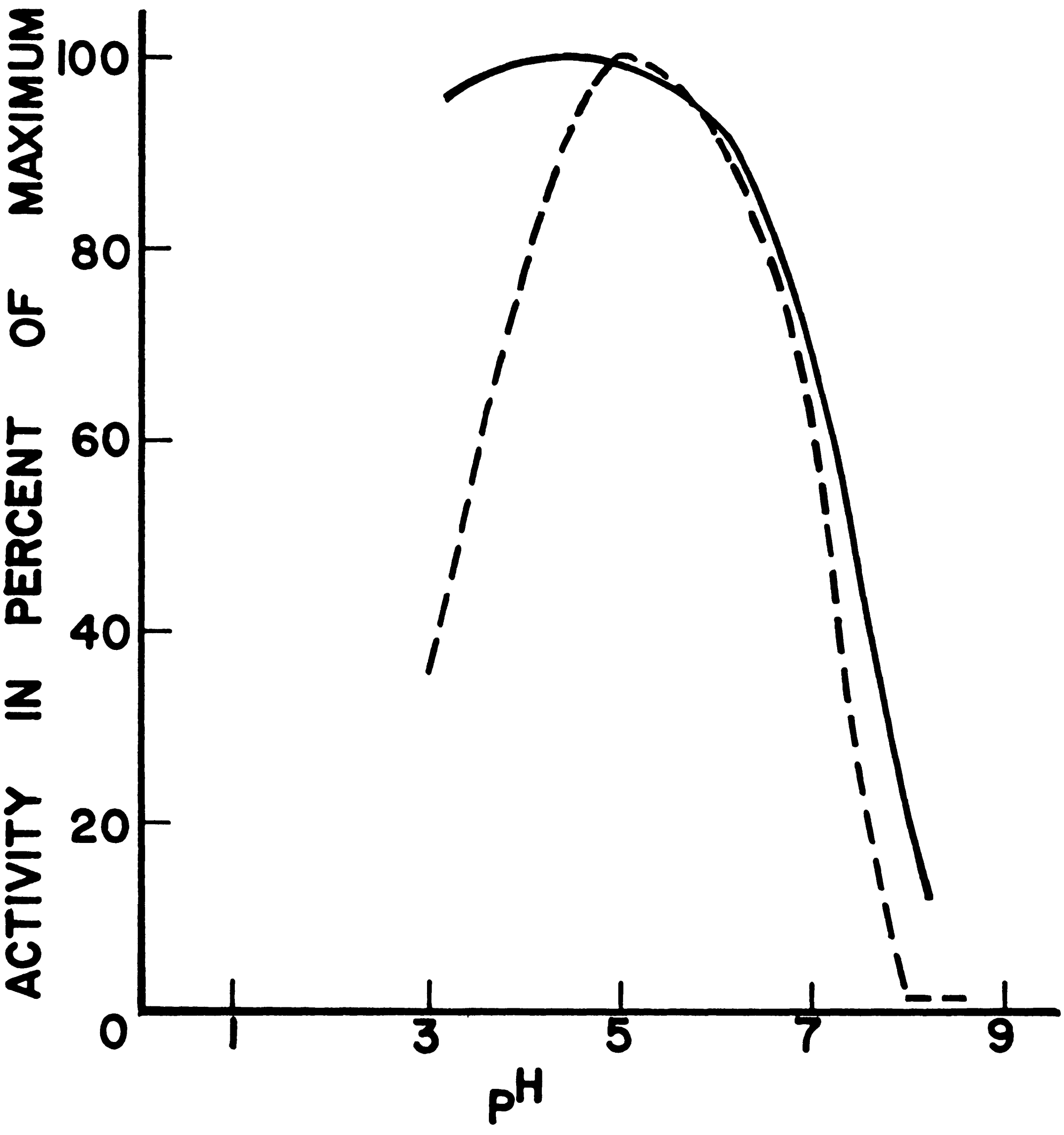

100
80
60
40
20
RELATIVE ACTIVITY (%)
0
30
60
120
240
INCUBATION TIME (MINUTES)

Table 4. The effect of pH on the activity of purified β-1,4-glucan 4-glucanohydrolase on both amorphous cellulose and CMC. CMC was used as substrate for reducing sugar assay. The conditions used in this experiment were exactly the same as described in Fig. 7.

On amorphous cellulose		On CMC	
pH	Percent of maximum	pH	Percent of maximum
3.20	95.4	3.0	35.6
3.65	98.8	3.5	56.8
4.12	100.0	4.0	78.5
4.58	100.0	4.5	93.8
5.05	98.8	5.0	100.0
5.55	96.5	5.5	96.6
6.05	93.0	6.0	92.2
6.45	83.7	6.5	80.7
6.85	72.1	7.0	60.0
7.35	55.2	7.5	25.4
8.00	18.6	8.0	0
8.20	11.6	8.5	0

Fig. 7. The effect of pH on the activity of purified β-1,4-glucan 4-glucanohydrolase on both amorphous cellulose and CMC. ————, amorphous cellulose used as substrate; ---------, CMC used as substrate for reducing sugar assay. CMC was adjusted to a given pH by the addition of 85% phosphoric acid and 1 N NaOH, 0.2 ml of purified enzyme after electrophoresis at pH 4 was incubated with this substrate at different pH's at 40°C. for 1 hour. The enzyme activity was determined according to the usual assay procedure. One ml of the purified enzyme after second alkali swollen cellulose column was incubated with 2 ml of amorphous cellulose suspension (4.4 mg/ml) and 2 ml of buffer of different pH's (using 0.1 M sodium citrate buffer and 0.1 M sodium barbital buffer) at 40°C. for 1 hour. The enzyme activity was determined turbidometrically. A trace of aryl-β-D-glucosidase appeared to contribute practically no effect in the decomposition of amorphous cellulose determined turbidometrically.


ACTIVITY IN PERCENT OF MAXIMUM
100
80
60
40
20
0
1
3
5
7
9
pH

determined with two different substrates, namely amorphous cellulose and CMC. For a period of 1 hour incubation, the optimum temperature appeared to be 57°C. for the breakdown of amorphous cellulose and 60°C. for CMC, the difference was not significant. The results are shown in Table 5 and Fig. 8.

By plotting these data according to the Arrhenius equation,

$$\log k = - \frac{E}{2.3R} (1/T) + C$$

a straight line was obtained with a negative slope of 0.219E from which the activation energy, E, can be calculated. In this equation, k is the rate constant, T the absolute temperature, R the gas constant, C an empirical constant. The activation energies for purified β-1,4-glucan 4-glucanohydrolase were 5.1 Kcal per mole for amorphous cellulose, 6.4 Kcal per mole for CMC between 40° to 60°C., and 16.7 Kcal per mole for CMC between 25° to 40°C. with a breaking curve and a downward bend at 40°C. The data are shown in Table 6 and Fig. 9.

As to the determination of Michaelis constant, the reducing sugar analysis of the hydrolytic products of water soluble cellulodextrins by this enzyme was found to be impractical. Because of a very high blank value, the data obtained were not reproducible. Although glucose appeared not to be the principal product of hydrolysis of cellulose and its derivatives by purified β-1,4-glucan 4-glucanohydrolase, it gave a quite satisfactory result for the calculation of K_m of this enzyme acting on the water soluble cellulodextrins. By means of the Lineweaver-Burk plot

Table 5. The effect of temperature on the activity of purified β-1,4-glucan 4-glucanohydrolase on both amorphous cellulose and CMC. CMC was used as substrate for reducing sugar assay. The conditions used in this experiment were exactly the same as described in Fig. 8.

	Activity of β-1,4-glucan 4-glucanohydrolase	
Temperature	on amorphous cellulose	on CMC
°C.	%	%
10	27.2	5.2
18	34.6	-
19	-	8.5
25	-	16.8
26.5	43.6	-
30	45.6	24.6
37	60.0	42.4
40	65.5	53.8
49	82.7	-
50	-	74.5
57	100.0	-
60	-	100.0
70	85.5	75.3

Fig. 8. The effect of temperature on the activity of purified β-1,4-glucan 4-glucanohydrolase on both amorphous cellulose and CMC. ————, amorphous cellulose used as substrate; — — — — —, CMC used as substrate for reducing sugar assay. CMC was incubated with a proper amount of purified enzyme after electrophoresis at pH 4 (for instance, 0.4 ml used between 25° to 40°C., 0.2 ml used at 50°C., and 0.1 ml used between 60° to 70°C.) at different temperatures for 1 hour. The enzyme activity was determined according to the assay procedure. One ml of the purified enzyme after second alkali swollen cellulose column was incubated with 2 ml amorphous cellulose suspension (4.4 mg/ml) and 2 ml sodium citrate buffer at pH 4 at different temperatures for 1 hour. The enzyme activity was determined turbidometrically. A trace of aryl-β-D-glucosidase appeared to have practically no effect on amorphous cellulose.

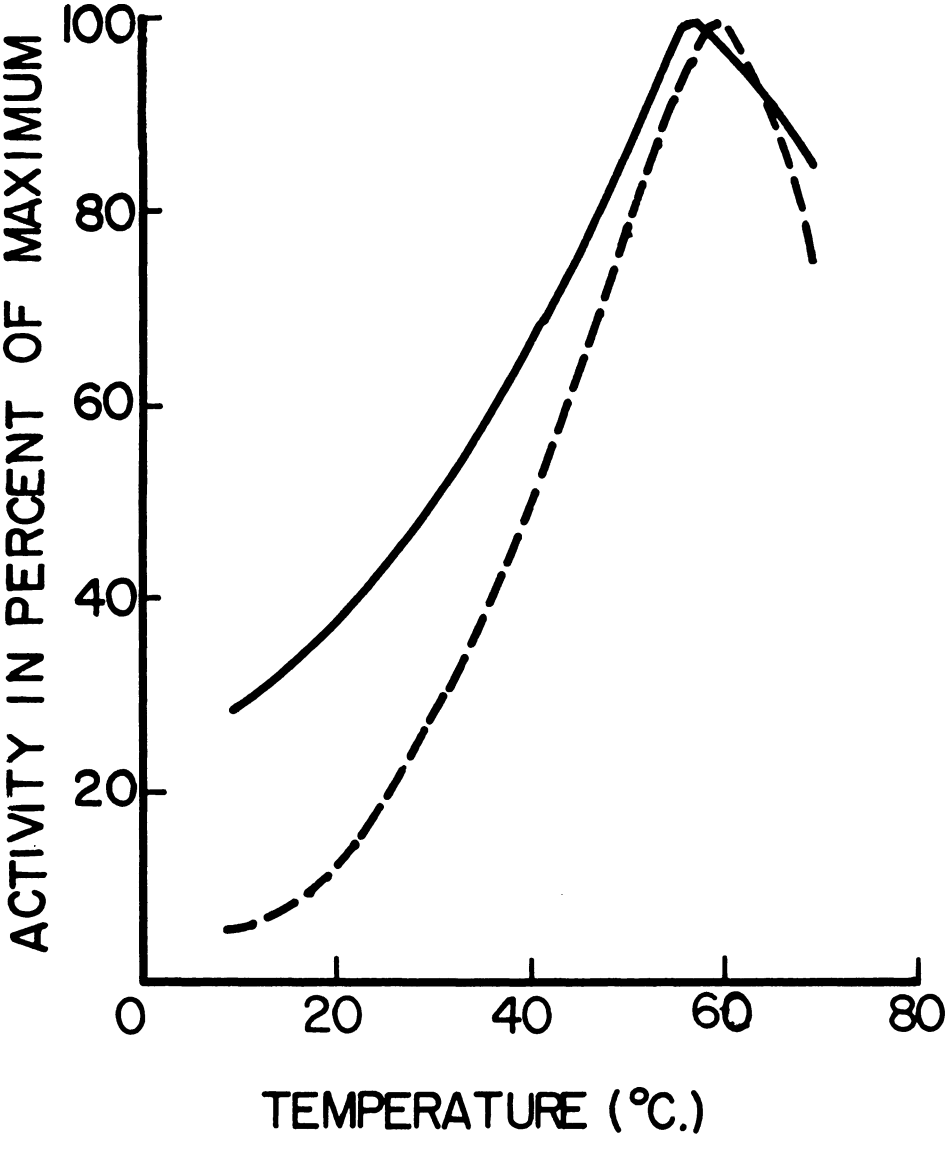
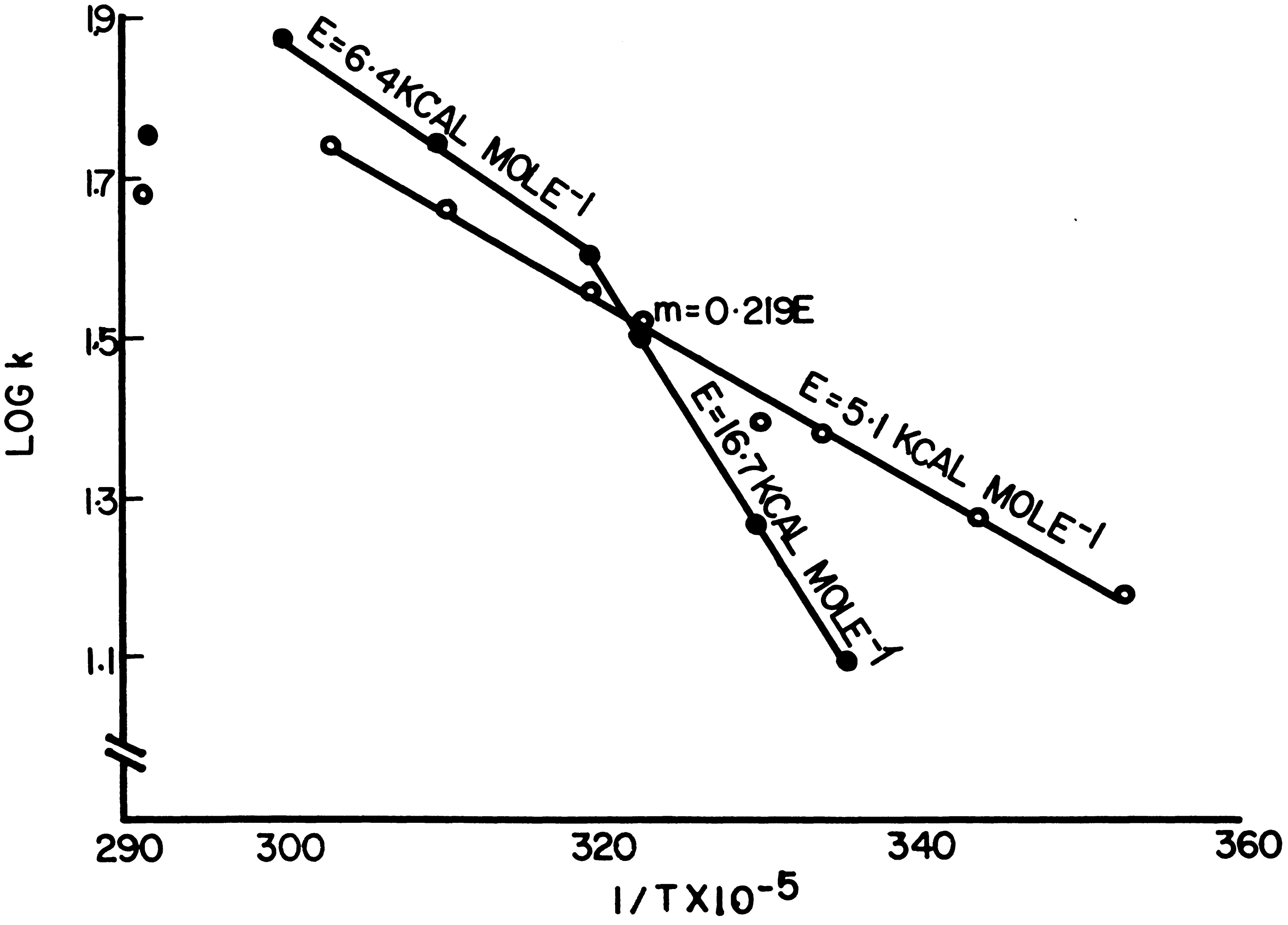

ACTIVITY IN PERCENT OF MAXIMUM
100
80
60
40
20
0
20
40
60
80
TEMPERATURE (°C.)

Table 6. Data for Arrhenius plot and the calculation of activation energy of purified β-1,4-glucan 4-glucanohydrolase attacking different substrates. k represents rate constant at each temperature in $\Delta Klett_{660\ m\mu}$ per hour for turbidometric assay on amorphous cellulose, and to $\Delta OD_{505\ m\mu} \times 10^2$ per hour for reducing sugar assay on CMC. The conditions used in this experiment were exactly the same as described in Table 5 and Fig. 8.

		Log k	
Absolute temperature	1/T	on amorphous cellulose	on CMC
T	1×10^{-5}		
283.0	353.0	1.176	-
291.0	343.5	1.279	-
298.0	335.5	-	1.097
299.5	334.0	1.380	-
303.0	330.0	1.398	1.263
310.0	322.5	1.519	1.498
313.0	319.5	1.556	1.602
322.0	310.5	1.658	-
323.0	309.5	-	1.744
330.0	303.0	1.740	-
333.0	300.0	-	1.872
343.0	291.5	1.672	1.748

Fig. 9. Arrhenius plot for calculating the activation energy of purified β-1,4-glucan 4-glucanohydrolase attacking different substrates. (o) amorphous cellulose, (•) CMC. The conditions used in this experiment were exactly the same as described in Table 5 and Fig. 8.



1.9
1.7
1.5
1.3
1.1
LOG k
290
300
320
340
360
1/T X 10-5
E=6·4 KCAL MOLE-1
m=0·219E
E=5·1 KCAL MOLE-1
E=16·7 KCAL MOLE-1

derived from the Michaelis-Menton equation,

$$1/v = K_m/V \cdot 1/[S] + 1/V$$

a straight line was obtained with a slope of K_m/V and an intercept of $1/V$, from which both K_m and V were calculated. The results are summarized in Table 7 along with the K_m data of purified β-D-1,4-exoglucanase obtained from King (51) in order to compare the difference between these two purified enzymes in the section of discussion. An example of the determination of K_m for the hydrolysis of cellopentaose by this purified enzyme is shown in Fig. 10.

With regard to the enzyme itself, the purified β-1,4-glucan 4-glucanohydrolase appeared to be quite stable at room temperature for a period of one or two hours, especially a concentrated solution. It was also found to be very stable at pH 6 to 7 and relatively stable over a wide range of pH at room temperature for a period of 2 hours. The results are shown in Table 8 and Fig. 11.

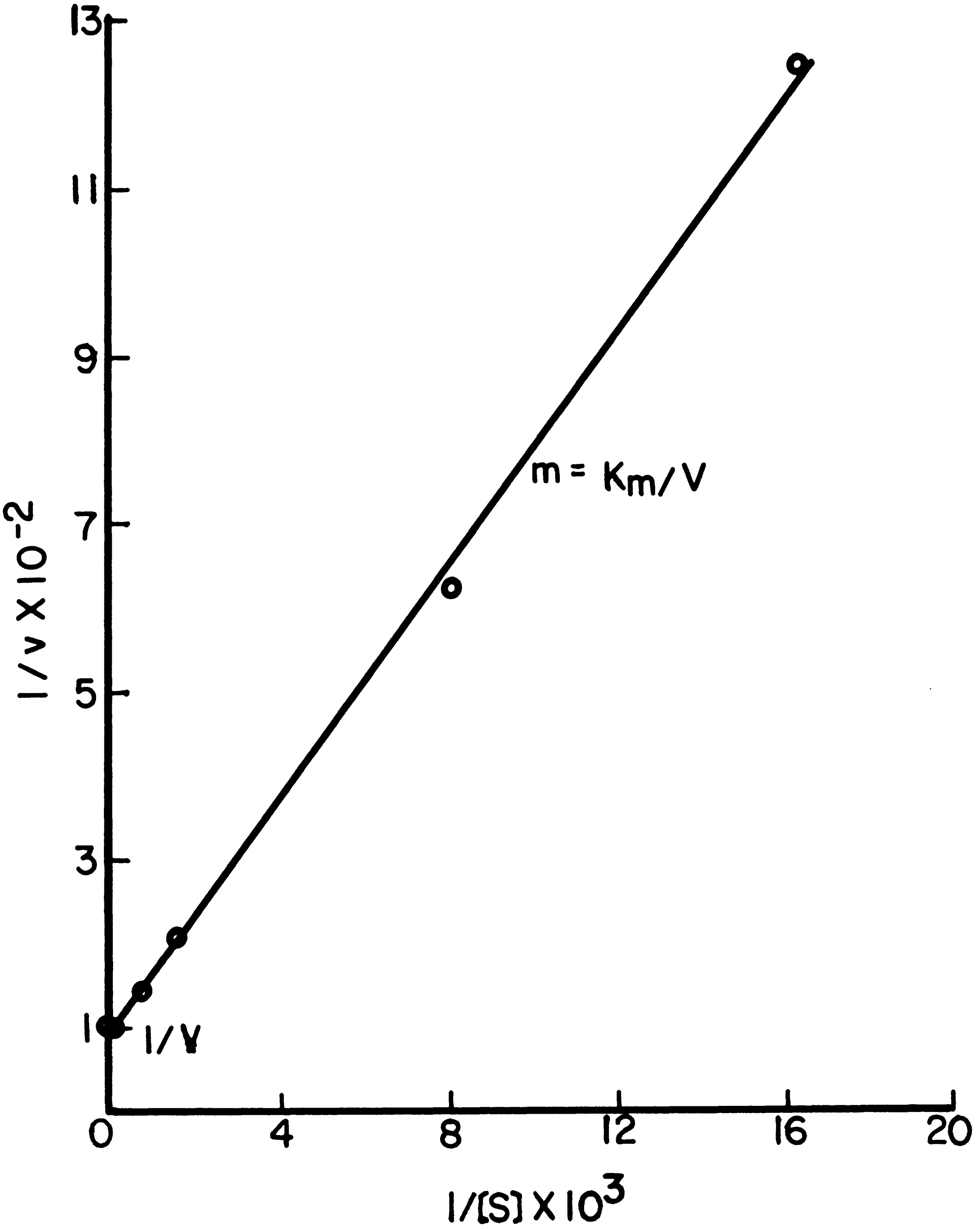
The amino acid composition of purified β-1,4-glucan 4-glucanohydrolase was analyzed in a Beckman-Spinco amino acid analyzer by using 1 ml of hydrolyzate of the purified enzyme after electrophoresis and 1 ml of norleucine as an internal standard. No cysteine and only a trace of methionine were detected. The data appear in Table 9.

Because of the difficulty in obtaining a large amount of this purified enzyme by paper electrophoresis, the determinations of molecular weight, and diffusion coefficient have not been carried out yet; however, a relative value of sedimentation coefficient was obtained as 3.5S.

Table 7. Determination of K_m for both purified β-1,4-glucan 4-glucanohydrolase and β-D-1,4-exoglucanase attacking water soluble cellulodextrins.

	K_m	
Cellulodextrin	β-1,4-glucan 4-glucanohydrolase	β-D-1,4-exoglucanase
	1×10^{-4}M	1×10^{-5}M
Cellobiose	190	220
Cellotriose	32	18
Cellotetraose	24	6.5
Cellopentaose	7.0	6.0
Cellohexaose	1.0	16

Fig. 10. Lineweaver-Burk plot for determination of the K_m of hydrolysis of cellopentaose by purified β-1,4-glucan 4-glucanohydrolase. The enzyme activity was measured by the glucose production using the notatin assay. K_m used as symbol for Michaelis constant, V for maximum reaction rate, [S] for substrate concentration, and v for reaction rate indicated by $\Delta Klett_{420\ m\mu}$ per hour at 40^oC.

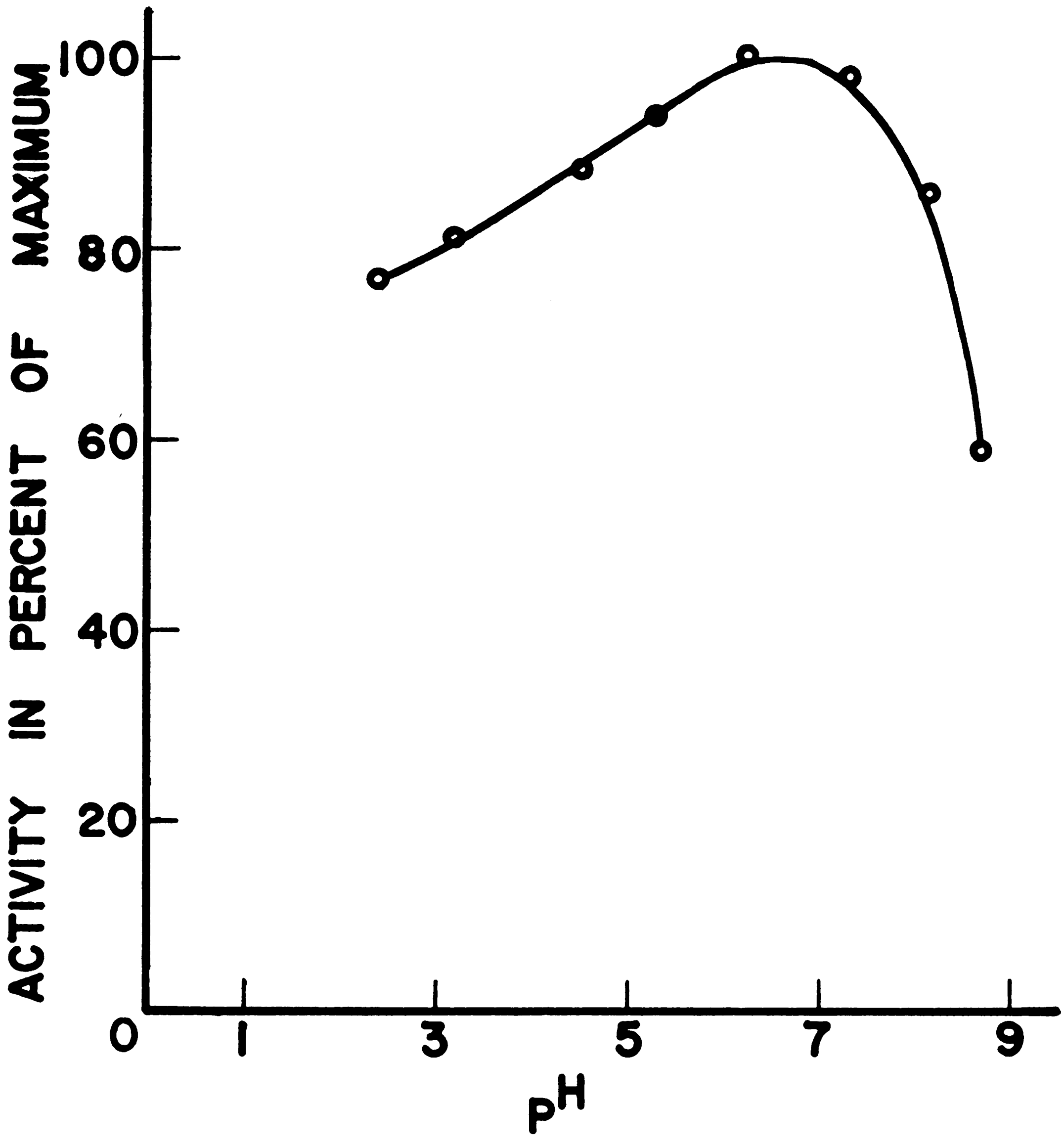


13
11
9
7
5
3
1
1/v X 10^-2
m = Km/V
1/V
0
4
8
12
16
20
1/[S] X 10^3

Table 8. Determination of pH stability of purified β-1,4-glucan 4-glucanohydrolase. 0.1 ml of purified enzyme after electrophoresis at pH 4 was added into 0.9 ml of ca. 0.01 M potassium phosphate buffer of different pH (adjusted to a given pH's by the addition of either 85% H_3PO_4 or 0.1 N NaOH) and held at room temperature for a period of 2 hours. CMC was used as substrate for the reducing sugar assay.

pH	Activity
	%
2.40	77.0
3.15	81.0
4.50	88.5
5.30	94.0
6.25	100.0
7.30	98.0
8.15	85.6
8.70	59.0

Fig. 11. Determination of pH stability of purified β-1,4-glucan 4-glucanohydrolase. 0.1 ml of purified enzyme after electrophoresis at pH 4 was added into 0.9 ml of ca. 0.01 M potassium phosphate buffer of different pH's (adjusted to a given pH by the addition of either 85% H_3PO_4 or 0.1 N NaOH) and held at room temperature for a period of 2 hours. CMC was used as substrate for reducing sugar assay.



ACTIVITY IN PERCENT OF MAXIMUM
100
80
60
40
20
0
1
3
5
7
9
pH

Table 9. The amino acid composition of purified β-1,4-glucan 4-glucanohydrolase.

Amino acid	μmole per ml	mole %
Lysine	.052	2.3
Histidine	.038	1.7
Arginine	.066	2.9
Aspartic acid	.335	14.7
Threonine	.202	8.9
Serine	.185	8.1
Glutamic acid	.210	9.2
Proline	.096	4.2
Glycine	.283	12.4
Alanine	.212	9.3
Cysteine	0	0
Valine	.136	6.0
Methionine	.009	0.4
Isoleucine	.136	6.0
Leucine	.162	7.1
Tyrosine	.076	3.3
Phenylalanine	.082	3.6
Total (Omitting NH_3)	2.280	100.1

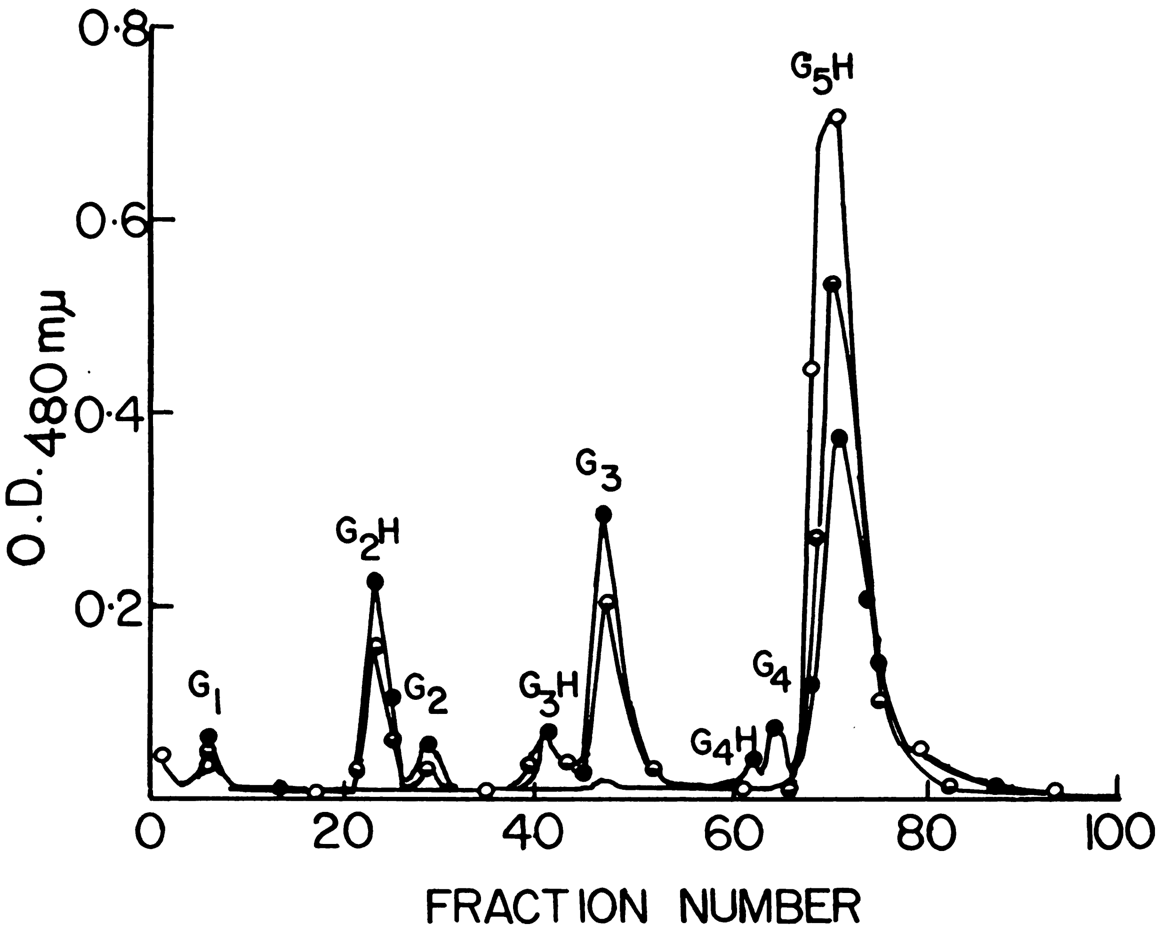
Mode of action

Mode of action of a cellulase is the study of the sites of hydrolysis in order to determine whether the decomposition of cellulose or its derivatives by the enzyme is in a random or an endwise fashion.

Since cellulodextrins of low DP appeared not to be suitable substrates for the purified β-1,4-glucan 4-glucanohydrolase as indicated from the K_m study of this enzyme, 5.1 mg of reduced cellopentaose (G_5H) was incubated with the enzyme in a 1.1 ml water at 40°C. Three-tenth ml of the sample was drawn out after periods of 0, 30, and 60 minutes of incubation, and then applied to separate charcoal-celite columns about 3.5 cm in length as described earlier. The results are shown in Fig. 12 and Table 10. They indicated that the hydrolysis of G_5H by this enzyme seemed to be more or less random. The third bond from the non-reducing end was preferentially attacked, and the second bond was also attacked. Therefore, G_2H and G_3 were principal end products along with considerable amounts of G_3H and G_2 and significant but small amounts of G_1H, G_1, G_4H, and G_4.

The detection of hydrolytic products from amorphous cellulose attacked by this purified enzyme was also carried out in attempting to understand the role of this enzyme in the hydrolysis of cellulose and its derivatives. The experiment was done as follows: One-tenth ml of the purified enzyme was incubated at 40°C. with 3.9 ml of an amorphous cellulose suspension (4.4 mg/ml of water) for a period of time required to reach 0, 6.7, and 43% hydrolysis, as measured by the decrease in

Fig. 12. Hydrolytic products from reduced cellopentaose by the action of purified β-1,4-glucan 4-glucanohydrolase from Trichoderma viride. (o) zero time, (◉) 30 minutes, and (●) 60 minutes incubation. 0.3 ml of each sample was applied on charcoal-celite column (1 cm x 3.5 cm). Sugars were desorbed by integrating 95% ethanol into water, and the effluents were collected in ca. 1 ml fractions. After drying at 110-115^{o}C., sugars were analyzed by phenol-sulfuric acid method.



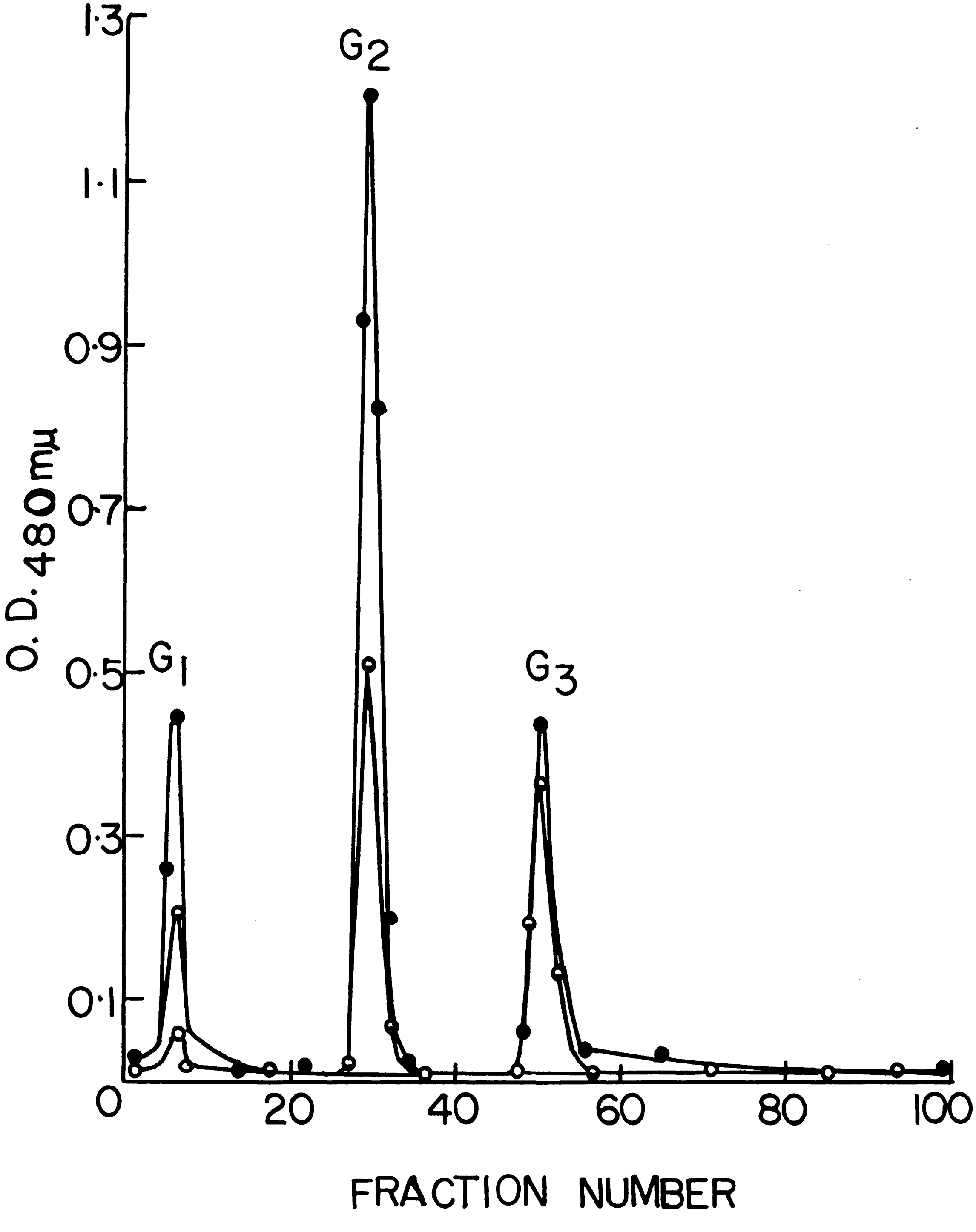
0·8
0·6
0·4
0·2
O.D. 480 mμ
G_5H
G_3
G_2H
G_1
G_2
G_3H
G_4H
G_4
0
20
40
60
80
100
FRACTION NUMBER

Table 10. Hydrolytic products from reduced cellopentaose by the action of purified β-1,4-glucan 4-glucanohydrolase. Values in the Table are given in μmoles calculated from the μg glucose-reacting material in each column peak (See Fig. 12).

Product	Incubation time: 0 minute	30 minutes	60 minutes
	μmole per ml	μmole per ml	μmole per ml
G_1H	0	0.010	0.097
G_1	0.019	0.090	0.093
G_2H	0	1.092	1.360
G_2	0	0.039	0.150
G_3H	0	0.082	0.149
G_3	0	1.110	1.310
G_4H	0	nil	0.022
G_4	0	nil	0.110
G_5	6.020	2.560	2.215

turbidity (ΔKlett$_{660\ m\mu}$). The reaction mixtures were filtered through millipore filters separately, and the filtrates were dried at 100° to 105°C. and redissolved in a given volume of water (0.5 ml for 0% and 6.7% hydrolysis, and 1.0 ml for 43% hydrolysis). Three-tenth ml of each concentrate was applied to a separate charcoal-celite columns. The data are illustrated in Fig. 13. By using a known amount of G_3, the end products were identified as glucose, cellobiose, and cellotriose; however, the yields of soluble sugars were less than the theoretical value (ΔKlett), especially in the case of the 43% hydrolysis as shown in Table 11.

Fig. 13. Hydrolytic products from amorphous cellulose. (o) zero % hydrolysis, (◐) 6.7% hydrolysis, (●) 43% hydrolysis. 0.3 ml of each reaction mixture was analyzed on the charcoal-celite column (1 cm x 4 cm). Hydrolytic products were eluted by integrating absolute alcohol into water, and the effluents were collected in ca. 1 ml fractions. After drying at 110-115°C., the samples were analyzed by phenol-sulfuric acid method.



O. D. 480 mμ
1·3
1·1
0·9
0·7
0·5
0·3
0·1
G1
G2
G3
0
20
40
60
80
100
FRACTION NUMBER

Table 11. Relationship between percentage of hydrolysis of amorphous cellulose and the production of soluble sugars. Values in this table are given in μg obtained from each column peak (see Fig. 13) as determined by phenol-sulfuric acid method. The quantity of amorphous cellulose in each assay tube was 18,876 μg. The percentage of hydrolysis and the theoretical yield of suluble sugars (assuming the hydrolytic products are 100% soluble sugars) was determined by turbidometric assay from the decrease of turbidity. The percentage of low DP sugars in the hydrolytic products was determined by total low DP sugars produced/theoretical total sugar produced.

	Percent of hydrolysis		
Soluble product	0	6.7	43
	μg	μg	μg
G_1	67	158	333
G_2	0	507	2,100
G_3	0	375	1,050
Total	67	1,040	3,483
Theoretical total	-	1,265	8,117
% of soluble sugars	-	82.2	42.9

V. DISCUSSION

During purification, both Avicel and alkali swollen cellulose were employed as adsorbents based on the idea that isolation of β-1,4-glucan 4-glucanohydrolase, or 3.2.1.4. as well as of hydrocellulase by column chromatography might depend on their catalytic specificity rather than on their general properties as proteins. The enzyme 3.2.1.4. was separated from hydrocellulase, since it was weakly adsorbed by Avicel and readily desorbed by sodium citrate buffer. Applying the same principle, the enzyme 3.2.1.4. was separated from most of the aryl-β-D-glucosidase and other contaminants by means of adsorption on alkali swollen cellulose columns on which the 3.2.1.4. was adsorbed and later eluted only by intetrating of water into potassium phosphate buffer. Having obtained the similar results from elution of aryl-β-D-glucosidase and other enzymes out of alkali swollen cellulose column by means of 0.05 M potassium phosphate buffer at pH 3.5, 5.5, and 8.5, pH 5.5 buffer was selected because it was closer to the optimum for cellulase activity.

In separating the enzyme 3.2.1.4. from a trace of aryl-β-D-glucosidase by electrophoresis, electroendosmotic flow was measured using parallel glass paper strips spotted with ionic and non-ionic dyes. The movement of β-1,4-glucan 4-glucanohydrolase was found to be toward the anode at the same position as that of non-ionic yellow dye, and both blue and red dye also moved toward the anode side under the conditions used, although red dye appeared to be positively charged. This indicated that a strong electroendosmosis might be involved. That aryl-β-D-glucosidase stayed close to the origin could be interpreted by either or both of the following: The enzyme might possess a cationic characteristic under these conditions and move toward the cathode, and it might also be strongly adsorbed on the surface of the glass paper. The latter assumption was indicated by the fact that very little enzyme activity appeared in water extracts being equilibrated overnight, but a great deal of activity was obtained by assaying the water extract along with paper strip from the same section. The exact reason for this behavior is not known.

By the application of Avicel column adsorption chromatography, alkali swollen cellulose column adsorption chromatography, precipitation with ammonium sulfate, rechromatography on alkali swollen cellulose column, and electrophoresis at pH 4, in that order, β-1,4-glucan 4-glucanohydrolase was purified 39-fold over the crude enzyme and the resulting preparation was completely free of hydrocellulase as well as of aryl-β-D-glucosidase. Actually the figure of purification might be much higher, because the crude cellulase obtained from Dr. Toyama (33) had been precipitated with ammonium sulfate and isopropyl alcohol. Several-fold in increase of specific activity over very crude cellulase might be expected. This enzyme may not be absolutely pure (Practically, there is no such thing in enzyme chemistry), but it may be considered as a highly purified β-1,4-glucan 4-glucanohydrolase which is pure enough for the determination of its physical and chemical properties, kinetics, and mode of action in order to understand the enzyme itself in terms of the action performed.

From the enzyme-time study, some inhibitors in the crude enzyme appeared to have been removed during the purification and hence there was no inhibitor involved in the action of the purified β-1,4-glucan 4-glucanohydrolase. The requirement of activators or cofactors by the crude enzyme was not exhibited by the purified enzyme, and the fact might be explained as the result of removal of cofactor-requiring enzymes in Trichoderma viride cellulase system which might have directly or indirectly effected the reducing sugar assay on CMC.

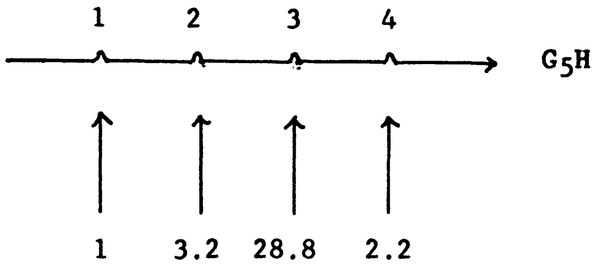
The optimum pH's for the action of this enzyme were found to be about 4.2 for amorphous cellulose and 5.0 for CMC. This may be accounted for by the difference of ionic characteristic between these two substrates. The data generally agreed with the values obtained by Toyama (29) except that he reported a wider range of pH from 4.0 to 5.2 for CMCase activity. The purified enzyme was found to be very stable at pH's of 6 to 7 at room temperature for a period of 2 hours. It was quite different from the optimum condition for its activity, since the optimum pH for the enzyme activity varied with different substrates.

The optimum temperature for the purified enzyme was found to be about 60°C. for both CMC and amorphous cellulose in a period of 1 hour incubation. These results are quite similar to those obtained by Toyama (29), i.e. the limiting temperature of thermostability over 10 minutes was 70°C. for CMCase.

The activation energies of the purified enzyme was 5.1 Kcal per mole for hydrolysis of amorphous cellulose calculated from the Arrhenius plot, 6.4 Kcal per mole for CMC between 40°C. and 60°C. and 16.7 Kcal per mole for CMC between 25°C. and 40°C. with a breaking curve and a downward bend at 40°C. The breaking curve is attributed to the fact that CMC solutions become highly viscous at temperatures below 40°C., and the substrate molecules could not freely diffuse toward the enzyme and vice versa. A low reaction rate resulting from this phase change of the substrate, therefore, was attributed to the great decrease in the chance of collision between substrate and enzyme molecules resulted in a steeper slope of the Arrhenius plot or a higher activation energy required.

After the final stage of purification i.e. electrophoresis at pH 4, the purified enzyme still gave a positive carbohydrate test as determined by phenol-sulfuric acid method. Whether this carbohydrate represents the hydrolytic product of alkali swollen cellulose collected with the enzyme fractions which apparently was not separated from the enzyme by electrophoresis or was an integral part of the enzyme needs to be further differentiated.

As to the mode of action, the hydrolysis of G_5H by the purified enzyme seemed to be more or less random because the third bond from the non-reducing end was preferentially attacked, and the second bond was also attacked. G_2H and G_3 were principal end products along with considerable amounts of G_3H and G_2 and significant but small amount of G_1H, G_1, G_4H, and G_4. The relative frequency of attack on each bond of G_5H by the purified enzyme calculated by the method of Cole and King (36) might be summarized in the following diagram.



Relative frequency of bond attack

These results are similar to those obtained by Whitaker (56) from *Myrothecium verrucaria*, although the *T. viride* enzyme appears to be more selective for internal bonds.

The soluble sugars produced from the hydrolysis of amorphous cellulose by this enzyme were identified as glucose, cellobiose, and cellotriose. However, the yield of total low DP sugars was less than the theoretical total sugar produced. At 6.7% hydrolysis, about 82% of the hydrolytic products was accounted for by low DP sugars, whereas in 43% hydrolysis, only 43% of the hydrolytic products was found to be low DP sugars. This might be interpreted according to Reese *et al*. (52), "Enzyme hydrolysis of both cellulose and hydrocellulose is effected by particle size. The rate of hydrolysis decreases rapidly as some constituents, presumably hydrated cellulosic fragments, are split off, leaving a residue that is quite resistant to further attack." In this case, the first 6.7% hydrolysis might have represented the hydrated cellulosic fragments of the substrate with medium chain length which were readily broken down to soluble sugars, such as glucose, cellobiose, and cellotriose. However, in the case of 43% hydrolysis, the breakdown of the portion of substrate containing long chain polymers and relatively rigid structure which was first decomposed randomly by the enzyme to medium-size chain intermediates which were in turn degraded to soluble sugar very slowly. Therefore, the medium chain oligosaccharide intermediates might be involved in the over-all hydrolysis of amorphous cellulose to soluble sugars in the case of 43% hydrolysis, which accounted for the requirement of long incubation time and less production of low DP soluble sugars.

The over-all possible nature of hydrolysis of amorphous cellulose to soluble sugars by the purified 3.2.1.4. might be summarized as follows:

Amorphous cellulose $\longrightarrow$ Oligosaccharides $\longrightarrow$ G_3, G_2, and G_1

Cellotriose in addition to cellobiose and glucose was found to be a principal end product from the hydrolysis of both G_5H and amorphous cellulose by the enzyme. The large amount of cellotriose might have been produced from a random type hydrolysis, which was indicated clearly from the study of the mode of action on G_5H of the purified 3.2.1.4. The accumulation of cellotriose and cellobiose can be explained by the absence of enzymes capable of hydrolyzing them rapidly, for instance, β-1,4-D-exoglucanase. This result appeared to be similar to the case of Streptomyces spp. reported by Reese et al. (53) except that they used a crude preparation.

There are at least three main components in the cellulase system from T. viride which have been isolated and purified to a certain extent in this laboratory, namely hydrocellulase, β-1,4-glucan 4-glucanohydrolase, and β-D-1,4-exoglucanase reported by Li, Flora, and King (54). The significant differences between them are summarized in Table 12. They are apparently different in molecular size and thermal stability from one

Table 12. Properties of purified components from *Trichoderma viride*.

Property	Hydrocellulase	β-1,4-glucan 4-glucanohydrolase	β-D-1,4-exoglucanase
Sedimentation coefficient	3.9S	3.5S	5.2S
Optimum substrate	Crystalline	DP $\geqq$ 6	DP $\leqq$ 5
Reaction product	Cellobiose	G_3, G_2, and G_1	Glucose
% loss, 70°C., 3 minutes	98	10	83

another. From the K_m study, it was indicated that the optimum substrates for the purified 3.2.1.4. and purified β-D-1,4-exoglucanase were different. With apparent endwise cleavage, the purified β-D-1,4-exoglucanase can break down cellulodextrins with DP of less than five to glucose predominately, and there was very little activity on either amorphous cellulose or hydrocellulose (51). On the contrary, purified β-1,4-glucan 4-glucanohydrolase can attack cellulodextrins with DP of more than 6 and amorphous cellulose rapidly though its activity on crystalline substrates is negligible.

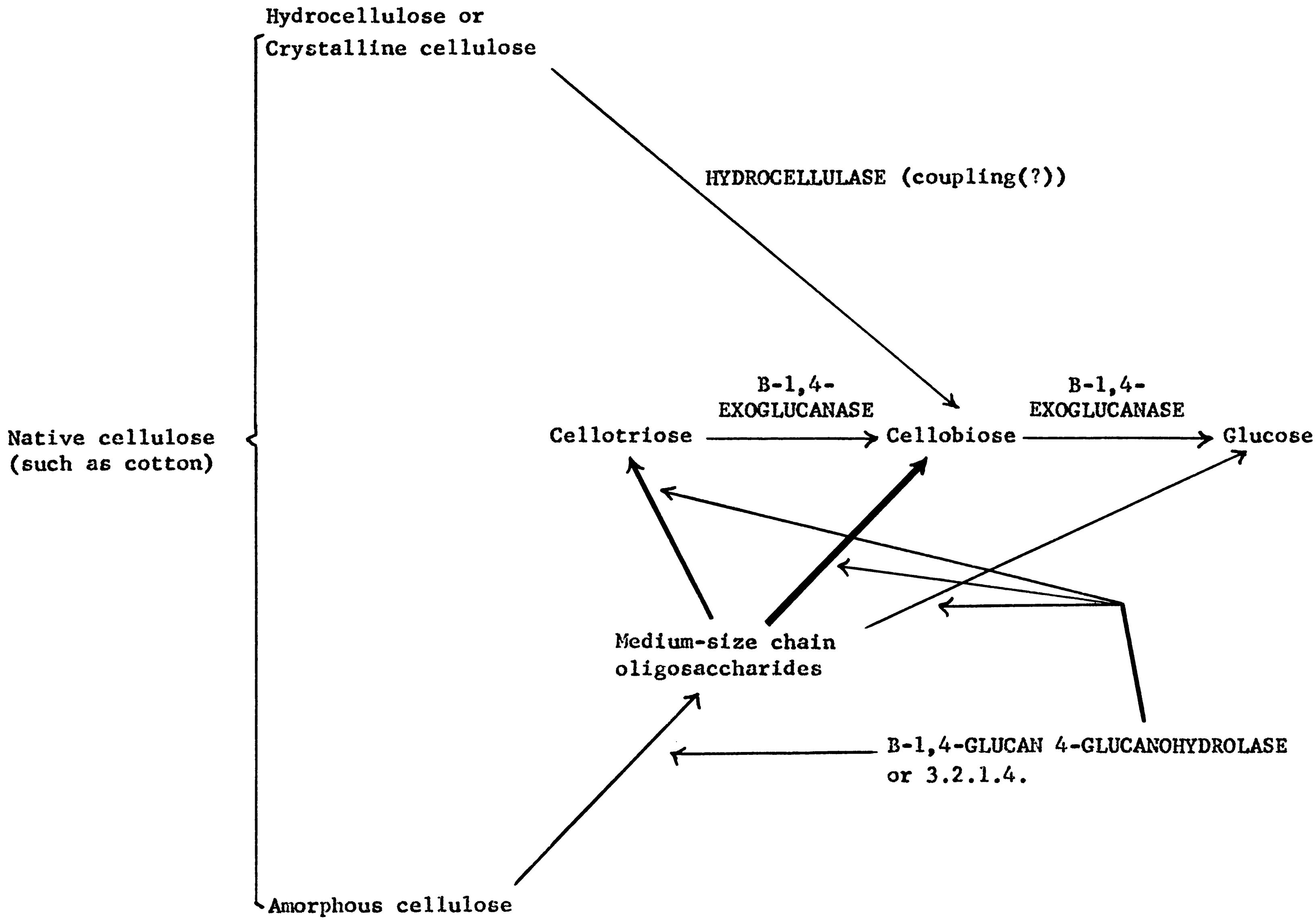
The purified β-1,4-glucan 4-glucanohydrolase is completely free of both hydrocellulase and β-D-1,4-exoglucanase activities and represents most of C_x-cellulase and/or CMCase activity in the cellulose system of _T. viride_, but it has been found to decompose neither hydrocellulose nor the crystalline portions of native cotton. It is much less capable of degrading cellulodextrins of DP less than three to glucose than the exoglucanase and hence cellotriose and cellobiose accumulated in addition to glucose from the hydrolysis of both amorphous cellulose and G_5H. The purified 3.2.1.4. alone could not account for decomposition of native cellulose to glucose. Similarly, neither hydrocellulase nor β-D-1,4-exoglucanase alone could do the job, since purified hydrocellulase appeared to attack mainly hydrocellulose and had little or no activity on amorphous cellulose and yielded predominantly cellobiose rather than glucose (37). Therefore, the concept of the complete decomposition of cellulose to glucose by a single enzyme developed by Whitaker (5) in 1953 does not seem to be the case here.

With the complete cellulase system, the nature of cellulose decomposition by the purified cellulases isolated from _T. viride_ is considered to indicate the concept of a multiple enzyme system which was put forth by Reese, Siu, and Levinson (2) in 1950. However, the sequence of breaking down native cellulose to glucose proposed by Reese _et al_. (2, 55),

$$\text{Native cellulose} \xrightarrow{C_1} \text{linear cellulose chains} \xrightarrow{C_x} \text{cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{glucose}$$

does not fit this case too well, since no synergistic or coupling effect on the decomposition of hydrocellulose was seen by the purified β-1,4-glucan 4-glucanohydrolase from a proportional recombination experiment of all 3 components isolated. This led the author to think that this purified 3.2.1.4. enzyme might not take part in the breaking down of hydrocellulose or crystalline portion of native cellulose (cotton). The main function of this purified enzyme appears to be decomposing amorphous portions of native cellulose. In the meantime, Flora (37) obtained data suggesting that there were no intermediates involved in the decomposition of hydrocellulose by the hydrocellulase which showed a very weak capacity to break down amorphous cellulose. From the above facts, a possible mechanism was proposed, as shown in Fig. 14, in attempting to explain the nature of decomposition of cellulose by the cellulase system of _T. viride_.

Fig. 14. Composite representation of the decomposition of cellulose by the cellulase system of *Trichoderma viride*.


Hydrocellulose or
Crystalline cellulose
HYDROCELLULASE (coupling(?))
Native cellulose
(such as cotton)
Cellotriose
B-1,4-
EXOGLUCANASE
Cellobiose
B-1,4-
EXOGLUCANASE
Glucose
Medium-size chain
oligosaccharides
B-1,4-GLUCAN 4-GLUCANOHYDROLASE
or 3.2.1.4.
Amorphous cellulose

VI. SUMMARY

1. By the application of Avicel column adsorption chromatography, alkaline swollen cellulose column adsorption chromatography, precipitation with ammonium sulfate, rechromatography on alkali swollen cellulose column, and electrophoresis at pH 4 in that order, a β-1,4-glucan 4-glucanohydrolase or 3.2.1.4. was isolated from Trichoderma viride which was purified 39-fold over the crude enzyme and was completely free of hydrocellulase and β-D-1,4-exoglucanase and aryl-β-glucosidase activities.

2. With optimum pH 5.0 for CMC and 4.2 for amorphous cellulose; optimum temperature 60°C. for both CMC and amorphous cellulose for a period of 1 hour incubation; an activation energy 5.1 Kcal per mole for amorphous cellulose and 6.4 Kcal per mole for CMC between 40° to 60°C., the purified enzyme involved neither activator nor cofactor nor inhibitor in its action. It was very stable at pH 6 to 7 at room temperature for a period of two hours.

3. The purified enzyme contained no cysteine, and only a trace of methionine. The sedimentation coefficient was 3.5S.

4. From K_m determination, cellodextrins with DP above 6 and amorphous cellulose appeared to be the optimum substrates for this purified enzyme which did not attack hydrocellulose.

5. A more or less random attack was indicated from the study of mode of action on G_5H, and medium chain oligosaccharides appeared to be intermediates for decomposition of amorphous cellulose by purified enzyme. Cellotriose in addition to cellobiose and glucose was found among

the principal products from the hydrolysis of both G_5H and amorphous cellulose.

6. The difference in the role of three main components obtained from T. viride was discussed in connection of with the concept of the complete decomposition of cellulose by a multiple enzyme system.

7. A scheme of the possible mechanism of decomposition of cellulose by the cellulase system of T. viride was proposed.

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Abstract of Doctor of Philosophy Degree Dissertation

of Li-Hsieng Li

PURIFICATION AND PROPERTIES OF A β-1,4-GLUCAN 4-GLUCANOHYDROLASE FROM TRICHODERMA VIRIDE

A β-1,4-glucan 4-glucanohydrolase or 3.2.1.4. from Trichoderma viride was purified 39-fold over the crude enzyme and completely freed of hydrocellulase, β-1,4-exoglucanase, and aryl-β-D-glucosidase activities by the application of Avicel column adsorption chromatography, alkali swollen cellulose column adsorption chromatography, precipitation with ammonium sulfate, rechromatography on alkali swollen cellulose column, and glass paper electrophoresis at pH 4 in that order. The optimum temperature was 60°C. for the action of this purified enzyme on both amorphous cellulose and CMC for a period of 1 hour incubation. An activation energy of 5.1 Kcal per mole for amorphous cellulose and 6.4 Kcal per mole for CMC between 40° to 60°C. was observed. The optimum pH appeared to be 4.2 for amorphous cellulose and 5.0 for CMC. The purified enzyme involved neither activator nor cofactor nor inhibitor in its action, and it was found to be stable at pH 6 to 7 at room temperature for a period of two hours. The sedimentation coefficient of this purified enzyme was 3.5S. It contained no cysteine and only a trace of methionine. From K_m determinations, cellulodextrins with DP above 6 and amorphous cellulose appeared to be the optimum substrates for this purified enzyme, but it did not attack hydrocellulose. A more or less random attack was indicated from the study of mode of action on reduced

cellopentaose. Evidence that medium-size chain oligosaccharides might be intermediates in the decomposition of amorphous cellulose by the purified enzyme was obtained. Cellotriose in addition to cellobiose and glucose was found among the principal products from the hydrolysis of both G_5H and amorphous cellulose. The difference in the role of three main cellulase components obtained from _T. viride_ was discussed in connection with the concept of the complete decomposition of cellulose by a multiple enzyme system, and a scheme of the possible mechanism of decomposition of cellulose by the cellulase system of _T. viride_ was proposed.